Yoram Vodovotz · Gary An *Editors*

Complex Systems and Computational Biology Approaches to Acute Inflammation

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Preface

We are now standing at the cusp of systems-based understanding of the processes that underlie, drive, and control the acute inflammatory response. Based on decades of painstaking progress, mostly driven by the reductionist methods that have served science well for hundreds of years, this new systems approach is poised to redefine how we approach diagnosis and therapy for sepsis/infection, trauma, and wound healing. The development of systems and computational biology over the past decade has brought a new understanding, new methods, and new terminology to the forefront of the biomedical enterprise.

These new tools and new understanding have begun to yield insights into basic biological mechanisms. What has lagged has been a clear understanding of how systems and computational biology could be applied systematically to change clinical practice. Indeed, we have not yet fully leveraged the power of computational and systems biology. There is currently no fully rational, computationally driven, pipeline for drug discovery, clinical trials, "smart" diagnostics, and patient-specific therapy driven by computational modeling (i.e., Translational Systems Biology). However, we are at, or near, an inflection point in this transition from reductionism to systems approaches applied clinically. Translational Systems Biology as a concept was formulated in an attempt to give initial definitions and directions to the biomedical community. A decade of Translational Systems Biology has resulted in increasingly realistic computational models that can recapitulate inflammation at the cellular, small animal, large animal, and human levels. As such, Translational Systems Biology is at an inflection point between early studies subjected to intense scrutiny and, to a degree, resistance from the research community vs. widespread adoption.

This book presents a snapshot of this inflection point.

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Chapter 1
An Overview of the Translational Dilemma and the Need for Translational Systems Biology of Inflammation

Yoram Vodovotz and Gary An

Introduction

The greatest challenge for the biomedical research community is the effective translation of basic mechanistic knowledge into clinically effective therapeutics, most apparent in attempts to understand and modulate "systems" processes/disorders such as sepsis, cancer, and wound healing. The United States Food and Drug Administration report: "Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products" (http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.html), clearly demonstrates the steadily increasing expenditure on Research and Development concurrent with a progressive decrease in delivery of medical products to market. This is the *Translational Dilemma* that faces biomedical research, and the current situation calls for a reassessment of the scientific process as an initial step towards identifying where and how the process can be augmented by technology [1].

This book is focused on systems approaches to the inflammatory response. It is now beyond doubt that inflammation, with its manifold manifestations at the molecular, cellular, tissue, organ, and whole-organism levels, drives outcomes following injury and infection and can lead to diverse manifestations of chronic diseases such as rheumatoid arthritis, neurodegenerative diseases, the metabolic syndrome, and cancer. Though properly regulated inflammation allows for timely recognition and effective reaction to injury or infection, acute inflammatory derangements such as those that accompany trauma/hemorrhage, sepsis, the wound healing response, and key aspects

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of host–pathogen interactions are manifestations of insufficient and/or disordered inflammation that in turn impairs physiological functions. It is critical to note that inflammation is not in and of itself detrimental. Well-regulated, self-resolving inflammation is necessary for the appropriate communication and resolution of infection and trauma and for maintenance of proper physiology and homeostasis. This paradox of a robust, evolutionarily conserved network of inflammation whose very structure may lead to disease [2–4], has resulted in its near ubiquitous involvement in those diseases that most dramatically manifest the Translational Dilemma. Indeed, most evidence suggests that either insufficient [5] or self-sustaining [6] inflammation drives the pathobiology of trauma/hemorrhage, sepsis, inadequate or exaggerated wound healing, and a breakdown of appropriate host–pathogen responses. Over a decade ago, there was recognition of the need complex interplay between inflammation and physiology in critical illness and of the need to apply complex systems approaches such as computational modeling to unravel this complexity [7, 8]. The advent of "omics" methodologies, with the theoretical capability of interrogating the complete responses of cells and tissues, spurred the application of these methodologies to critical illness following injury or infection [9–16], wound healing [17, 18], and host–pathogen interactions [19–21].

It is now increasingly recognized that merely suppressing inflammation is an ineffective therapeutic strategy for most diseases, and that controlling and reprogramming inflammation in order to reap the benefits of this evolutionarily conserved process is the future therapeutic paradigm. However, there is currently no rational, reductionism-based approach by which to accomplish this goal. In addition to the multiscale complexity inherent in its organizational structure, inflammation manifests very differently based on personalized factors. These factors include individual features of the initial inflammatory perturbation, the individual's demographic and disease histories (including genetic predispositions and setpoints/thresholds for inflammatory processes), and the impact of environment and clinical care. We assert that mathematical modeling and computational biology can help decipher this multidimensional puzzle, and that, when geared towards practical applications, these methods hold the potential to transform the entire process of healthcare delivery from preclinical studies, through clinical trial design and implementation, to personalized diagnosis and therapy, and ultimately to long-term care.

Progress in Translational Systems Biology of Inflammation

We and others have suggested a rational, systems engineering-oriented, computationally based framework, Translational Systems Biology, for integrating data derived from basic biology experiments as well as preclinical studies and clinical studies, and ultimately leading to rational inflammation reprogramming [22–25]. Translational Systems Biology involves using dynamic mathematical modeling based on mechanistic information generated in early-stage and preclinical research to simulate higher level behaviors at the organ and organism level, thus facilitating

the translation of experimental data to the level of clinically relevant phenomena. This book introduces and demonstrates the Translational Systems Biology approach.

This book brings together leaders from the interdisciplinary field of inflammation modeling as well as thought leaders in the fields of trauma/hemorrhage, sepsis, wound healing, and host–pathogen responses. This book is divided into five sections, covering recent progress in Translational Systems Biology as applied to disease states involving acute inflammation. In Part I (*Complex Systems Methods and Applications*), the relevant methods for computational modeling of inflammatory diseases are discussed. In Part II (*Translational Modeling of Sepsis and Trauma*), the relevant clinical and experimental features and challenges of systemic inflammation in trauma/hemorrhage and sepsis are detailed, along with recent progress in computational modeling of these diseases. Similarly, in Part III (*Translational Modeling of Wound Healing*), the relevant clinical and experimental features and challenges of wound healing are discussed, along with computational modeling studies in this field. Part IV (*Translational Modeling of Host–Pathogen Interactions*) discusses challenges and systems/computational biology approaches to host–pathogen interactions and systemic disease, including examples from gut-derived sepsis, necrotizing enterocolitis, and malaria. Finally, in Part V (*Future Perspectives: Translation to Implementation*), we discuss the challenges that remain in order to fully implement the vision of Translational Systems Biology of Inflammation.

Indeed, as summarized in this book, *in silico* modeling has yielded both basic insights and translational applications in critical illness [2, 3, 23, 25–30]. Indeed, key translational applications, such as *in silico* clinical trials, were pioneered in the arena of critical illness [31, 32]. Recent studies show the potential to predict the individual inflammatory and pathophysiologic outcomes of human subjects [33] and large, outbred animals [34] subjected to acute inflammatory stress. Such studies highlight the maturity of computational modeling in the clinical arena and suggest the possibility of predicting the outcomes of—and possibly tailoring therapy for—individual critically ill patients [25, 26, 35].

Early studies utilizing complex systems approaches in critical illness suggested the concept of "coupled oscillators" that become uncoupled as inflammation becomes dysregulated and organ dysfunction progresses [7], which has led to the explosion of studies on the use of physiological waveforms (e.g., those derived from heart rate or breathing pattern) to diagnose outcomes in sepsis and trauma/hemorrhage. More recent in silico modeling work has posed specific hypotheses with regard to the mechanisms by which inflammation is coupled nonlinearly to physiological (dys)function at multiple scales [2, 23, 27–32, 34, 36–46].

Challenges and Future Perspectives

As we detail in the final section of this book, many challenges remain for the field of Translational Systems Biology. As useful as mechanistic computational modeling has been in integrating known interactions gleaned from the literature,

this approach is inherently biased, given the tremendous volume of information that could, in theory, be incorporated into models and that is deemed irrelevant or unnecessary for the degree of abstraction chosen by the modeler. In recent years, there has been an attempt to couple the less-biased data-driven approach with mechanistic mathematical modeling of the acute inflammatory response [3, 24, 25, 27–30, 47]. In these studies, mechanistic computational simulations were created based on biology abstracted from "omics" data [45, 46, 48–51] or inferred from data-driven analysis of principal drivers [34]. This type of combined data-driven and mechanistic modeling reflects the maturity of computational modeling in acute illness and is likely to be the area of study with most growth in coming years due to the inherent appeal of unifying—and gaining testable mechanistic insights from—the growing repository of "omics" data.

At the most practical level, in silico modelers must also prove the translational benefit of this technology through prospective clinical studies and ultimately through the development of computationally based diagnostics or therapeutics for critical illness. The central challenge in this field is to integrate the multiscale, multisystem nature of acute inflammation. Translational Systems Biology must therefore rise to the challenge of integrating inflammatory, neuroendocrine, and physiologic processes in order to unravel the multidimensional, multicompartment, and highly dynamic landscape of trauma/hemorrhage, sepsis, wound healing, and host–pathogen interactions.

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Part I
Complex Systems Methods and Applications

Chapter 2
Translational Equation-Based Modeling

Gilles Clermont

Biological systems are complex and evolving. Because of the intense network of interaction present, intuition often fails to predict the system-level impact of altering one of a few components of this system. At a preclinical level, gene knock-out mice often result in phenotypes that are more complex than a mouse with the inability to express a given gene. Rather, knock outs are more typically highly adapted survivors of this gene deletion, where other mechanisms have compensated for what is otherwise an important biological function. Yet, biological knowledge of isolated mechanisms, such as ligand–receptor dynamics, transcription-factor binding, second-messenger cascades, and myriad other cell, tissue, and organ-level interactions, has expanded immensely in the last few decades. Basic knowledge of causal links has improved, but tools to interpret and predict the integrated effect of the combined dynamics are limited in number.

A pragmatic approach, therefore, is to create a simplified representation of the system, a model, and to define rules that describe the presence, nature, and intensity of interactions present between components of this model. We focus on the use of differential equations as the mathematical implementation of this set of rules and describe the time evolution of the components included in the model. At a given point in time, the state of the system is described by the actual values of all components included in the model, a vector of real numbers. Solving the equations yields a description of the time evolution of the system. In other words, the solution of this system of equation describes the trajectory of the system as its state evolves in time. Once a computational model has been developed, it will be used to generate predictions and evaluate the potential effect of perturbing the system.

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Equation-Based Models of Biological Systems

Historical Perspective

Equation models of biological systems have been used for several decades, initially applied in ecology, infectious disease, and organ physiology. More recently, considerable efforts have been devoted to computational models of the immune response. Yet, the discipline of computational systems biology and the extensive use of models as a discovery tool in modern biology is less than two decades old [1]. Although computational models have been used for several decades in pharmacokinetics, their use as tools to enhance drug discovery and clinical trial simulation is less than a decade old [2, 3]. Therefore, computational models is just emerging as a potential tool in translational science [4–6], and in particular in personalizing treatments [7, 8]. The vast majority of these models have been implemented using differential equations as mathematical formalism, partly for historical and familiarity reasons, and also because methods to enhance the scalability and computational efficiency of alternative modeling methods are still very much in the process of being developed.

Types of Equation-Based Models

The simplest mathematical expression of is in the form of a difference equation $X(t+1) = f(X(t))$, where $X(t+1) = \{x_1(t+1), x_2(t+1), \ldots, x_n(t+1)\}$ and $X(t) = \{x_1(t), x_2(t), \ldots, x_n(t)\}$ are vectors representing the state of the system, that is counts of each of n components $\{x_1, x_2, \ldots, x_n\}$ at consecutive times t and $t+1$, and the function f embodies the underlying biology of interactions dictating the evolution of the system. Such models describe the evolution of the system in discrete time steps and are common in mathematical biology and chemical reactions. For example, in such systems, the state of the system is simply a series of numbers representing the number of individuals of different species in an ecosystem, or the number of molecules of each single chemical species in a network of chemical reactions. An important ecological problem involves modeling the population size of a given species, for example dividing cells or bacteria. The change in population size during the interval between these times is given by the following growth equation, also known as the logistic map $x(t+1) = rx(t)(1-x(t))$, where $x(0)$ represents the initial population at time 0, r is a positive number corresponding to an overall growth rate, and the last negative term represents increased competition as the population grows. Similar models have been used to describe the dynamics of an epidemic, where, for a standard Susceptible-Infected-Recovered (SIR) model, the system state is the number of individuals in the susceptible, infected, and recovered pools at a given point in time. All large-scale simulation of epidemic disease are sophisticated

implementations of this construct [9], with geospatial information and constraints also included in more realistic, large-scale models. Discrete models are typically computationally intensive to simulate, but they have a clear advantage in the limit of small numbers, when spatial information is so detailed that alternative forms of spatial models, such as partial differential equations, are impractical.

Ordinary differential equations (ODE), the most popular mathematical implementation of biological models, express the rate of change of each component of a model $\mathrm{d}X/\mathrm{d}t = f(X(t), \Delta, t)$ as a function of other components in the model, a vector of parameter Δ and time t. Also, implicit in this formulation is that the state of the system at time t is typically dependent on the initial state of the model $X(t=0)$. So, the simplified mathematical representation of the biological system includes the components of the model one chooses to include, their initial values of each component, and the form and strength of the interaction one chooses to represent.

Yet, if the system evolves for a long enough time, it may evolve toward a basin of attraction, which may be a single state (fixed point), an orbit of states (limit cycle or limit tori), a set of states within a defined region of state space with no particular structure (strange attractor), a feature of chaotic systems. A feature of ODE is that the evolution of states is deterministic. In other words, for given initial conditions, the trajectory will always be the same. Although this feature can be perceived as a weakness of this formalism and inconsistent with real-life behavior of biological systems where randomness and uncertainty often plays a key role in the dynamics, techniques have been developed which incorporate elements of randomness in the formalism of ODEs. Therefore, ODEs are currently the main platform used by most for modeling the acute inflammatory response in a variety of contexts, cellular networks, and models linking inflammation to macroscopic observables such as blood pressure or heart rate variability [10–14].

Equation-based models can also describe processes where physical compartments or the spatial characteristics of the biological system are important. Examples of such system include wound progression and healing [15], propagation of infection, inflammation, or gas exchange [16]. Generally however, biological processes are compartment specific, and communication between compartments is often limited and regulated. Preserving compartmentalization is often, but not always, required for a reasonable computational simulation of the underlying biology. ODEs are particularly well suited to compartmental modeling, while partial differential equations, which explicitly include continuous spatial evolution, are more appropriate for certain processes such as wound healing, tumor growth, and other systems where there is not a clear concept of compartment. More recently, alternative modeling formulations, such as rule-based or agent-based simulations, have grown in popularity and are more appropriate for the simulation of systems where it cannot be assumed that individuals of a species (e.g., molecules of a chemical species, people, etc.) are distributed homogeneously within a compartment, and where there is a sufficient number of individuals to express interactions as reaction rates [17].

Advantages of Equation-Based Models

Differential equations, as a modeling approach, have enormous appeal. They (1) provide an intuitive implementation of causal mechanisms into a mathematical framework, (2) can be analyzed using a large body of existing techniques, (3) can be numerically simulated easily and inexpensively on a variety of computing platforms including portable devices, (4) provide *both* qualitative and quantitative predictions, and (5) allow for the systematic incorporation of higher levels of complexity and uncertainty. Furthermore, this modeling framework integrates existing knowledge embodied within the structure of the model, yet admits the flexibility of being data driven and stochastic. Therefore, knowledge gaps are readily identified, unlike alternative modeling approaches. Further, the speed of existing numerical solvers for differential equation-based models allows for massive experimentation with parameters that may not be determined experimentally, leading to the development of hypotheses on the roles of individual parameters, reflecting the presence and relative importance of biological processes or interactions.

Disadvantages of Equation-Based Models

An equation-based representation of a moderately complex biological system usually depends on a large number of parameters that quantify biological interactions, and identifying these parameters can be a challenging and often impossible task. Although some of these parameters might have been determined experimentally a priori, this is rarely the case and several parameters are therefore poorly understood and constrained. Methods of inferring parameters must integrate the fact that limited data will be reflected in limited knowledge of model parameters. This is however a general issue with models, irrespective of their mathematical implementation.

As mentioned above, equation-based representations violate basic assumptions of their underlying mathematical theory when the number of instances of model components is small, of when components cannot be assumed to be well mixed within explicitly modeled compartments [18]. For partial differential equations, this must also hold approximately true within the limits of the spatial resolution of the model because of numerical solving schemes, and boundary effects and phenomena must be considered with care. When these assumptions are significantly violated, alternative modeling frameworks should be used.

Models Big and Small

Small models are highly simplified representations of a system and are comprised of a very limited number of components [19, 20]. These components will typically

not represent measurable biological entities but rather lumped biological actions or principles. Several small models of the inflammatory response aggregate a complex network of cytokines and immune active cells under the general categories of proinflammatory and anti-inflammatory mediators (e.g., [20, 21]). Because the mathematical theory of differential equations is mature, small models allow formal mathematical analysis and the derivation of broad conclusions such as long-term stability and exhaustive enumeration of all possible types of evolution of such models. Empirical evidence will often restrict this range of possible behaviors, which in turn may limit the range of possible interaction between components (model parameter values), or impose restrictions on how interactions are coupled. Small models are not meant to be calibrated to experimental data, but to ensure that, from the outset, the proposed model structure is biologically supported.

If more insight is sought into biological details and in particular, if experimental data is explicitly available, then a larger model must be synthesized such that specific quantitatively verifiable predictions are formulated. The larger model must preserve empirically observed or plausible time evolutions. It is generally useful to initially construct the simplest model representation of a system such that expected behavior is indeed possible in this simplified representation. Models, much like pieces of a puzzle, are enhanced by the inclusions of modules that offer a more biologically realistic description of aspects of the biological system the modeler wishes to focus on. A cellular pathway for which empirical data are available or which is the potential target of a drug intervention would constitute a good candidate for more detailed representation. As general rule, a model should be as detailed as the data it wishes to describe or explain, but no more. Simple models can provide insight as to broad therapeutic strategies such as structured interruption of antiretroviral therapy in HIV patients [22]. Yet, it is imperative that realistic models are used to offer quantitative predictions which can be validated experimentally, or offer specific insight on potential therapeutic targets, or on specific timing and dosage of a potential therapeutic agent.

Validating Equation-Based Models

When applied to statistical models, the concept of model validation is intuitive and well characterized: are the predictions of a statistical model developed from experimental data verified in a different set of observations gathered when the experiment is repeated? For example, the experiment could be an observational cohort of patients exposed to a disease or treatment. The model provides predictions on to a cohort of patients different from the cohort used to develop the model. A valid model will yield predictions within statistical error of observed predictions. The validity of the prediction is typically judged on the entire cohort, on not on any single individual.

On the other hand, validation of a dynamical system is uncharted territory. The burden of proof is unclear, since the claims as to what computational models are attempting to accomplish are admittedly more extensive than for statistical

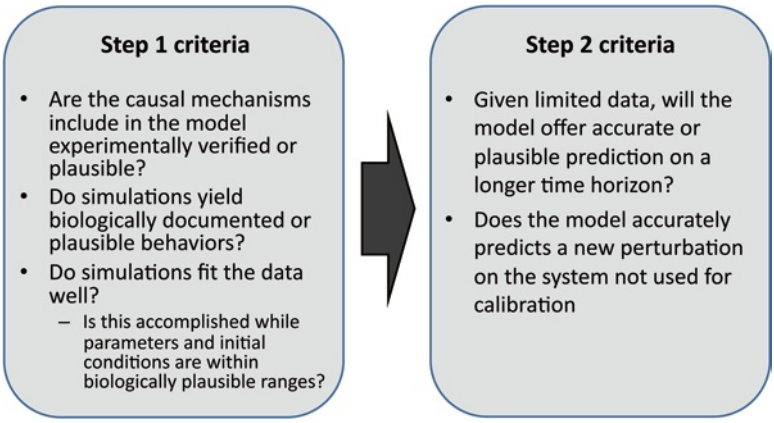

Fig. 2.1 Roadmap for the validation of equation-based models of biological systems

models [23]. However, a roadmap could be constructed and applied to equation based, and other simulation platforms for complex systems (Fig. 2.1). A first set of criteria could be constructed as follows. First, because computational models are knowledge rich and meant to relate components of a system causally, it should include biologically verified or plausible interactions. Second, simulation of the computational model should result in biologically plausible behaviors under a wide range of initial conditions and perturbations representing realistic experiments. Third, when a model generates time courses for experimentally accessible data, deviations between model predictions and observed data should be statistically insignificant. And fourth, parameter ranges should fall within biologically verified or plausible values. A second set of criteria which are more directly related to model predictive ability and external validity are then considered. First, given limited data (e.g., the first few hours), can the model offer accurate predict the future evolution of the system, at least within a biologically or clinically relevant time horizon. A similar criterion exists for statistically based longitudinal models and is key to validating weather forecasting models [24]. Second, and a unique expectation from mechanistic models, is the ability of the models to predict the results of an experiment not used for estimating parameters. For example, what is the expected impact of exposing the system to a drug at a specific concentration and time duration on model components or ultimate behavior of the system? Will drug X decrease mortality? Such predictions are not easily formulated with standard statistical models and represent a unique challenge to mechanistic models. At the very least, attempts at validation should include an effort at externally validating the model in a different experimental system. It is apparent that model validation may be an iterative exercise, where failure of external validation leads to a reformulation of the basic mechanisms included in the model, while preserving desirable behavior and predictions. Further expansion of this roadmap would be a useful addition to the current modeling literature.

Using Equation-Based Models as a Prediction Tool

Parameter Estimation and the Inverse Problem

In its simplest expression, the inverse problem consists of reconstructing model parameters given observed data. In statistical linear regression model, there is only one optimal solution to the inverse problem, which is the set of parameters minimizing the sum of square residuals. In such situations, the inverse problem is said to be well posed. Equations-based models, especially larger, more complex models include a number of parameters, which are poorly known. Formal identifiability analysis will confirm that many such models are not structurally identifiable [25]. In other words, even if provided with perfect and infinitely rich data, not all parameters of the models can be identified. It behooves the modeler to develop a priori sound models where such issues are minimized. A second obstacle lies in the sparsity of data available to estimate model parameters and in the experimental uncertainty associated with experimental measurements. This type of problem contributes to the practical identifiability of the model. A third obstacle is that nonlinear systems may admit a large number of good solutions, and thus potentially a large number of parametrizations are compatible with observed data. The lack of a unique solution to the inverse problem is referred to as ill-posedness [26]. Therefore, in complex models, it is rarely realistic to identify a set of parameters that describes a system uniquely, while preserving the ability of such a parametrized model to offer robust predictions. Indeed, although this single parameter set may create a good fit to the data at hand (e.g., maximum likelihood in the immediate vicinity of this parameter set) or display all the require biological behaviors, it will in all likelihood not hold to extended validation (see above).

Approaches to Solving the Ill-Posed Inverse Problem

In an equation-based framework, a satisfactory solution to an ill-posed inverse problem is tantamount to identify a set of model structures (the equations themselves), and for each structure, as set of parameters and initial conditions that will provide a good enough explanation of the data available on the biological system being studied. In the simpler case when only one model structure is under consideration, that is, the set of mechanisms represented by the equations is believed to be well understood, solving the inverse problem is limited to the identification of parameters and initial conditions that will produce a good solution, while maintaining biological fidelity of simulations under a variety of input to the model, as described above in the discussion on model validation. A good solution can be defined using a variety of metrics, or cost functions, expressing how close predicted trajectories are from observed data. A simple cost function would be, for example, the sum of squared residuals.

Ideally, all system variables are observed at a level of time granularity sufficient to provide a good description of the longitudinal dynamics, up to restoration of homeostasis or stabilization to a different state. In the absence of fine-grained data obtained over a wide range of experimental conditions, the next step is to complement the dataset with a set of heuristic rules that will define a priori the plausible ranges of observations and limiting behavior. Heuristic rules are based on prior knowledge and expert opinion of system behavior. These rules are of particular importance to system variables for which data are sparse or missing. For example, there almost always exists literature or other empirical evidence where some variables of the system were measured under a different set of circumstances, or in a somewhat different biological system (e.g., endotoxemia in human vs. sepsis in humans). Yet, data from human endotoxemia may still be of use in a computational model of human sepsis in constraining the potential range of unmeasured variables in this model. More generally, such data can be used "qualitatively" to create rules that restrict the range of plausible behaviors of these variables. These rules are used in the calibration of computational models as (1) models and parameter combinations for which the system violating a rule are excluded a priori or (2) a number expressing the severity of the deviation from a rule is added to the cost function (the function that quantifies numerically the difference between experimental data/heuristics and predicted behavior), decreasing the likelihood of this model which is minimized by the optimization process.

In the discussion that follows, we use the notion of a generalized parameter $\mathbf{p}$ vector of a model as encompassing both standard parameters Δ and initial conditions $X(0)$, $\mathbf{p} = \{\Delta, X(0)\}$. It is apparent from the discussion above that standard algorithms searching for local minima are not well suited for addressing ill-posed inverse problems. The ill-posedness may open the possibility to a large, possibly infinite, number of local minima. A practical approach is to initiate the calibration process from a large number of initial points in parameter space, selected in such a way as to offer reasonable coverage of parameter space such as through Latin hypersquare sampling. This approach is referred to as multistart optimization and yields a set of local minima, many of which may fit data relatively well and display suitable heuristic behaviors [27]. Yet, many regions of the cost function landscape may be very flat and consequently such algorithms may not converge to realistic solutions. This is fact to be the case for larger models when data constrains the plausible parameter distributions to a submanifold of lower dimension than the number of parameters including the model, demonstrating the practical nonidentifiability of the model. It does not necessarily follow that such models are useless and they may in fact offer good, albeit probabilistic, predictions if one considers the entire set of parameters. Indeed, each parameter set leads to a deterministic prediction, but the ensemble of parameter vectors produces a variety of plausible predictions. Although some problems, such as the tertiary structure of a macromolecule, plausibly admit a single global minimum of the cost function, which in this case is the conformational energy for a given local microenvironment, there is neither assurance nor intuition that this is generally true of out of equilibrium complex dynamical systems constantly adapting to changing environments and under varying energetic constraints.

It would seem unwise therefore to think of calibration of a complex model as the identification of an optimal parameter set, but rather of an ensemble of parameters.

There is a growing tendency to conduct stochastic searches of parameter space as a solution to ill-posed problems [28–30], typically following a Bayesian estimation procedure. In such a scheme, the calibration process also involves the construction of an ensemble $E(\mathbf{p})$ of a large number of good solutions, each characterized by a generalized parameter vectors $\mathbf{p} = \{\Delta, X(0)\}$ as described above. For a given dataset $Y = \{y_i(t_j)\}$ or i variables collected at j time points and model $\mathrm{d}X/\mathrm{d}t = \mathbf{f}(X, \mathbf{p}, t)$, the distribution $\rho(\mathbf{p})$ will be computed using $w(\mathbf{p}) = \prod_{ij} P_{ij}(x_i(t_j; \mathbf{p}))$, where it is understood that $w(\mathbf{p}) \equiv w(\mathbf{p} \mid Y)$. If prior knowledge on parameters $P(\mathbf{p})$ is available, a Bayesian scheme is adopted where the posterior distribution $w(\mathbf{p}) = \dfrac{\prod_{ij} P_{ij}(x_i(t_j; \mathbf{p}))P(\mathbf{p})}{P(Y)}$ is known within a normalization fraction, $P(Y)$. Yet, this normalization factor is generally not required for practical computations as one is typically interested in ratios of probabilities in the process of selecting suitable parameter sets. The probability functions P_{ij} are determined by the data accuracy. If the data point $y_i(t_j)$ has Gaussian uncertainty σ_{ij}, then $P_{ij}(x_i(t_j; \mathbf{p})) = Q\exp(-(x_i(t_j; \mathbf{p}) - y_i(t_j))^2 / 2\sigma_{ij}^2)$. By normalizing the weight function, we obtain a probability density $\rho(\mathbf{p}) = w(\mathbf{p}) / \int w(\mathbf{p})\mathrm{d}\mathbf{p}$ over the space of parameters. Efficient sampling of parameter space, although alleviated by dimensional reduction methods, remains computationally challenging and will be approached using stochastic sampling methods such as parallel tempering [30, 31]. A posteriori analysis of the distribution is conducted to determine the width of the distribution, modality (number of local maxima), separation of local maxima, approximate dimension, etc. Simulation of the ensemble model produces not only a single trajectory but also an ensemble of trajectories parameterized by $\mathbf{p}$ with weights proportional to $\rho(\mathbf{p})$. These trajectories will provide probabilistic prediction of the outcome of the model as a time-dependent distribution of values of system variables $\phi(\mathbf{x}, t) = \int \delta(\mathbf{x} - \mathbf{x}(t; \mathbf{p}))\rho(\mathbf{p})\mathrm{d}\mathbf{p}$. This distribution can be used to compute the average trajectory $\mathbf{x}(t) = \int \mathbf{x}\phi(\mathbf{x}, t)\mathrm{d}\mathbf{x}$, the time-dependent variance of average trajectory $\sigma^2(t) = \int (\mathbf{x} - \mathbf{x}(t))^2 \phi(\mathbf{x}, t)\mathrm{d}\mathbf{x}$, the probability $P(x_j(t) < x_j^0)$ that a value of a given variable drops below a prescribed threshold at time t, and various other quantities of interest.

Hybrid Models

A growing number of computational models simulate phenomena observed at different scales, for example, intracellular pathways and cellular phenotypes, intrahost models of viral infection and population epidemic models. Scales are typically physical and be best approaches using different simulation platforms. Hybrid models consist of computational models comprising two or more simulation platforms.

Such models may be of particular interest in translational applications, where the largest scale of the simulation is at the individual or population levels, and the lowest scale determined by the type on perturbation we wish to impose on the system. It could be, for example, an antiviral medication with a pharmacodynamics profile dependent on the age or preimmune status on an individual. Simulations using coarse distributional assumptions "average" behavior of individuals may lead to result very significantly different from more-detailed simulations of drug action within an individual. Hybrid models raise considerable computational challenges. Expanding on the example above, representing each individual in a population using an equation-based model of intrahost infection (e.g., [32]) is not feasible within the framework of a large population scale agent-based simulation. Creative solutions exists however to mitigate the additional computational cost associated by multiscale hybrid simulations [33]. Use can be made of large differences in temporal dynamics that exist at different scales of the simulation. A method our group has implemented in the context of epidemic simulation is to create, from intrahost models of Influenza A infection, algebraic response surfaces using preexisting simulations of the equation-based model to generate an algebraic input–output map [34]. Consequently, given a patient's age, preexisting immune status and initial viral load, a daily profile of infectivity and symptomaticity is generated by applying a regression equation rather than embedded simulation of an equation-based model. The incremental computational cost was negligible and such an approach is imminently scalable.

A class of applications of particular interest in inflammatory diseases relate organ function to specific anatomic intricacies as the playground for molecular or cellular inflammatory effectors [16, 35–38]. Such models may not be explicitly hybrid in nature but typically present very similar challenges in that efficient computation require creative solution to bridging anatomical scales.

Translational Applications

The Interdisciplinary Perspective

Computational models are practical instantiations of the state of current knowledge and a representation tool for competing hypothesis of processes driving biological systems and therefore constitute a framework for hypothesis generation and efficient experimental design for testing these [1, 7, 39]. The truly impactful concept is that this approach allows a model-centered discussion between clinicians, biologists, and modelers [7]. Hypotheses and thought experiments can be pushed computationally to their logical outcome as well as regions that may offer unexpected clinical benefit. This is extremely difficult to achieve from discussions only, without an existing quantitative model of disease. We believe that computational models will become a powerful and standard tool that promotes effective interdisciplinary research and scientific epistemology.

Enhancing Current Trial Design

The ultimate purpose of basic mechanistic research is to improve human health. The mantra of pharmacological intervention remains that the right drug at the right dose must be administered to the right patient at the right time. In addition to basic lack of efficacy of biologics tried for sepsis, several have raised the issue that the design of clinical trials itself was to blame [40], namely that patients were enrolled too late given the biological rationale of the intervention, that phenotype-based enrolment may not be ideal, or that dosing might have been inappropriate. For example, patients presenting with community-acquired sepsis already have elevated circulatory biomarker levels upon enrolment [41, 42]. These circulatory biomarkers generally peak between 3 and 36 h postadmission and diminish over the subsequent 72 h [41]. The vast majority of immunomodulatory trials have enrolled patients well after key biomarkers have peaked [43].

The US food and Drug administration has published a report on the use of adaptive clinical trial design to maximize information extracted for clinical trials and minimize sample size required to detect real treatment benefit [44]. Recent methodological developments in adaptive clinical trials design, such as sample size reestimation as proposed in the recent failed confirmatory trial of drotrecogin alfa [45], are for the most part only tangentially applicable to sepsis trials [44, 46–49]. However, computational models of human sepsis could contribute to an adaptive design in two distinct ways. Every large trial has prespecified interim analyses. If at this point in trial execution, a computational model calibrated from data accrued up to the interim analysis was more sensitive than standard data analysis to identify treatment effect as we suspect if would be, of lack of a difference in biological activity as suggested by extensive overlap in extensive overlap of model ensembles, one could envision (1) consolidating trial arms that show no difference in biological activity and thus improve power to detect differences between residual arms of the trial or (2) declare futility with greater confidence. A second potential application of computational models to augment adaptive design resides in their ability to identify cohorts of patients with a better probability to respond to the proposed intervention. Adaptive patient enrichment design allows the modification of enrolment criteria as the enrolment accrues as it becomes clear that some types of patients clearly do not benefit from the intervention [50]. Both types of contribution are potentially promising for future trials of immunomodulatory intervention in acute and chronic inflammatory diseases.

In Silico Clinical Trials

Clinical trials of immunomodulation in acute inflammatory disorders have a generally dismal track record. This is particularly true for sepsis, a clinical syndrome arising

from the systemic host response to infection with clinical manifestations that span a broad set of inflammation-related signs and symptoms [51]. Although the host's response to sepsis strives to contain infection and promote repair, the intensity of the inflammatory response often leads to compromised tissue function, uncontrolled inflammation and/or profound immune suppression, organ failure, and death [52, 53]. Severe sepsis accounts for between 2 and 11 % of all admissions to hospitals or ICUs, approximately 750,000 cases a year, with an associated mortality of 35 %, most often from progressive organ failure in an ICU [54]. An intense effort by the critical care community to raise sepsis awareness and provide evidence-based recommendations is ongoing. The Surviving Sepsis Campaign (http://www.surviving-sepsis.org) published guidelines in 2004, 2008, and 2013 [55–57]. These recent guidelines support the implementation of "care bundles," sets of care decisions that constitute generally accepted competent critical care, but all recommendations regarding immunomodulation have been withdrawn. Care environments, which have implemented sepsis bundles, have seen a modest improvement in outcome paralleling that of general ICU care [54, 58–62]; yet, recent spectacular failures of confirmatory trials of immunosuppressive agents [63, 64] have further contributed to the profound consternation, skepticism, and soul searching permeating the critical care community regarding breakthrough treatments for sepsis [65, 66]. Presumably, major reasons for this dismal record are the failure to integrate the complexity of sepsis pathophysiology towards mechanistically sound therapeutic rationales and the failure to translate knowledge acquired from in vitro and preclinical experiments to clinically and genetically diverse human beings [67]. Computational simulations of immunomodulatory agents in sepsis will hopefully contribute to bridge the gap between a reasonably well-known pathophysiology, generally favorable preclinical data, and clinical trial results. In addition, such simulation may contribute to proper patient selection, dosage selection, and duration of intervention. A computational simulation of an acute inflammatory disease, such as sepsis, would provide recommendations on the basis of point-of-care measurements of biomarkers, a platform which is currently not commercially available, presumably because there is currently no market for such methods.

There have been few prior attempts at computational simulations of clinical trials [2, 68, 69]. A simulation of an anti-TNF intervention pointed out such of the potential advantages underlines above. A potential for harm was identified in patients who were predominantly immunosuppressed at the time of initiation of therapy, in patients offered high doses for longer than 48 h, and in those with particularly aggressive bacterial infection [2]. These predictions are teleologically plausible, and the computational model identified, early in the course of treatment, responders from nonresponders with high probability. Although such conclusions are speculative until verified in the clinic, animal data supported many of the predictions of this computational model [70]. Opportunities for computational models to gain legitimacy in the clinical realm are ongoing, extend well beyond acute inflammatory disease to cancer and cardiovascular interventions, and are encouraged by registration entities such as the Food and Drug Administration in the USA [44].

Parameter Ensembles vs. Data: Different Worldviews

One typically expresses differences in terms of statistically significant levels of biomarkers, incidence of organ failure, or mortality. The computational model-based alternative is to express differences in terms of differences in the distributions of model parameters that characterize important phenotypes such as survival or death. For example, in a recent publication, statistical tools were compared the parameter ensembles of a computational models fit to animals that survived a septic challenge to the distribution obtained from a model fit to data of animals that died [31]. Analyzing parameter distributions rather than data provides insight on mechanisms explaining the difference in outcome, not merely a description of what is different. Although both the "data world" and the "parameter world" can be used to produce predictive models, only a parameter-based description can offer insight on mechanistically motivated therapeutic strategies that may alter outcome.

Novel Approaches to Personalized Therapies for the Critically Ill

Personalized, or precision medicine is often confused with genomic medicine and an inference is often made that gene-level understanding of biological processes is sine qua non to the development of personalized therapies [7]. Standard approaches have had some, yet limited success in linking gene expression profiles to local and circulatory protein levels, to clinical disease severity and outcome [70–72]. Care givers, and acute care physicians have been offering effective, individually titrated care to their patients based on a different, yet complementary premise of recognizing patterns of organ function and injury, framed in a conceptual framework of mechanistic pathophysiology, and establishing individualized therapeutic targets to mitigate these mechanisms. Computational approaches offer the possibility of reconciling these concepts of translating differences in data, which does not easily lead to hypothesis generation, into differences in mechanisms, which typically have a more direct interpretation and may suggest individualized, time-dependent, therapeutic approaches.

Two features of equation-based computational models may contribute to the development of personalized approaches to treatment. Computational models, and equation-based models in particular, map data to mechanism and thus offer a way to identify mechanistically based phenotypes. As more data becomes available and model parameters reestimated, mechanistically based phenotype becomes better defined. Interventions in acute care, pharmacologic or physical organ support, interact directly with mechanisms. Indeed, practitioners constantly select therapies based on perceived mechanisms. Therefore, a mechanistically based decision support system suggesting which mechanisms are particularly important in a given individual at a given time might enhance data interpretation, diagnostic ability, and

treatment selection [26]. The recent rise of control-based approaches in cancer pharmacotherapy of glucose control in type I diabetes offers a second application for equation-based models applied to personalized care. The underlying concept is that, if a biological process is well modeled computationally, then using tools of control engineering facilitates achieving a preset therapeutic objective, such as a specific drug concentration, or glucose zone target much as an artificial endocrine pancreas would achieve [73, 74]. Such approaches have already been ushered in clinical practice.

Conclusions

Equation-based models of translational relevance are recent. Their acceptance as useful knowledge discovery and decision support tools, although unquestioned in the basic sciences, has met with considerable criticisms in the translational arena. The immediate task of modelers and clinicians alike is to build and disseminate success stories. There are more likely to emerge from cancer research, diabetes, or immunomodulation of chronic or acute inflammatory disorders.

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Chapter 3
Agent-Based Modeling in Translational Systems Biology

Scott Christley and Gary An

The Translational Dilemma and the Need for Dynamic Knowledge Representation

As noted elsewhere in this book, the Translational Dilemma, the inability to translate the successes at obtaining basic mechanistic knowledge about biological processes into clinically effective therapeutics, is the greatest challenge facing the biomedical community [1]. The Translational Dilemma consists of two primary barriers that need to be breeched (1) the need to accelerate the scope of hypothesis testing necessary to deal with the multiplicity of possible explanations of high-resolution data (the experimental throughput problem) and (2) the ability to adequately evaluate the consequences of highly complex, multicomponent, multihierarchical integrative hypotheses (the multiscale problem). Both of these issues are directly related to this requirement: biomedical researchers must greatly increase their ability to evaluate the *plausibility* of mechanistic hypotheses and their manifestation at the systemic level. Meeting this requirement will almost certainly involve harnessing the power of advanced computational modeling and computer hardware for the dynamic knowledge representation of biological systems in such a way that hypotheses can be instantiated and evaluated in silico. The ability to execute in silico experiments offers potentially the only viable path to substantially accelerate and enhance the Scientific Cycle by providing a plausibility filter for putative hypotheses. This will substantially reduce the set of possible mechanistic explanations for a particular observation and will help direct and focus the design of traditional laboratory experiments to further refine the set of possible hypotheses. This chapter discusses the

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use of agent-based modeling (also known as individual-based modeling) for dynamic knowledge representation with an explicit translational goal in the area of acute inflammation.

Dynamic Knowledge Representation with Agent-Based Modeling

Agent-based modeling is an object-oriented, discrete-event, rule-based computational modeling method [2–6]. Agent-based models (ABMs) consist of virtual environments populated with objects (agents) that execute behaviors based on programmed rules that govern their interactions with the local environment and other agents. An ABM represents a system as populations of components ("agents") where the simulation agent level of the ABM corresponds to the primary component level of the system being studied; for instance, a cell-level ABM uses agents that primarily represent biological cells. An ABM *agent class* is defined by a specification of the properties, characteristics, and rules of an agent type that govern its identity and behavior. As an ABM is executed, it creates a population of individual computational instances (an agent) of each agent class, where each individual agent possesses the behavioral rule sets and defined properties of its agent class but once created can have diverging behavioral trajectories based on the different inputs it receives within a heterogeneous simulation environment. ABM rules are often expressed as conditional statements ("if-then" statements), making ABMs suited to expressing the hypotheses that are generated from basic science research, though it should be noted that the general conditional nature of simulation agent rules does not preclude the encapsulation of other types of mathematical or computational models (i.e., differential equation, stochastic, or network) as rule systems [7–9]. A standard conditional agent rule for a cell agent interacting with its environment might have the following format:

if Compound A (in the environment) is present, then bind to and activate Cell-Surface Receptor B (in the cell-agent)
if Cell-Surface Receptor B is activated, then increase Signal Transduction Enzyme C (in the cell-agent) by x
if Signal Transduction Enzyme C is increased beyond threshold y, then activate Transcription Factor D
if Transcription Factor D is activated, then express Gene E
and so on…

As noted above, the rule sets for agents can be of any formal type, such as a series of logical statements or a differential equation. Regardless of the specific ABM rules, ABMs allow a close mapping between the natural language expression of hypotheses present in publications (the current means by which this knowledge is communicated within the community) and the rule structure of ABM [10, 11]. As results can be readily used for dynamic knowledge representation, particularly for

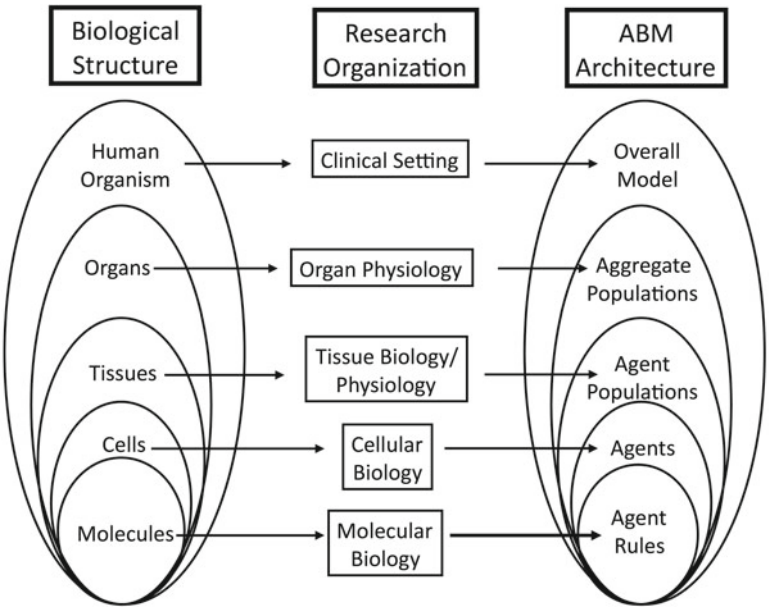

Fig. 3.1 The mapping between scales of biological organization, research community structure, and agent-based models. This diagram maps the similar structure of organizational scales present in biological systems, the research communities studying them, and the architecture of an ABM. Note that scales of organization are nested in the biological system and the ABM, reflecting the trans-scale coupling seen in both systems. Alternatively, the research community structure is disparate and compartmentalized, arising from both social and pragmatic logistical factors. Reprinted with permission from [11]

researchers not expressly trained in either computational or mathematical modeling by allowing them to more easily translate their biological knowledge into a computational form.

ABMs also intrinsically cross multiple scales of biological organization by necessarily involving at least three levels of system organization. Scale #1 is the lowest level of system process represented, and this is accomplished by the agent's behavioral rules. Scale #2 is the "middle" level corresponding to the primary component level chosen, and processes at this level are represented by the behavior of an individual agent. Scale #3 is the "system" level consisting of the global phenotype under investigation and is generated by the aggregate behavior of populations of agents. To use an example of a cell-as-agent ABM, Scale #1 then represents molecular events associated with signaling and protein synthesis, Scale #2 represents the behavior of an individual cell as it changes state, secretes something or moves, and Scale #3 represents tissue behavior arising from the interactions between populations of cellular agents. Furthermore, these levels can theoretically be nested, to provide a comprehensive depiction of a multiscale biological system (see Fig. 3.1), making ABMs well suited for creating modular models [6, 7, 12–14].

Related Modeling Methods

Given the description above, it is clear that agent-based modeling is actually a very general means of system representation and as such is viewed as quite similar to many other modeling methods. In fact, many of these types of modeling methods can be considered as subtypes of ABMs, leading to a great deal of variability in the use of the term "ABM." As such, it is useful to clarify the distinctions between certain other commonly used modeling methods and agent-based modeling as the term is used in Translational Systems Biology. One of the most closely related modeling methods is cellular automata (CA), particularly two-dimensional CAs. Cellular automata involve a discretely divided space into a series of "cells," such that the state of each particular cell is defined by a set of rules dependent upon the states of some defined neighborhood of cells. Classical examples of two-dimensional CAs are Conway's Game of Life [15] and Kaufman's N-K System [16]. These systems can be seen as ABMs where there is a single agent class (the basic unit "cell"), which does not move, and a set of agent rules that govern an agent's state transitions. Another closely related modeling method is the Cellular Potts Model (CPM), developed by Glazier and Graner, where the states of points on a lattice are determined using probabilistic rules, and membership in a particular group of points is used to define superstructures representing cells or aspects of tissue [17]. Each of these methods has its own benefits and uses, most often governed by a combination of the resulting model's use and the data available to construct the model. For instance, while "movement" can be simulated using a CA, it is often less intuitive for a biologist to think of a cell's movement as a progression of cellular variables across a grid as opposed to a specific computational object that changes its position. As another example, while a CPM can allow cells to change their size and shape (where a "cell" is defined by a group of lattice points), the means by which a lattice point's membership in a particular cell, often expressed as a Hamiltonian representing an effective energy function, does not readily map to a biologist's knowledge set (as evidenced by the relative incomprehensibility of the prior terms!). At one level (i.e., in terms of the actual execution of the binary code), the distinction between these methods and agent-based modeling may be a distinction without a difference; however, in terms of facilitating knowledge representation, the component-centric emphasis of agent-based modeling is more consistent with how most biological systems are conceptualized (i.e., "things doing things").

Agent-Based Models Versus Multiagent Systems

In addition to closely related modeling methods, there is also ambiguity in the use of the term "agent." The distinction between an "agent-based model" and a "multiagent system" is just such a situation. Both terms are widely used in the computer science and the modeling and simulation community and are often used to mean the

same thing: a computer program that utilizes multiple computational agents. However, in terms of the types of systems they usually describe these two methods actually represent very different types of computational tools. Therefore, for purposes of comparison, we define a distinct difference between these two entities (noting that the following distinction is not intended to be a definitive description of the distinction but rather is intended to clarify the differential usage of the term "agent" in the context of Translational Systems Biology).

We consider "agent-based modeling" as a simulation method, where the model constructed is intended to mimic or represent some other reference system, which is the subject of investigation. The computational agents making up the ABM are intended to represent specific types of components in the real world where selected characteristics of the real-world object are reflected in the nature of the rules incorporated into the simulation agent. Since a main benefit of agent-based modeling is the ability to represent populations of real-world objects at the individual level with simulation agents, in many circumstances ABMs consist of a large number of individual instances of simulation agents derived from a single agent class.

Alternatively, an "agent-directed" or "multiagent system" is generally used to describe a computer system design solution, where computational agents perform tasks related to the implementation of a particular computing goal. These computational agents generally have some decision-making capacity, which may be augmented using artificial intelligence approaches, that allows them to manage the information flow within a particular software implementation. In multiagent computer systems, the computational agents generally do not have a specific real-world reference object for a computational agent, rather there is a set of recognized tasks in information flow management that can be expressed as a set of algorithms and packaged for execution by a computational agent.

Properties of Agent-Based Models

As noted above, ABMs are related to other spatially discrete modeling methods, most notably cellular automata, though the mobile capability ABM agents and ability to represent a wider range of model topologies could lead to consideration of cellular automata as a special type of ABM. However, in practice, many ABMs have several characteristics of agent-based modeling that set it apart from other object-oriented, rule-based modeling systems (such as Petri nets, Boolean, or Bayesian Networks), even though at its purest definition, they could all be potentially viewed as ABMs.

Representation of Spatial Relationships

Agent-based models (ABMs) readily incorporate *spatial relationships*, be they manifest in an actual spatial topology or a topological interaction neighborhood linking

individual agents. In an ABM agent, behavior is driven by interactions determined by agent neighborhoods defining the communication and interaction network for each agent. An agent neighborhood can be represented as a two-dimensional square grid (very common), a three-dimensional cubic space [7, 12], two- or three-dimensional hexagonical space [18, 19] or as a network topology, as a neighborhood does not necessarily mean physical proximity but rather the configuration of some set of other agents with whom an agent can interact. This definition of an agent neighborhood is consistent with the bounded nature of the sense-and-respond and message passing capabilities of biological objects. This may also be used to represent physical interactions and forces between agents that affect their subsequent behavior.

Representation of Parallelism and Concurrency

ABMs simulate *parallelism*. In general, each ABM agent class has multiple computational instantiations that form a population of agents, each capable of having different behavioral trajectories. These heterogeneous behaviors produce population dynamics that are the observable, system-level output of the ABM. A classic example of this phenomenon is the behavior of flocks of birds, in which simulations utilizing relatively simple interaction rules among birds can lead to sophisticated flocking patterns without an overall controller [20]. This property is well suited to the tendency in biology towards classification: the grouping of similar biological entities that share some set of properties and behaviors. Biological systems are then readily characterized as being composed of some types and numbers of these entities. This type of conceptual representation exactly suits the architecture of an ABM.

Incorporation of Stochasticity and Randomness

ABMs readily incorporate *stochasticity*. Many biological systems have behaviors that appear to be random [21, 22]. Whether these behaviors are truly random, or just merely appear to be due to a lack of finer grained knowledge is, from an operational standpoint, often irrelevant as long as the probabilities of a particular behavior can be determined for the population as a whole experimentally. These probabilities are then used to generate a probability function for the behavior of a single agent that is then incorporated into the agent's rules. As a population of agents executes their rules during the course of a simulation, each agent follows a particular behavioral trajectory as its behavior rules' probabilities are resolved as the simulation progresses. A set of behavioral outputs is thusly generated from a single ABM, producing system behavioral state spaces representing the set of population-level biological observations.

Modular Architecture

ABMs are *modular*. Agents represent a distinct and circumscribed modular level into which new information can be added either through the introduction of new

agent types or by the modification of existing agent rules without having to reengineer the entire simulation. Agent classes representing generic cell types can be subdivided and expanded to include a finer degree of detail with respect to subcategories of cells while the remainder of the ABM remains essentially intact. New mediators can be similarly added by creating new cellular state or environmental variables and rules. Multiple ABMs can be aggregated, providing that their points of contact and interaction are consistent across the incorporated ABMs [12, 19].

Generation of Non-Intuitive System-Level Phenomenon

A central hallmark of ABM is that they generate system-level behaviors that could not have been reasonably inferred from, and often may be counter-intuitive to, examination of the rules of the agents alone. This is our definition of *emergent* behavior. ABMs are able to generate this type of behavior due to the locally constrained and stochastic nature of agent rules, and the population effects of their aggregated interactions. For example, in the bird flock, an initial observation would suggest an overall leader, thereby requiring a means of determining rules for flock-wide command and control communication. This, however, is not the actual case; birds function on a series of locally constrained, neighborhood-defined interaction rules, and the flocking behavior emerges from the aggregate of these interactions [20]. The capacity to generate nonintuitive behavior is a vital advantage of using ABM for conceptual model verification, as often the translation of generative mechanisms to system-level behavior produces paradoxical and unanticipated results that break a conceptual model.

Facilitation of Useful and Detailed Abstraction

ABMs provide for high-fidelity component abstraction of system structure. ABMs can be readily constructed using incomplete and abstracted knowledge, yet produce surprisingly highly "realistic" system level behavior. Because of this property it is advantageous in the initial steps of developing an ABM to keep the rules as simple and verifiable as possible, even at the expense of some detail. As such, meta-analyses of existing basic research often guide the development of an ABM [23]. ABMs constructed with admittedly incomplete and uncertain mechanisms representing statements of hypotheses can provide qualitative verification of those hypotheses [24]. As with all computational models, the greater fidelity of mapping between the ABM and its biological counterparts enhances the correlation between simulation results and the real-world behaviors. An iterative process of refinement of an ABM will lead to increased detail, possibly a stronger correlation to real-world data and a greater confidence in the ability of the ABM to describe observable phenomena.

Agent-based modeling is an integrative modeling framework that can readily be used for communicable dynamic knowledge representation [10–12, 25] (see Fig. 3.1). Agent-based modeling, because of its emphasis on "things doing things,"

is generally more intuitive for nonmathematicians/computer scientists than more formal mathematical modeling methods such as ordinary differential equations, partial differential equations, and their stochastic variants. Agent-based modeling presents a lower threshold barrier for researchers to "bring to life" their conceptual models and integrate in silico methods with traditional in vitro and in vivo experiments [2].

Since ABMs are knowledge-based models, constructed by instantiating bottom-up mechanisms (as opposed to inductive models, where mechanisms are inferred with the goal of explaining data), agent-based modeling addresses different modeling questions than equation-based inductive models. For instance, ABMs are not readily developed directly from a mass of raw data; they require that the modeler have a mechanistic hypothesis that, when instantiated in an ABM, can be used to generate simulated data, which can then be compared to the real-world data set. One can envision an iterative process by which inductive models are applied to large data sets, wet lab experiments are carried out to investigate the mechanisms inferred from the inductive model, and the experimentally confirmed mechanisms are used as a basis of an ABM, which would close the discovery loop by recapitulating the original data set.

Agent-based modeling was pioneered in the areas of ecology, social science, and economics, but since 2000 they have increasingly been used to in the biomedical arena to study sepsis [11, 12, 26, 27], cancer [7, 18, 28–30], cellular trafficking [31–35], wound healing [36–38], and intracellular processes and signaling [8, 25, 39–44]. The majority of biomedical ABMs utilize cells as the primary simulation agent level, though there are several exceptions of modeling intracellular processes from [8, 25, 39–44], and we consider the use of agent-based modeling in epidemiology, with its extremely rich background [45], as a separate discipline. From the standpoint of addressing the Translational Dilemma, cells form a ready level of "encapsulated complexity" that is both highly studied as a unit (i.e., cellular biology) and can be addressed with relatively straightforward input–output rules [6]. As noted above, while ABM agent rules are often logical or algebraic statements, rules can be a mathematical model in itself. There are multiple examples of embedding complex mathematical models within a cell-level ABM agent [6–9, 14, 38, 46]. These examples emphasize the potential unifying role of agent-based modeling as a means of "wrapping" different simulation methodologies. This suggests that the metastructure of an ABM can be used as a template into which structured biomedical knowledge can be integrated to facilitate the instantiation of multiple mechanistic hypotheses [47].

Tools for Agent-Based Modeling

Agent-based modeling environments require addressing certain software issues beyond the basic capabilities of more traditional object-oriented programming tools. These issues include emulating parallel processing to represent the actions of

Table 3.1 Freeware agent-based modeling toolkits

Toolkit name	Language/ Platform	Degree of programming expertise?	Degree of flexibility?	Website
Swarm	Objective C, Java	High	High	http://www.swarm.org
Netlogo	Windows, Macintosh, Linux	Low	Low	http://www.ccl.northwestern.edu/netlogo/
Starlogo	Windows, Macintosh, Linux	Low	Low	http://education.mit.edu/starlogo/
Repast	Java	Moderate/high	Moderate	http://repast.sourceforge.net/
MASON	Java	Moderate/high	High	http://cs.gmu.edu/~eclab/projects/mason/
SPARK	Java	Moderate/high	Moderate/high	http://www.pitt.edu/~cirm/spark/

multiple agents within populations, dealing with associated execution concurrency issues within those populations, establishing means of defining model topology (i.e., agent interaction neighborhood), and the development of task schedulers to account for the multiple iterations that constitute an ABM run. As a result of these issues, along with the case that many researchers who utilize ABMs are not trained computer scientists or programmers, many biomedical ABMs are created using existing ABM development software packages. These agent-based modeling environments attempt to strike a balance between representational capacity, computational efficiency, and user-friendliness. A noncomprehensive list of such ABM toolkits can be seen in Table 3.1. All these platforms represent some trade-off among the triad of goals mentioned above. For an excellent review and comparison of many of these agent-based modeling toolkits, see [48].

Agent-Based Modeling of Inflammation

The difficulty in engineering safe and effective therapeutic agents directed at inflammation is a primary example of the Translational Dilemma in biomedical research. Because of these characteristics inflammation represents perhaps the ideal target for systems biology and computational modeling with agent-based modeling. The use of agent-based modeling has dramatically increased since the year 2000 and is now a generally accepted means of performing computational biology. As is the case when discussing any specific modeling method, it should be reemphasized that agent-based modeling is only one of an array of methods that can be used to represent and investigate biological systems (such as those covered in other chapters in this book). Each of these modeling techniques has its strengths and weaknesses, and

potential modelers need to recognize that the modeling method chosen should be tailored to the question(s) being asked of the model [49]. One of the most effective ways of communicating the capabilities (and limitations) of a particular modeling method is through the use of examples. Since the rest of this book includes detailed descriptions of several ABMs involved in Translational Systems Biology, this chapter presents a few examples of types of ABMs not explicitly covered elsewhere in this book.

ABMs of Inflammation-Related Intracellular Processes

The characterization of intracellular pathways is the traditional focus of systems biology, with a long history of work and achievement in the development of mathematical models of cellular signaling and metabolic control. These models are generally biochemical kinetic models, utilizing deterministic and stochastic differential equations. However, the use of discrete-event, particle-based modeling, exemplified by agent-based modeling, has certain applications in this arena. With increasing awareness of the influence of the complex, compartmentalized environment of the intracellular milieu on intracellular dynamics, there is a need to account for issues of molecular crowding and spatial heterogeneity of the reaction milieu and how they affect enzymatic reactions within the intracellular environment. Additionally, the presence of subcellular structures, cytoskeletal elements, organelles, and compartments call for the increasing incorporation of spatial properties and detail. Ridgway et al. [42] used an ABM of intracellular signaling to demonstrate that the biochemical reaction kinetics in the prokaryotic cytoplasm was reduced from three dimensions to nearly two dimensions, with significant consequences for the dynamic modeling of control loops in which subtle changes in feedback determine the direction of a molecular switch. Pogson et al. [41] developed an ABM of control pathways affecting the transcription factor Nuclear Factor kappa B (NF-kB). These studies demonstrating the importance of the spatial distribution in terms of nuclear translocation of the constitutive inhibitor of NF-kB, I-kappa-B (IkB), and the binding of IkB to actin, a cytoskeletal protein, a mechanism subsequently identified in their laboratory [40]. We developed an agent-based architecture called Spatially Configured Stochastic Reaction Chambers to demonstrate that even an abstract representation of enzyme kinetics could, if sufficient pathway component detail was included, reproduce canonical behavior at the cellular level, as in the effect of preconditioning on the behavior of the Toll-like Receptor 4 (TLR-4) signaling pathway [25]. A screenshot of the SCSRC for TLR-4 can be seen in Fig. 3.2. Similarly, an ABM of NF-kB response to endotoxin utilized molecular level agents nested within “mega-agents” representing different inflammatory cell types to reproduce recognizable dynamics of endotoxin response, including priming and tolerance at both the transcription factor and cellular activation level [44].

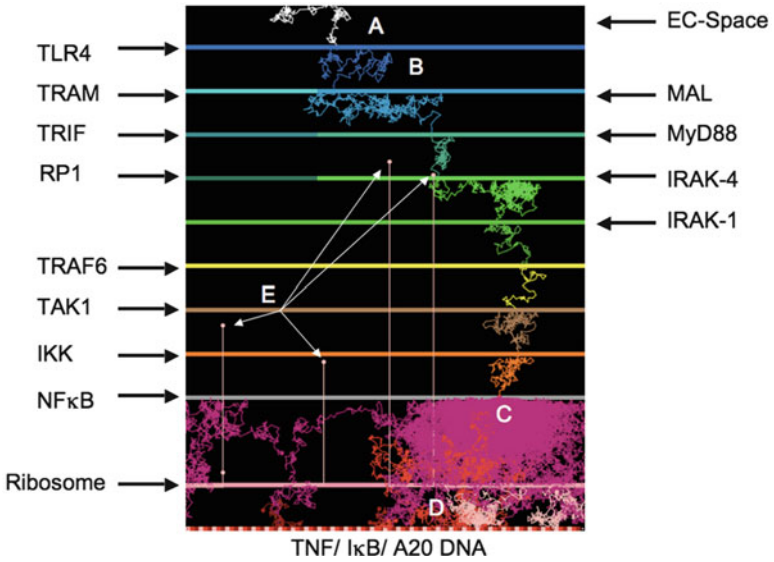

Fig. 3.2 Screenshot of spatially configured stochastic reaction chamber (SCSRC) model of TLR-4 signaling. This figure demonstrates the underlying architecture of the SCSRC as well as the signal trajectory of a single LPS signal agent. Reaction chambers are oriented vertically, and TLR-4 signaling propagates from the "top" of the model (representing extracellular space) towards the "bottom" (DNAs). The various signal transduction proteins are represented as *horizontal bars* across the model. The trajectory of a single LPS signaling agent as it passed through the various layers of signaling. Note the irregular path of the agent, reflecting the random movement rules that reflect the stochasticity in molecular dynamics. Letter "A" denotes the initial extracellular space where the LPS agent is introduced. Letter "B" denotes the first intracellular reaction space immediately under the TLR-4 border. Letter "C" demonstrates the signal amplification at the NF-κB activation site, as the single signal agent results in multiple NF-κB agents. Letter "D" denotes the DNA reaction space, as additional amplification can be seen in simulated transcription. Letter "E" labels synthesized TNF molecules in the process of transport to the extracellular space, seen as the straight trajectories. This figure is reprinted with permission from [25]

Cell-Level ABMs of Systemic Inflammation and Simulated Clinical Trials for Sepsis

The cell-as-agent level of component representation provides perhaps the most intuitive link between the laboratory-derived basic mechanistic knowledge and the structure of an ABM. Some of the earliest examples of biomedical ABMs were focused at this level leading to the realization that even abstract agent rules could produce very recognizable dynamics that could provide deep insights into the essential characterization of a disease process [26, 29]. For example, an early ABM of systemic inflammation and sepsis viewed the inflammatory process as being governed by interactions at the endothelial blood interface [26]. This ABM generated four clusters of distinct trajectories of model-system behavior purely by altering the degree of initial perturbation, trajectories that matched the four primary clinical scenarios

associated with systemic inflammatory response. This ABM also demonstrated that the mechanistic basis of inflammation was the same whether the initiating insult was infectious, as in classical sepsis, or tissue damage, as in severe trauma.

The endothelial-surface systemic inflammation ABM was further extended to perform in silico clinical trials based on published and hypothetical inflammatory-mediator-based interventions [27]. Published pharmacologic properties of a series of mediator-targeting compounds were inputted into the ABM simulating a sepsis population. The efficacies of the interventions were then evaluated against a simulated control population. None of the mediator-directed interventions led to a statistically significant improvement in simulated patient outcome, including a set of immune augmenting interventions (e.g., addition of Granulocyte Colony Stimulating Factor) and combination anticytokine therapy (intended to overcome possible pathway redundancy). While these results were not totally unexpected, the exercise demonstrated that the ABM could be used as a means of assessing the veracity of the proposed intervention, i.e., what are the global consequences of intervening in a particular pathway, and is it actually a good idea to intervene at this point? The confirmation that what appeared to be intuitively plausible points of mechanistic intervention did *not* in fact behave as expected when placed in a systemic context demonstrated the potential usefulness of agent-based modeling and dynamic knowledge representation for hypothesis verification. We suggest that one of the primary roles of dynamic knowledge representation is exactly this type of hypothesis evaluation and verification, intended to reduce the set of plausible hypotheses and thereby help direct future investigation by eliminating therapeutic dead-ends.

ABMs of Multiorgan Inflammation and Failure

The structural/anatomic approach to multiscale modeling can be taken one step further by using the modular property of agent-based modeling to link individual organ ABMs in a multiscale architecture. The approach was introduced in an ABM of the gut–lung axis of systemic acute inflammation and multiple organ failure [12]. This ABM incorporates multiple structural and anatomic spaces, e.g., endothelial and epithelial surfaces as aggregated by cell-type into organ-specific tissues and finally to organ-to-organ interconnections and crosstalk (see Fig. 3.3). This architecture also *translates* knowledge across domain specialties (molecular biology to clinical critical care), representing molecular and cellular mechanisms and behaviors derived from in vitro studies, extrapolated to ex vivo tissue experiments and observations, leading to patterns of organ-specific physiology, and finally simulating clinically relevant, interconnected, multiorgan physiology including the response to ventilator support of acute respiratory failure. This ABM also posited certain characteristics of the gut-derived proinflammatory compound that is circulated in the mesenteric lymph and induces pulmonary inflammation. Examining the time course of pulmonary inflammation and comparing that to generated factors following intestinal ischemia suggested that the mesenteric lymph inflammatory compound was neither an initial inflammatory cytokine nor a translocating luminal compound

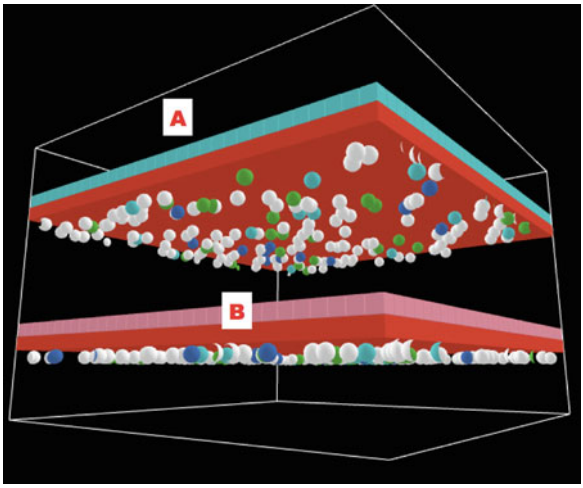

Fig. 3.3 Screenshot of multibilayer gut–lung ABM of systemic inflammation. The multiple bilayer topology of the gut–lung ABM is seen with the upper bilayer (letter A) representing the pulmonary bilayer, with *aqua cubes* representing pulmonary epithelial cell agents, *red cubes* representing pulmonary endothelial cell agents, and below are spherical inflammatory cell agents. The lower bilayer (letter B) represents the gut bilayer, with a similar configuration, the only difference being that gut epithelial cell agents are *pink*. Circulating inflammatory cell agents move between these two bilayers on a time schedule calibrated to the rate of systemic circulation and gut lymph flow. This ABM represents an aggregate of several submodels including endothelial-based inflammation and epithelial tight-junction protein metabolism. This ABM was able to reproduce the effects of gut ischemia in propagating the development of acute respiratory failure, the salvaging effects of mechanical ventilation, and posited the nature of the gut ischemic product driving respiratory failure as being tied to endothelial cell/tissue damage. Figure reproduced with permission from [12] under the creative commons license

manifesting decreased intestinal permeability, but rather a substance reflecting cellular damage of gut tissue with properties consistent with damage-associated molecular patterns (DAMPs). This last hypotheses remains to be completely confirmed by the sepsis research community, but at this time appears to be consistent with ongoing research in this area [50].

Moving Forward: Scaling Dynamic Knowledge Representation, the Agent-Based Modeling Format

As noted in the Introduction, the Translational Dilemma arises not only difficulties in multiscale representation and instantiation but is also a throughput problem. While computational modeling (including agent-based modeling) can potentially address the former, generating these models, even with a relatively intuitive method as agent-based modeling, is currently a highly specialized, laborious, and

time-consuming task. Therefore, developing a scalable global strategy to overcome the Translational Dilemma will require substantially lowering the threshold for the general researcher to engage in computational modeling. We suggest that the process of constructing dynamic computational models can be augmented by leveraging ongoing work in bioinformatics and knowledge representation, primarily related to ontologies [10, 47, 51].

Ontologies are knowledge classification systems that provide a structured vocabulary and taxonomy for a particular scientific domain [52]. Ontologies utilize taxonomic class structures, their properties, and the relationships between the constitutive concepts to organize information within the domain. The use of ontologies is well established in bioinformatics, and many bio-ontologies are currently found in an online repository called BioPortal [53], which is managed by the National Center for Biomedical Ontologies (NCBO) [54].

However, despite their usefulness, ontologies/bio-ontologies remain primarily classification systems that define identity relationships between concepts but have difficulty expressing dynamic and functional relationships than can be used to represent mechanistic rules; this gap is the transition from a descriptive model to a simulation. There has been work converting ontology-based knowledge representations into dynamic mathematical models of molecular signaling pathways [36, 55–59]. However, while useful for representing the behavior of specific pathways, these approaches focus on working within a single ontology (namely the Gene Ontology) and do not deal with the multiscale aspects of biology, i.e., the transition of molecules to cells to tissues to the whole organism. Alternatively, ABMs are well suited to be an integrating modeling paradigm since they capture the multiscale organization of biological systems (see Fig. 3.1). We suggest that an ABM-based framework can be used to integrate the knowledge from multiple ontologies describing different aspects of a biological system (components, functions, space, etc.) in order to construct a dynamic multiscale, translational model.

We propose the agent-based modeling format (ABMF) as a framework that leverages and integrates ontological descriptions of biology to facilitate the construction of dynamic, executable knowledge representations with multiscale representational capacity [47]. The ABMF integrates terms and metadata from BioPortal ontologies into three-level modules formatted around the information and data needed to construct an ABM. These levels are centered on the level of the simulation agent in a "middle-out" configuration [6]. A schematic of an ABMF module can be seen in Fig. 3.4 and is organized in a series of orthogonal descriptive class structures that can be populated with terms extracted from BioPortal ontologies. The modular structure of the ABMF allows for nesting of modules and a recursive description of biological systems; this multiscale organizational recursion has been noted as a property of biological systems [60].

We emphasize that the ABMF is *not* "the" format for an executable ontology layer; we hope that there will be development of similar types of tools, using different modeling paradigms. However, we believe that an agent-based modeling paradigm demonstrates a robust, evolvable approach that can be spur future development.

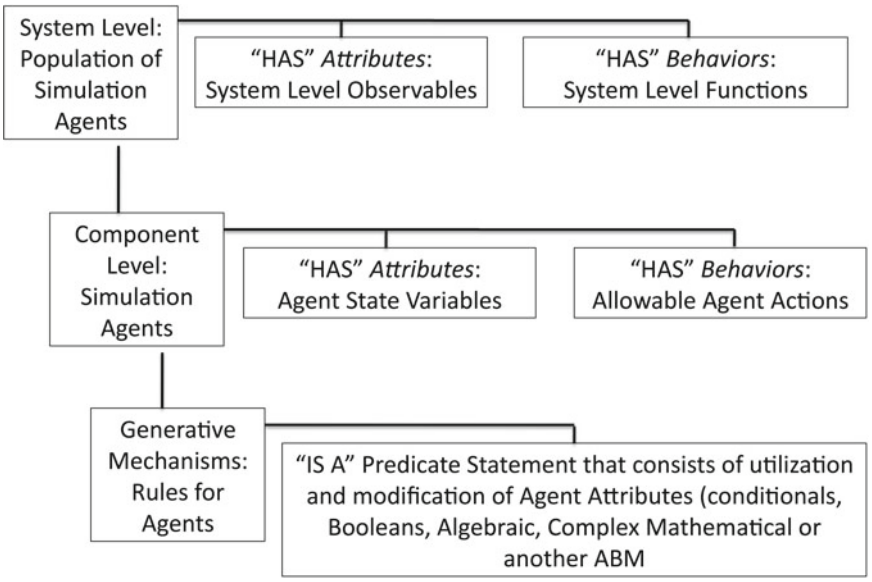

Fig. 3.4 A schematic description of an agent-based modeling format (ABMF) module. The ABMF module incorporates three levels of system representation centered on the simulation agent level, which corresponds to the classical agent level in an ABM. The system level corresponds to agent population behavior (including so-called emergent phenomenon), and the lowest level of organization, generative mechanisms, corresponds to agent rules. Inputted generative mechanisms can be in the form of any formal model system including another ABM. This gives the ABMF a recursive structure that allows nesting of ABMF modules and makes it a potential pathway to hybrid computational models that concurrently employ multiple modeling and simulation methods. Reprinted with permission from [47]

We further note that the ABMF is not a modeling platform, but rather a metastructure that helps collect and organize the components needed to construct an ABM from a biological hypothesis. There is still a significant gulf between the formatting of a biological hypothesis and the ability to construct a computer simulation of that hypothesis. The ABMF provides a pathway towards automation by leveraging the structured vocabulary and inference capabilities of ontologies. Additional text analysis and information extraction technologies can be integrated with an ABMF constructor and provide a semiautomated way to collect potential parameter values with which to populate a simulation program.

The expression of a conceptual biological model in the ABMF places that biological model into computable form, perhaps facilitating conversion into an executable simulation through the use of a semi-intelligent computational agent. There has been recognition of the importance of ontologies in the development of intelligent system-aided model and simulation generation, with several proposed schema for the development and use of ontology-driven processes [61–63]. The repetitive nature of certain steps of model construction suggests that these steps in the creation and programming of a simulation model can be expressed as task-based algorithms

embedded into an intelligent computational agent, which then treats simulation construction as a planning task using formal logical inference. Computational agents have been used in this fashion in bioinformatics for data integration and information flow management [64–68]. We have proposed that the task of converting biological conceptual models into executable simulations, including those associated with the population of the ABMF and subsequent conversion into ABMs, could be carried out by an intelligent computational agent, which we term a "computational modeling assistant (CMA)" [51]. We envision that this type of agent-directed process can semiautomate the specification-mapping work of model construction through the use of ontology-based/traditional predicate logic inference structures to generate simulation code. This will move towards achieving the translational research goal of high-throughput instantiation of conceptual models. Treating the steps of the composition process as a planning task can improve the modularity, robustness, and scalability of knowledge integration by creating a "middle-ware" discipline, i.e., *modeling*, and thereby focusing future development on the algorithmic expression of the mapping rules used in model development that form the CMA's inference instruction set. This allows expansion of the CMA's capabilities and expressiveness while maintaining interoperability with established but ongoing development in the areas of formal semantics/knowledge representation and modeling and simulation methods. We believe that this type process automation advances offered by the CMA will lead towards the development of cyberenvironments providing scalable high-throughput hypothesis instantiation and evaluation.

Challenges to the Use of Agent-Based Modeling

As with all modeling methods, agent-based modeling is not without its limitations. One common issue shared with all computational and mathematical modeling methods is that the quality and reliability of the models are directly related to the reliability of the underlying assumptions of the model and the quality of their implementation during construction of the model. This issue can be addressed by emphasizing transparency of both underlying assumptions and implementation details with respect to the construction of an ABM. The ODD protocol, while not developed specifically with biomedical ABMs in mind, provides a useful reference point with respect to documenting the structure and goals associated with an agent-based modeling project [69].

One shortcoming of agent-based modeling is the difficulty in applying formal analysis to the relationship between the agent rules and the behavior of the system. Due to the combined stochastic behavior of agents and the difficulty in assigning scalar metrics to account for the spatial aspects of an ABM's output, it can be very challenging to evaluate the effect of parameter values and model structure on an ABM's behavior. Alternatively, equation-based models have well-established procedures for analytical tasks such as parameter sensitivity analysis, bifurcation analysis, and behavior-state-space determination. Work on developing mathematical descriptions of ABMs offer the prospect that formal analysis may be available in the

future [70]. In the meantime, ABM researchers use a variety of strategies, such as heuristics [5, 27], literature-based constraints [31, 34] and Latin Hypercubes [9, 71], for parameter estimation and sensitivity analysis.

Some of the apprehension associated with the analysis of ABMs can be addressed by viewing ABMs as objects more akin to wet lab experimental platforms rather than more traditional, equation-based mathematical models. Pattern-oriented analysis, in which corresponding patterns of dynamic behavior are used to relate the computational ABM to its real-world referent, allows ABMs to be evaluated much in the same way as wet lab systems or model organisms [24]. From this regard, the stochastic and emergent properties of ABMs reinforce their ability to capture the robustness of dynamic behavior seen in complex systems, thereby allowing more insight into their core organizational structure.

ABMs are, in general, more computationally intensive than equation-based models. The increased computational requirements place constraints on both the size of ABMs in terms of number of agents as well as the complexity of their internal rule systems. The natural solution to this bottleneck is to implement very large-scale ABMs on current high performance computing platforms. However, there are intrinsic properties of ABMs, primarily related to the high degree of dynamics in the agent-to-agent interaction and communication network that challenge the ability to implement ABM on highly distributed memory systems. Certain types of model architectures, mostly incorporating limited or relatively static interaction neighborhoods with a high ratio of intra-agent computation (i.e., very complex mathematical rules) to interagent communication, are more suited to implementation on these massively parallel computer architectures. These types of models are also suited to implementation using Graphical Processing Units (GPUs), which offers the possibility of "supercomputer on a desk" computational power for selected types of ABMs [72–74]. It should be noted that there are also nontrivial modeling issues associated with parallel implementation of ABMs, aside from the computer science challenges just noted above. The selection of the scale of process to be distributed across multiple processors may have consequences with respect to concurrency and event scheduling and to the mapping of the simulation behavior back to the biological referent, for instance, attempting to distribute a single agent's rules over a series of processors. Thus, far parallel ABM implementations have not explored the distribution of a single agent's execution across multiple processors and have opted for a more organizationally defined distribution strategy that expands the overall size of the ABM (i.e., more agents) and keeps the implementation of agent-scale behavior at the processor and subprocessor level.

Conclusions

The Translational Dilemma is the greatest challenge facing the biomedical research community today. Future operational procedures for biomedical science should involve technological augmentation of all the steps of the scientific cycle and allow

the knowledge generated from such research to manifest in multiple areas. These include the development of highly predictive, personalized simulations to streamline the development and design of therapies, simulating the clinical application of these therapies in population studies (in silico clinical trials) and predicting the effects of drugs on individuals. We suggest that the agent-based paradigm, incorporating knowledge encapsulation, modularity, and parallelism can play an important role in the development of this metaengineering process. Agent-based modeling can provide an integrative architecture for the computational representation of biological systems. Expanding the tools for AI-augmentation of computational dynamic knowledge representation (such as the ABMF and the CMA) can significantly reduce the threshold for the general researcher to utilize computational modeling and allow investigators to "see" the consequences of a particular hypothesis structure/conceptual model, such that the mechanistic consequences of each component of the hypothesis can be probed and evaluated. Dynamic knowledge representation enables the instantiation of "thought experiments": the exploration of possible alternative solutions and identifying those that are plausible, i.e., consistent with the observed data. These models can aid in the scientific process by providing a transparent framework for this type of speculation, which can then be used as jumping off points for the planning and design of further laboratory experiments and measurements. It is hoped that the increasing use of this type of knowledge representation and communication will foster the further development of "virtual laboratories" and in silico investigations.

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Chapter 4
Analysis of Heart Rate Variability

Patrick R. Norris

When Einthoven published the first electrocardiogram (EKG) recordings in 1902, he would not have imagined the range of EKG applications being explored by clinicians, scientists, and engineers more than a century later. Certainly, Einthoven and his contemporaries appreciated EKG morphology—the shape of the waveform itself including presence or absence of particular features and relationship of these features to one another. Alterations in these shapes can be linked directly to various cardiac disorders, and the biology and physics behind most of these phenomena are straightforward. They would nonetheless marvel at the widespread application of the EKG enabled by technical advancements; since the days of Einthoven's 600-pound galvanometer, improvements have enabled high-fidelity EKG recordings to be obtained inexpensively in a variety of settings, by medical professionals and laypersons alike. For example, automated external defibrillators rely on the EKG to appropriately deliver electrical shocks to defibrillate a patient, possibly at the hands of someone with no formal training. The cardiac electrophysiologists of the early 1900s appreciated the anatomy, physiology, and physics behind the EKG but could not have predicted its widespread application in the twenty-first century fostered by technology.

Even more surprising to early cardiac electrophysiologists is the newfound potential of the signal to reflect whole-organism response to acute illness, systemic inflammation, and other pathophysiology of noncardiac origin. While an elevated heart rate has long been recognized as a sign of infection (and heart rate changes with numerous other physiological states such as exercise and sleep), heart rate also varies in a more subtle way, with each individual beat. These "beat-to-beat" differences are detectable only via precise measurements of the cardiac cycle such as the distance between R-wave peaks (Fig. 4.1) or other sentinel points on the EKG. The magnitude and pattern of these subtle variations, and their relationship to various

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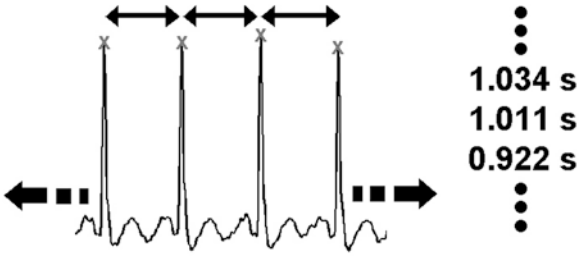

Fig. 4.1 Beat-to-beat heart rate variability. HRV typically refers to the subtle variation in duration of subsequent cardiac cycles measured between R-wave peaks or other fiducial points

states of health and disease, comprise modern heart rate variability (HRV) analysis. HRV has been studied since the 1960s, especially with respect to fetal monitoring and cardiac applications [1], but only recently have these techniques been applied to inform clinical decision making in acute illness and inflammation. For example, changes in HRV have been correlated with development of sepsis in neonates; a large randomized trial recently showed significant reduction in mortality attributed to a novel sepsis monitoring system based on HRV [2].

Many techniques used to measure and understand HRV draw from the fields of complex systems and chaos theory. These disciplines began to emerge at the same time as early formal analyses of HRV began [3] and provide appealing conceptual frameworks in the view of the body as a complex system, or a collection thereof. A host of analytic tools, with origins in applying chaos theory to other domains such as weather forecasting and information transmission, are directly applicable to analyzing series of cardiac interbeat intervals. Entire books have been dedicated to tools, methods, and applications in the broad field of HRV analysis [4, 5]. This chapter provides a short introduction to the rationale, methods, and applications of HRV analysis for those interested in applying such techniques to the study of acute inflammation and critical illness. It reviews selected methods of studying HRV, their application to acute illness and inflammation, and concludes with future directions for this exciting field. Throughout, the term "HRV" will be used to reference any analysis of cardiac interbeat intervals including *complexity* measurements, not being restricted to spectral or other analyses from which "HRV" historically derives. The following section briefly describes the biological basis of human HRV and complexity, prior to detailing selected HRV methods and applications.

The Biological Basis of Heart Rate Variability

Multiple regulatory mechanisms have evolved to ensure adequate blood pressure via changes in heart rate and contractility. While a complete description of these mechanisms is beyond the scope of this chapter, a brief discussion of cardiac regulation will underscore several important concepts in the application of HRV to acute inflammation or other domains: First, the regulatory mechanisms relevant at time scales of interest in acute inflammation or critical illness can generally be understood

as feedback control systems with associated inherent variability; second, the heart itself contains sufficient regulatory control to be able to serve the body well (albeit imperfectly) absent any external input; and third, complexity in cardiac beat-to-beat intervals likely arises out of interplay between relatively uncomplicated but nonlinear regulatory mechanisms both internal to the heart itself, as well as involving the central nervous system and other exogenous control pathways.

Negative feedback control, i.e., the magnitude of the control signal being attenuated as the output approaches a desired setpoint, is the hallmark of both excitatory and inhibitory cardiac regulation. Several excellent primers to heart rate regulation can be found online (e.g., [6]), and the reader unfamiliar with these mechanisms is urged to review a more complete and pictorial source to augment this brief description. Essentially, heart rate is directly mediated by sympathetic and parasympathetic (vagal) inputs: increased parasympathetic input tends to decrease heart rate via inhibition of pacemaker cells, and increased sympathetic input tends to increase heart rate via pacemaker cell stimulation. Changes in heart rate due to parasympathetic versus sympathetic inputs typically occur at different time scales, reflecting the evolution of the sympathetic nervous system to mediate "fight or flight" responses to external stimuli and the parasympathetic to mediate internal processes, when external threats or other stimuli are absent. Note that while parasympathetic processes are typically thought of as being slower due to their visceral nature, organs including the heart often respond more rapidly, and/or at higher regular frequencies, to these inputs. Thus, the time course of parasympathetic-mediated changes in heart rate—decreases in heart rate with increased parasympathetic activity, or vice-versa—is typically more rapid than changes mediated by sympathetic activity. This becomes important when interpreting *spectral analysis*: signal processing methods that can discern the various contribution of changes at different time scales to the overall signal. In the case of interbeat intervals, parasympathetic activity has been attributed to variation in heart rate that occurs more quickly, while both parasympathetic and sympathetic inputs drive changes that occur more slowly, at lower frequencies (see Methods below). The sympathetic and parasympathetic branches of the autonomic nervous system provide most, but not all, of the inputs to heart rate control (others include thermoregulatory and endocrine mechanisms [7]), and operate as feedback control systems to control rate in response to external stimuli and changes in blood pressure.

Indirect regulation of heart rate becomes considerably more complicated but can still be understood in terms of individual, albeit overlapping, feedback mechanisms. Because blood pressure is central to cardiovascular regulation, nearly any factor which alters blood pressure can impact heart rate via feedback from baroreceptors (pressure sensors located in the carotid sinus) to central nervous system centers that generate excitatory and inhibitory inputs to heart rate via sympathetic and parasympathetic nerves, respectively. Further complicating the milieu are stretch receptors within the myocardium that relay input to the central nervous system about how much the heart is filling with each cardiac cycle, which in turn stimulates sympathetic activity to increase rate and contractility. Finally, diverse biochemical phenomena and intrinsic mechanisms also can affect heart rate. Fundamentally, heart rate is dependent upon cyclic ion flux across pacemaker cell membranes, and

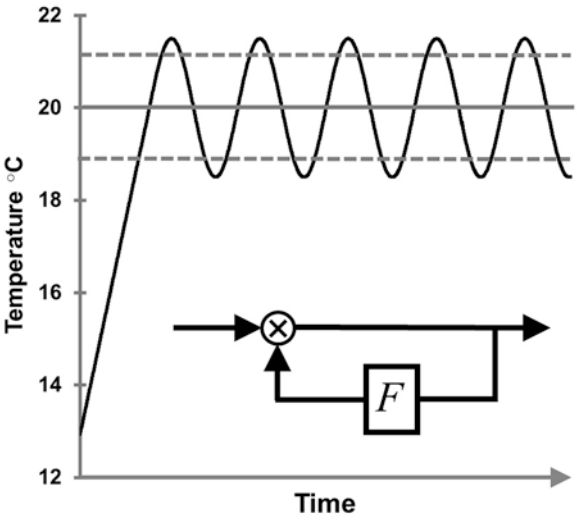

Fig. 4.2 Feedback control gives rise to variability. In this example, temperature varies in a room controlled by a thermostat, illustrating the inherent variability arising from feedback control. *Inset*: Simple feedback control system diagram. The output of the system at any time is a function *F* of previous output and initial conditions

alterations to either these passive channels or active ion pumps can alter heart rate, or change the responsiveness of pacemaker cells to external excitatory or inhibitory input. Properties of the myocardium itself can modulate heart rate, including the fact that increased stretch tends to increase contractile force (i.e., Frank–Starling mechanism) which in turn is coupled with heart rate (i.e., the Bowditch effect).

This intrinsic regulation allows the heart to function well even in the absence of external neural inputs. Since Beck and colleagues' early case studies of heart rate in transplant patients [8], many have investigated the remarkable ability of the heart to sufficiently maintain blood pressure without connection to the autonomic nervous system. In the months and years following transplant, both sympathetic and parasympathetic fibers gradually reinnervate the heart [9–11], providing an excellent opportunity to characterize regulatory mechanisms such as the effect of sympathetic reinnervation, which seems to occur prior to or is more robust than parasympathetic regrowth [12]. While feedback loops regulating heart rate can be isolated and studied in nontransplant patients using medications to block various pathways and/or artificial stimuli such as baroreflex pressure cuffs or tilt tables, studying transplant patients and the gradual return of individual central regulatory mechanisms has been key to understanding heart rate control. At particular points in the neural regrowth in some patients, heart rate responses can be observed which represent a simple (i.e., first order) feedback system [12].

Variability is inherent to such feedback control systems, even when the system being controlled is in a constant or restful state. Consider for example a simple thermostat, designed to trigger a heater to maintain a constant room temperature during winter. The outside temperature is constant, and there are no other sources of heating or cooling. The room temperature over time might vary as shown in Fig. 4.2. When the thermostat is first set, the heater is turned on and room temperature increases. As the room reaches the thermostat setpoint the heater is turned off, temperature overshoots, then falls. When the temperature drops below setpoint the

heater is again activated (thermostats often implement hysteresis—essentially delays and/or slightly different temperatures for turning the heater on and off as shown by the dotted lines—to prevent rapid cycling). Similarly, healthy cardiac regulatory mechanisms result in a heart rate that varies around a given set point, even when a subject is resting quietly. The facts that heart rate is influenced by multiple inputs simultaneously, and that feedback signals are continuous versus simple "on–off" signals, makes the pattern of oscillation much less predictable than the temperature in a thermostat-controlled room. Despite being difficult to predict, heart rate varies subtly around a setpoint, or multiple setpoints simultaneously, and mathematical models have predicted these complex patterns arising from multiple negative feedback processes [13, 14].

It is both the concurrent action of multiple individual feedback control systems, as well as nonlinear behavior of these individual pathways, that give rise to *complexity* in the heart rate signal. Continuing the above example, the heater simply being turned on or off is not particularly representative of physiologic control pathways, as they typically exhibit more continuous behavior (e.g., heart rate is signaled not to simply go fast or slow, but to change in proportion to the degree of need perceived by the body). A straightforward feedback system exhibiting such behavior has been described by Gleik [3] and others and is an excellent example of how complexity arises from such systems. The output (e.g., temperature or heart rate) changes in proportion to the output at some prior time, adjusted by the difference between that prior output and some value, times a constant (gain term). In other words, the temperature "x" at any given time "t" depends on what temperature the room was before (time "$t-1$") and the output of the heater since that time, where the output of the heater is proportional to the deviation of the temperature from a reference point. This can be described mathematically as:

$$x_t = ax_{t-1}(1 - x_{t-1})$$

Note that the interval to the prior point in time ("$t-1$") is arbitrary and is simply the duration over which the heater output will not change while the system is sampling and processing the next value. The next value is easily calculated from the previous one. From this straightforward system, surprisingly complex patterns of outputs can be obtained depending on the value of a (Fig. 4.3). When a is below about 2.9, the system converges to a constant value, sometimes after a brief oscillation depending on the initial value of x (Fig. 4.3a). As a increases, interesting patterns emerge in the output: first, it converges on two discrete values (Fig. 4.3b), then four (Fig. 4.3c), then eight, until many different values emerge (Fig. 4.3d). While the signal might at first appear random, upon closer observation similar (but not identical) repeating patterns can be seen. Modifying the value of a will produce a signal of increasing complexity. It is not random, yet without knowledge of the underlying equation and *precise* value of the output at any given time point, the signal is very difficult to predict. The degree of complexity in a signal can be described as extent to which it is unpredictable, but not necessarily random, and various analytic methods can quantify this "nonrandom unpredictability."

The following section details selected methods of assessing HRV (including complex variability) that arise naturally from cardiovascular feedback control

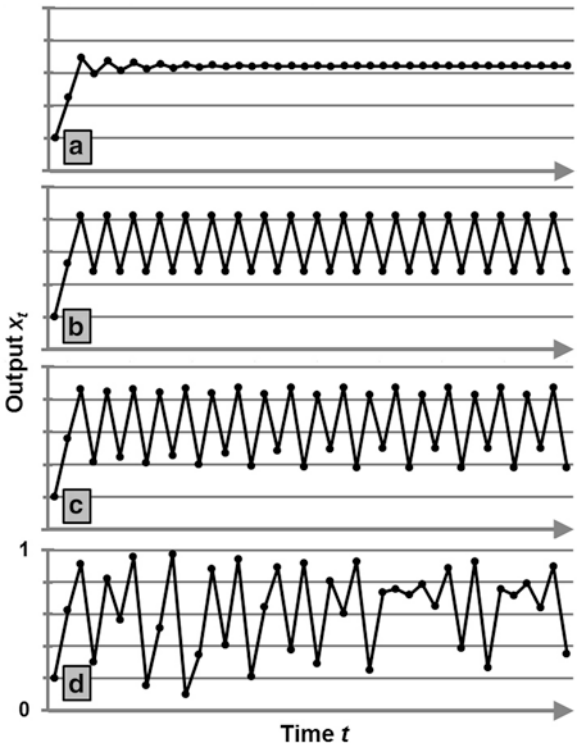

Fig. 4.3 A straightforward feedback control system produces complex output. As a increases output becomes increasingly unpredictable. With $a = 2.8$ the output converges to a constant value (**a**). The output converges to two oscillating values when $a = 3.3$ as shown in (**b**), and four values when $a = 3.5$ (**c**). Finally, when $a = 3.9$ output is unpredictable, but not random (**d**)

systems. Again, the individual control pathways are generally straightforward and well understood but can give rise to surprising patterns of heart rate changes, just as the relatively simple feedback control equation above generates complex patterns that seem to belie the simplicity of the underlying computation. HRV analyses can detect changes in theses control mechanisms, or in the ability of the heart to respond to their signals, providing new opportunities to understand human response to acute inflammation or other sequelae of critical illness or injury. In clinical applications, the challenge is to detect changes in the complex output of a system and relate them to faults in particular feedback loops to inform medical decision making in meaningful, cost-effective ways. Prior to exploring these applications below in "Applications of Heart Rate Variability in Critical Illness and Acute Inflammation," selected methods to characterize HRV by measuring subtle changes in cardiac interbeat intervals are described below.

Introduction to Techniques for Measuring Heart Rate Variability

Various techniques have been devised to measure HRV, and lack of standardization combined with the research community's healthy desire to innovate has resulted in an extensive array of HRV analyses. Many HRV measurements overlap in the

information they provide and/or the types of algorithms used. Furthermore, choice of a particular method is often determined not only by the particular phenomena or application of interest but also by available expertise, technology, and personal preference. Given the wide array of choices and a selection process that can seem somewhat arbitrary when reviewing published reports, choosing a particular technique can be daunting. This section outlines steps common to all HRV measurement methods and presents examples of selected techniques to highlight some general issues in choosing and applying particular methods. Rather than attempt to list and describe the many possible algorithms for HRV analysis (notable summaries of HRV methods, guidelines, and interpretive caveats can be found in the literature [15–19] and online [20]), several techniques are presented in detail and in order of increasing technical expertise needed to understand and apply them successfully. Finally, software tools for capturing and preprocessing EKG signals and computing various HRV measurements are briefly reviewed.

Regardless of the particular HRV measurement under consideration, analysis consists of the following general steps:

1. Data acquisition: The EKG must be digitally sampled and stored along with associated information about the subject and conditions during the recording. Signal metadata—information about the collection method itself, such as sampling frequency, signalduration, and precision—may also be stored.
2. Signal preparation: Most methods require some degree of filtering, feature detection, or data transformation prior to processing. This step may include peak detection, noise rejection, removal of ectopic beats, and so on.
3. Processing: The HRV algorithm(s) are applied to the prepared signals, yielding HRV measurements. Results can be expressed numerically, but graphs are increasingly commonplace to describe HRV as a function of *scale, frequency, or duration*.
4. Interpretation: Results are evaluated for technical, mechanistic, or clinical implications.

The first step, acquiring the EKG signal, is usually accomplished by interfacing to a commercial medical EKG monitor. These devices have become increasingly reliable, inexpensive, and straightforward to interface and can serve all but only a few special-purpose HRV analyses or applications. Signals should be captured via a digital interface to the monitor; it is usually more difficult and expensive to adequately sample analog outputs. Commercial software exists for interfacing to common monitors (e.g., solutions from Capsule Technologie or Excel Medical) and vendors increasingly provide documentation, sample software, or even built-in data export functionality via USB hardware or network connections. Various academic efforts [21–24] have described systems designed to capture, store, and/or manage EKG signals from inpatient medical monitoring systems; while such efforts may be technically beyond the scope of most HRV research, they can inform data capture and storage decisions or provide samples of EKG data for those unable to collect their own signals. Regardless of the data capture method used, it is often useful to document—and sometimes control—the conditions under which the EKG recordings are made. Generally speaking, short-term recordings should be made when the subject is in a quiet, relaxed state, undisturbed by sudden movement, abrupt changes

in noise or lighting, or other stimuli. Signals should be of sufficient length to allow for trimming artifacts that may occur when recordings are started or stopped or to allow for a sufficient baseline period. Finally, in lieu of recording EKG samples, investigators without capability of acquiring their own data can take advantage of an ever-increasing number of publicly available EKG signal libraries such as those available via the Physionet project [25, 26] and MIMIC II database [24].

Once signals are captured, most HRV methods require some degree of *preprocessing* prior to applying algorithms that will compute variability metrics. For nearly all HRV analyses, an R–R tachogram is generated: a series of interbeat intervals between successive peaks detected in the EKG (Fig. 4.1). The R-wave peak or other fiduciary point in the cardiac cycle can be used. Some HRV methods utilize integer heart rate data exported by most modern medical monitors along with the EKG waveform; while less accurate and precise then interbeat intervals extracted from high-fidelity waveform recordings, integer heart rate is considerably easier to capture, store, and process automatically, thus may be useful for preliminary analyses or when extremely large datasets must be analyzed. Rarely, the EKG waveform is analyzed directly with little or no preprocessing.

Regardless of the type of heart rate signal used, a crucial step during preprocessing is for the investigator to understand the impact of various types of noise or other artifacts on the HRV technique being used, and possibly take corrective steps. For example, algorithms vary greatly in their sensitivity to ectopic or missed beats, which may result from actual abnormalities in the subject's heart rhythm or arise from technical issues in beat detection and artifact rejection. Some of the more straightforward methods that make use of standard deviation or pattern matching algorithms are relatively insensitive to a small proportion of missing/extra beats, while spectral analyses can be significantly affected and thus require careful work in preprocessing to remove ectopic beats and interpolate missing ones. Detrending is often performed, and more complex underlying patterns (such as respiratory sinus arrhythmia, the natural phenomena of heart rate increasing slightly during inspiration) might intentionally be removed, or possibly enhanced. Ideally, trained personnel manually review the original EKG signal, R–R tachogram, and filter output to ensure data quality prior to applying HRV algorithms.

The remainder of this section describes three types of methods for assessing HRV, with illustrative examples, in order of the expertise needed to easily implement the techniques and successfully interpret results. The first techniques are straightforward *time-domain* methods that can be implemented using built-in functions of most common spreadsheet applications. Next, simpler nonlinear methods are described, which require some degree of programming but could nonetheless be implemented using commonly available functions in a spreadsheet application. Finally, frequency-domain analyses are covered, which typically require more involved programming and interpretation. Advantages and pitfalls of each methodology are presented, as are other considerations in selecting a particular analytic method such as signal length, sampling frequency, and other recording conditions. Finally, software tools for performing these and other types of HRV analyses are reviewed.

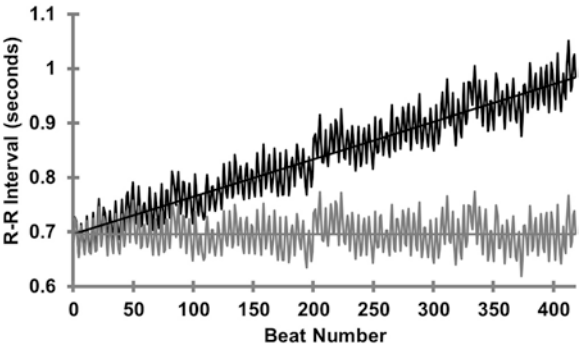

Fig. 4.4 Importance of detrending in HRV analyses. R–R intervals plotted over time showing a significant underlying trend (*black line*), and the same data after detrending (*gray line*). The standard deviation, SDNN, of the data prior to detrending is 0.089 s, compared to 0.030 s in the detrended sample

Some of the most straightforward measurements of HRV are based on standard deviation. As with many time-domain methods, the standard deviation of all R–R intervals, or SDNN, reflects overall variability irrespective of frequency (i.e., slow vs. more rapid changes in heart rate) and can be calculated simply by using built-in spreadsheet functions such as STDEV in Microsoft Excel®. It is important to note that standard deviation is influenced by signal length, especially when underlying trends are present as shown in Fig. 4.4. Thus, choice of recording duration, and whether or not to detrend the data, are important when using HRV methods based on standard deviation. Another essential aspect of choosing recording duration, regardless of analytic method, is the fact that the EKG should be recorded long enough to adequately capture the time course of variation of interest. Furthermore, recordings should be taken at times that minimize bias or noise in the signal. Practically, the example of diurnal variation highlights these points: recordings for such purposes should be at least 24 h long (ideally, exactly 24 h long), and if they cannot be recorded for 24 h at least the start/stop times should be similar in all subjects. For shorter signals, recordings should be obtained when the subject is in a relatively quiescent state (unless of course the effects of particular stimuli on HRV are the subject of the investigation). In the case of methods that rely on standard deviation, the shortest recording duration typically used is 5 min, with some methods (e.g., SDANN) dividing longer signals up into 5-min segments in order to analyze relatively short-term contributions to HRV. Some of the largest studies in HRV in clinical populations thus far have utilized standard deviation in integer heart rate over 5-min intervals, the distribution of which during the first hours of intensive care unit (ICU) stay can predict trauma patient mortality [27, 28]. Numerous other methods have been described that compute relatively simple statistics of R–R intervals, such as pNN50, the proportion of intervals that differ by more than 50 ms from each other [29].

Advantages of these straightforward time-domain methods like SDNN, SDANN, or pNN50 include the fact that they are simple to compute using everyday software and are relatively insensitive to noise unless very short signals are considered or large outliers or underlying trends are not filtered. Any noise present is likely to increase reported variability metrics; the investigator should be aware of effects of

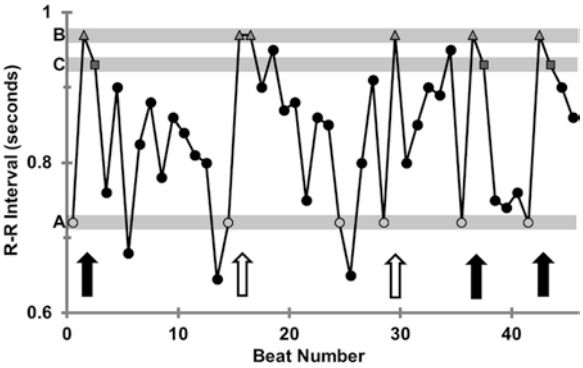

Fig. 4.5 Calculating sample entropy. First, data points are assigned based on ranges of values defined by the variance of the data series. This example shows the first three data points being assigned to ranges A, B, and C, and corresponding subsequent points are identified by *circles*, *triangles*, and *squares*, respectively. The number of A, B sequences is counted (*arrows*) and divided by the number of times the sequence A, B, C occurs (*filled arrows*), essentially measuring how predictable a third point in a sequence is based on the first two. These counts are aggregated over all possible sequences in the series and the natural logarithm is taken of the ratio to compute sample entropy

noise or other phenomena such as baseline shifts on these or any analyses, especially if EKG recordings were obtained in clinical versus controlled laboratory settings. The main disadvantage of these methods is that results inherently represent all variability in aggregate, regardless of underlying time course or frequency. As such, it is difficult to postulate about contributions to or causes of variability. Also, these methods cannot distinguish between complex and noncomplex variability, since the ordering of the R–R intervals is relatively unimportant in computing the standard deviation or other similar analyses. As the following paragraphs will describe, other methods can identify both underlying time course and complexity of HRV.

The complex, nonlinear variation of cardiac interbeat intervals has been well described [30–33], and several now-popular methods for characterizing this variability were developed by Goldberger, Peng, Costa, and colleagues at the Rey Institute for Nonlinear Dynamics in Medicine. Analytic methods include *detrended fluctuation analysis* [34] and *multiscale entropy* (MSE) [35], which complement a number of other tools developed elsewhere including fractal dimension [36], approximate entropy [37, 38], Lyapunov exponents [39, 40], and many others. This section highlights MSE, as it has been applied in several clinical domains [41–47] and illustrates two important concepts in HRV analysis: signal complexity and scale.

Signal complexity can be thought of as the degree of unpredictable variation, and measurements like *entropy* have been developed to reflect complexity in a time series based on Shannon's seminal work in communication theory [48]. Variations on the calculation of entropy have been proposed; the idea of *sample entropy* [49, 50] is straightforward to describe and comprises the entropy measurement used in MSE. Essentially, given a signal like the one depicted in Fig. 4.5, sample entropy is

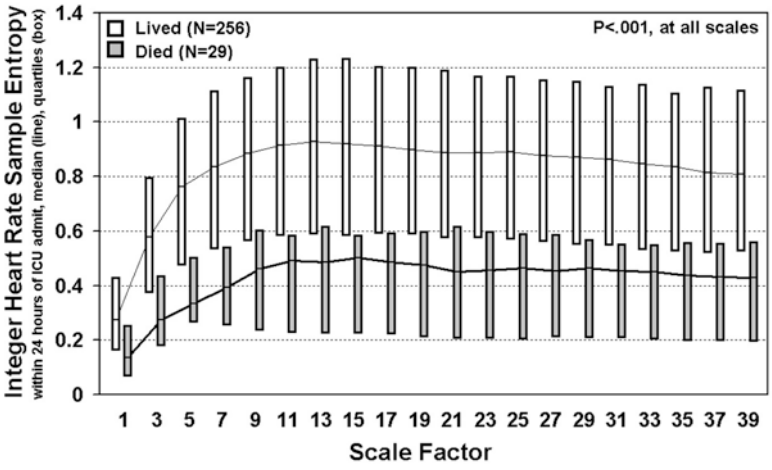

Fig. 4.6 Multiscale entropy in trauma ICU patients. When neighboring points are successively averaged and sample entropy computed as in Fig. 4.5 after each such averaging, a value of entropy for each such averaging (scale) is produced. The graph shows MSE for trauma ICU patients stratified by in-hospital mortality, using the first 24 h of heart rate data from the ICU stay. From [47], used with permission

computed for each sequence of N points by first counting the number of the first $N-1$ points that exhibit a similar pattern, and then the number of times that the last (Nth point) matches that first pattern. The figure illustrates the straightforward case of three points, where there are X matching sequences of two points (values alpha and beta), and Y matching sequences of three points (values alpha, beta, and gamma). The predictability of this and subsequent sequences in the signal can be described in terms as the ratio of the number of times it was possible for the sequence A, B, and C to occur over the number of times it actually was observed. In other words, how well does the sequence A followed by B predict C? Aggregating analysis over all possible sequences of a given length and taking the natural log of this ratio yields the sample entropy. There are several methods of entropy calculation, but the fundamental concept of assessing predictability remains the same.

Furthermore, the technique of MSE performs such measurements over multiple scales, providing an assessment of signal complexity over various time courses. MSE is simply the sample entropy computed on signals obtained following successive averaging of adjacent points. The sample entropy at scale 2 is obtained by first averaging each point with its neighbor to produce a signal with half the number of samples as the original, then applying the same sample entropy algorithm. This process is repeated as many times as desired, usually limited by the number of points available in the original signal, as the number of available points decreases by 50 % with each successive increase in scale. MSE will yield a graph of sample entropy values over different scales as shown in Fig. 4.6. Sometimes, values over multiple scales are summed to provide a single metric for comparative purposes.

Pattern-matching methods of computing HRV, such as MSE, are appealing from several perspectives. First, while they cannot be immediately implemented using a single built-in function of readily available spreadsheet programs, entropy algorithms are not overly complicated as illustrated by the above example. The computation can indeed be implemented by successive application of built-in spreadsheet functions on a series of R–R intervals. (Ideally, for more than a few signals a more automated approach would be used as provided by some of the tools mentioned below.) Furthermore, entropy differentiates between predictable and unpredictable variability and as such reflects complexity of the signal shown to be important in HRV analysis. Finally, MSE enables assessment of variability at multiple scales, which may provide insight into the time course of underlying regulatory processes of interest.

The main disadvantage of MSE and similar pattern-matching methods (at multiple or single scales) is that output is sensitive to choice of similarity criterion; successive points in a series do not have to match a pattern exactly but can vary within a range as illustrated by the dotted lines in Fig. 4.5. However, this similarity criterion is typically chosen by the investigator and computed as a function of the overall variance of the signal. Thus, signals containing significant nonlinear trends or baseline shifts can have different similarity ranges applied by the same algorithm, and thus different entropy, even if the signals are otherwise quite similar. As with any method of computing HRV, careful review of underlying data and a thorough understanding of the algorithm and impact of noise or particular signal characteristics on results are crucial to successful application and valid interpretation.

The final type of HRV analysis covered in this section assesses how well the signal matches a predefined template, or series of templates (as opposed to matching existing arbitrary patterns within the signal itself). The classic example, described in more detail below, is *frequency-domain* or *spectral* analysis using the Fourier transform. Some of the earliest frequency analyses of HRV were performed in the late 1960s on patients with heart disease patients [51], with modern spectral analysis beginning in the 1970s [52]. These techniques are still widely used and are now much easier to implement as a result of automated software, some of which is freely available and listed at the end of this section. While a number of different methods have been proposed for identifying and applying template functions to the signal (e.g., wavelets [53–55], which may better reflect instantaneous changes in HRV), the basic Fourier transform decomposes the heart rate signal into a series of overlapping sine and cosine waves of different frequencies. At any particular frequency, the constituent periodic function has an amplitude and phase that best fits the original signal. In theory, this allows underlying periodic processes that generate a signal to be isolated and characterized based on the frequencies at which they operate. In the case of heart rate, the fact that heart rate responds on different time scales to sympathetic, parasympathetic, and other inputs means that these individual regulatory processes can be identified and studied using spectral analysis. The result of these techniques is typically a power spectral density function as shown in Fig. 4.7, which plots the relative energy (contribution) of underlying constituent frequencies of the heart rate signal.

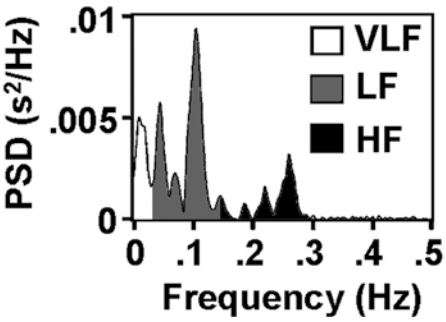

Fig. 4.7 Power spectral density function. The output of classic frequency-domain HRV analysis, the graph shows signal energy as a function of frequency. In other words, it depicts how much variation in the signal is due to rapid versus slower oscillations. Heart rate variation is typically computed as the energy (area under the *curve*) in several frequency bands, for example very low frequency (VLF, 0.003–0.04 Hz), low frequency (LF, 0.04–0.15 Hz), and high frequency (HF, 0.15–0.40 Hz). These can be different regulatory mechanisms and combinations thereof, although caution is needed in interpretation

In practice, however, application and interpretation of spectral HRV methods are nontrivial [16, 19]. Technically speaking, algorithms are complicated and require either use of third-party tools (which tend to distance the investigator from a thorough understanding of methods and effects of artifact or noise) or significant programming skills to implement. Additionally, spectral measurements can vary more than tenfold simply as a result of artifacts or outliers [56], or due to attempts to correct such imperfections [57], so high-quality signal capture and preprocessing is crucial. Results can also be impacted by other aspects of the analysis including use of zero padding and windowing, techniques to help overcome limitations of the discrete Fourier transform. In terms of sampling, the duration of recording and sampling rate inherently will determine the range of frequencies that can be studied; longer duration recordings are important for investigating lower-frequency components, and higher sampling rates are needed for exploring higher-frequency phenomena. Interpretation of results in terms of underlying regulatory mechanisms is still debated, despite well-established cutpoints to measure of power at various frequency ranges in the heart rate PSD (see Fig. 4.7 legend) [16]. Generally speaking, power in the LF band is attributed to both sympathetic and parasympathetic modulation of heart rate, while the HF band is restricted to parasympathetic modulation. The ratio of power at low and high frequency bands (LF/HF) is usually accepted to reflect relative contribution of sympathetic and parasympathetic regulation. However, comparing these measurements between subjects, or even within the same subject, is not straightforward, in part because individuals may differ in optimal frequency cutpoints. Other factors can also confound spectral analyses, including the requirement that the underlying processes responsible for heart rate variation are *stationary* during the recording interval. While the technical definition of stationarity is somewhat involved, essentially this requirement means that the fundamental regulatory mechanisms of heart rate must remain consistent during the recording, and the R–R

tachogram is free of baseline shifts, acyclic trends, and other such changes. For example, sudden exercise, an intravenous fluid bolus, or administration of a beta-blocking agent to mask sympathetic regulation would all likely introduce significant nonstationarity into the heart rate signal. Short-term EKG recordings captured under controlled resting conditions can usually be assumed to be stationary; longer-term recordings or those collected in uncontrolled environments need to be carefully evaluated and perhaps analyzed piecewise around known perturbations.

Despite these difficulties, spectral analysis remains commonly used for HRV assessment and can discern different relative contributions of regulatory mechanisms (e.g., sympathetic versus parasympathetic) to heart rate variation in a given recording. However, newcomers would be well served to also consider other, simpler methods of HRV analysis that may be less vulnerable to noise or misinterpretation, and to proceed carefully in use of available tools that automate spectral analysis (or other HRV metrics). A few commonly available resources and software tools are listed in the following paragraph, which can simplify preprocessing and analytic steps of various HRV methods including those described above.

Prior to downloading software or even collecting EKG recordings, newcomers to HRV analyses would be well served to consult more-detailed review articles and reference sources for additional guidance on choosing and applying method(s), some of which are cited herein. Once a particular method or methods are selected, investigators can select from a number of readily available software tools and should also weigh the costs and benefits of developing processing algorithms themselves. The first step in using any software tool (whether written by the investigator or obtained from a third party) is to validate use of the software by processing an existing dataset with known HRV characteristics and verifying output. The Physionet project [26] contains a wealth of data, software, and guidance for HRV analysis and should be a first and frequent destination for anyone pursuing HRV analyses. It contains not only freely downloadable datasets, open-source tools, and reference materials but also news on related research activities. However, many of the tools available from the Physionet site require a modicum of technical expertise to compile or run on common PC platforms. Numerous other toolkits, both open source and proprietary, are available, some of which provide "point-and-click" ease of installation and EKG processing. For example, the Kubios HRV software package [58, 59] based on MATLAB® (but not requiring MATLAB to run) can be downloaded, installed, and running in just a few minutes by someone with no software engineering skills. Kardia [60] is a similar tool, also written using MATLAB, and many other HRV tools or function libraries have been written for this popular platform (e.g., Ecglab [61], POLYAN [62]). At least one package is available for the popular R statistical package, RHRV [63]. Finally, commercial offerings are emerging such as CIMVA [64], a package targeted for use in clinical applications to analyze variability from multiple organ systems including HRV.

The proliferation of online HRV software, datasets, and technical guidance has significantly lowered technical barriers to HRV research. Investigators studying acute inflammation and other pathophysiologic processes can easily augment their research projects with HRV analyses, especially if EKG capture is part of existing

experimental protocols. The HRV techniques highlighted in this brief introduction are representative starting points for further study; regardless of method, the most important factor to success is the investigator's understanding of how the particular analytic technique produces a measurement that reflects underlying phenomena of interest. Knowledge of how the measurement is affected by noise, atypical signals (especially signals that were not examined or considered by original developers), and how comparisons within or between subjects may be biased by technical or experimental factors, is likewise crucial for many studies. Ultimately, continued research and successful application of HRV may provide clinicians with powerful new tools to monitor patients and inform therapeutic decision making. The following section highlights selected efforts toward this objective, focusing on the domain of acute inflammation and related areas of critical illness.

Applications of Heart Rate Variability in Critical Illness and Acute Inflammation

Practically, the goal of applying HRV to care of patients suffering from acute inflammation or other critical illness is to be able to efficiently inform meaningful healthcare decisions. In other words, the effort to implement the novel HRV measurements, including research and development work, device technology, regulatory approval, provider education, maintenance, and other costs, must be outweighed by the value of improved medical decision making attributed to HRV. While this goal remains largely unrealized today, a number of efforts promise to ultimately bring HRV to the clinical bedside, perhaps in concert with other novel patient monitoring or clinical processes. After briefly discussing the rationale for HRV's potential utility, this section describes recent work to explore and develop such applications in inflammation and three overlapping clinical domains where inflammation may play a major role: infection, trauma, and organ failure.

The basis for HRV's utility in these and other areas is the autonomic nervous system's role in mediating processes crucial to response and recovery from physiologic insult. As described above in "The Biological Basis of Heart Rate Variability," HRV can reflect both parasympathetic and sympathetic regulation, among other things. Thus, changes in autonomic activity might be detectable as alterations in HRV. Assuming such changes exist and are indeed measurable with sufficient sensitivity and specificity in the heart rate signal, the body's autonomic response to insult could be measured, thus allowing clinicians to characterize the potential impact on an individual case-by-case basis. Patients who exhibit a relatively large response to a small insult might be managed differently, as might patients who exhibit a relatively small autonomic response to a large pathophysiologic event. Furthermore, the first clues to impending patient deterioration might be provided by changes in sympathetic or parasympathetic activity. HRV provides a noninvasive way to potentially identify significant changes in autonomic function, using a commonplace signal which is routinely and inexpensively captured in most clinical settings.

HRV measurements, some of which were covered above in "Introduction to Techniques for Measuring Heart Rate Variability," have been studied in humans and animal models since the late 1960s. Early human studies were performed in heart transplant and myocardial infarct patients, which remain active areas of research today [65, 66]. These efforts were focused on characterizing cardiac abnormalities and raise an important point: changes in HRV may not be due to systemic regulatory processes, but due to changes in the ability of the heart itself to appropriately respond to neural and other inputs. Thus, cardiac abnormalities can certainly confound applications of HRV focused on characterizing systemic processes. Fortunately, in many patient populations such abnormalities are uncommon and usually detected early in the course of care, if not already known from the patient's medical history. Despite the potentially confounding impact of cardiac abnormalities and other factors, diverse efforts have successfully illustrated the potential of HRV to provide relevant information in a number of pathophysiologic states.

The inflammatory response, for example, is now known to be tied to autonomic nervous system activity. Animal studies, in which vagal nerve activity is artificially stimulated [67–69] and/or interrupted [70], have clearly defined this correlation. One underlying mechanism, the cholinergic anti-inflammatory pathway and corresponding afferent inflammatory signals, in which parasympathetic activity inhibits cytokine release and resulting inflammation has been well established through decades of work by Tracey and colleagues, and other groups [67, 70–74]. Clinically, isolating effects of acute inflammation on HRV in humans is challenging as the inflammatory response is usually accompanied by varying uncontrolled stimuli in patient populations (e.g., trauma, infection, or organ failure). However, use of an endotoxin challenge in both humans [75–80] and animal models [81, 82] to induce (or stimulate) an inflammatory response clearly demonstrates reduced HRV as part of this response. Additionally, study of individuals suddenly exposed to changes in pollution levels [83, 84], other environmental particulates [85], or vaccine [86] confirm effects on HRV in a variety of inflammatory responses. Finally, changes in HRV have been correlated with a number of inflammatory markers such as C-reactive protein, elevated leukocyte counts, and cytokines in various populations [87–96]. The above examples highlight an impressive body of evidence supporting the use of HRV to monitor ongoing response to inflammation. Potentially, another exciting future application would be to use HRV to monitor effects of direct vagal stimulation to appropriately modulate inflammatory response. However, prior to widespread clinical application, the efficient and meaningful use of HRV for these purposes must be established. Possibly, the cholinergic anti-inflammatory pathway and inflammatory reflex are responsible for the relationships between HRV and outcomes seen in clinical populations, described in more detail below.

One of the earliest clinical applications of HRV to critical care was the study of traumatic brain injury (TBI) by Lowenshon in 1977 [97]. In more than three decades since then, reduced HRV measured in the ICU has been associated with poor outcomes in larger TBI adult [98–100], and pediatric [101] inpatient populations, and similar associations have been noted when HRV is measured from TBI patients in the emergency room [102, 103]. In populations of patients with diverse critical

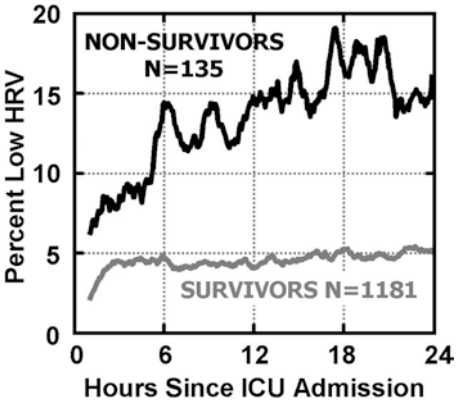

Fig. 4.8 HRV in the first 24 h of trauma ICU care by outcome. Specific ranges of the distribution shown in Fig. 4.8 most predictive of mortality were used to define a critically low range for standard deviation of integer heart rate. The percent time each patient spent in this low range, from 0.3 to 0.6 beats per minute, was measured each hour then averaged over all patients and plotted by hospital mortality. Patients who ultimately did not survive show increasing proportion of heart rate variability in this critically low range in the first day of their ICU stay. From [27], used with permission

injuries, HRV measurements early [41] and throughout the ICU stay [104] or during prehospital air medical transport [105, 106] are associated with patient mortality, and these relationships are relatively unaffected by degree of injury or mechanism [44]. Figure 4.8 shows how the difference in average HRV potentiates over the first day of ICU stay in a large population of trauma patients. However, the reader is cautioned that HRV of individual patients typically varies substantially, and in this population there is significant overlap in HRV measurements from survivors and nonsurvivors. Work at the US Army Institute of Surgical Research has characterized HRV in trauma patients during prehospital transport, animal models of injury [107–109], and in a human hemorrhagic shock model where lower body negative pressure is induced [110, 111]. Many of the HRV references from the Army Institute include comprehensive analyses incorporating a wide variety of HRV techniques, and thus may be of particular interest to the newcomer looking to compare methods. While inflammatory mechanisms are certainly relevant in severe injury, especially closed head injury, it is likely that other regulatory processes are at least partially responsible for the observed effects on HRV. For example, baroreflex activity and other circulatory regulatory mechanisms incorporate sympathetic or parasympathetic feedback. In the case of severe brain injury, autonomic tone may be compromised due to central defects, or patients may have spinal or other injury that severs the vagus or other neural pathways. These phenomena are particularly relevant to the study of inflammation using HRV in trauma patients, especially TBI patients [112], as successful clinical application for inflammation monitoring may need to account for confounding effects of injury.

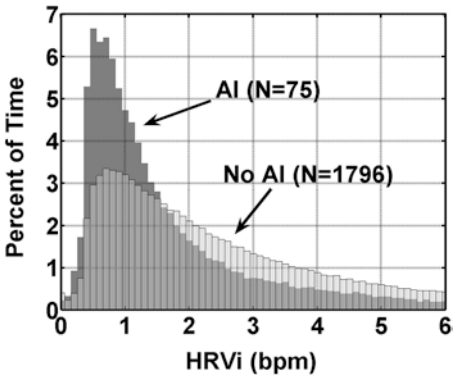

Fig. 4.9 HRV in trauma ICU patients with adrenal insufficiency. Patients with adrenal insufficiency exhibit lower HRV (measured using methods similar to Figs. 4.8 and 4.10). From [120], used with permission

Changes in HRV related to inflammatory processes may be more easily observed in patients who have not suffered physical trauma, or whose autonomic activity has begun to normalize following injury or acute illness. In these cases, unexpected changes in HRV might be more attributable to inflammatory processes associated with infection [113] or organ failure [114]. Changes in HRV can predict septic shock and in-hospital mortality in emergency department patients with sepsis [115, 116] and has shown promise to predict sepsis and outcomes of sepsis in several other adult patient populations [92, 117, 118]. Similarly, HRV has been shown to change with organ failure [119, 120] and is predictive of outcomes in these populations. For example, Fig. 4.9 shows differences in integer HRV among trauma patients with and without adrenal insufficiency. The key differences in these distributions are similar to those differences observed early in the trauma ICU stay between survivors and patients who ultimately die (e.g., Fig. 4.10). While these and many other efforts have illustrated that changes in HRV may reflect various infectious and/or inflammatory processes in patient populations, the clinical value of such measurements remains largely unproven, with a notable recent exception in neonatal monitoring. Beginning nearly two decades ago, Moorman and colleagues at the University of Virginia began measuring HRV in neonatal ICU patients [121]. Recently, they published results from the first randomized multicenter trial to define the value of HRV in predicting neonatal sepsis, in which use of HRV monitoring technology resulted in one life saved for each 22 patients monitored [2]. The history of this effort, from initial observations to development of technology and methods, to rigorous clinical trials and commercialization [49, 122–124], illustrates the challenges of establishing efficient and meaningful measurements of HRV. More importantly, they show how these obstacles to applying HRV at the clinical bedside can be successfully overcome.

Diverse efforts, only a handful of which are mentioned here, have and will continue to define HRV's utility in diagnosing and treating acute inflammation and related pathophysiology. The studies mentioned above, and the vast majority of work in applied HRV, clearly show that decreased HRV is associated with infection, poor outcomes, and/or pathophysiologic response. However, it is worth noting that

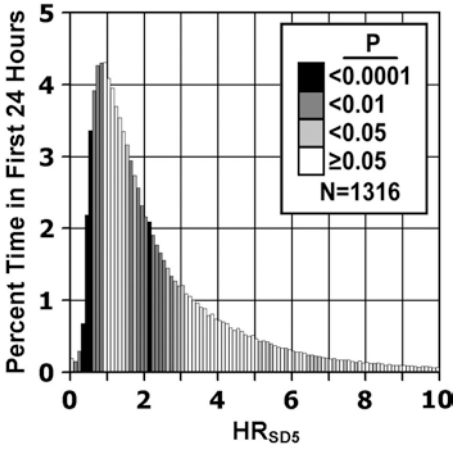

Fig. 4.10 Distribution of a simple HRV metric in trauma ICU patients. The standard deviation of integer heart rate was measured in 5-min intervals (HR_{SD5}), during the first 24 h of trauma ICU care. Each *bar* represents a one-tenth range of HR_{SD5}, e.g., 0.3–0.4 beats per minute. Shading represents statistical significance of a logistic regression model using the percent time within that range as the only input variable and hospital mortality as the outcome. From [27], used with permission

some evidence exists to the contrary [125] or suggests that HRV undergoes transient increases and/or decreases prior to a longer-term response. For example, data from animal models suggest that HRV may increase initially for a short time following a sudden infectious insult [126]. Potentially, early, rapid modulation in the cholinergic anti-inflammatory axis in response to a large bacteria inoculation explains this effect. It underscores the fact that decreased HRV may not *always* be the earliest sign of poor prognosis; in certain situations, an increase in HRV could signal early response to severe insult. Thus, standardizing the time at which HRV measurements are made relative to particular phenomena of interest is crucial. Ideally, a consistent reference point can be selected across the study population, and HRV measurements made continuously before and after that point to characterize the entire time course of any HRV response, whether increased or decreased, transient or long term. Ultimately, as technology and methods continue to improve, HRV will become easier to continuously measure, interpret, and apply to a wide variety of clinical and research problems. The challenge will be to *prove* the value of these new applications, amidst an ever-growing milieu of data and other innovations that promise to hasten our understanding of acute inflammation and related clinical pathophysiology.

Conclusions

As noted above, evidence is growing for HRV's utility in acute inflammation and other areas of critical illness. A wealth of methods and tools have been developed to measure HRV, many of which are now easy to apply without extensive technical knowledge. However, much work remains to determine if findings can be substantially validated and incorporated into clinical practice. With the possible exception of neonatal ICU physicians, today's critical care practitioners use HRV no more regularly in bedside decision making than those who marveled at the early EKG tracings

from Einthoven's galvanometer. Nonetheless, the role of autonomic regulatory mechanisms and associated opportunities for diagnosing and even treating pathophysiology of acute illness are increasingly apparent. In clinical practice (e.g., the use of beta-blockade in head injury patients [127]) and research (e.g., cholinergic anti-inflammatory axis mentioned above), the monitoring and modulation of autonomic activity has clear implications for patient care aside from the ability of HRV to predict clinical outcomes or monitor autonomic activity. The difficult next step is to reliably associate changes in HRV with specific—and potentially treatable—derangement of various regulatory mechanisms in real time, at the clinical bedside.

Taking the crucial step of translating HRV research findings into practice will require new approaches and continued collaboration among and between scientists, clinicians, and engineers. Key challenges include understanding and accounting for confounding effects of healthcare processes; potentially, mechanical ventilation [128–130], medication [102, 131, 132], nursing procedures [133], and a host of other factors affect HRV. Furthermore, individual patients may exhibit different HRV characteristics due to genetic differences [134, 135] or other phenotypic variation. Traditional reductionist strategies are unlikely to efficiently address these and other issues, not only due to the complicated array of possible confounders and their interactions (i.e., the curse of dimensionality) but also because of the complexity inherent in the underlying systems. A more fruitful approach for unraveling such systems may be to identify particular *classes* of output that define underlying states of the system. Returning to the example of Fig. 4.3, rather than focusing on individual values of the output per se, it may be more informative to understand of the effects of changing the multiplier a on the patterns of output observed. Physiologically, the notion of discrete states [136], and transitions [137] through these states, is a hallmark of conceptual models proposed over the years that apply complex systems approaches to understanding inflammation and other pathophysiology of acute illness [138–140].

Can HRV identify these states in a meaningful way? For the first time, large multiscale models are being developed that represent complex physiological variability in clinically important domains like inflammation [141, 142]. It is clear that HRV will play a role as these models move from concept to application, especially considering HRV's ability to provide rapid, noninvasive, assessment of autonomic function in some scenarios. However, the information reflected in HRV—central autonomic regulation, neural transmission of these signals, and cardiac rate response—is almost certainly insufficient to define all states of interest. Thus, HRV's utility to understand and potentially treat pathophysiology will be maximized when used in concert with other measurements [143] and efficient models that use these data to define states, and in turn to map these states and their transitions to clinically meaningful decisions. Increasingly, efforts to measure and apply HRV will occur in conjunction with nascent work to produce clinically relevant models reflecting the complexity inherent in most biological processes. In doing so, scientists, clinicians, and engineers may together strike a new, more efficient path from measurements to models to meaningful clinical decisions, broadening our understanding of acute inflammation and other pathophysiology of critical illness.

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Chapter 5
Analysis of Ventilatory Pattern Variability

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Introduction

"Variability is the law of life …" was written by William Osler in 1903 [1]. This was written 5 years before William Gosset introduced the *t*-test to determine significance in the differences between two sample means. The recognition of variability

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as a fundamental property of biologic systems was stated by Osler in the context of "individualized" medicine but has progressively led to the development and utilization of tools to measure variability and to improve—not only beer—but also health. Chapter 4 introduced the concepts of physiologic (especially heart rate) variability. In this chapter, we examine the tools that we recently developed to assess variability and quantify ventilatory pattern variability (VPV).

Heart rate variability (HRV) was the first characterized physiologic system in which variability could be considered as "a law of life." Hon and Lee (1963) associated decreased HRV with increased mortality in fetuses [2, 3]. But even here, ventilation and respiration could be considered. In identified at risk infants, the heart rate was steady without a respiratory sinus arrhythmia (RSA). This has been verified in frequency analyses examining the frequency component of heart rate using the power spectral density (PSD) of heart rate. Decreased HRV and absence of RSA is apparent as the loss of the high-frequency (HF) component in the PSD. Thus, decreased HRV and HF component of PSD depends on the magnitude of respiratory modulation of vagal nerve activity [4–12]. But, does VPV influence cardiorespiratory coupling? On the other hand, does VPV like HRV reflect changes in neural control of the cardiorespiratory system? These questions determined our interest in quantifying VPV.

Clinical Relevance for Quantifying Ventilatory Pattern Variability

Ventilatory pattern variability changes with aging and health status [13–18]. In their classic studies, Tobin and coworkers distinguished variability in patterning from "normal" ranges ventilation in noting changes in the coefficient of variation were present even when mean values of frequency and tidal volume were different from normal [16, 17]. These initial studies have been replicated and developed by Tobin and others in the field. Variability in ventilation decreases in restrictive lung disease and is associated with dyspnea [19, 20]. In contrast, increases in respiratory pattern variability have been observed in patients with obstructive lung disease [20–22]. In an example study, ventilation in humans was measured using inductance plethysmography and the variability in breath-to-breath tidal volume (VT) was calculated. Using the coefficient of variation (CV) of male subjects ($n=26$) without pulmonary disease as a reference [CV = (standard deviation/mean) × 100] for VT was 26 ± 7.5 % (mean ± SD); they reported that the CV was less in patients with restrictive lung disease (CV = 17.5 ± 4.6 % in old pulmonary tuberculosis and 18.9 ± 9.3 % in pneumonitis; $n=17$); and greater in patients with obstructive lung disease (CV = 43.2 ± 13.0 % in pulmonary emphysema with hypercapnia, 33.0 ± 7.5 % in normocapnia and 35.8 ± 9.4 % in asthmatic attack; $n=29$) [20]. Differences in CV between patients and subjects without lung disease were significant; showing decreased versus increased VPV in patients with restrictive versus obstructive lung disease. Thus, variability changes in the respiratory pattern, whether an increase or a decrease, reflect the development of disease.

Changes in the variability of ventilation are also indicative of disease processes even when the lungs are not the primary site of pathology. Classic examples involving the controller and sensors are Cheynes–Stokes [23–26] and Biot's breathing [27–31] as well as sleep-disordered breathing [32–36]. These ventilatory patterns result from changes in the gain of sensory input from the effect of state of consciousness on the central pattern generator. Cheynes–Stokes respiration is a waxing and waning of ventilation and the pattern can be replicated by increasing circulation time and increasing the gain of the chemosensory inputs [23–26]. Types of anxiety disorders have been associated with disordered breathing marked by increases in variability [37–39]. Finally, recent emphasis on genetic diseases, like Rett's, and Prader–Willi syndromes have increased awareness of ventilatory variability in characterizing the deficits in these diseases [40–52]. Recently, we, as part of a larger consortium have hypothesized that VPV could complement HRV as a biomarker in septic and acutely ill patients [53, 54].

Recording the Ventilatory Pattern

Quantifying variability, particularly distinguishing stochastic from nonlinear deterministic sources variability, is a developing field. First, the advent of powerful personal computers with enhanced multicore processors and increased random-access memory address space, providing the capability to handle long strings of data have proven critical in this development. Second, the ventilatory pattern lends itself to this analysis with limitations and opportunities that are distinct from the frequency analysis of HRV. The primary limitation is that the respiratory rhythm is about 4–5 times slower than heart rate so an adequate stable sequential data set can be difficult to record. The electrocardiogram is the electrocardiogram, a consistent signal or event independent of electrode placement. However, the ventilatory pattern can be recorded many different ways and the method of recording ventilation can influence the pattern. Further, the analysis depends on the signal characteristics. Common methods for recording ventilatory patterns measure (1) *airflow*, which is more accurate if the airway is intubated or covered with a tight face mask than using the less invasive method of a nasal thermistor or a newly developed noninvasive photoplethysmogram [55], (2) *chest wall/abdominal circumference*, using inductance band plethysmography [56], (3) *ambient pressure* increases in a closed chamber that result from the heating of tidal volume from room to body temperature using whole-animal, flow-through plethysmography (a common research tool for small animals but typically not for humans), and (4) *neuromuscular signals*, phrenic electroneurogram, (ENG) or diaphragmatic electromyogram (EMG) represent a "fictive" ventilation or respiratory motor pattern. In the *in situ* brainstem-spinal cord preparation (a common research model to study the neural control of respiration), these primarily inspiratory signals have a long silent during expiration which would bias the analysis. In this model, activity from the vagus nerve can be analyzed because it has activity

throughout the cycle because it is a compound motor nerve, which innervates both laryngeal abductors and adductors. The laryngeal abductors are active during inspiration; the adductors, during early expiration. Given this, we present our approach as it was applied first to an "integrated" (full-wave rectified and smoothed using a 100-ms sliding window) diaphragmatic EMG signal and then to ventilatory waveform as recorded from the whole-animal, flow-through plethysmographic chamber.

Analytic Approach

Variability is a rhythmic signal and has both stochastic and deterministic sources [57]. While these components of variability can neither be measured directly nor completely separated, we have developed an approach based upon comparing the original data set to surrogate data sets that abolishes the non-linear but retains (autocorrelation) serial dependency between points. In this way, we can attempt to distinguish linear stochastic from nonlinear deterministic sources of variability. Thus, through a consistent and disciplined approach incorporating multiple methods and suitable surrogate data analysis, a comprehensive understanding of signal variability and its relationship to health and disease can be achieved.

Our overall goal is to understand how different components of biological variability interact during health and disease using a balanced application of computational methods while recognizing the strengths, assumptions, and limitations of each approach. For example, reliance on a single technique may lead to overinterpretation [58], whereas a comprehensive view enables the formation of mechanistic hypotheses that evaluate the sources of variability more fully. To help formalize this process, we define a set of conceptual properties, each describing an aspect of variability (Fig. 5.1). Our ability to characterize these properties of biologic variability depends on the development of analytical tools that are used to assess these properties (Fig. 5.1). Rather than use a single approach, we apply multiple complementary tools to a data set to assess VPV.

Distribution properties addresses the question "Is the dispersion of point within the data set independent of temporal relationships?" Thus, signal variability is assumed to be stochastic and common measurements such as mean, standard deviation, and coefficient of variation (variance-based measurements) are used to characterize statistical properties of the distribution. This approach is traditional and has evolved over the past 100 years.

Linear properties asks the question "Are the temporal and frequency relationships defined by a linear-affine model of the form $y = ax + b$?" Measurement tools include autocorrelation, single- and multiorder histograms, Poincaré plots (circle-return maps), and power spectral density.

The Poincaré plot is commonly used to display dynamic oscillator behavior. In the Poincaré plot, a characteristic in the current cycle (n) is plotted against that characteristic in the next ($n+1$), or previous ($n-1$), or any constant ($n+x$) cycle.

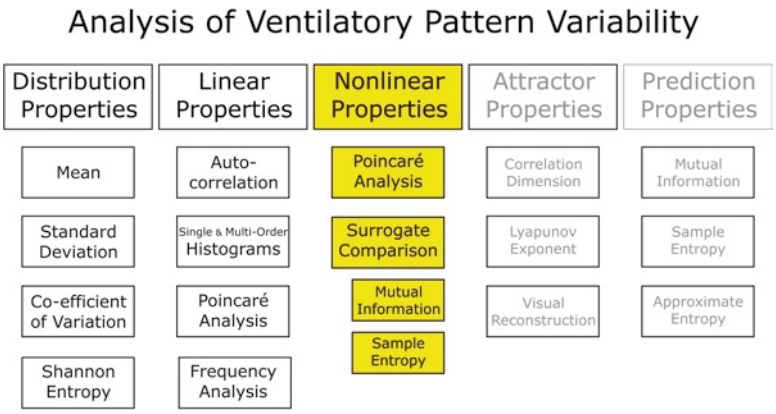

Fig. 5.1 Properties of biologic variability and analytic tools that assess these properties. Distribution properties: the dispersion of the data set independent of temporal relationships between data points. The variability in the signal is assumed to be stochastic and uses common measurements such as mean, standard deviation, and coefficient of variation as well as Shannon Entropy. Linear properties: temporal and frequency relationships that are linear. Measurement tools include autocorrelation, single- and multiorder histograms, Poincaré plots (circle-return maps), and power spectral density. Nonlinear Properties (highlighted in *yellow*): deterministic temporal relationships; for example, repeating patterns in the data. Poincaré analysis provides insight into temporal pattern variability. Distinguishing nonlinear properties depends on generating surrogate data set that accounts for the linear properties of the original data set. This boot-strapping method compares the original to surrogate data for mutual information and sample entropy. Attractor properties: reconstruction of the attractor (if one exists) and characterizes invariant measures such as correlation dimension and Lyapunov exponents. Prediction properties: characterization of the amount of information contained in the measurements and time series using information theory. Adapted from [57]

Thus, it reveals recurrence, self-similarity, or periodicity over time and provides both qualitative and quantitative information about the data set [59].

Nonlinear properties (Fig. 5.1, highlighted in yellow) describe deterministic temporal relationships; for example, repeating patterns in the data. We have worked to develop tools that distinguish nonlinear properties. The approach is to generate surrogate data sets that retain the linear autocorrelation properties of the original data set. In other words, a boot-strapping method using the same amplitude distribution of the original data set in combination with the iterated amplitude adjusted Fourier transform (iAAFT) that progressively narrows the discrepancy in the autocorrelation function. Thus, the surrogate data sets account for the linear autocorrelation properties of the variability in the original data set. Then, to identify nonlinear determinants of respiratory pattern variability using mutual information (MI) and sample entropy (SampEn), we compare results for the original to surrogate data sets. We expect a low amount of nonlinear variability with neighboring data points, where $\tau = 1$. In our analysis, τ is the time delay, a multiple of the sampling interval and, thus, depends on the sampling frequency. To compare across time, respiratory rates, and animals, we normalized the difference in SampEn by dividing this value

by the number of τ in which the difference is significant. We refer to this value as the nonlinear complexity index (NLCI).

We have also developed a tool to examine temporal pattern variability (TPV) in Poincaré plots. Previously, analytical tools required a scatter plot that formed a single cluster of points, did not account for overlapping points, and measured simply the length of the major (parallel to the line of identity) minor (perpendicular to the line of identity) axes of a hypothetical ellipse. Our analytical approach accounts for multiple overlaying points, allows multiple cluster analysis, and provides insight into nonlinear properties. TPV is calculated point by point, capturing time-dependent information in a sequence of values. Time-delayed TPV (TPVTD) measures the similarity of an interval to a successor, where average TPV (TPVA) measures the similarity of an average of multiple intervals to a successor average [59].

Attractor properties (Fig. 5.1, gray to indicate that these analyses will not be presented thoroughly in this chapter): reconstruction of the attractor (if one exists) and characterization of invariant measures such as correlation dimension and Lyapunov exponents. We and others have used the correlation dimension, which is an index of pattern variability that quantifies the degrees of freedom required to capture the dynamics of the system on an attractor [60]. Although we have used correlation dimension as a complementary analysis to verify an increase in deterministic variability measured by SampEn in the fictive ventilatory pattern after vagotomy [6], we have not used it extensively for VPV.

Prediction properties characterize the amount of information contained in the measurements and time series using information theory. As stated under Nonlinear Properties, we have applied MI and SampEn in the context of distinguishing nonlinear properties by comparing original data to surrogate data sets. It is worth noting, that in our analyses, the absolute values for these measures have been shown to have meaning.

Mutual Information

Mutual information (MI) quantifies the statistical dependence in a time series (see Fig. 5.2). In Fig. 5.2a, we graphically explore MI and illustrate how knowing coordinate $x(t)$ (presented as yellow dot) reduces the amount of uncertainty associated with a time-shifted coordinate $x(t+t)$ (Fig. 5.2a). In our application, τ is equal to a number of samples from $x(t)$ and since the waveform is sampled at a constant rate, τ is related to time and to a portion of the average cycle length. We present three representative time intervals $\tau = 1$, the next point, which is highly linearly correlated to $x(t)$ or the reference point (Fig. 5.2b1), $\tau = 20$ or the first minimum (Fig. 5.2b1), and $\tau = 30$ or the first regional maximum before the cycle length (Fig. 5.2b1).

MI is distinct from the autocorrelation function in that MI quantifies statistical dependence and not simply autocorrelation. Hence, both linear (e.g., autocorrelation) and nonlinear correlations contribute to MI, which could also be defined as the similarity between points as a function of the time interval or τ between them.

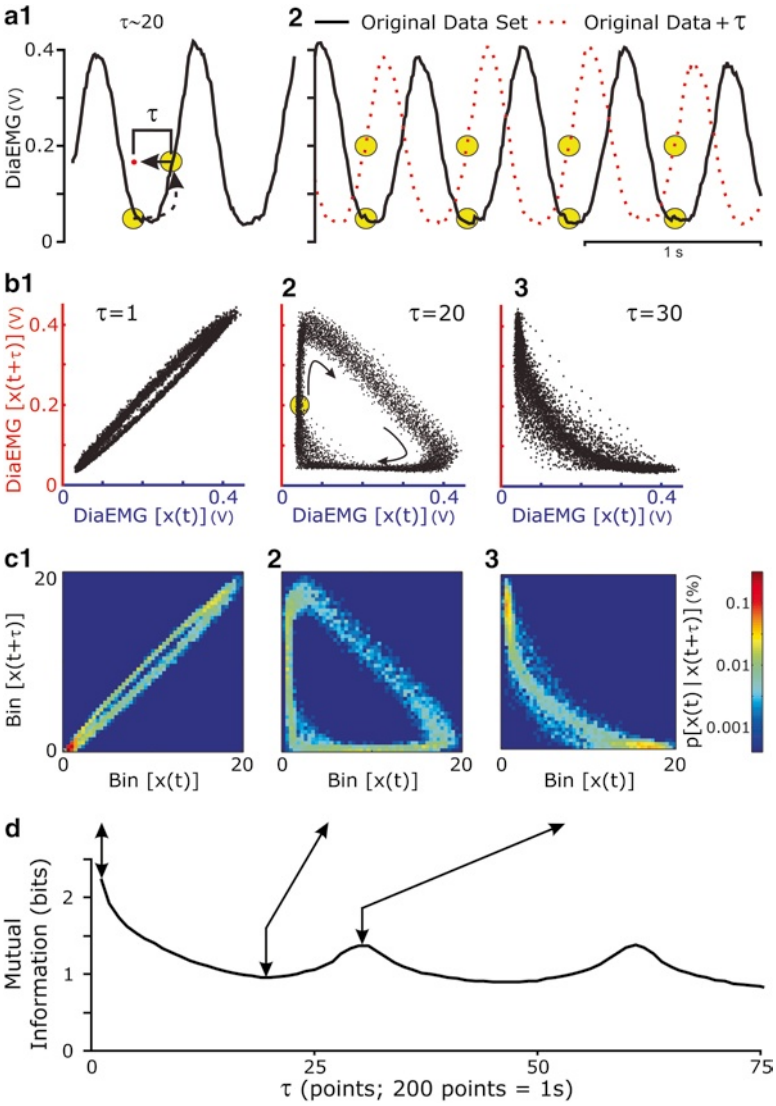

Fig. 5.2 Determination of mutual information (MI) across multiple time delays (τ). (**a1** and **2**) Representative tracings of original data of "integrated" diaphragmatic electromyogram (DiaEMG) from a spontaneously breathing rat. For MI, a time-delayed series is constructed from the original data set by first (**a1**) determining the value of a point at time $t+\tau$ and then (**a2**) shifting this time series to the left by τ points and aligning the points at time τ. This is done for all τ from 1 to 1-cycle length; in this example, τ is equal to one-third of the cycle length, when MI is at its first local minimum. (**b1–3**) Return maps from the original and time-delayed data sets at three representative τ's are: (**b1**) $\tau=1$, (**b2**) $\tau=20$, (**b3**) $\tau=30$ or approximately ½-cycle length. (**c1–3**) To compute MI, return maps are divided into bins to generate a two-dimensional probability histogram. Individual probability distribution functions are generated by summing each column or row for the original or time-delayed time series, respectively. Thus, a single measurement of MI summarizes the histogram. (**d**) The computation is repeated for all τ from neighboring points (5 ms, 200 Hz) to those separated by 1-cycle length. Adapted from [6]

As we published in our adaptation of MI [6], its equation is:

$$\mathrm{MI}[x(t), x(t+\tau)] = \sum_i \sum_j P[x_i(t), x_j(t+\tau)] \log \left[\frac{P[x_i(t), x_j(t+\tau)]}{P[x_i(t)] \bullet P[x_j(t+\tau)]} \right]$$

where $P[x(t), x(t+t)]$ is the joint probability of $x(t)$ and $x(t+t)$ (in Fig. 5.2c, the joint probability distribution function is plotted color coded, on a logarithmic scale) and $P[x(t)]$ and $P[x(t+t)]$ are the marginal probabilities of $x(t)$ and $x(t+t)$ for the original or time-shifted time series, respectively. We begin the computation by constructing a return map (Fig. 5.2b) from the original and time-shifted time series. Then, we plot the joint probability distribution function by dividing the graph into bins and determining the frequency of points in a given bin (Fig. 5.2b). We use a constant number of bins (40 bins) and scale bin size by the variance of the data set. The individual probability distributions are determined by summing each column or row of the joint probability distribution for $x(t)$ or $x(t+t)$ time series, respectively. Individual probability distributions of the original and time-shifted, time-series data sets are the same. We use the MI equation to generate graphs for MIs for τ equal to 1 (neighboring points) to 1 cycle length.

Sample Entropy

Sample entropy (SampEn) is a measure of a pattern's predictability and provides an index of reproducibility within a signal (see Fig. 5.3) [61–63]. Specifically in time-series data, the "order" of the points or samples matters and can become predictable in rhythmic oscillations. This deterministic quality in the data waveform is not captured by mean and variance so repeated patterns in the data have to be identified using techniques designed to capture them. SampEn is a technique used to quantify the amount of regularity or "self-similarity" in time-series data. We apply SampEn to measure the repeatability of a 3-point template in the respiratory pattern, the simplest; but a template consists of "m" points plus one more point that has to "match" the pattern's criteria. In this approach, we construct the template 2-point base (each separated by a time interval, τ) (Fig. 5.3a, $m=2$, and are represented by red and green squares separated by a $\tau=10$. See the black-outlined rectangle located at the start of the time series). Then, this and subsequent possible template matches are identified based on these first two points (Fig. 5.3a, red and green points). A pair of points is considered a potential template match if its member points are within a tolerance "r" of the corresponding point in the original template. Next, an additional point (Fig. 5.3a, black square within the black-outlined rectangle) completes the 3-point template. Then the set of potential matches is searched with this extended template of $m+1$ points (Fig. 5.3a, each series of a red, green and black point). This searching algorithm is repeated for all possible starting points to compute the total number of m-point "potential template matches" (Fig. 5.3a, red and green boxes) and $(m+1)$-point matches (Fig. 5.3a, green boxes). Thus, SampEn is computed as a ratio of the number of template-matches to the number of potential template-matches, excluding self-matches.

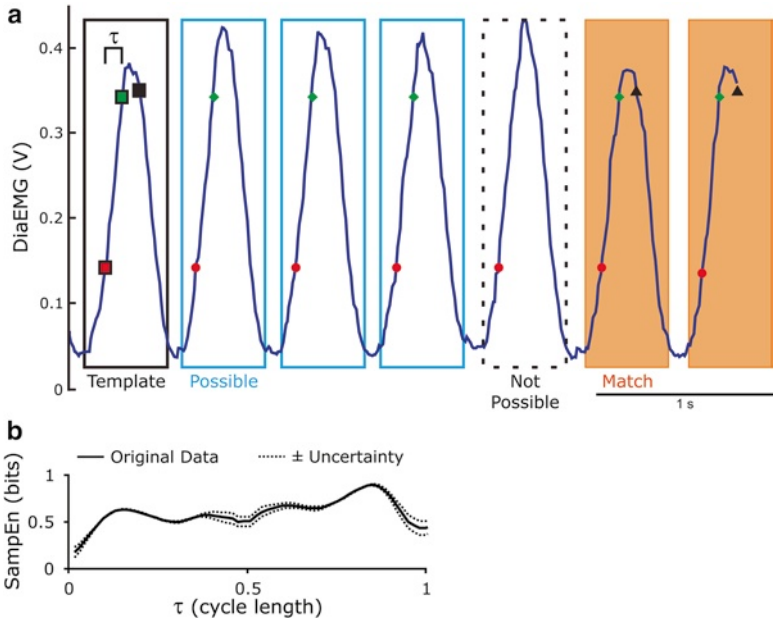

Fig. 5.3 Determination of sample entropy (SampEn) across multiple time delays (τ). (**a**) Superimposed on a representative tracing of DiaEMG is a template of m points (each separated by t) (*red* and *green squares* within the *black box*; $m=2$ and $\tau=10$). Then, the time series is searched to identify possible template matches (each series of *red* and *green points*). A pair of points was considered a template match if its member points were within 20 % of the standard deviation of all points in the epoch relative to the corresponding point in the original template ($r=0.2$ XSD). If the point is outside of this tolerance, then it is excluded as a possible match (*dashed box*). Next, the template of m points is extended to include an additional point (*black square* within the *black box*). Then, the time series is searched again for matches of this extended template of $m+1$ points (each series of *red*, *green*, and *black points*). This searching algorithm is repeated for all possible starting points to compute the total number of m-point matches and $(m+1)$-point matches. Informally, SampEn is a permutation of the ratio of the number of matches for the $(m+1)$-point templates (*green boxes*) to the number of matches for the m-point templates (*red* and *green boxes*). This process excludes self-matches (the *black box* is not scored as a match). (**b**) We repeat the computation for all τ from neighboring points (5 ms due to a 200 Hz sampling frequency) to those separated by one cycle length. Adapted from [6]

Again, we published our adaptation of SampEn [6], its equation is:

$$\mathrm{SampEn}(m,r,\tau) = -\ln\left(\frac{(N-m)^{-1}\sum_{i=1}^{N-m} A_i^m(r,\tau)}{(N-m)^{-1}\sum_{i=1}^{N-m} B_i^m(r,\tau)}\right)$$

where $A^m(r, \tau)$ is the probability that $(m+1)$-point sequences match, $B^m(r, \tau)$ is the probability that (m)-point sequences match, N is the number of points in the epoch, m defines the template length, r is the tolerance (defined as a fraction of the standard deviation of the amplitude envelope), and τ is the time interval (time delay) between

points in a template. Note that SampEn is expressed as a negative of the natural log of the fraction of matches divided by the total number of possible matches. Thus, a higher SampEn indicates a shift in this ratio either a fewer number of $(m+1)$-point template matches or a greater number of m-point template matches (i.e., a lower SampEn indicates greater regularity in the temporal patterns of the time series, and as such, more predictability and less complexity).

Surrogate Data Sets

MI and SampEn measure both linear and nonlinear properties of time-series data sets. This complicates data interpretation and obfuscates results for comparative analysis of time series that exhibit short- or long-range correlations. This partially depends on the rate of decay in the autocorrelation function. For signals with a slowly decaying autocorrelation function, linear properties of the signal contribute to the predictability in the signal.

To distinguish between linear and nonlinear sources of variability using these techniques, we compare the values of MI or SampEn of the original data set with those of surrogate data sets [6]. The surrogate data sets are designed to preserve the amplitude distribution and autocorrelation functions and to eliminate nonlinear correlations of the original data set [64, 65]. The amplitude distribution of the original data set is easily preserved in the surrogate data set because we use all the points of the original data set. To preserve the autocorrelation function, we apply the iterated amplitude adjusted Fourier transform (iAAFT) method [6, 66] and accept only those surrogate data sets that have autocorrelation functions that are consistent with the autocorrelation function of the original data set (Fig. 5.4c). For testing statistical significance, we create 19 surrogate data sets and test for differences in the statistics (mean and standard deviation) of the MI and SampEn of the original data and the surrogate data sets (Fig. 5.4). We have displayed a representative waveform pattern of the surrogate data sets below that of the original data (compare Fig. 5.4a, b).

Application of Our Approach

To demonstrate the applicability of our approach to the ventilatory pattern, we did what every individual interested in the neural control of respiration would do: we analyzed the respiratory pattern before and after vagotomy. We combined the vagotomy with bilaterally blocking synaptic currents mediated by the NMDA receptors in the dorsolateral pons, which has been referred to as "the internal vagus" [67]. We hypothesized that vagotomy diminishes nonlinear ventilatory pattern variability and these changes can be identified by our analytical approach [6].

A representative figure (Fig. 5.4) of our results details the different steps that we perform in the analysis of ventilatory pattern data, which in this example is the rectified and integrated recording of diaphragmatic EMG (Fig. 5.4a, original data are shown in black tracings). The next step is the formation of surrogate data sets ($n = 19$,

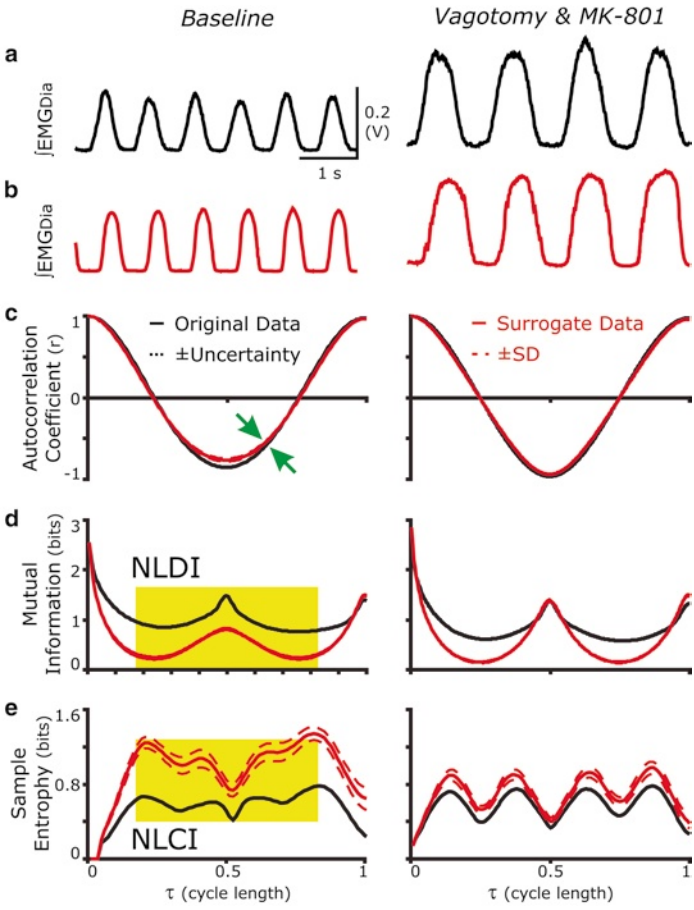

Fig. 5.4 Representative examples from a single rat of the effects of bilateral vagotomy followed by bilateral MK-801 injections in the KFn on nonlinear dependence and complexity of the respiratory patterns. The traces are: (**a**) òDiaEMG, (**b**) surrogate data, (**c**) autocorrelation function, (**d**) mutual information and (**e**) sample entropy. (**a**) Representative trace of the recorded integrated diaphragmatic activity representing the ventilatory patterns at baseline (*left panel*) and after vagotomy and bilateral injections MK-801 in the KFn (*right panel*, see [6] for injection sites). Respiratory frequency decreases and inspiratory burst amplitude increases after the intervention. (**b**) Representative traces of surrogate time series (*red traces*) generated from the original data set in (**a**). The surrogate data sets preserve the general character of the original time series including the periodicity and amplitude distribution. (**c**) Autocorrelation functions of the original (*black*) and the surrogated (*red*) data sets. The autocorrelation function overlaps (note between *green arrows*) indicating that iAAFT surrogate data sets maintained the linear correlation of the original time series. (**d**) Mutual information (MI) for the original (*black*) and surrogate (*red*) data sets. At baseline (*left traces*), the difference between MI curves suggests the presence of significant nonlinear dependence at many time delays across the respiratory period. After vagotomy and subsequent MK-801 injections (*right traces*), nonlinear dependence decreases as evidenced by a smaller difference between traces (highlighted in *yellow*). (**e**) Sample entropy (SampEn) between original and surrogate data sets. The differences between the original and surrogate data complement those of MI. The difference in SampEn between original and surrogate data sets (in *yellow highlighted area*) is significant at baseline (*left traces*) and not significant after vagotomy and MK-801 injections (*right traces*). Adapted from [6]

a tracing from one (Fig. 5.4b, surrogate data are shown in red tracings). In particular, the autocorrelation functions for the original and surrogate data sets are nearly equivalent (Fig. 5.4c between green arrows). We interpret these equivalent autocorrelation functions as indicating that the linear (autocorrelation) properties of the original data set are maintained in the surrogate data sets. On the other hand, MI shows a clear difference between the original and surrogate data sets (Fig. 5.4d). The probability of knowing the value of a second point based on the value of the reference point diminishes drastically after destroying the nonlinear deterministic properties in the original data set (Fig. 5.4d, yellow highlight). To present a single value for the nonlinear contribution to MI, we calculate the difference between the curves at each τ between 17.5 and 82.5 % of cycle length and divide by the sum by the number of τ between 17.5 and 82.5 % of cycle length (Fig. 5.4d, yellow highlight). We analyze SampEn as MI (Fig. 5.4e, yellow highlight). Of course, the SampEn increases with the elimination of the nonlinear deterministic properties in the surrogate data sets. Finally, we normalize SampEn in the same manner as that for MI.

In the manuscript [6], we refer to the normalized MI value as the nonlinear detection index (NLDI) and to the normalized SampEn as the nonlinear complexity index (NLCI). For the group of rats ($n=11$) studied, NLDI and NLCI decreased significantly to a similar value (by approximately 30 % of baseline) after vagotomy or bilateral microinjections of MK-801 in the dl pons or after both interventions together and was independent of which intervention was performed first [6]. Thus, these complementary analyses agreed in their results and revealed the importance of the integration of sensory input into the pattern generator in determining VPV. In particular, the balance between pontine and peripheral inputs to the respiratory rhythm generator determines VPV.

Our team's first application of the approach [68] was to a clinically relevant animal model and occurred actually before we had refined the formation of surrogate data sets (In Fig. 1 of [68], the examples of surrogate data sets show considerable variation from the original data. Subsequent to this study, we restricted the tolerance for deviation from the autocorrelation function in the iAAFT (see Fig. 5.4) Nevertheless in this manuscript [68], we were able to show that ventilation pattern variability became more deterministic following stroke in mice. In these studies, ventilatory pattern was measured using whole-body flow-through plethysmography and compared between two groups of mice (male, A/J, Jackson Laboratory, Bar Harbor, ME): one group received a middle cerebral artery occlusion (stroke model $n=7$); the other had a sham procedure ($n=7$). The data were analyzed using traditional approaches [mean ± SD and coefficient of variation were computed for respiratory frequency, tidal volume (VT) and minute ventilation (VE)] and using our approach for nonlinear measures of (mutual information, sample entropy and a nonlinear complexity index) comparing original to surrogate data sets. Even though both approaches showed changes in the variability of the breathing pattern, the breathing pattern 24 h after the mice had received a stroke had more deterministic variability than those that only had the sham procedure. Using the traditional measures, the mice that received the stroke breathed slower and with greater variability than they did prior to the occlusion and compared to the sham [68]. In particular, the autocorrelation function was lower and CV was higher for VT and for VE. Despite

this increase in the variability in the signal, mutual information and the nonlinear complexity index were higher in the ventilatory pattern following stroke. These data indicate that nonlinear deterministic sources contributed to the increased variability following stroke that this increase in variability was not simply stochastic.

Furthermore, these data indicate that VPV in conditions like stroke following cerebral infarction increased variability but with increased deterministic characteristics. These sources may be mediated by reflexes—as noted in Cheynes–Stokes breathing, which has increased variability due to increased gain of chemoreceptors. This and the prolonged feedback are ideal sources for mediating changes in the nonlinear components of variability.

Cheynes–Stokes breathing pattern is a rhythmic waxing and waning ventilatory pattern. Previously, this pattern was evaluated by a "ventilation oscillation strength index" [69, 70]. This index is the ratio of the difference in the maximum and minimum minute ventilation to the sum of these measures for a ventilatory cycle length. Two limitations of this index are: it assumes there is a pattern and it is not a statistical measure of probability. With regard to the pattern, the index assumes a waxing and waning pattern, but the index makes no distinction as to whether the maximum wanes to a minimum or if the maximum neighbors the minimum. The index is not a statistical measure. If a pattern exists, the index measures neither the consistency nor strength of the pattern. In contrast, our approach could be adapted to reflect a pattern developing over multiple breaths and pathophysiologic states.

Variability Versus Stability

Variability is an aspect of stability but changes in variability do not necessarily indicate a change in stability. Stability of a limit-cycle oscillator is defined by its ability to return to its limit-cycle pattern after a perturbation. If the oscillator is stable, the "relaxation time" is the time required for the oscillator to return to its "attractor" and is an approximate measure of its degree of stability. Measure of a variable oscillation like the ventilatory pattern requires pseudorandom perturbations [71–73]. Given this distinction, the ease of measuring variability makes it not only tractable but also a plausible biomarker. The issue is in the case of established illness would tracking VPV provide insight into the effectiveness of therapeutic approaches and weaning strategies.

Other Methodologies

A technique referred to as noise titration was designed to test for the presence of nonlinear deterministic dynamics within a noisy time-series data and was initially applied to the respiratory signal [74]. Briefly, the technique tested for the presence of nonlinear dynamics by comparing the fit of the time series to a linear versus a nonlinear Volterra series model. An Akaike cost function was used to determine whether the fit was better for the nonlinear model. If so, then Gaussian noise with increasing standard deviation was titrated into the time series until the nonlinear

model was no longer better than the linear model. The value of the standard deviation of the noise at this point divided by the standard deviation of the signal multiplied by 100 was defined to be the noise limit of the data, which has been correlated with the degree of chaos [74].

This technique has been applied to human respiratory patterns to identify chaotic dynamics in the pattern [75, 76]. While controversial this group defines chaotic-like complexity as long-term unpredictability modulated by low-dimensional nonlinear deterministic process [77]. Noise titration indicated a chaotic dimension to ventilatory flow in normal humans during tidal breathing [76]. Further, the technique was able to identify that hypercapnia affected chaos, which was reflected in decreased VPV [75]. In a creative application of noise titration, the technique was used to identify changes in the complexity of respiratory pattern as a tadpole matured into a frog [77]. In their study, linear models fit the respiratory pattern of tadpoles but postmetamorphosis, frogs displayed complexity in their patterns [77]. The authors interpreted these data to indicate that the respiratory pattern generator accounts for ventilatory chaos-like complexity [77].

Consistent with tadpole study, the noise titration technique was used to analyze the ventilatory flow during assist-controlled ventilation and patient-initiated, inspiratory-pressure support ventilation [78]. Even though assist-controlled ventilation can be initiated by spontaneous respiratory motor activity, this became suppressed in two-thirds of the subjects. So, their ventilatory flow was determined by the ventilator. This pattern was regular, with stochastic variability and lacked complex nonlinear dynamic patterns of variability. In contrast, ventilatory flow was chaotic during pressure support in 95 % of the subjects with spontaneous activity (both before spontaneous activity became suppressed and with persistent spontaneous activity). Clearly, the complex dynamics associated with breathing has a neural rather than mechanical origin. The neural origin would be both the respiratory pattern generator and sensory afferents including vagal PSR feedback. Our data support that nonlinear dynamics within the CPG arise from the interactions of the peripheral inputs feeding into the network.

A basic point of the review is that entropy measures provide more insight into properties of variability than traditional variance-based measures (like standard deviation or coefficient of variation); entropy measures provide quantification of network complexity and signal predictability, thus providing a sensitive, complementary measure when applied in conjunction with traditional signal analysis methods. Here, we compare the Irregularity Score [79, 80], a measure of the breath-to-breath variability to Shannon entropy of the intervals that is used to quantify the predictability of interval length (Fig. 5.5). We compared intervals of hypoglossal (XII) nerve discharge in the in vitro rodent "bursting" slice preparation at different levels of extracellular potassium concentration ($[K^+]_o$).

The in vitro rodent medullary slice preparation (Fig. 5.5a) contains the preBötzinger complex (PBC) and premotoneurons, these are necessary and sufficient neural elements for the generation of spontaneous rhythmic activity. This preparation also contains hypoglossal (XII) motoneurons, which permits the recording of rhythmic output from XII nerve rootlets and allows direct access to brainstem neurons and their milieu. Changes in $[K^+]_o$ alter ionic equilibrium potential and reproducibly changes the excitability of cells embedded within the neural network.

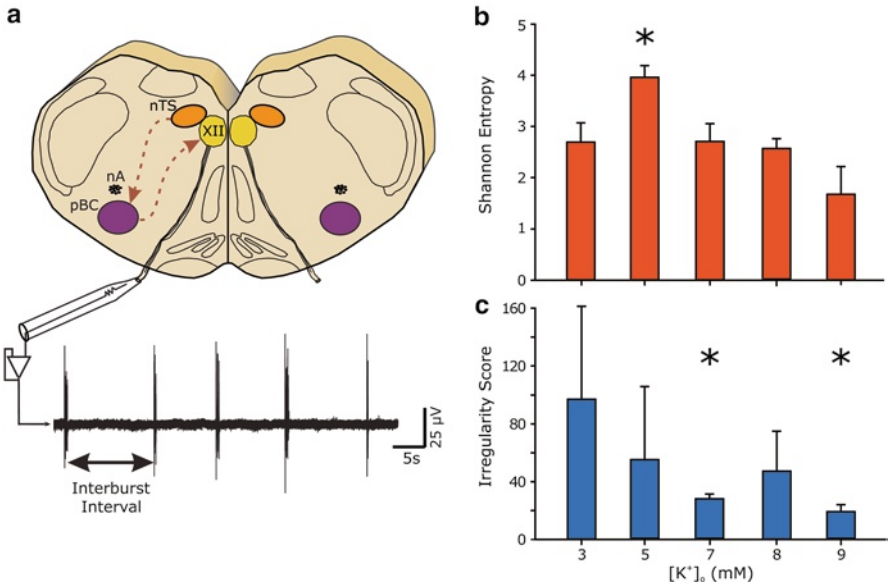

Fig. 5.5 Comparison of interval analyses. (**a**) Medullary rhythmic slice containing PreBötzinger Complex (pBC). Other labeled components of the rhythmic slice are: nucleus Tractus Solitarius (nTS), nucleus Ambiguus (nA), and hypoglossal motor nucleus (XII). The cells in the pBC are intrinsically rhythmic, they receive inhibitory input from the nTS (*dashed arrow*) and project to XII (*dashed arrow*). The *lower panel* shows spontaneous rhythmic bursting hypoglossal electroneurogram (XII-ENG). (**b**) Shannon Entropy was calculated on the interburst interval (IBI), the interval of quiescence between bursts of XII-ENG. The pattern with the greatest Shannon Entropy, i.e. the IBI with the greatest predictability occurred when the bath contained 5 mM extracellular potassium concentration ($[K^+]_o$). (**c**) Irregularity score was calculated on the cycle duration (interburst interval and a burst). In contrast, to Shannon Entropy, the intervals with the least breath-to-breath variability in cycle duration occurred at 7 and 9 $[K^+]_o$ (Wilson, unpublished data). Asterisks denote a significant difference to the other values. In C the irregularity scores for 7&9 are not significantly different but are significantly different than the others

Shannon Entropy of the intervals had a peak at 5 mM $[K^+]_o$ (Fig. 5.5b); and the irregularity score, a nadir at 9 (7 was not statistically different from 9) mM $[K^+]_o$ (Fig. 5.5c). These measures are not negatively correlated because they measure complementary properties of the time series. The irregularity score captures breath-to-breath variability serial dependency similar to Poincaré plots, whereas Shannon entropy captures the statistical properties of the distribution of interburst intervals. A constant interburst interval would have minimal, whereas a uniform distribution has maximal Shannon entropy. By comparing entropy measures to other currently used analyses (i.e., coefficient of variation, power spectral density, and irregularity score), we showed that entropy provides information about signal complexity that is not evident using standard methods. Analysis methods and tools may quantify different aspects of the same signal. To interpret a biological signal, we recommend applying a variety of different algorithms that encompass as many features of the signal—nonlinear and linear—as accurately as possible.

Limitations

We advocate that multiple analytical approaches should be applied to data sets to complement each other. These techniques have limitations. Mostly, the complexity of colored or non-Gaussian noise can create ambiguities in interpretation. Freitas and coworkers reported that noise titration fails to distinguish nonchaotic signals from low-dimensional deterministic chaos, especially in the context of colored noise [81].

Indeed even with our surrogate data sets, we cannot distinguish stochastic non-Gaussian or deterministic nonlinear variability. In our case, we examined the correlation integrals for both original and the surrogate data sets. Correlation integrals showed convergence for the original but not surrogate time series confirming the existence of nonlinear deterministic structure in the original but not surrogate data sets [6].

Variability as Biomarker and Our Current Application

In our Introduction, we argue for VPV having potential clinical relevance. An underlying issue in accepting and appreciating heart rate and ventilatory pattern variabilities as biomarkers is the poor understanding of what determines pattern variability in the neural control mechanisms and of how these change from health to disease. For instance, we know that the high-frequency component of the power spectral density of heart rate is related to respiration and to the prevalent respiratory modulation of autonomic innervation of the heart. But we neither know how VPV influences HRV nor how these systems become uncoupled during these diseases.

The expectation is that now we have an analytical approach that can provide consistent and interpretable results in measures of VPV. We, especially Frank Jacono and Yee-Hsee Hsieh, have been addressing how and why the VPV changes in human and animal models of lung injury and sepsis. Recently, we published an association between the expression of proinflammatory cytokines in the lung and brainstem and dramatic increases in deterministic properties in VPV [82]. We identified changes in both linear and nonlinear components of VPV, but these occurred over a different time course after acute lung injury [intratracheal instillation of 3 U of bleomycin (or saline) in adult male rats ($n=12$)]. The breathing pattern, for instance respiratory cycle duration (T_{TOT}), changed, T_{TOT} decreased, at 24 h. Surprisingly, this change in the breathing pattern was not accompanied by changes in the linear component but rather the nonlinear component of VPV. For instance, CV of T_{TOT} and even the autocorrelation coefficient of the pattern at one cycle length did not change significantly until 48 h after acute lung injury, whereas the NLCI had increased significantly already at 24 h and remained increased at 48 h [82]. In this initial study, bronchoalveolar lung fluid (BALF), lungs, serum, and brain tissue were harvested only at the 48 h time point. At this time point, the proinflammatory cytokines IL-1β and TNFα were increased in the BALF and the homogenate of pulmonary tissue but not the serum. We stained the brainstem for these cytokines and noted increased IL-1β expression in the *area postrema* and nuclei of the solitary tracts, where pulmonary primary afferent terminate [82] (Fig. 5.6).

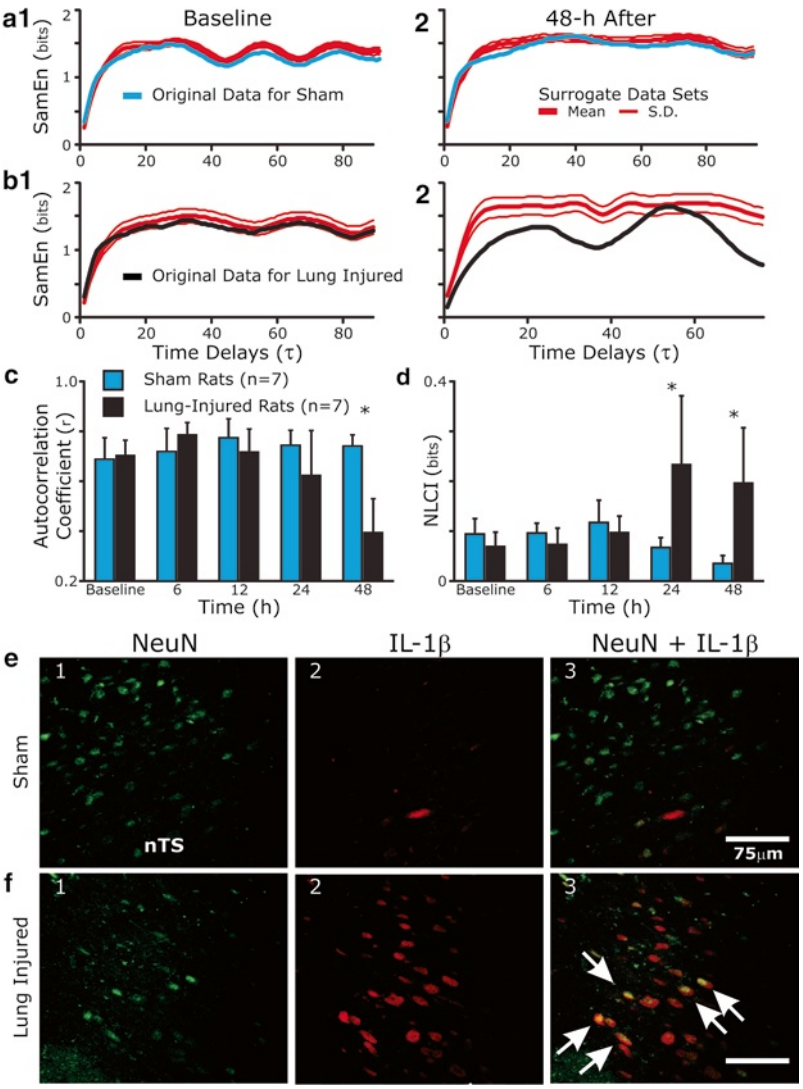

Fig. 5.6 (**a** and **b**) Representative examples of Sample Entropy measurements from single rats (**a**, sham; and **b**, acutely lung injured). (**c** and **d**) Group data of the autocorrelation coefficient (*r*, **c**) and nonlinear complexity index (NLCI, **d**). (**e** and **f**) Histological data showing the nucleus solitary tract (nTS) in a sham (**e**) and lung-injured rat (**f**). (**a** and **b**) Nonlinear variability of the ventilatory pattern as measured by SampEn before (1) and after (2) intratracheal installation of saline (sham, **a**) or 3 U of Bleomycin (lung injured, **b**). (**a1** and **2**) In the sham rat, SampEn of the original and surrogate data sets overlap both before and 48 h after intratracheal instillation of saline. (**b1** and **2**) In the lung-injured rat, the baseline SampEn is similar to that of the saline-treated rat at baseline, but SampEn separates between the original and surrogate data sets 48 h after induction of lung injury. These data indicate an increased contribution of nonlinear and/or non-Gaussian sources of variability. (**c**) Linear determinants of VPV, as indicated by the *r*, did not change in significantly until 48 h after lung injury when it decreased. (**d**) In contrast to the linear determinants, nonlinear determinants of VPV, as indicated by NLCI, increased at 24 h when the breathing pattern changed in the lung-injured rats. (**e** and **f**) Brainstem tissue was sectioned coronally and sequential sections were stained immunohistochemically with or without the primary antibody for IL-1β (Abcam) or with NeuN conjugated to FITC to identify neuronal nuclei (Millipore) or in the lung-injured rat, IL-1β was colocalized with neurons in the nTS as identified by fluorescent staining. Adapted from [82]

Conclusions

While many have reported changes in VPV with respiratory disease [19, 20, 76, 83–91], we are focused on the consistent measurement of VPV, on dissociation of linear and nonlinear sources of variability, on distinguishing deterministic from stochastic sources of VPV, and on the mechanisms that determine VPV. We propose that changes in VPV depend not only on vagal sensory afferents but also on altered brainstem mechanisms, specifically neuroimmune interactions regulating neurotransmission.

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Part II
Translational Modeling of Sepsis and Trauma

Chapter 6
Disorder of Systemic Inflammation in Sepsis and Trauma: A Systems Perspective

Kent R. Zettel II and Timothy R. Billiar

Introduction

The inflammatory response is the host's response to a perceived threat in the form of an invading pathogen or tissue damage [1] and is defined as upregulation of inflammatory cytokines and chemokines, leukocyte adhesion molecules, and infiltration of neutrophils and other immune cells into tissues [2]. This response involves multiple factors that detect the stress response, and many cell types to create the physiologic reaction to stress (Table 6.1). Homeostasis of the inflammatory response must be closely regulated. Without the inflammatory response, the host would succumb to the threat, while excessive responses will cause cell and tissue damage, and death. Trauma and sepsis are distinct entities with some overlapping features. In trauma, the inciting event is tissue damage often associated with local or systemic hypoperfusion. Sepsis, which often complicates trauma, is initiated by an infectious agent. Both trauma and sepsis induce systemic changes most often manifested by interrelated immune and physiologic changes. Many of the overlapping features are likely due to the involvement of immune-sensing mechanisms both trauma and sepsis have in common.

When the inflammatory response becomes severe and generalized, the pathologic sequelae are manifested in a condition called the systemic inflammatory response syndrome (SIRS). SIRS is diagnosed when the patient presents with two

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Table 6.1 Host injury inflammatory response

Sensors	Cells	Effectors	Consequences
PRR—DAMP, PAMP	Neutrophils	Neuroendocrine mediators	Beneficial
Heat shock proteins	Macrophages	Cytokines/chemokines	1. Adaptive cell stress responses
Hypoxia sensors	Lymphocytes	Complement	2. Initiate tissue repair
Mitochondria	Dendritic cells	Coagulation cascade	3. Immune defenses
ROS/RNS targets	NK/NKT	ROS/RNS	Detrimental
	Mast cells		1. Immune dysfunction
	Epithelial cells		2. End-organ damage
	Platelets		3. Impaired repair processes

Participants in the systemic inflammatory response divided into sensors of the noxious stimuli, the cells involved, the systemic mediators of the inflammatory response and the consequences of such response

PRR pattern recognition receptor, *DAMP* danger-associated molecular pattern, *PAMP* pathogen-associated molecular pattern, *ROS* reactive oxygen species, *RNS* reactive nitrogen species

or more of the following criteria: temperature > 38 °C or < 36 °C, heart rate > 90 beats per minute (BPM), respiratory rate > 20 breaths per minute or $PaCO_2 < 32$ mm Hg, or white blood count (WBC) > 12,000 or < 4,000 mm^{-3} or > 10 % bandemia. Sepsis is defined as the SIRS response to a septic focus [3].

Trauma and severe sepsis are associated with a high rate of immune irregularities. Patients can succumb early after the onset of trauma or sepsis due to a cytokine storm characterized by a hyperinflammatory state, which can lead to early multiorgan failure (MOF). The appearance of early MOF correlates with the development of nosocomial infection and prolonged ICU stays. Recent human data examining the phenotype of circulating leukocytes in severely injured blunt trauma patients indicates that both the upregulation of innate immune pathways and suppression of adaptive immune pathways occur simultaneously. These responses are exaggerated and sustained in patients that go on to develop infectious complications [4].

Billions of dollars have been invested to create therapies for severe sepsis and injury. Despite some early promise, there has been only disappointment. This is most likely due to the failure to completely understand the mechanisms involved in the host response to injury and severe infection. Systems biology holds great promise as an approach to develop an integrated and predictive model of the human immuno-inflammatory response.

Sensing Mechanisms

Evidence continues to mount that the host is programmed to initiate inflammatory and cell stress-signaling pathways in response to threats to tissue homeostasis through highly specific sensing mechanisms. These threats are often in the form of invading microorganisms, tissue damage or reduced oxygen and nutrient delivery. Many of the sensing mechanism have been identified and some are listed in Table 6.1.

Table 6.2 Toll-like receptors and their respective stimulants

Toll-like receptor	Exogenous molecule	Endogenous molecule
TLR-1	Triacyl lipoproteins	
TLR-2	Lipoproteins	HSP-60, 70
	Peptidoglycan	HMGB-1
	Lipoteichoic acid	
TLR-3	Double-stranded RNA	mRNA
TLR-4	Lipopolysaccharide	HSP-22, 60, 70
	Fusin protein	HMGB1
	Envelope protein	Surfactant protein A
TLR-5	Flagellin	
TLR-6	Lipoteichoic acid	
	DNA zymosan	
TLR-7	Single-stranded RNA	
TLR-8	Single-stranded RNA	
TLR-9	Unmethylated CpG-containing DNA	DNA–HMGB1 complexes

Examples of microbial factors and endogenous moieties that are both recognized by pattern recognition receptors
HSP heat shock protein, *HMGB1* high mobility group box 1, *DNA* deoxyribonucleic acid

Infections

In order for the human immune system to respond to infectious agents rapidly, the system recognizes a subset of molecular motifs that are uniquely expressed by microorganisms but are not expressed by host cells. These molecules are known as pathogen-associated molecular patterns, or PAMPs. PAMPs are recognized by pattern recognition receptors (PRRs), which include several families of receptors that are expressed on both immune and nonimmune cell types. The toll-like receptors are prototypic PAMP sensors and the microbial molecular motifs recognized by many of the 13 TLRs have been identified. Examples of PAMPs that initiate signaling through TLRs are listed in Table 6.2. TLR signaling involves a number of adapter molecules. Notable among these are the downstream adapters TIR-domain containing adaptor-inducing interferon-β (TRIF) and myeloid differentiation primary response gene 88 (MyD88). These adapters link TLRs to downstream signaling pathways including NF-κB, MAP kinases, and IRF3. The LPS receptor complex, comprised of TLR4 and MD2, is unique among TLRs in that it can signal through both TRIF and MyD88. TLR signaling regulates a range of cellular responses including cytokine/chemokine production to cell stress responses such as autophagy [5].

Tissue Damage

Not long after the identification of the role of PRR in the recognition in PAMPs, it became apparent that many of the PRRs were involved not only in the detection of

microbes but also the sensing of tissue damage [6, 7]. In the setting of tissue damage or even cell stress, PRR recognize molecules of host origin referred to as damage-associated molecular pattern molecules, or DAMPs. These are molecules within cells or tissue matrix that are normally not available to PRR. However, in the setting of tissue damage or stress, DAMPs are released in quantities adequate to trigger signaling through PRR. Examples of DAMPs that trigger signaling through TLRs are shown in Table 6.2. It is notable that the concentrations of DAMPs required to trigger signaling through TLRs are often at least an order of magnitude higher than that seen for PAMPs. This suggests that the threshold for the detection of microbes by PRR is much lower than the detection of tissue damage and perhaps correlates with the magnitude of the threat to the host. However, it seems reasonable to conclude that a common set of receptors are used to detect both infection and tissue damage and that this feature could account for the similarities in the inflammatory response induced by these two very different threats to the host.

Hypoxia/Ischemia

Hypoxic and ischemic conditions also represent a major threat to the host by leading to cellular dysfunction and injury. Whether local or systemic, sustained reductions in perfusion or oxygen delivery lead to the activation of inflammatory and cell stress signaling. Therefore, it is not surprising that several oxygen sensing mechanisms are linked to inflammation.

Oxygen tension is detected by oxygen sensors within the cells, which hydroxylate proline residues on the hypoxia-inducible transcription factor (HIF) [8]. This hydroxylation creates a binding site for the von Hippel–Lindau gene product, which leads to the proteasomal degradation of the α subunit [8]. Hypoxia inducible factor-1 (HIF-1) is one of the main mediators of homeostasis in the hypoxic environment [9]. HIF-1 plays a key role in the inflammatory response including the metabolism, migration, inducible nitric oxide synthase (iNOS) expression, and antimicrobial activity of polymorphonuclear neutrophils (PMN) and macrophages [8–10]. The expression and release of inflammatory protein-1B by macrophages, which protects PMNs from apoptosis and thereby extending their lifetime, is through HIF-dependent NF-KB activation [8, 10]. Increased HIF-1α production in T cells induces a Th1 to Th2 phenotype shift.

The mitochondrion acts as a sensor during episodes of ischemia and reperfusion with resultant production of reactive oxygen species. Under normal circumstances of respiration, mitochondria leak a small quantity of reactive oxygen species (ROS) along the electron transport chain in the form of superoxide radicals ($O_2^{\bullet -}$). Most of these reactive oxygen species are reduced by superoxide dismutase to hydrogen peroxide and further reduced by peroxidase and glutathione. Ischemia leads to alterations to the mitochondrial electron transport chain and electron leak causing increased superoxide radical formation [11]. Under prolonged ischemic episodes, the capacity of the cell to reduce the superoxide becomes overwhelmed, resulting in oxidative stress. Under these states, ROS activate NF-κB and activator protein 1.

NF-κB regulates inflammatory factors such as inducible nitric oxide synthase (iNOS) and cyclooxygenase II [12]. Activator protein 1 and NF-κB are essential for induction of many inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-2, IL-6, IL-8, macrophage chemoattractant protein (MCP-1), interferon-β, granulocyte-macrophage colony stimulating factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES), and E-selectin [1, 12].

Cellular Factors of the Inflammatory Response

The inflammatory response to an acute threat involves a response coordinated by cells. The roles of immune cells in driving the initial inflammatory response are well recognized. Less appreciated are the roles of nonimmune cells, which can participate in the response not only by responding to signals from the immune cells but also by responding through the same range of sensing mechanism expressed within immune cells. Here, we briefly consider the dominant known roles of specific cell populations in the early inflammatory response.

Tissue macrophages are among the first cells to send signals in response to infection or injury. These signals include chemokines and cytokines that begin to integrate an influx of cells into the threatened tissue. Monocytes enter the tissues and differentiate into macrophages by the cytokine milieu. These cells play a dual role in the immune response by phagocytizing pathogens and destroy them through oxygen-dependent and oxygen-independent mechanisms, then presenting antigens to T cells, promoting cell-mediated immunity [13]. These cells secrete proinflammatory cytokines to delay neutrophil apoptosis and further enhance the inflammatory response.

After stimulation, a massive PMN infiltration occurs within 4–6 h, followed by monocytes in 24 h and lymphocytes in 48 h [14]. Neutrophils clear the infection by phagocytosis and intracellular killing mechanisms involving reactive oxygen species and the release of proteases, elastase, cathepsins, and matrix metallopeptidase-9 [13]. They also secrete cytokines to further attract and activate the immune response, including TNF-α, IP-10, macrophage inflammatory peptide-1α (MIP-1α), IL-12, IL-8, B-lymphocyte stimulator (BLyS), and vascular endothelial growth factor (VEGF) [15]. PMN also release DNA nets to snare bacteria in response to TLR4 activation [16, 17]. They have a short half-life of 8–12 h unless extended by IL-8 and TNF-α, bacterial components, and complement [13].

In part of the inflammatory response to trauma, the spleen becomes infiltrated with $CD11c^{+}/Gr\text{-}1^{+}$ myeloid cells that are known to suppress T-cell function, Myeloid-derived suppressor cells (MDSCs). These cells express the enzyme arginase, which converts arginine to ornithine and urea. In trauma, these cells localize with T cells in the germinal centers of the white pulp in the spleen where they suppress T-cell function. The expressed arginase activity depletes the local environment of available arginine, which is required for the T-cell receptor ζ-chain [18]. T-cell proliferative dysfunction ensues and can be reversed by arginine supplementation or inhibition of arginase activity.

Lymphocytes can be divided into T cells and B cells. T cells infiltrate the inflammatory site and secrete cytokines that increase neutrophil infiltration (GM-CSF and IL-3), the macrophage and neutrophil response to pathogens (TNF-α and GM-CSF), and delay neutrophil apoptosis (GM-CSF) [19]. Subsets of T cells, regulatory T cells (Tregs), have been implicated in the suppression of T-cell response to antigen and immunosuppression. B cells were once thought to have the sole function of producing antibody, but more recent evidence demonstrates that B cells play an active role in modulating the immune response. Regulatory B cells (Breg) are a subset of B cells that produce interleukin-10 (IL-10) and induce an anti-inflammatory immune response [20].

Severe injury and sepsis leads to a suppressed T-cell response. Multiple mechanisms regulate this immune suppression, including certain cytokines and regulatory T cells (Tregs). The topic of regulatory T cells is too extensive to be completely covered in this chapter. These cells play a role in the inflammatory response and immune modulation after injury and sepsis. Tregs suppress $CD4^+$ T-cell proliferation and activation after trauma and sepsis and mitigate LPS and PGN-induced TNF-α, IL-1β, and IL-6 production [21, 22]. Tregs inhibit T-cell proliferation by cell contact-mediated pathways [23]. Tregs produce high levels of the immunosuppressive cytokine IL-10 after injury [23]. Tregs are upregulated in sepsis and suppress $CD4^+$ T-cell proliferation; however, Treg depletion did not lead to a survival advantage in CLP-induced sepsis [22].

Natural killer cells are bone marrow-derived lymphocytes that attack cells through apoptosis-inducing mechanisms. Unlike T and B cells, they do not express clonally specific antigen receptors, but rather detect self-antigens on MHC I molecules [24]. They are producers of chemokines and proinflammatory cytokines TNF-alpha, TNF-beta, and IFN-gamma [25]. They are activated by the proinflammatory cytokines IL-12, type-1 interferons (IFN-alpha and IFN-beta), through toll like receptors, and through direct contact with dendritic cells [25]. NK cells, through IFN-gamma production, promote Th1 polarization of $CD4^+$ T cells and activate dendritic cells through IFN-gamma, TNF-alpha production, and cell–cell contact [26]. In addition to intensifying the inflammatory immune response, NK cells can also dampen the response through depletion of immature myeloid dendritic cells and activated T cells [26].

Natural killer T (NKT) cells are thymus-derived T cells that contain the T-cell receptor, but are dissimilar from their $CD4^+$ and $CD8^+$ T cells because they detect antigens expressed on CD1d and contain markers for natural killer cells [27, 28]. NKT cells can be divided into subsets by the expression of IL-18R or ST2L that can direct an immune response toward a Th1 or Th2 response by secretion of the cytokines IFN-gamma or IL-4 and IL-5, respectively [29]. These cells have been implicated in the T-cell-mediated immune suppression after burn injury by high production of IL-4 and lower MHC-II and costimulatory CD40 expression [30, 31]. Reversing the inhibitory role of NKT cells has been demonstrated by blocking the CD1d signaling [30–32].

Dendritic cells (DC) play an important role in bridging the innate and cell-mediated immune response. These cells phagocytize antigen, migrate to secondary

lymphoid organs, differentiate into a mature phenotype, and activate naïve T cells [13]. DCs have much higher capability to present antigen to T cells than macrophages. These cells can be divided into plasmacytoid DCs and myeloid DCs. Plasmacytoid DCs resemble plasma B cells and are involved in autoimmunity and are the major subset of DCs responsible for IFN-α response to infection. Myeloid DCs play a major role in IL-12 production to stimulate macrophages and natural killer cells and induction of the Th1 response [13]. Sepsis induces an acute expansion of splenic follicular DC, with an interval decrease through caspase-3-mediated apoptosis [33, 34]. Sepsis also induces an acute decrease in interdigitating DCs through caspase-3-mediated apoptosis [33]. The immature rather than the mature DC population are the targets for apoptotic death [35]. The depletion of DC populations seen in sepsis is also witnessed in trauma/hemorrhage [36]. Dysfunction, as indicated by suppressed antigen presentation, is seen in both sepsis and trauma [33, 36]. Trauma and hemorrhagic shock suppress DC response to LPS with downregulated MAPK activation and suppress LPS-induced proinflammatory TNF-α and IL-6 cytokine production, which is likely due to diminished TLR-4 expression [36, 37]. However, there was no change in DC anti-inflammatory IL-10 production [37].

Often underappreciated is the role of nonimmune cells in the host response to acute infection or injury. Take, for example, the fact that most PRR are widely expressed in nonimmune cell types [38–41]. The roles of PRR on parenchymal cells are an underexplored area of inflammation biology. Evidence that PRR on nonimmune cells is important in the setting of acute injury and infection comes from studies using chimeric mice. In fact, the majority of the studies using chimeric mice deficient in TLR4 signaling in either the bone marrow or non-bone marrow derived cells shows that TLR4 on non-bone marrow derived cells plays an important role in the acute pathobiology of infection [42, 43], ischemic injury [44–46], tissue trauma [47], and hemorrhagic shock [47]. We have shown that redox stress and hypoxia can lead to a TLR4 dependent release of HMGB1 from hepatocytes [48] raising the possibility TLRs may serve as a sensor of redox stress.

Of the nonimmune cells that participate directly in the inflammatory response, endothelial cells are probably the best characterized. In acute inflammation, the endothelial cells induce leakage of plasma and leukocytes and increase local blood flow. These features of endothelial cells make up the signs of *tumor*, *rubor*, and *calor* associated with inflammation. In response to activation of PRR, endothelial cells upregulate leukocyte adhesion molecules that direct the accumulation of leukocytes into the tissues [41, 49]. Endothelial cells can produce inflammatory mediators and activate the coagulation cascade through the upregulation of tissue factor through interactions with monocytes [50].

TNF-alpha and IL-1 are strong activators of the endothelial cells. These induce Cox2 for prostaglandin synthesis for edema, and chemokines such as IL-8 and E-selectin, ICAM-1, VCAM-1 for leukocyte attraction adhesion, and integration into the underlying tissues [51, 52]. Endothelial cell injury from inflammation is a consequence of both the influx of the leukocytes and the toxic effects of their secretory granules, as well as the cytokine production from these cells [51, 53]. Interferon-gamma in combination with TNF-alpha and IL-1 activates endothelial cell death [51].

Effectors of the Inflammatory Response

Effectors of the inflammatory response can be broadly classified as pathways activated and mediators produced in response to the sensing mechanisms. The effectors carry out the steps intended to remove the threat and reestablish tissue homeostasis. These induce capillary permeability, fever, tissue injury, and immune cellular responses that are responsible for the phenotype of the inflammatory response. The coagulation and complement cascades, cytokine and chemokines, and neuroendocrine responses are all effectors of the inflammatory response.

ROS and RNS

Reactive oxygen species are not only rapidly produced as part of the sensing mechanisms that initiate the inflammatory response but can also be viewed as effectors when produced in a sustained manor. As previously mentioned, ROS are formed from mitochondrial stress activate the inflammatory response through NF-κB and activator protein 1. Reactive oxygen species are also actively produced and released by immune cells during respiratory burst. In this process, from nicotinic adenine dinucleotide phosphate (NADPH) oxidase produces superoxide [2]. This can be further reduced by superoxide dismutase to hydrogen peroxide, which myeloperoxidase converts to hypochlorous acid. The major sources of these reactive species through this mechanism are from phagocytic cells, such as macrophages and PMNs with the purpose to eradicate infectious agents, but the nonspecific release of reactive oxygen species also results in tissue injury.

Reactive nitrogen species (RNS) become an effector when nitric oxide (NO) is produced by the high output inducible NO synthase (iNOS). iNOS is not expressed in resting cells but can be upregulated by cytokines and hypoxia in many cell types [2, 54]. Once expressed, iNOS produces NO in a sustained manor and the NO produced can have cell signaling functions to protect cells through promoting perfusion or inhibiting apoptosis or induce cell toxicity through nitrosative or oxidative stress. When produced in proximity to superoxide, NO can lead to the formation of peroxynitrite, a potent oxidant [55]. We have shown that iNOS contributes to inflammation, organ injury, and immune dysfunction following hemorrhagic shock and trauma [56, 57].

Coagulation Cascade

The most extensively studied component of the coagulation cascade in the setting of sepsis is protein C. Activated protein C (drotrecogin-alfa activated, Xigris) has anti-inflammatory, as well as antithrombotic and profibrinolytic properties. Drotrecogin-alfa was initially considered the boon for treating excessive proinflammatory

response, but in time was found not to improve survival. This pharmacologic agent was developed from the long-known relationship between the coagulation and the inflammatory response in that inflammation activates coagulation and correspondingly, coagulation modulates inflammation.

Sepsis impairs anticoagulation by TNF-α downregulation of thrombomodulin, which then reduces protein C activation by thrombin–thrombomodulin complex [58].

TNF-α and IL-1 reduce the endothelial cell expression of thrombomodulin [58]. TNF-α not only reduces thrombomodulin transcription and translation, but also with the aid of neutrophils induces endothelial cell release of thrombomodulin [58, 59]. TNF-α and IL-1, along with IL-6 also have procoagulant properties [60, 61]. IL-6 infusion in human subjects increases both plasma thrombin–antithrombin III complexes and thrombin activation fragment F1 + 2, with no change in fibrinolysis [60]. Recombinant TNF-α and IL-1 promote procoagulant activity in vascular endothelium analyzed through a plasma recalcification clotting assay [61]. This cytokine activity on the vascular endothelium may be beneficial in hemostasis in hemorrhage and may be associated with disseminated intravascular coagulation in the setting of sepsis.

Tissue factor affects levels of IL-6, and IL-8 either directly or through thrombin [62]. Thrombin induces E-selectin in endothelial cells, as well as monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-1, and IL-6, IL-8 in both fibroblasts and endothelial cells and enhances leukocyte migration [62]. It induces monocyte production of IL-6 and IL-8. Fibrinogen is an acute phase reactant, upregulated in times of stress, injury, infection, or other inflammation [62]. Fibrinogen split products, such as D-dimer, are used in clinical practice as indicators for inflammation and coagulation activity. Degradation products from fibrin formation act as chemoattractants for leukocytes [62]. Fibrin binds to the CD11b/CD18 integrin receptor on leukocytes, the CD11c/CD18 integrin on dendritic cells, leukocytes, and some B cells [62], and the $\alpha_{IIb}\beta_3$ integrin, which activates mast cells.

Neuroendocrine

The major components of the stress response to injury include the corticotropin-releasing hormone (CRH) and locus ceruleus-norepinephrine systems. In response to injury, CRH release through the hippocampus and norepinephrine release through the hypothalamic–pituitary–adrenal axis cause increased cardiac output with elevated blood glucose, heart rate and blood pressure, respiratory rate, and leukocytosis. α-Adrenergic stimulation via the sympathetic response leads to NF-κB pathway activation and subsequent production of IL-6, TNF-α, IL-1β, and TGF-β1 production [63]. IL-1β enhances hypothalamic activity and the level of corticotropin releasing hormone (CRF release), increases sympathetic activity and splenic norepinephrine release, while some studies demonstrate conflicting results with diminished splenic sympathetic activity [64].

The catecholamine surge during hemorrhagic shock and resuscitation stimulates the release of the inflammatory modulator, HMGB1, which induces bone marrow neutrophil mobilization through IL-17 and IL-23 induction [65]. The addition of beta-blockade can alleviate the bone marrow mobilization and HMGB1 release, as well as hematopoietic cell growth suppression after hemorrhagic shock [65, 66]. Beta receptors are also expressed on B and $CD4^+$ T cells. Of the T cells, naïve and Th1 T cells express of beta-adrenergic receptors [64]. Although early studies have demonstrated that beta-adrenergic stimulation of T cells promotes proliferation, later investigations have demonstrated a suppressed T-cell proliferation [64]. There has been similar controversy in the T-cell cytokine production through beta-adrenergic receptor stimulation [64]. B-cell proliferation in response to bate-adrenergic stimulation also varies depending on the costimulating agent [64].

There is antagonism between catecholamines and inflammatory modulators. For example, nitric oxide, the product of iNOS, which is upregulated in sepsis and trauma, causes vasodilation, while catecholamines cause vasoconstriction. Using hepatocyte culture, catecholamines have demonstrated an antagonistic effect on the production of nitric oxide by hepatocytes by both $alpha_2$ and $beta_1$-mediated pathways [67]. These inhibitory effects are greater with epinephrine than with norepinephrine and through $beta_1$ more than $alpha_2$-mediated mechanisms [67]. The mechanism of this inhibitory role is uncertain, though thought to be posttranslational.

The cholinergic neuroendocrine response is a vagal-mediated anti-inflammatory pathway of the immune response that suppresses the release of TNF-α, IL-1β, IL-6, and IL-18 [68, 69]. This anti-inflammatory response is initiated through central muscarinic acetylcholinergic receptors, transmitted through the vagus to the periphery, where it promotes its anti-inflammatory effects through nicotinic acetylcholinergic receptors on immune cells [68, 70]. This peripheral nicotinic-cholinergic response has been used for the treatment of chronic inflammatory conditions such as ulcerative colitis [71, 72]. In vivo models have further analyzed the cholinergic anti-inflammatory pathway in sepsis. Anticholinesterase administration once at the time of cecal ligation and puncture (CLP) reduced the serum IL-6, IL-10, TNF-α levels, though did not influence survival [73]. Survival benefit was demonstrated in other studies when either anticholinesterase or choline itself was administered 2–3 times daily after CLP [69, 74]. This indicates that the initial anticholinergic effect is beneficial at reducing the cytokine storm, while prolonged anticholinergic treatment is necessary to produce a survival benefit.

Cytokines and Chemokines

Cytokines are hormones that mediate the inflammatory response through a cell–cell communication and often with overlapping functions. Following injury, cytokines are produced in response to pattern recognition receptor activation to DAMPs or PAMPs leading to a vigorous inflammatory cytokine response with major contributions from IL-1, IL-2, IL-4, IL-6, IL-8, IL-18, and TNF-α [75] (Table 6.3). Chemokines are a subclass of cytokines that induce chemotaxis in nearby responsive cells.

Table 6.3 Inflammatory cytokine and chemokine functions

Cytokine	Function
TNF-α	Induces fever. Stimulates NK cells and macrophages/monocytes. Induces synthesis of NO, products of selectins, cell survival, apoptosis, cytokine secretion, PAI, ICAM, thromboxane A2, prostaglandin E2. Delays neutrophil apoptosis
IL-1	Induces fever. Stimulates T cells and macrophages. Induces PMN release from bone marrow. Increases adhesion molecules. Stimulates MCP-1 and MIP-1 α and IL-6 production
IL-2	Proliferation and differentiation of T cells into effector T cells, and survival of antigen-specific CD^{4+} and CD^{8+} T cells promoting memory T cells
IL-4	Stimulates B-cell and T-cell proliferation. Promotes B-cell differentiation into plasma cells and class switching to IgE. Decreases Th1 cells and the production of IFN-γ and IL-12
IL-6	Induces fever. Regulates growth and differentiation of T and B cells. Increases antibody production by B cells. Inhibits apoptosis of PMNs and mediates hepatic acute phase response
IL-12	Induces T-cell differentiation to Th1. Activates NK cells
IL-18	Promotes natural killer (NK) cells and T cells to release IFN-γ. Promotes cell-mediated immunity and inhibits IL-4-dependent IgE production
Chemokine	**Function**
IL-8	Induces neutrophil and granulocyte chemotaxis and phagocytosis. Delays neutrophil apoptosis
MIP-1α	Activate PMNs. Stimulates IL-1, IL-6, and TNF-α production
MCP-1	Promotes monocytes, T cells, and dendritic cells to the site of inflammation

Examples of cytokines of the inflammatory response to trauma and sepsis and their functions
PAI platelet activator inhibitor, *PMN* polymorphonuclear neutrophils, *MIP-1α* macrophage inflammatory protein, *MCP* monocyte chemotactic protein

Macrophage inflammatory protein-1 alpha (MIP-1α) mediates both the acute and chronic inflammatory response by recruiting inflammatory cells and stimulating the production of TNF-α, IL-1, and IL-6 by peritoneal macrophages [75]. Other chemokines involved in the inflammatory response include monocyte chemoattractant protein (MCP-1), MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES), monokine induced by gamma interferon (MIG), and IL-8.

Complement

The complement cascade is a defense mechanism activated by antigen–antibody complexes (classical pathway) or microbial surface (alternative pathway) that has primary antimicrobial properties in creating the membrane attack complex (MAC) to lyse invading pathogens and assist in phagocytosis by opsonizing bacteria. The complement system also augments the inflammatory response. Cleavage products of these enzymes in this cascade, C3a and C5a, increase capillary permeability and are powerful neutrophil chemoattractants. The classical and alternative complement

pathways are both activated after trauma and implemented in the inflammatory pathway [76–78]. Macrophages are a major source of complement activation through factor B synthesis in response to LPS and DNA [79, 80]. After activation through injury or infection, there is a nonspecific amplification of the cascade through a positive feedback loop, thus mounting a rapid inflammatory and immune response. When complement activation becomes excessive, it can lead to organ injury [3, 76–78]. We have shown that compliment activation is a driver of the early inflammatory response and organ injury in a mouse model of hemorrhagic shock and trauma [81].

Consequences of the Inflammatory Response

In the setting of acute infection or tissue injury, the adaptive roles of the early inflammatory response will remove the infectious threat, initiate repair processes, and reestablish tissue homeostasis. In trauma, the activation of innate immune pathways probably also occurs to remove microorganisms that invade when barriers are disrupted. There is clearly a threshold at which these processes designed to be adaptive and improve survival become maladaptive and can contribute to adverse outcomes. These adverse outcomes are seen as excessive and sustained inflammation leading to end organ dysfunction and immune dysfunction rendering the patient more susceptible to nosocomial infections. The end effect is a prolonged ICU stay, more days on the ventilator, and increased likelihood of death.

Derangements of Systemic Inflammation

Pioneers in the field of immune dysfunction after sepsis and trauma initially proposed a biphasic immune response [82]. This work was largely based on animal studies, which revealed an early proinflammatory response or SIRS followed by a delayed and sustained immune dysfunction. Recent results from a large multi-institutional observational study in trauma patients have refined the paradigm for the immune response in patients suffering from severe blunt trauma [4] (Fig. 6.1).

In gene array analysis or peripheral blood leukocytes of 167 severe blunt trauma patients, severe injury is shown to induce a “Genomic storm” involving 80 % of the leukocyte transcriptome within the first 4–12 h, which remains altered for days to weeks [4]. The genes that had the greatest increase in expression after trauma involved the innate immunity and the inflammatory response, including integrin signaling, leukocyte extravasation, Fcγ receptor-mediated phagocytosis, IL-10 signaling, TLR signaling, Ephrin signaling, IL-6 signaling, TREM1 signaling, actin cytoskeleton signaling, and B-cell receptor signaling [4]. Those with the greatest decrease in expression involved T-cell activation and antigen presentation [4]. On gene analysis comparing uncomplicated to complicated course after trauma, upregulated pathways associated with complicated recovery include IL-10, IL-6, and p38

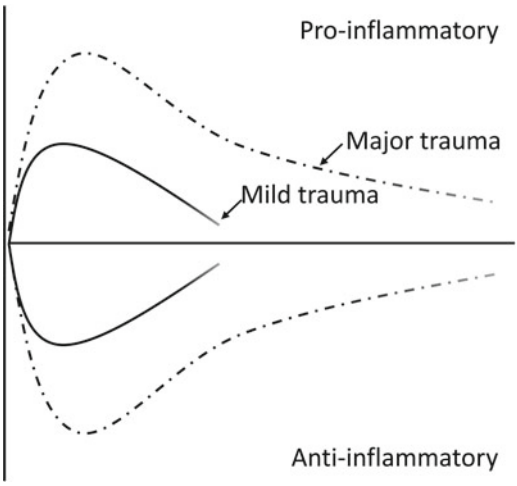

Fig. 6.1 Inflammatory response after trauma. Coexisting proinflammatory and anti-inflammatory responses are rapidly upregulated in circulating leukocytes after injury. Major injury induces a more pronounced response. Adapted from Xiao W et al (2011) A genetic storm in critically injured humans. J Exp Med 208:2581–2590

MAPK signaling [4]. Antigen presentation and T-cell proliferation and apoptosis, T-cell receptor signaling, and NK cell function were the most downregulated pathways associated with complicated recovery [4]. The difference in gene expression between uncomplicated and complicated recovery was the magnitude of the early response and the time required for gene expression to return to baseline levels [4]. There were no genes that are exclusively expressed or suppressed in complicated vs. uncomplicated recovery [4]. The adaptive immune response alterations occur simultaneous with the acute proinflammatory response to injury.

Excessive Inflammation from Severe Injury

Factors that drive the excessive and sustained immune responses after injury are only partially understood. These include magnitude of insult, gender, age, and some gene polymorphisms [4, 83–86].

Immunosuppression

The delayed response to severe trauma is characterized by suppression of both the cell-mediated and adaptive immune responses. This suppression of the adaptive immune response has been associated with regulator T cells, myeloid-derived suppressor cells, and apoptosis. Suppression of both the cell-mediated and adaptive immune responses has been associated with the conversion of the cytokine milieu from a Th1 to a Th2 response. The conversion of a proinflammatory to an anti-inflammatory response is thought to be beneficial to limit the injury induced by the proinflammatory response, though a prolonged anti-inflammatory response seen with severe injury leads to increased susceptibility to infection.

Apoptosis

Apoptosis has been implicated as a cause for depleted lymphocyte response after trauma and sepsis. The degree of T-cell apoptosis directly correlates with the degree of sepsis and occurs by both mitochondrial-mediated and receptor-mediated mechanisms in human populations [87]. The T cells most susceptible to apoptosis include the effector memory helper T cells, while the central memory helper T cells remain relatively spared [88]. Blocking mitochondrial-mediated T-cell apoptosis is by overexpressing Bcl-2 in transgenic mice improved survival in sepsis [89].

Th1 to Th2 Conversion

Helper T cells activate and direct the immune response through cell-mediated and cytokine-directed mechanisms. The two major pathways have been described as a Th1 and Th2 responses. Th1 responses are proinflammatory and promote the cellular immune response, including macrophages, neutrophils, and $CD8^+$ T cells. IL-2, IL-12, and IFN-γ mediate this response. Th2 responses promote antibody-mediated immunity and are mediated by IL-4 and IL-10. Th2 responses are generally considered anti-inflammatory due to the inhibitory effect of IL-10 on proinflammatory cytokines such as IL-2 and IFN-γ. Injury and sepsis causes an alteration in T-cell function from a proinflammatory Th1 to an anti-inflammatory Th2 response [90]. It has been speculated that the function of the Th2 response is to compensate for and neutralize the proinflammatory-induced tissue injury. When produced in excess, as seen in severe injury, the Th2 response can counteract the Th1 response, making the host susceptible to infection.

Immune Response with Age

Advanced age is well known to be an increased risk factor for mortality and multiorgan failure after trauma and sepsis [91–96]. The exact etiology for this has yet to be elucidated but is thought to be due to altered or excessive immune and inflammatory response to these stresses in the aged. The effect of age on inflammation is characterized by a complicated expression of derangements in cytokine production and response, cellular number and activity, and tissue response.

Elderly individuals have a baseline hyperinflammatory state with increased C-reactive protein and cytokine levels of TNF-α, IL-6, and soluble TNF receptor. Elderly also have elevated baseline neutrophil counts [97]. Monocytes in elderly are in a preactivated state, which release a larger initial amount of cytokines, though there is no difference in the peak cytokine production compared to younger individuals [97]. When healthy elderly and young human subjects were given IV endotoxin, the elderly subjects demonstrated a larger initial TNF-α, soluble TNF receptor,

and C-reactive protein response than the younger subjects [98]. Elderly subjects also had a more rapid decrease in monocyte populations and slower resolution of monocyte number and fever after endotoxin exposure [98]. The delayed recovery of leukocytes after infection may make elderly more prone to leukopenia during severe protracted infections. Despite the elevated cytokine level and response to injury, neutrophils appear to be less responsive in the aged. The proliferative response to G-CSF and the ability of GM-CSF to delay apoptosis in neutrophils are blunted in the elderly [99, 100]. The etiology for this diminished response is unknown but could be related to the immune system's tolerance to inflammatory signals from continual immersion in the inflammatory environment of ageing.

The combination of atrophy of the thymus with age, which limits the production of new T cells, in addition to replicative senescence due to telomere shortening in the memory T cells causes a decreased pool of T cells that are less capable to respond newly and previously encountered pathogens [19, 101]. Elderly have decreased proinflammatory IFN-γ and increased anti-inflammatory IL-4 and IL-10 production by T cells suggesting a natural tendency to an immunosuppressive Th2 response compared to young counterparts [102, 103].

Treatment Considerations

The clinical course of sepsis and trauma differs between individuals. This suggests variable activation of inflammatory mediators or different expression in protective mechanisms lead to poorer outcomes. As both excessive proinflammatory and anti-inflammatory processes are maladaptive, modulating either or both processes are viable options for treating the derangements in the inflammatory response. An integrated and predictive model of the immune response using systems biology should be the focus therapeutic approaches, but at this time, such a model has yet to be developed. This could be the underlying reason why attempts to reel-in the derangements in the immuno–inflammatory response have been unsuccessful. There are two straightforward strategies to attempt to "normalize" the magnitude of the host immune response. These include suppression of the initial hyperinflammatory response or the reversal the delayed immunosuppressive state.

Mitigating the Hyperinflammatory Response

It is reasonable to hypothesize that suppressing certain key inflammatory pathways early in the host response to injury could have a beneficial effect on both the proinflammatory and counterinflammatory responses. Complete inhibition, however, may not be desirable since this could result in a loss of the adaptive aspects of a specific mediator or pathway. It is hoped that systems biology analysis will lead to the identification of high value targets for selective therapies. Below are two examples of potential targets.

Nitric oxide, NO, is produced in the setting of inflammation by the high output iNOS by numerous cell types [104]. Nonselective inhibition of iNOS has caused increased injury to liver, while selective inhibition of iNOS has been shown to reduce injury to liver and lung following trauma/hemorrhage and ischemic injuries [2, 104, 105]. The damage associated with nonselective inhibition is thought to be due to inhibition of eNOS, which is organ protective. By selectively inhibiting iNOS, while preserving eNOS, the deleterious inflammatory effects can be suppressed, while maintaining the cytoprotective effects of eNOS. *N*-[3-(aminomethyl) benzyl]acetamidine (1,400 W) is a selective iNOS inhibitor that when administered prior to trauma/hemorrhage, attenuates the hepatic damage, and reduces inflammatory markers of liver tissue myeloperoxidase activity and normalizes the levels of TNF-α and IL-6 after trauma and hemorrhagic shock in Sprague-Dewey rats [105]. iNOS inhibition also decreases the HIF-1α expression, which plays an important role in the inflammatory response to hypoxic stressors [105].

Blocking the actions of proinflammatory cytokines or chemokines in the early inflammatory state after trauma and sepsis is another therapeutic approach. One such cytokine studied in animal models is IL-6. Anti-IL-6 administration prior to burn injury improved survival and reduced translocation of gut bacteria acutely after injury [106]. Anti-IL-6 has also been shown to improve delayed type hypersensitivity and splenocyte proliferation after burn injury [107].

Reverse Immunosuppression

Sepsis and severe trauma are associated with a reduction of T-cell Th1 phenotype. One method to reverse this immunosuppression would be to replace Th1 cytokines. IL-12 is a cytokine produced by macrophages and dendritic cells that induces a Th1 phenotype. Administration of IL12 in a murine model of CLP after burn injury resulted in improved survival [108]. IL-12 supplementation enhanced IFN-γ production and decreased IL-4 production. IFN-γ supplementation after burn injury also improved survival after subsequent peritoneal sepsis by CLP, though less effective than IL-12 supplementation [108]. The excessive toxicity of IL-12 therapy in clinical oncologic trials has limited its clinical usefulness [109].

Another method to reverse the immunosuppression would be to inhibit the Th2 response after trauma or infection. IL-4 and IL-10 are known Th2 cytokines, promoting anergy and inhibiting the cell-mediated response. Treatment with anti-IL-10 antibody restores the Th1 cytokine response by T cells to antigen stimulation after burn injury [90]. Though this may have important role in immunomodulation after trauma and sepsis, IL-10 has been shown to have a dichotomous role in survival after infection. IL-10 is critical to survival of mice in models of endotoxemia and peritonitis while it impairs bacterial clearance and survival in murine *Klebsiella* pneumonia and chronic *Klebsiella* peritonitis models [110–113]. Thus, the dual activities of IL-10 may limit its usefulness as a therapeutic target. Whereas it has a

proinflammatory role early, its prolonged expression after the initial injury renders patients more susceptible to infection. For example, delayed inhibition of IL-10 in a two-hit model of CLP and pseudomonas pneumonia has shown to benefit survival and bacterial clearance [114].

One theory to explain immunosuppression following sepsis is the apoptotic loss of immune mediators. IL-7 is an antiapoptotic cytokine that is currently being studied in multinational clinical trials for HIV, cancer, and hepatitis C and has demonstrated to induce a greater than twofold increase in CD4 and CD8 T lymphocytes in human subjects. In a murine model of peritoneal sepsis, recombinant human IL-7 improved survival, blocked apoptosis of $CD4^+$ and $CD8^+$ T cells, restored IFN-γ production, and improved immune cell recruitment to the site of infection [115]. IL-7 also improved the innate cellular response by increasing the expression of leukocyte adhesion molecules LFA-1 and VLA-4 to improve leukocyte infiltration to the infection site [115]. In vivo, IL-7 is able to restore the loss of delayed type hypersensitivity to recall antigen during sepsis [115]. Other mechanisms to prevent the apoptotic loss of helper T cells include inhibiting proapoptotic factors, such as Bid and Bim, increasing antiapoptotic factor expression such as Bcl-2, and caspase inhibitors. The use of caspase inhibition by *N*-benzyloxycarbonyl-Val-Ala-Asp(*O-methyl*) fluoromethyl ketone (z-VAD) has been shown to prevent T-cell apoptosis and improve survival in septic mice [116].

Conclusions

The inflammatory response to sepsis and trauma is a highly integrated and multifaceted interaction of sensors, cells, and effector responses. As an adaptive response, it is designed to promote tissue repair and immune defenses to impending pathogens during times of stress. The inflammatory response is also self-limiting due to the anti-inflammatory component of the response. Individuals who succumb to proinflammatory events such as major trauma and sepsis have a prolonged anti-inflammatory response that leaves them susceptible to nosocomial infections. It was once thought that the anti-inflammatory response follows the initial proinflammatory response to infection or trauma, but recent evidence points out that the anti-inflammatory response is initiated at the time of the proinflammatory response in injured humans. Under circumstances of severe sepsis and trauma, either the proinflammatory or the anti-inflammatory response may become excessive and prolonged leading to multiorgan failure, immune dysfunction, and further infectious complications. Decades of clinical and experimental research have enhanced our understanding of the host response. However, the overwhelming complexity of the immune response is the most important barrier to progress toward effective therapies. By taking a systems approach, it is hoped that future targets can be identified to modify the immune–inflammatory response to suppress the deleterious effects while maintaining its benefits.

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Chapter 7
Multiscale Equation-Based Models: Insights for Inflammation and Physiological Variability

Jeremy D. Scheff, Steve E. Calvano, and Ioannis P. Androulakis

Introduction

Inflammation is a critical component of the stress response. In response to stressors such as injury and infection, inflammation activates the initial physiological responses aimed at returning to homeostasis. The failure to restore homeostasis subsequent to an inflammatory response can be caused by either an insufficient response that is not strong enough to address the root cause of stress or an overwhelming inflammatory response that leads to further damage in addition to the original stressor. Dysregulation of the inflammatory response is a component of many pathological conditions such as sepsis. Although the incidence of sepsis is increasing, leading to approximately 215,000 deaths per year and healthcare expenditures of $17 billion in the USA alone [1], the only drug approved specifically to treat severe sepsis (activated protein C) was recently withdrawn from the market after failing to show improved outcome in a clinical trial [2]. There is clearly a need for more effective clinical tools for the management of inflammatory dysregulation, and novel approaches may be required to achieve this goal [3].

The pervasiveness of nonlinearity, redundancy, and pleiotropy in components of the inflammatory response leads to challenges in reductionist approaches and motivates systems-level approaches towards understanding inflammation [4]. Mathematical modeling is a promising technique because it allows for studying the dynamics of multiple interacting components of a complex system while integrating research from disparate disciplines with the ultimate goal of gaining insight into disease progression and therapeutic interventions [5, 6]. Thus, the potential exists for significant

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translational innovations based on models of inflammation in optimizing patient care, designing clinical trials, and rationalizing drug development [7–10]. In all of these areas, issues related to physiological variability are important to consider.

Many components involved in the inflammatory response, such as cytokines, hormones, and autonomic signaling, contain homeostatic rhythmic variability at a wide range of time scales. The disruption of physiological rhythms is often associated with disease, such as changes in patterns of heartbeats preceding the onset of sepsis [11, 12] and alterations in circadian variations in plasma cortisol, which are associated with depression [13], obesity [14], psychological stress [15], and cancer [16, 17]. Through studying the origins of these rhythmic signals in homeostasis and their disruption in inflammation, we can work towards understanding their underlying mechanisms and the potential diagnostic utility embedded in physiologic variability. Therefore, when investigating translational applications of systems biology of inflammation, physiological variability represents an important factor influencing the state of the host. For instance, given the circadian time structure underlying many of the physiological responses dysregulated in sepsis, novel therapies should be tested with circadian rhythms in mind because the same treatment given at different times of day could have very different results [18, 19].

In the following sections, we discuss mechanisms through which biological rhythms can exert physiological regulatory effects; relationships between physiological variability and inflammation; and our work on systems-level mathematical modeling of human endotoxemia, as a surrogate model for systemic inflammation, with a particular focus on accounting for the effects of physiological variability in endotoxemia.

Multiscale Modeling of Human Endotoxemia

The human endotoxemia model is an experimental model that can be applied to evaluate issues related to physiological variability in inflammation. Human endotoxemia consists of injection of endotoxin (lipopolysaccharide, LPS) to healthy human volunteers, allowing for the study of systemic inflammation in vivo in humans [20]. LPS is a component of the outer membrane of Gram-negative bacteria that is recognized by the innate immune system and instigates an inflammatory response. The response to LPS initiates from LPS binding to Toll-like receptor 4 (TLR4), leading to the activation of the innate immune system through the transcription of inflammatory mediators, eventually propagating to the systemic level to induce a wide range of physiological changes characteristic of systemic inflammation such as the release of immunomodulatory hormones, the activation of the autonomic nervous system, and increased body temperature [21]. Human endotoxemia reproduces many of the inflammation-linked physiological changes that occur in critical illness such as sepsis [20, 22], acute respiratory distress syndrome (ARDS) [23], and trauma [24]. Additionally, endotoxemia alters biological rhythms at multiple time scales, ranging from circadian rhythms [25] and short-term HRV [26–34], allowing for investigation into the relationship between systemic inflammation and biological rhythms. HRV is driven largely by rhythmic patterns in the variability of heartbeats [35], and diminished HRV is correlated with disease severity in sepsis

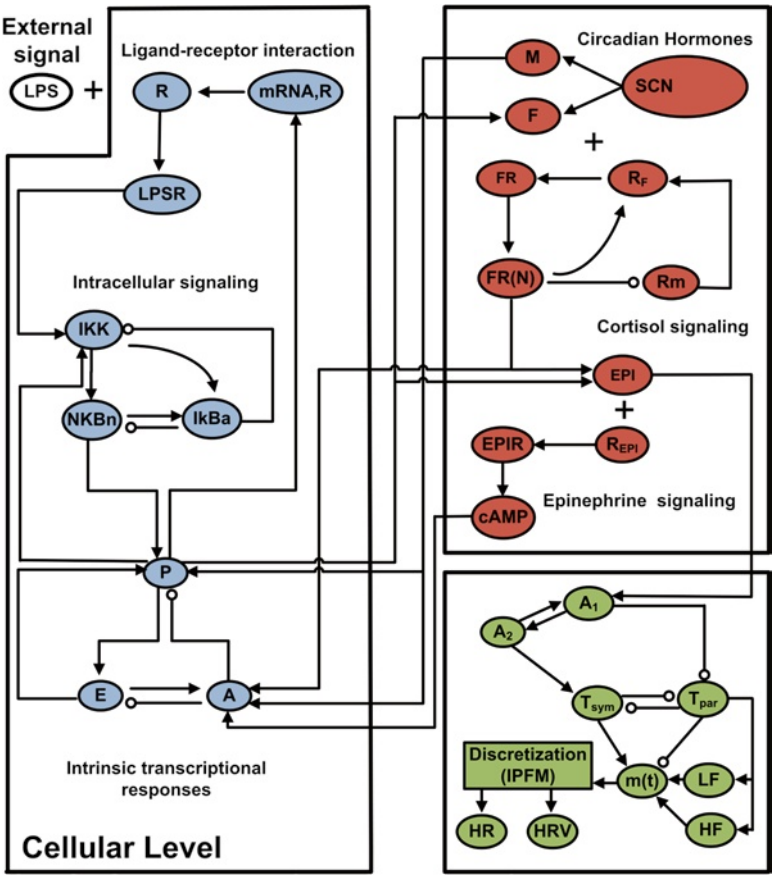

Fig 7.1 Network diagram of the components of a multiscale model of human endotoxemia at three levels. At the cellular level (*blue*), LPS binds to its receptor TLR4 (R) forming the activated complex LPSR. LPSR stimulates NF-kB activity (IKK, NfKBn, and IkBa), which modulates the transcriptional response to inflammation, consisting of proinflammatory (P), anti-inflammatory, and energetic (E) components. At the central level (*red*), hormonal output both responds to and modulates the progression of the inflammatory response to LPS through both cortisol (F) and epinephrine (EPI) signaling. Furthermore, circadian rhythms in the immunomodulatory hormones cortisol and melatonin (M) impose circadian patterns on many components of the inflammatory response. Finally, at the autonomic level (*green*), changes in sympathetic (T_{sym}) and parasympathetic (T_{par}) signaling are reflected in modulated patterns of heart rate (HR) and heart rate variability (HRV)

[36–41]. In addition to serving as a useful biomarker (see Chaps. 4 and 5), the loss of HRV may give insight into disease mechanisms. This reduction in HRV may be driven by a loss of interorgan coupling and communication [21, 27, 42, 43].

Here, we describe the iterative development of multiscale models of human endotoxemia, starting with the binding of LPS to its receptor on immune cells and growing to encompass hormonal responses and changes in heartbeat patterns. These three compartments are depicted in the network diagram in Fig. 7.1. Additionally, we identify and discuss areas where physiological variability may play an important role in either governing the response to endotoxemia or giving insight into the state of the system.

Immune Cells

While there are many levels to consider in a model of human endotoxemia, a critical aspect is modeling the initial recognition of LPS and the production of signals that lead to a systemic response. In general, pathogen-associated molecular patterns (PAMPs) bind to Toll-like receptors (TLRs) expressed by cells of the innate immune system, leading to transcriptional responses including the production of proinflammatory cytokines; LPS, in particular, activates the TLR4 signaling pathway leading to broad transcriptional changes driven by inflammation-related transcription factors such as NF-κB [44]. The initial transcriptional response to human endotoxemia has been studied experimentally through high-throughput DNA microarrays, which simultaneously quantify the state of thousands of gene transcripts in blood leukocytes [45]. This wealth of experimental data allowed for data-driven modeling of the transcriptional responses to LPS without the a priori postulation of which genes are most important in endotoxemia. From this, a dynamical model was constructed to represent transcriptional changes in leukocytes during human endotoxemia [8, 9].

Identification of Key Transcriptional Responses

In order to discover the critical transcriptional motifs in high-dimensional time-course microarray data, a systematic computational framework was recently proposed that decomposes the data into elementary set of clusters representing key temporal responses [46]. Given the availability of such high-dimensional data in human endotoxemia experiments [45], we applied this computational approach based on the hypothesis that a specific underlying network structure gives rise to the dynamics of the inflammatory response. Therefore, we sought to identify a set of core transcriptional responses to endotoxemia representative of the dynamic evolution of the host response to LPS under the assumption that related genes responsive to endotoxin undergo concerted changes in their expression profiles.

The clustering algorithm is based on a symbolic discretization of time-series data, which labels similar temporal expression profiles with the same symbolic motif [47]. Having assigned each gene to a motif, the next task is to select motifs that are so highly populated that the temporal patterns of the genes in that motif are very unlikely to arise by chance. In other words, the goal is to identify highly non-random patterns in gene expression profiles putatively caused by a coherent transcriptional regulatory mechanism, thus generating a subset of transcriptional motifs, which characterize the host response to LPS. The next step is to reduce this relatively large subset of transcriptional motifs into a smaller elementary set that best characterizes the deviation from homeostasis in human endotoxemia. The global nature of gene microarray data generally results in a roughly log normal distribution

of gene expression values [48]. Consistent deviations from this distribution generally result in a motif that is highly enriched relative to baseline. Based on this concept, the transcriptional state of the system is defined as the distribution of expression values at a time point; by comparing the transcriptional state of the system as a whole with the distribution of expression values in a subset of motifs, the motifs that lead to a maximal deviation can be identified. This was done by applying the Kolmogorov–Smirnov test at each time point for subsets of highly populated (as defined above) motifs and searching through the potential combinations of motifs to identify the minimum number of motifs that maximally deviate from the overall transcriptional state distribution. This defines a combinatorial optimization problem that was solved through a stochastic simulated annealing optimization algorithm.

Applying the algorithm described above to human endotoxemia, data revealed three critical expression motifs, all enriched in genes participating in physiologically relevant pathways (1) an early upregulated proinflammatory response containing genes related to TLR signaling and members of the NF-κB/RelA family; (2) a late upregulated anti-inflammatory response including components of the JAK-STAT and IL-10 signaling cascades; and (3) a downregulated energetic response comprised largely of genes involved in cellular bioenergetic processes. All of these expression patterns return to baseline within 24 h.

This approach for identifying the key transcriptional signals in human endotoxemia through high-dimensional data analysis is appealing for several reasons. First, it provides in vivo data from a relatively accessible source (blood samples). Thus, the gene expression data reflects complex regulatory properties of the human inflammatory response, such as hormonal and autonomic responses, which cannot be recapitulated through analysis of human cell lines; some of these interactions will be described in later sections of this chapter. Additionally, the ability to gather this type of experimental data in humans rather than in animals means that the results are likely closer to human clinical data, although of course animal studies would allow for a wider range of experimental perturbations and analysis techniques.

Indirect Response Modeling

Having identified the critical components of the transcriptional response to endotoxemia, the next step was to account for these transcriptional patterns in a dynamical model. This is a challenging problem because the precise signal transduction steps leading to the activation of specific genes are not always known, and even if they were, the parameters governing that signal transduction pathway are generally not known. In pharmacokinetic/pharmacodynamic modeling, this problem is often approached through indirect response modeling to quantify indirect relationships between model components [49]. In general, if a compound is modeled by a 0th order production term and a 1st order degradation term, such as x in Eq. (7.1a), then

indirect effects on these productions and degradation terms can be modeled as in Eq. (7.1b), where $f(y)$ is function (typically sigmoidal) of y representing y's indirect stimulation on the production rate of x.

$$\frac{dx}{dt} = k_1 - k_2 \cdot x \tag{7.1a}$$

$$\frac{dx}{dt} = k_1 \cdot \left(1 + f(y)\right) - k_2 \cdot x \tag{7.1b}$$

Depending on the sign and placement of this indirect modulation term, it can represent either stimulation or inhibition of either the production or degradation rate of x. Furthermore, it allows for several factors on production or degradation to be combined multiplicatively. Through indirect response modeling combined with a simple model of LPS recognition, we developed an eight equation model of the transcriptional responses to endotoxemia. Equation (7.2a) shows the three core transcriptional responses described above, the proinflammatory, anti-inflammatory, and energetic responses, respectively [8, 9].

$$\frac{dP}{dt} = k_{in,P} \cdot \left(1 + H_{P,DR^*}\right) \cdot \left(1 + H_{P,E}\right) / A - k_{out,P} \cdot P \tag{7.2a}$$

$$\frac{dA}{dt} = k_{in,A} \cdot \left(1 + H_{A,P}\right) \cdot \left(1 + H_{A,E}\right) - k_{out,A} \cdot A \tag{7.2b}$$

$$\frac{dE}{dt} = k_{in,E} \cdot \left(1 + H_{E,P}\right) / A - k_{out,E} \cdot E \tag{7.2c}$$

$$H_{X,Y} = k_{X,Y} \cdot Y \tag{7.2d}$$

This results in a model which has the ability to produce dose-dependent responses to LPS as shown in Fig. 7.2. In response to a low dose of LPS, as is given in human endotoxemia experiments, the transcriptional responses are activated acutely and return to baseline within 24 h. Yet, in response to a higher dose of LPS, the anti-inflammatory controls are overwhelmed by self-stimulatory proinflammatory signaling, leading to a persistent inflammatory state.

Equation (7.2a) represents the core transcriptional responses of the innate immune system to human endotoxemia, and indirect response modeling allows for the extension of this core to interact with other systems as described in subsequent sections.

Central Control of Immunomodulatory Hormones

In systemic inflammation and in endotoxemia, inflammatory mediators produced by immune cells in response to LPS recognition are secreted into systemic circulation

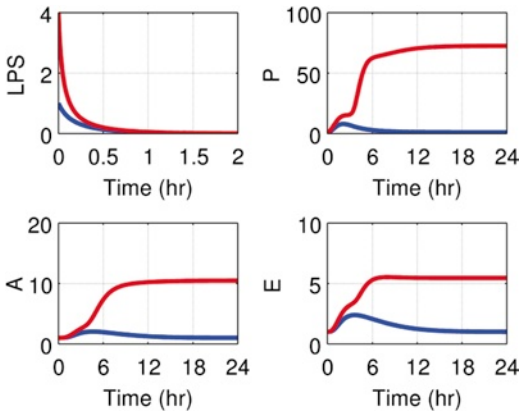

Fig. 7.2 The cellular-level model described more completely in Foteinou et al. [9] predicts two classes of responses to acute endotoxemia. In response to relatively low doses (*blue lines*), a resolving response is generated where all components return to baseline within 24 h. Yet, in response to larger doses of LPS (*red lines*), the self-stimulatory nature of the proinflammatory response dominates and leads to a persistent inflammatory state. *P* proinflammatory transcriptional response, *A* anti-inflammatory transcriptional response, *E* energetic transcriptional response

and recognized by the central nervous system, which responds with the release of immunomodulatory hormones such as cortisol (an endogenous glucocorticoid in humans) and epinephrine. Both of these hormones normally undergo circadian rhythms. Glucocorticoids are also commonly used anti-inflammatory drugs. Therefore, there is value in an integrated model relating the pharmacodynamics of glucocorticoids and other hormones with the progression of the human endotoxemia response [8, 9, 50]. Glucocorticoids exert their immunomodulatory effects through binding to the glucocorticoid receptor in the cytosol, translocating to the nucleus, and then acting as a transcription factor for a wide range of glucocorticoid-responsive genes. This glucocorticoid signal transduction pathway has been studied from the perspective of pharmacodynamics, resulting in well-established mathematical models such as the model by Ramakrishnan et al. shown in Eq. (7.3a) [51].

$$\frac{\mathrm{d}R_{\mathrm{m}}}{\mathrm{d}t} = k_{\mathrm{syn\_Rm}} \cdot \left(1 - \frac{\mathrm{FR}(N)}{\mathrm{IC}_{50\_\mathrm{Rm}} + \mathrm{FR}(N)}\right) - k_{\mathrm{deg\_Rm}} \cdot R_{\mathrm{m}} \tag{7.3a}$$

$$\frac{\mathrm{d}R}{\mathrm{d}t} = k_{\mathrm{syn\_R}} \cdot R_{\mathrm{m}} + R_f \cdot k_{\mathrm{re}} \cdot \mathrm{FR}(N) - k_{\mathrm{on}} \cdot F \cdot R - k_{\mathrm{dgr\_R}} \cdot R \tag{7.3b}$$

$$\frac{\mathrm{dFR}}{\mathrm{d}t} k_{\mathrm{on}} \cdot F \cdot R - k_T \cdot \mathrm{FR} \tag{7.3c}$$

$$\frac{\mathrm{dFR}(N)}{\mathrm{d}t} = k_T \cdot \mathrm{FR} - k_{\mathrm{re}} \cdot \mathrm{FR}(N) \tag{7.3d}$$

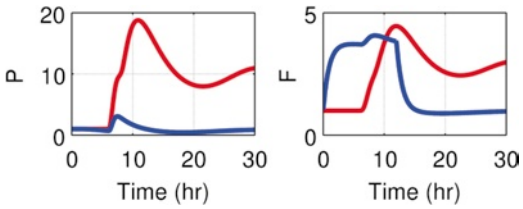

Fig. 7.3 After incorporating the effects of immunomodulatory hormones on the inflammatory response [8], the response to cortisol treatment was evaluated. The *two lines* show responses to identical doses of LPS at 6 h, but the *blue lines* represent a system that has been infused with cortisol for 6 h prior to LPS, while the *red lines* represent a simulation of the response to only LPS. This produces divergent outcomes to the same dose of LPS, with cortisol pretreatment exerting a protective effect leading to a self-limited response rather than a persistent inflammatory state

The variables in Eq. (7.3a) represent glucocorticoid receptor mRNA (R_m), free cytosolic receptor (R), cytosolic glucocorticoid-receptor bound complex (FR), and nuclear glucocorticoid-receptor complex [FR(N)], driven by a glucocorticoid concentration F. FR(N) is then the component that acts as a transcription factor, which we can account for in our core transcriptional response equations by altering Eq. (7.2b) to account for the effect of glucocorticoids on anti-inflammatory gene transcription as shown in Eq. (7.4).

$$\frac{dA}{dt} = k_{in,A} \cdot \left(1 + H_{A,P}\right) \cdot \left(1 + H_{A,E}\right) \cdot \left(1 + H_{A,FR(N)}\right) - k_{out,A} \cdot A \tag{7.4}$$

Through this integrated model, upstream changes in glucocorticoid levels (either endogenously produced in response to inflammation or exogenously given) propagate through the model and modulate transcriptional processes in immune cells. This allowed us to evaluate the relationship between endotoxemia and cortisol treatment, given either before or after LPS [8, 9, 50]. For example, Fig. 7.3 shows how cortisol infusion prior to LPS can have a protective effect, allowing for a resolving response to a large dose of endotoxemia that otherwise would perturb the system to the persistent inflammatory steady state. It also allowed for the evaluation of the effects of circadian rhythms on human endotoxemia [52].

Circadian Rhythms

Circadian rhythms are present in many components of the inflammatory response [53] including plasma cytokine [54–58] and cortisol concentrations. Melatonin, another hormone with both circadian and immunomodulatory properties, may play a key role in mediating communication between peripheral components of the immune system and the central circadian clock in the suprachiasmatic nucleus [53]. Furthermore, given that melatonin levels peak in the night, roughly at the same time as cytokines, and melatonin stimulates the production of cytokines [59–61],

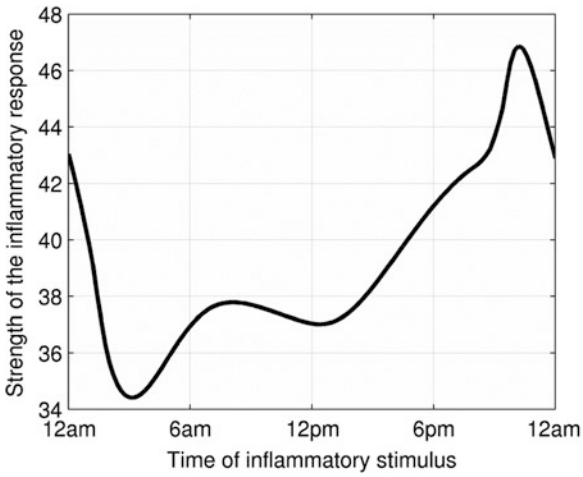

Fig. 7.4 The magnitude of the inflammatory response to LPS has a circadian dependence as illustrated here by the maximal response of proinflammatory signaling to identical LPS doses given at different times throughout the day [52]

melatonin signaling serves as a plausible mechanism for synchronization between central and peripheral circadian clocks in the immune system, and indirect response modeling can be applied to represent this relationship as described above. Circadian hormone production is then imposed by varying the production rates of cortisol and melatonin throughout the day [62]. In total, this produces circadian rhythms that propagate throughout the model, in line with homeostatic experimental data [52]. In response to endotoxemia initiated at different times throughout the circadian cycle, this model predicts that responsiveness of the innate immune system to LPS has a circadian dependence as shown in Fig. 7.4, for instance decreasing when the anti-inflammatory hormone cortisol is at high levels in the morning.

Ultradian Rhythms

The circadian pattern in cortisol secretion is driven by patterns in rhythmic cortisol secretion at a faster time scale, known as ultradian rhythms. This refers to the pulses of cortisol released roughly hourly, whose magnitude imposes a circadian rhythm. We recently studied a combined model of the hypothalamic–pituitary–adrenal (HPA) axis and glucocorticoid pharmacodynamics [62]. This work revealed differences in mean levels of homeostatic gene expression in response to constant or oscillatory hormone levels as well as a correlation between homeostatic ultradian rhythm amplitude and peak responsiveness to stress. Similar computational results were identified by Rankin et al. [63]. The importance of glucocorticoid ultradian rhythms is further supported by experimental studies showing differences in expression of glucocorticoid responsive genes when exposed to oscillatory or constant patterns of glucocorticoid concentration [64, 65] as well as more general relationships linking effective physiological function with rhythmic variability in HPA axis output [66].

Heart Rate and Heart Rate Variability

It has long been recognized that critically ill patients, such as those with sepsis, tend to exhibit diminished physiological variability as quantified by HRV [36–41] (see also Chaps. 4 and 5). Despite this, there is a lack of mechanistic understanding as to why the phenomenon of decrease HRV in disease exists, which limits the translational applications of HRV metrics to observation-based prognostic and diagnostic analysis rather than the development of novel therapies. Given the similarities in physiological changes occurring in endotoxemia and sepsis [20, 22], including the loss of HRV in endotoxemia [26–34, 67], human endotoxemia represents an excellent platform for studying the mechanistic origins and implications of inflammation-driven diminished HRV by applying both experimental and computational techniques.

Modeling changes in HRV driven by inflammation necessitates a multiscale approach. HRV can be quantified by a diverse array of metrics operating on discrete data, a series of heartbeat intervals. However, this discrete signal is modulated by continuous variables such as concentrations of inflammatory mediators. Thus, a significant challenge in mechanistic modeling of HRV in endotoxemia is reconciling continuous inputs (such as hormone and cytokine concentrations) with discrete, noisy output (the beating of the heart). We approached this problem through a continuous model of autonomic influences on the heart combined with a discrete model to output a series of heartbeats, which were then postprocessed to assess HR and HRV [68].

Autonomic Origins of Heart Rate Variability

Cyclic contractions of the heart initiate at the sinoatrial (SA) node, also known as the pacemaker of the heart. The sympathetic and parasympathetic branches of the autonomic nervous system converge at the SA node, allowing for appropriate regulation of heart rate. The SA node is also exposed to oscillations in the output of the autonomic nervous system, which typically occur in characteristic frequency ranges. *High frequency (HF) rhythms* in the frequency range of 0.15–0.4 Hz [69] are largely the manifestation of the breathing pattern and are transduced to the heart by the vagus nerve [70]. *Low frequency (LF) rhythms* in the frequency range of 0.04–0.15 Hz [69]. LF oscillations are generally interpreted as reflecting fluctuations in both sympathetic and parasympathetic activities [70]. At a much longer time scale, *circadian rhythms* in autonomic activity are also apparent in HRV [71].

These periodic signals modulate the firing pattern of the SA node, leading to rhythmic components in HRV. Thus, to mechanistically link inflammation with changes in heartbeat patterns, a model of the autonomic modulation of the heart is required. Based on prior work considering the effects of endotoxemia on the autonomic nervous system [72], we constructed a continuous algebraic equation

representing effective autonomic modulation of the SA node in homeostasis and in endotoxemia [68] as shown in Eq. (7.5).

$$m(t) = \underbrace{\underbrace{\mathrm{HR}}_{\text{constant activity level}} + \underbrace{k_{\mathrm{circ}}\left(T_{\mathrm{sym}} + \frac{1}{T_{\mathrm{par}}}\right)}_{\text{circadian variability}} + \underbrace{k_{\mathrm{osc}}\left(1 + k_{\mathrm{par,LF}}T_{\mathrm{par}}\right)\sin\left(f_{\mathrm{LF}}t\right)}_{\text{LF oscillations}}}_{+\underbrace{k_{\mathrm{osc}}\left(1 + k_{\mathrm{par,HF}}T_{\mathrm{par}}\right)\sin\left(f_{\mathrm{HF}}t\right)}_{\text{HF oscillations}}} \tag{7.5}$$

Equation (7.5) accounts for a baseline activity level modulated by the three rhythms discussed above: HF, LF, and circadian rhythms. The effective modulations imposed by each of these components are driven by levels of sympathetic (T_{sym}) and parasympathetic (T_{par}) activity, which respond to the levels of inflammatory mediators [72].

However, to assess HRV, a series of discrete heartbeats is required. Therefore, the continuous Eq. (7.5) must be discretized to output distinct heartbeats whose period is modulated by this effective autonomic activity.

Discrete-Continuous Modeling

Cells at the SA node respond to the autonomic nervous system by recognizing the concentrations of autonomic neurotransmitters, leading to altered firing rates. In the absence of autonomic modulation, the heart still beats, just with a regular pattern unperturbed by autonomic rhythms. This type of system can be represented with an integrate-and-fire model, where fluctuations in the propensity for the SA node initiating a contraction depend on the effective autonomic modulation [68, 73, 74]. This is done by repeatedly integrating under the curve defined in Eq. (7.5) and recording the time of a heartbeat occurring whenever a constant threshold is reached. The translation from continuous input signals to a discrete output system is a fundamental aspect of mechanistic modeling of HRV, as physiologically a similar discretization process is occurring. Homeostatic circadian output of this model is shown in Fig. 7.5, illustrating how oscillatory autonomic input produces patterns in both HR and HRV. Based on mechanistic modeling, the resulting discrete list of heartbeats can then be analyzed with the same algorithms that are used for real data. HRV is quantified by time domain, frequency domain, and nonlinear metrics, all aimed at either gaining some specific physiological information from the heartbeat data or optimizing the correlation of the HRV metric with some relevant clinical outcome. Different HRV metrics thus have different information content and have different practical applications. Based on the discrete output of our model, we were similarly able to apply diverse HRV metrics, revealing discrepancies in responsiveness of different HRV metrics to endotoxemia.

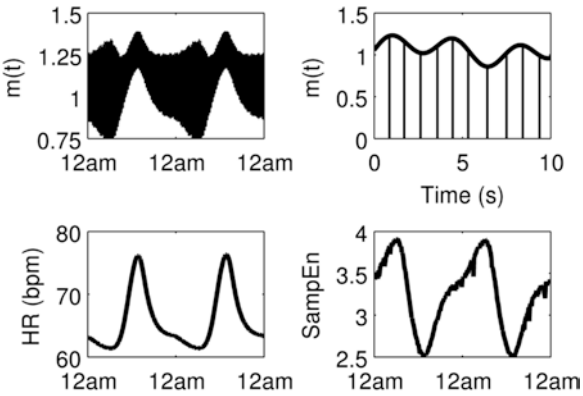

Fig. 7.5 Multiscale rhythmic effects on the heart are depicted here. At the *top*, $m(t)$ represents the effective autonomic modulation of the heart, which contains circadian (24 h), HF (0.15–0.4 Hz), and LF (0.04–0.15 Hz) components. These combined rhythmic effects produce variability in the discretized beating of the heart as illustrated by *circadian patterns* in both heart rate (HR) and heart rate variability (HRV) as quantified by sample entropy (SampEn). These rhythmic components of the output of the heart are altered in response to endotoxemia [68]

Challenges in Translational Modeling of Heart Rate Variability in Endotoxemia

Although it is difficult to precisely identify how changes in HRV relate to changes in underlying physiology [75], there are already practical clinical applications of HRV analysis. Even going beyond purely phenomenological approaches, our growing mechanistic understanding of the origins of HRV and the dysregulation of physiological rhythms is leading to further insights. For instance, the baroreflex negative feedback loop maintains homeostatic blood pressure while also producing oscillations in blood pressure that are reflected in HRV. In sepsis, the baroreceptor function is altered, producing changes in autonomic output that can be assessed through HRV [38]. Thus, some of the decrease in HRV in sepsis may result from dysregulated baroreflex activity. Studying the mechanisms driving rhythmic patterns in HRV will eventually allow for the maximization of information retrieval from the heartbeat signal, which is particularly of interest when considering changes occurring in the stress response. However, several challenges remain in more broadly developing and leveraging mechanistic model-based approaches in the context of inflammation-linked diseases.

While endotoxemia experiments have explored changes in HRV in response to LPS [26–34, 67], even in this controlled environment a broad understanding of the mechanisms is lacking. Given the importance of sympathetic and parasympathetic oscillations in driving HRV, changes in autonomic activity in endotoxemia likely play a role in the altered HRV patterns that have been seen experimentally. It is often assumed that sympathetic activity increases in endotoxemia, based on the

characteristic physiological changes observed such as increased HR. It is also known that the parasympathetic nervous system plays a role in endotoxemia via the cholinergic anti-inflammatory pathway [76]. However, experimental data on HRV changes in endotoxemia shows decreases in both HF and LF rhythms, which is indicative of a decrease in autonomic modulation of the heart [77]. Further investigation into this apparent paradox sheds light on our lack of understanding of autonomic function in endotoxemia and thus also in systemic inflammation in general. Mounting experimental evidence suggests that an answer to this paradox may lie in the sensitivity of the heart to autonomic activity during inflammation, because if the responsiveness of the heart to autonomic signaling is diminished, then HRV will generally decrease. Fairchild et al. showed that pathogen-mediated effects on cardiac function desensitize the heart's response to vagal signaling in mice [77]. In human endotoxemia, Sayk et al. found decreased sympathetic activity measured through microneurography at the peroneal nerve [30]. While peroneal nerve activity may not precisely reflect sympathetic activity at the heart [32], Sayk et al. also found that sensitivity of the heart to drug-induced sympathetic modulation is diminished in endotoxemia [30]. In vitro experiments show that, independent of the autonomic nervous system, interactions between inflammation and cardiac tissue can produce altered beating patterns and also play a role in altering the sensitivity of the heart to autonomic activity [34, 78, 79]. These results suggest that the changes in HR and HRV in endotoxemia may be driven not by changes in autonomic output, but by nonautonomic interactions between inflammation and the heart and decoupling between the heart and the autonomic nervous system.

Godin and Buchman hypothesized reduced physiological variability, such as reduced HRV, is representative of diminished interorgan communication [26, 27]. Given the experimental evidence discussed above, this hypothesis is as relevant as ever. Even in a well-studied and controlled environment like human endotoxemia, the existence of biological rhythms with multiple sources and regulators makes it challenging to determine what precisely a change in HRV means. However, this complexity also means that there is potentially a wealth of information from a wide range of sources embedded in the HRV signal, representing an opportunity to apply computational techniques to reveal as much as possible of the physiologically importance of HRV. Through representing the mechanistic background of HRV in a mathematical model, we can evaluate hypotheses concerning the origins of HRV in homeostasis and the loss of HRV in the stress response by evaluating the relationship between endotoxemia and HRV.

Conclusions

While the lack of mechanistic understanding of the relationship between HRV and endotoxemia has not fully impeded the clinical application of HRV analysis in inflammation-linked disorders such as spesis [12], increased mechanistic knowledge, backed both by systems-level experimentation and mechanistic multiscale

models, will likely lead to improvements in diagnostic and prognostic applications of HRV as well as potentially novel therapeutic strategies. Just as the realization that the vagus nerve modulates inflammation [80] resulted in the conception of novel therapies based on vagus nerve stimulation [81], greater understanding as to why HRV correlates with disease state may reveal other pathways for therapeutic intervention.

HRV is a particularly appealing metric of physiologic variability due to the ease of noninvasive measurement and well-established correlations with disease state. However, biological rhythms at other time scales are also of importance. For example, LPS given at different times of day both suppresses and synchronizes circadian clock gene expression in peripheral blood leukocytes [25], representing another scale at which the decoupling between oscillators may play a role in inflammation, given the observed relationship between disease and circadian rhythms [82]. Rhythmic oscillations in NF-κB activation [83] and ultradian rhythms in regulate the inflammatory response.

The relationships between physiological variability, interorgan communication, and disease suggest that monitoring variability may reveal disease state; thus, decreases in variability would correspond with disease progression and increases in variability (towards homeostasis) would correspond with recovery. However, the specific molecular mechanisms driving the loss of variability may be disease specific, paralleling how the presence of variability itself may exert physiological effects through several different mechanisms. Increasingly detailed mechanistic modeling will be required to understand the underlying molecular processes driving the loss of variability, and studying these processes in endotoxemia represents a good first step towards this goal. If these lower level processes can be linked to readily available observables such as HRV, then this will lead to advances in translational medicine.

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Chapter 8
Integrating Data-Driven and Mechanistic Models of the Inflammatory Response in Sepsis and Trauma

Nabil Azhar, Qi Mi, Cordelia Ziraldo, Marius Buliga, Gregory M. Constantine, and Yoram Vodovotz

Introduction

Inflammation is an essential process in maintaining health and responding to disease. Acute inflammation is driven largely by the innate immune system, which not only serves as the first line of defense against invading pathogens but also functions to resolve tissue damage and restore homeostasis upon a variety of inflammatory conditions including sepsis, trauma, wound healing, and many more. However, when inflammation is either insufficient to address the original disruption of homeostasis, or becomes dysregulated and systemic, it can contribute substantially to morbidity and mortality in these conditions. Dysregulated systemic inflammation also

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plays a significant role in the pathophysiology of diseases that are not primarily attributed to innate immunity such as cancer and diabetes. Although the list of diseases is broad and the processes important to each setting may differ in certain respects, the core architecture of the inflammatory response to biological stress is highly conserved [1].

The systemic inflammatory response syndrome (SIRS) is a major driver of morbidity and mortality in the settings of sepsis and trauma/hemorrhagic shock. Sepsis is one of the leading causes of death in the USA and is responsible for nearly $17 billion in health care costs annually [2]. Trauma/hemorrhage is the most common cause of death for young people in the USA, costing over $400 billion every year [3]. In both sepsis and trauma, the acute inflammatory response is concomitant with physiologic manifestations including changes in heart rate and body temperature, responses that act in a concerted fashion in order to help optimize host defense while minimizing tissue damage. Indeed, although a well-regulated inflammatory response is crucial for effective healing and host defense, an excessively vigorous response can become self-perpetuating and lead to organ dysfunction and death [4, 5]. Both sepsis and trauma patients are particularly susceptible to multiple organ dysfunction syndrome (MODS), a poorly understood syndrome that may be partly attributed to excessive and dysregulated inflammation [5]. These vastly different outcomes can be explained by the overall framework of the immune response, which includes a positive feedback loop from inflammation → damage/dysfunction → inflammation that can drive pathophysiology in inflammatory diseases [6–8].

The adverse effects of self-sustaining inflammation are likely responsible for the general perception of inflammation as an intrinsically harmful process [9, 10]. However, in addition to the aforementioned beneficial roles of inflammation in the resolution of tissue injury, recent studies suggest that morbidity and mortality are worse in animals with low levels of early proinflammatory signals [11]. The emerging view of inflammation is indeed more nuanced, casting inflammation as a highly coordinated communication network that allows the body to sense and respond to challenges and subsequently restore homeostasis [6, 12]. One may consider the complexity resulting from this coordination to be an indicator of a well regulated and properly orchestrated response, and consequently a less complex response would be indicative of a pathological dys- or mis-connectivity of the network. Guided by insights from studies on the dysregulated physiology characteristic of sepsis and trauma/hemorrhage, which have reported that a decrease in variability/complexity of heart rate can presage increased morbidity and mortality, we have suggested that well-organized dynamic networks of mediators are crucial to an appropriate inflammatory response [2, 13]. Indeed, such networks are induced early in the response to experimental surgical trauma in mice, and these networks become disorganized and less complex with the addition of hemorrhagic shock to this minor trauma [13].

The current paradigm for acute inflammation, based in large part on studies in response to trauma, hemorrhage, or infection, involves a dynamic cascade of cellular and molecular events. Innate immune cells, such as mast cells, neutrophils, and macrophages, are activated directly by bacterial endotoxin or indirectly by various stimuli elicited systemically upon trauma and hemorrhage [14–17], including the

release of damage-associated molecular pattern molecules (DAMPs) [7, 18, 19]. Both DAMPs and proinflammatory cytokines—primary among them tumor necrosis factor-α (TNF-α) [20–26]—further activate both parenchymal and immune/inflammatory cells and can affect tissue/organ physiology adversely. These stressed tissues/organs feed back positively to promote further production of inflammatory mediators. We have hypothesized that this behavior could lead to multicompartment and multiscale inflammatory "tipping points" [27–29].

A Systems Approach to Inflammation

The complexity and nonlinearity of the acute inflammatory response as described above has largely stymied the development of novel therapies for trauma/hemorrhage and sepsis. Systems biology is an emerging paradigm for tackling complex biological systems in a holistic fashion [30]. Approaches in systems biology span a broad range of techniques and can be categorized roughly into correlative or causative methods, with focus on either learning basic principles of system organization and function [31–33] or building predictive computational models [31, 34]. Although there is overlap between these areas, most efforts at elucidating biological mechanisms from high-dimensional data have traditionally focused on particular points along this spectrum of computational approaches. We suggest that gleaning translationally relevant insights into the inflammatory response and its interconnected (patho)physiology will require integration of methods from across this spectrum [13–17, 35–38], in order to progress from data to models to actionable knowledge and prediction (ideally in an in vivo or clinical context) [18, 27].

Data-Driven (Correlative) Approaches to Dynamic Inflammation Data

Statistically based approaches, with which most biologists and clinicians are generally familiar, include regression techniques that build models predictive within the conditions of the data on which the models were trained [39]. Although these methods cannot provide detailed mechanistic insights, they can be used to understand abstract features of the response such as the presence of nonlinearities or the identification of factor interactions that affect the response. The main drawback of this class of models is the fact that they often are devoid of mechanistic insight, and their linearity in the parameters can overfit to the data on which they were trained. Associative methods, such as hierarchical clustering, may be used to highlight the natural variability, as well as any overlap, across experimental or clinical conditions. Hierarchical clustering is a simple and unbiased clustering method, which aims to build a hierarchy of clusters. The limitation is the cluster must be built pairwise; since it is purely based on the similarity between the data, the cluster may lack

biological relevance [20]. Hierarchical clustering is used extensively in the genomics field and was used to discern patterns and coregulated clusters of gene expression associated with sepsis and trauma/hemorrhage in both animals [40–43] and humans [44–48].

A less-utilized data-driven method is principal component analysis (PCA), which reduces a high-dimensional dataset into a few principal components that account for much of the observed variance in the data. When applied to time-series data, PCA may identify the subsets of the variables under study (genes/proteins/etc.) that are most strongly representative of the response. Thus, these principal components may be interpreted as the principal drivers of the observed response and can give some mechanistic insights into the underlying process [13, 49]. In the setting of inflammation, correlative approaches, such as PCA, could facilitate the development of therapeutics by yielding insights into the mechanisms by which these therapeutic modalities may function [50]. Similarly, PCA may aid the development of diagnostics by analyzing the cytokine milieu in the blood resulting from inflammatory spillover in order to identify the health state of individuals and possibly inform patient-specific interventions [51].

Nonetheless, principal components, being linear combinations of the original mediator variables, often do not lend themselves to clear biological interpretations [32]. Principal components do, however, greatly ease dimensionality issues and provide a compact and efficient explanation of the data in terms of meaningful groups of mediator variables. Successful implementation of PCA within this context requires some adjustments. Mediators are measured on widely different scales which need to be appropriately adjusted for meaningful comparisons. This may be done in several ways, taking into account known biological effects. Two mediators may show significant variation within their possibly very different ranges, in which case we can rescale them appropriately. However, this should not be done, for example, if one of the two hypothetical mediators has small variation simply because it is an inert factor. Rescaling inert factors would simply amplify the error in the data. Once this rescaling issue is settled, a PCA can be carried out. In our own studies, we augmented such analysis in two additional ways. We reevaluated the importance of a specific mediator as follows: deem k principal components as being significant (by explaining, as usual, a certain fraction of the total variance). Next, assess the importance of each mediator in view of these k principal components, by adding the absolute values of the weights associated to that mediator within the k principal components. The higher the sum, the more relevant the mediator. This allows us to rank the relative importance of the mediators. A word of caution: a mediator that is naturally very noisy may be ranked as important by the PCA method, but it need not necessarily be highly relevant to the phenomenon under study. The last point we make is that it is often more convenient to work with only biologically intuitive linear combinations of mediators rather than principal components. Such intuitive linear combinations are usually suggested by the principal components themselves from which we may delete certain mediators that appear nonintuitive. This still reduces the dimension, offers good biological interpretation, but the analysis that results is more complicated, since these linear combinations become correlated [18, 32].

One clinically important area in which we have carried out data-driven modeling is traumatic brain injury (TBI). Inflammation induced by TBI can lead to both morbidity and mortality [3, 52]. We obtained both clinical data and data on the dynamic changes in multiple inflammatory mediators in the cerebrospinal fluid of TBI patients. The clinical data on each patient consisted of one-dimensional variables such as age, gender, presence of infection, bleeding, decompression, presence of subarachnoid hemorrhage, and Glasgow Coma Scale (GCS), which quantifies the nature of the initial brain injury on a numerical scale. The Glasgow Outcome Score (GOS) is the outcome variable; we view it as the response variable to study and predict as a function of the other input variables. The GOS quantifies the state of health of the subject when hospital treatment ceases. Our initial approach involved extracting orthogonal polynomial trends from each cytokine's time series, up to a specific degree d. The degree d was constant across both cytokines and subjects. The trends, by merely encapsulating linear, quadratic or cubic growth, have the distinct advantage of not being dependent on the actual length of the time series (which generally have widely different lengths). We then used these polynomial trends, quantified as one-dimensional variables, as predictors for the GOS and explored multinomial logistic as well as probit models. The models emerged upon fitting to data, and subsequent selection of the statistically significant clinical predictors as well as the orthogonal polynomial time trends of cytokines. Upon extracting polynomial trends, we carried out a study of the residuals. The model was obtained by using 80 % of the available data and was tested on the remaining 20 %. Ultimately, a logistic model was found as an optimal predictive tool (unpublished observations).

We next hypothesized that changes in the probability of survival vs. nonsurvival are related to the dynamics of the inflammatory response, the factors intrinsic to the patient (i.e., key demographic indicators) as well as to metrics related to the injury itself. To test this hypothesis, we developed a method which we call "Dynamic Profiling," as a means of assessing the dynamic course of a TBI patient within the hospital environment (Fig. 8.1). In the TBI application of Dynamic Profiling, a cluster is a subset of TBI patients that share similar characteristics. The set of clusters, recalculated after each set of cytokine readings, forms a partition of the TBI patients. To a given cluster, we associate three statistics based on the GOS score: the number of GOS scores equal to 1 in the cluster (this is the number of patients that died, to which we refer as "red flags"), the average GOS score of the subjects in the cluster, and the standard deviation of the GOS scores in that cluster. The vector of these three statistics is called the "weight" of the cluster. A cluster has a favorable weight if it has a small number of deaths, a high GOS average and a low GOS standard deviation. A useful statistic for the cluster is the probability of death of a patient belonging to that cluster (a "red flag"); it is derived as the ratio of "red flags" to the total number of subjects in the cluster. During the hospital stay, the aim is to diagnose, and ideally, reduce the probability of death (as we pass from stage i clusters to stage $i+1$). The data on which clustering is based consists of vectors in Euclidean space, with the most natural metric to use being the usual Euclidean distance. Hartigan's k-means routine is particularly well suited to clustering such

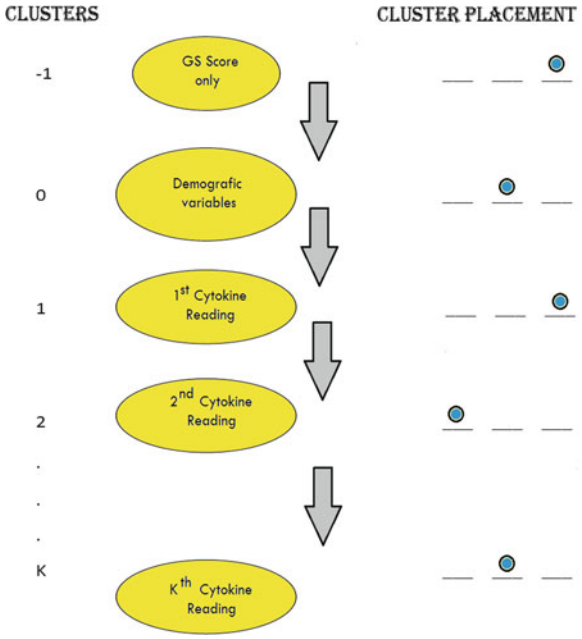

Fig. 8.1 Patient data is used to generate clusters. The initial cluster is based solely on the GS initial injury. A cluster adding in demographic data follows. Upon the reading of cytokine data, successive clusters are produced. The stage K cluster yields the probability of survival at that stage of the hospital stay

high-dimensional Euclidean data. We used the following variables to obtain the clusters: GCS, the subset of statistically significant demographic and clinical variables, the statistically significant polynomial trends in the time series of inflammatory mediator readings up to stage $i-1$ clustering (inclusive), and the inflammatory mediator readings during the current time interval. We note that the number of variables used to cluster on does not increase as we move to higher stage clustering. Indeed, we only use polynomial trends of degree at most d, irrespective of the length of the time series, or, equivalently, irrespective of the stage of clustering. This yields robustness to the clustering process while simultaneously bounding the dimension in which clustering takes place. The clusters' weights offer the opportunity of identifying patterns in the inflammatory mediators that yield favorable GOS scores. At each clustering stage, the fraction of "red flags" (deaths) in the cluster, in which the new patient falls, estimates the probability of death of the patient. The procedure lends itself easily to a Bayesian approach by placing a prior distribution (of probability of death) on existing clusters based on known medical expertise not pertaining to the data at hand. This is then updated by the observed data through the Dynamic Profiling method described above. The resulting posterior distribution encapsulates both the medical expertise as well as the observed probabilities of death within the data. Using this method, we were achieved a 72 % success rate in prediction of outcome post-TBI, a rate considerably higher than that of 50 % obtained by assigning the outcome to Low or High randomly (unpublished observations).

Like most biological processes, inflammation proceeds as a series of interacting cascades of signaling events that are often reflected in the production and secretion of

inflammatory mediators that likely form well-coordinated networks [13, 47, 53–59]. In order to better discern organizational aspects of interacting networks of inflammatory mediators, such as coregulation or autoinduction, a variety of methods have been developed. Hierarchical clustering and Bayesian methods use high-throughput genomic or proteomic data of several time points and/or conditions to correlate gene expression patterns with function and infer regulatory networks of correlated genes [60, 61]. Several developments in these methods over the last 15 years have yielded more informative networks that can be more easily translated into mechanistic models [62, 63]. A key point is that any network analysis method must reflect, and yield insights into, the dynamics of a given inflammatory response. For example, we have utilized a relatively simple network analysis method employed over discrete intervals of data to analyze the commonality and differences between experimental surgical cannulation trauma + hemorrhage in mice vs. the sham procedure (surgical cannulation only). This analysis suggested that the circulating mediators produced in response to the sham procedure were characterized by a high degree of interconnection/complexity at all time points, while the response to trauma/hemorrhage consisted of different central nodes, and exhibited zero network density over the first 2 h with lesser connectivity vs. sham at all time points [13].

Among network methods, dynamic Bayesian networks (DBNs) are particularly suited for inferring directed (causative) networks of interactions based on the probabilistic measure of how well the network can explain observed data. DBNs provide a good platform for incorporating biological knowledge alongside data in order to increase our knowledge of connectivity in biological processes and may be supplemented by additional experimental evidence and expert knowledge to hypothesize mechanistic models. As an example of the application of this methodology to the acute inflammatory disease, we have begun to examine the systemic inflammatory responses of pediatric acute liver failure (PALF) patients (unpublished observations). PALF is a complex, catastrophic, rapidly evolving clinical syndrome. The clinical trajectory of PALF is dynamic and the precise onset of disease rarely identified, with an exception being acute ingestions (e.g., mushrooms and acetaminophen). Patient outcome is reflected, in part, by the interaction among etiology, disease severity, supportive management, and treatment. Yet, outcomes vary among children with seemingly similar etiology, disease severity, and treatment; thus, additional factors are likely involved to explain these variations. Such factors likely include a complex interaction among the inflammatory milieu, end-organ damage, immune activation, potential for liver regeneration, and interventions [64, 65]. We hypothesized that dynamic networks of immune/inflammatory dysregulation drive outcomes in PALF, and that DBN analysis would shed insights into the structures of these networks. We assayed 26 inflammatory mediators on stored serum samples obtained from 49 children in the PALF study group (PALFSG; http://www.ccm.pitt.edu/research/projects/multi-center-group-study-acute-liver-failure-children) collected over 7 days after enrollment. Data were subjected to DBN analysis to suggest how inflammatory mediators are connected over time in spontaneous survivors, nonsurvivors, and PALF patients who received liver transplants (outcomes were assessed within 21 days of enrollment). Whereas raw inflammatory mediator levels assessed

over time did not distinguish among PALF outcomes, DBN analysis revealed distinct chemokine-related networks that distinguished spontaneous survivors from those who died. The DBN pattern identified in patients who underwent liver transplantation was more like that seen in spontaneous survivors than in those who died. Thus, we suggest that DBN may have general utility in other complex diseases with an inflammatory etiology.

Dynamic, Mechanistic Modeling of Inflammation

Mechanistic computational models are derived from more-detailed biological and physical descriptions of a system and have a rich set of tools for both analysis and simulation. These models, based on causative interactions, can be constructed as ordinary differential equations (ODEs), rules-based models (RBMs), and agent-based models (ABMs) among other methods (including hybrid methods) and have the advantage of potentially being predictive outside the range of conditions/time-points on which they were calibrated. Although it is often difficult to parameterize such models, they can unveil emergent phenomena not immediately obvious from the interactions that are encoded in the model. There are several analytic tools, for ODE models especially, that have been developed and used to decipher the organizational principles of networks (or subnetworks), the properties that explain the dynamics and robustness/sensitivity of a given complex system, and, perhaps most importantly, the critical points of control in the system [33]. These tools are particularly important in order to help define the complex interplay between the inflammatory mediators in the blood and other compartments both within the host (organs/tissue) and without (e.g., in the case of interactions with blood-feeding vectors). Tools from dynamical systems theory allow identification of the possible steady state(s) of a system as well as the dynamics of the system's time evolution. These tools have been used extensively to explain (or predict, depending on the context) diverse behaviors such as bistability, hysteresis, and oscillations in a variety of biological systems [66]. Bifurcation diagrams, in particular, can be used to map out the effects of a particular parameter on the possible steady-state behaviors of a system and to indicate the transition from a healthy steady state to a pathological one [16, 35, 67, 68]. The relative importance of parameters can also be quantified by calculating the change in the model output in response to changes in the parameter values using sensitivity analysis [33, 69]. These methods work in a complementary fashion to identify the key points that can be modulated to change the behavior of a system.

The analysis of ODE models of biological systems can be approached from a control theory perspective as well. Achieving robustness and efficiency are core principles of both evolution as well as engineering. Indeed, feedback, a pervasive biological phenomenon, is also a fundamental component of control strategies [70]. An ODE model is the equivalent of a state space representation of a control system. Thus, it is possible to decompose the biological system into a control structure and analyze the role of each component using control theoretic tools that characterize

their robustness and identify the key mediators that modulate the performance of such a control system [71]. These analyses are especially relevant given that the "tipping point" phenomenon in the inflammatory response is likely the result of a failure of the body's control structure to handle stress.

While we wish to navigate through the process of data → data-driven model → mechanistic model → prediction and understanding of the innate immune response, we seek to put it in the perspective of translational applications with a focus on clinical and preclinical settings. Much of the work in systems biology has understandably been in simpler, well-studied model organisms, but even among studies focused on preclinical science, there has been an overall lack of translation to the clinical arena. *Translational Systems Biology* is a framework with a focus on translational insights for novel diagnostic or therapeutic purposes and predictive mathematical models that inform in silico clinical trials [6, 72, 73]. Initially formulated to deal with the clinical challenge of integrating acute inflammation and organ dysfunction in critical illness, this work expanded to include healing of acute and chronic wounds and infections in various diseases, and rational dynamic modulation of inflammation.

We and others have created mechanistic computational models of acute inflammation in sepsis [16, 37, 74–76], endotoxemia [14, 35, 36, 77–88], and trauma/hemorrhage [14, 15, 17, 36]. In large part, these models (both ODE and ABM) are based on the typical progression of the inflammatory pathway described in the preceding section. Some of these models are purely theoretical (e.g., [16, 35, 37, 74–76]), while others are based on data either at the protein [14, 15, 17, 36] or mRNA [78, 79, 84–86] level. Similar mechanistic models have focused on related diseases such as necrotizing enterocolitis [89, 90].

Inflammation is an inherently multiscale process that manifests at the molecular, cellular, tissue/organ, whole organism, and population levels [28]. Early models of acute inflammation at the cellular level highlighted the nonlinear responses to multiple exposures to the same stimulus (Gram-negative bacterial lipopolysaccharide) [78, 80–82, 84, 88]. Some of these computational studies based on in vitro data suggested molecular control mechanisms that lead to the phenomena of nonlinear responses to repeated inflammatory stimulation at the cellular level [80–82, 84, 88]. One recent in vitro study involved mouse macrophages treated with extracellular β-nicotinamide adenine dinucleotide (NAD^+), a ubiquitous intracellular molecule that is anti-inflammatory when given extracellularly [91]. In that study, we hypothesized that extracellular NAD^+ would modulate the anti-inflammatory cytokine transforming growth factor (TGF)-β1. Indeed, NAD^+ led to increases in both active and latent cell-associated TGF-β1 in mouse macrophages. The time and dose effects of NAD^+ on TGF-β1 were complex and biphasic. A statistical model suggested that the effects of NAD^+ on TGF-β1 were nonlinear and this model was capable of predicting not only the levels of active and latent TGF-β1 but also the biphasic dose effect of NAD^+. Based on these data-driven modeling studies, we inferred that the effects of NAD^+ on TGF-β1 are nonlinear. Accordingly, we created a nonlinear ODE model of interactions we considered the most parsimonious and yet still capable of recapitulating the complex biological phenomena observed experimentally.

Model-predicted levels of TGF-β1 protein and mRNA were not only largely confirmed experimentally but also suggested the presence of other mechanisms of regulation of TGF-β1 by NAD^+ [92]. These studies highlight the utility of traditional biochemical/pharmacological studies coupled with computational modeling in defining novel biological mechanisms.

Combining Data-Driven and Mechanistic Modeling of Inflammation

We have utilized dynamic data, data-driven modeling, and dynamic mechanistic modeling in diverse contexts. We also utilized both correlative (transcriptomic analysis, PCA, and regression) and causative (ODE) models in our in vivo studies on the role of trauma in the murine response trauma/hemorrhagic shock. Initial studies using a literature-based, in vivo-calibrated mechanistic ODE model suggested that the underlying trauma is central in driving the inflammatory response to combined trauma/hemorrhage, both systemically and in the liver [15]. Transcriptomic data supported these model predictions as indicated by a large overlap between the genes and pathways induced in trauma alone vs. those induced in the setting of experimental trauma/hemorrhage [15]. This ODE model was extended to include details of experimental trauma/hemorrhage in mice (e.g., bleeding rate and target blood pressure) and further validated using a unique, computerized platform for automated hemorrhage that was constructed specifically to test the behavior of this mathematical model [17]. Later, multivariate regression, hierarchical clustering analysis, PCA, and dynamic network analysis all suggested that despite a large overlap at the level of unprocessed inflammatory mediator data (as shown by inconclusive hierarchical clustering of these data), there were major mechanistic differences between surgical trauma alone vs. trauma/hemorrhage [13].

In addition to the data-driven modeling work on TBI described above, we also carried out combined data-driven and mechanistic modeling in TBI using the same data on TBI patients described above (unpublished observations). Initially, we carried out PCA, which suggested that primary drivers of inflammation in this TBI cohort. Based on this analysis, we created patient-specific, mechanistic ODE models that were fit to each patient's data. These modeling-based studies raise the possibility of personalized modeling for TBI patients during their hospital stay.

In a similar fashion, we created a two-compartment mathematical model of porcine endotoxemia [83], based on an existing mathematical model of mouse endotoxemia [14, 15, 17, 36], in order to further test the hypothesis that a conserved inflammation framework could have radically individual manifestations. PCA of circulating inflammatory mediators suggested a central role for the cytokine IL-1β in this inflammatory response. Based on this analysis, we constructed a two-compartment ODE mathematical model that encompasses inflammation, lung (patho) physiology, and a damage variable that recapitulates the health of the animal [83]. This mathematical model could be fit to both inflammatory and physiologic

data in the individual swine, whose outcomes ranged from a self-resolving inflammatory response with fairly normal lung histopathology and function through various degrees of dysregulated inflammation and lung damage to death accompanied by severe lung injury [83]. More recently, we augmented this pig-specific two-compartment ODE model to include a third "tissue" compartment. This three-compartment mechanistic model was initially calibrated with data from individual surviving trauma patients, data that were used to produce 10,000 in silico patients subjected to virtual trauma/hemorrhage. This study raises the possibility of individualized outcome prediction for trauma patients as well as showing the potential for in silico clinical trials based on a small, but representative, cohort of actual patients.

Conclusions

We have increased our understanding of the inflammatory response beyond description of its symptoms and unveiled an ever-increasing complexity underlying this evolutionarily conserved internal communication mechanism [2, 7] that manifests at multiple biological scales [27, 28]. Clinically, translational systems approaches to inflammation have the potential for the identification of novel, rationally designed therapies and diagnostics—as well as for gaining new basic mechanistic insights—via combined data-driven and mechanistic modeling.

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Chapter 9
In Silico Trials and Personalized Therapy for Sepsis and Trauma

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Abbreviations

ABM	Agent-based model
ICU	Intensive care unit
ISS	Injury Severity Score

Inflammatory Diseases: A Pox on All Our Houses

We are currently faced with a barrage of complex diseases that often coexist in the same patient [1]. In the developing world, the modern disease landscape is a constellation of acute and chronic infections, traumatic injuries, and nonhealing wounds; diseases that are made even more complex due to the impact of malnutrition, war, and displacement [2, 3]. In the industrialized world, we face some of the same challenges with regard to infections, trauma, and wounds, but these diseases are complicated by lifestyles of excess and the attendant metabolic irregularities (diabetes and obesity). In addition, the generally longer life spans now being experienced around the world have paradoxically resulted in the rise of aging-related

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diseases such as cancer and various neurodegenerative diseases [4]. Given the degree and extent of medical care in the first world, it is virtually guaranteed that a common pathway for patients with this range of diseases is to spend at least some time in an intensive care unit (ICU) with critical illness manifesting with multicompartment pathophysiological derangements and organ failure. Critical illness can result directly from trauma, hemorrhagic shock, and bacterial infection (sepsis). On its own, trauma/hemorrhage is a leading cause of death worldwide, often leading to inflammation-related late complications that include sepsis and multiple organ dysfunction syndrome (MODS) [5–7]. Sepsis alone is responsible for more than 215,000 deaths in the USA per year and an annual healthcare cost of over $16 billion [8], while trauma/hemorrhage is the most common cause of death for young people in the USA, costing over $400 billion annually [9–11]. **There is currently not a single approved pharmacological therapy for critical illness**.

It is now clear that the acute inflammatory response, with its manifold manifestations at the molecular, cellular, tissue, organ, and whole-organism levels, drives outcomes in all the aforementioned diseases, and is central to the pathophysiology of critical illness. Properly regulated inflammation allows for timely recognition and effective reaction to threats to an individual, be it tissue damage resulting from injury or infection from pathogenic microbes. However, when the insult is too great, or repetitive in nature (as seen in chronic inflammatory and autoimmune diseases), we have suggested that inflammation can become disordered and result in ongoing tissue damage and organ dysfunction. We assert that critical illness is the most dramatic manifestation of disordered, dysregulated, and miscompartmentalized inflammation [12–14]. Thus, the presence of a robust, evolutionarily conserved network of inflammation [15–17], able to respond to heterogeneous insults and tuned for effective containment yet paradoxically capable of driving and propagating host tissue damage, results in disease states that are fundamentally resistant to reductionist characterization. This property of critical illness is the basis for the lack of effective mechanism-based pharmacologic therapies and accounts for the fact that even life-saving/perpetuating measures, such as mechanical ventilation or hemodialysis, may have detrimental effects through the induction of additional inflammation [18–20].

Insufficiencies in the Current Process of Drug/Device Design and Executing Clinical Trials

In order for a therapeutic drug or device to reach its ultimate end-user—the patient—a multistep process must be carried out, culminating in approval by regulatory agencies. This process generally consists of years/decades of basic research to identify candidate therapeutic targets, followed by sequential studies to demonstrate safety and some acceptable degree of efficacy (e.g., dosage or timing that results in greatest therapeutic benefit with least harm) in both experimental animals and humans. This process typically concludes with a pivotal (Phase III) clinical trial, which is randomized (i.e., subjects that meet predecided inclusion and exclusion criteria are

recruited into either a placebo or treatment arm in a random fashion) and double blinded (i.e., neither the clinician nor the patient knows a priori the study arm in which the patient is enrolled) [16, 21–24]. The enrollment into this Phase III trial is usually not individualized in any fashion beyond the set inclusion and exclusion criteria (and, of course, the withdrawal of a patient from the study if certain predecided adverse events occur). This process is considered the sine qua non of the scientific method, and it has indeed resulted in numerous drugs and devices available to physicians to treat diseases.

However, there are many problems with this approach. To begin with, the disease being targeted is usually thought of in a reductionist, static way as a series of discrete "stages" or "syndromes" rather than as a dynamic, stochastic progression of biological events driven by initial conditions and genetically determined parameters that, upon reaching certain multidimensional thresholds, leads to multiple outcomes. This discrepancy leads to the design of drugs that are targeted to ostensibly diagnostic symptoms rather than to underlying causes of the disease as a whole. Next, a highly linear (cause–effect) view of the biological pathways is presumed to underlie the various discrete symptoms, leading to the generation of drugs absent any consideration (at this initial stage of drug development) of impact on other pathways, cells, tissues, and organs. Finally, the statistical approaches commonly used to structure and analyze clinical trials typically make a number of questionable assumptions, e.g., that variables are normally distributed, that a marker of patient state is equivalent to a mechanistic driver of that state, and that such a marker of patient state will be altered in a statistically significant fashion as a function of therapeutic efficacy. Below, we discuss how these general features of the healthcare delivery process manifest in therapies for acute inflammatory diseases, with a focus on critical illness.

Inflammation in Critical Illness: Rational Systems Approaches for a Complex Therapeutic Target

The flaws in—and the fragmented nature of—the current healthcare delivery paradigm have led to the recognition of the need to address complex interplay between inflammation and physiology in critical illness, manifesting in divergent group outcomes and heterogeneous individual trajectories [12, 25, 26]. Initially, there was hope for some improvement in this situation through the adoption of "omics" methodologies, with their theoretical capability of interrogating the complete responses of cells and tissues in individuals (and thereby both improving the mechanistic understanding of critical illness in general and enhancing diagnostic and treatment capacities in individuals) [27–34]. While this approach has resulted in key contributions to the understanding of molecular pathways induced by injury and infection in humans [35, 36], as these techniques have become more commonplace there has been a growing recognition that more data does not necessarily lead to better—or any—explanations for the phenomena from which those data are derived. Thus, these "omics"

methods have not proven to be the panacea for the design of drugs, clinical trials, and diagnostics that they were projected to become. In addition, from a practical standpoint, there are multiple challenges to implementation of these purely data-driven, descriptive approaches in the healthcare delivery chain [7, 13, 14].

In contrast to data-driven, descriptive modeling, mechanistic computational simulations depict the behavior of biological interactions (e.g., among cells, their products, and the outcomes that result under a given set of conditions) dynamically. Such dynamic computational models and simulations may be used as "knowledge stores" that may be queried as to the emergent behavior of the sum total of known or hypothesized reductionist biological interactions [37–41], to suggest novel interactions not yet described by experimental data [42], and to address controversies based on diverse experimental/clinical conditions or other experimental differences among groups studying any given complex biological system [43]. Unlike data-oriented, descriptive models, dynamic mechanistic models offer the possibility of prediction outside of and beyond the data on which they were developed [7, 13, 14, 44]. We have extended the classical systems biology approach to that of Translational Systems Biology as systems and computational biology methods have matured and begun to take on characteristics, features, and operating principles of engineering [24, 44–46].

Indeed, the computational modeling toolset now available for integration into the healthcare delivery pipeline is rich and suited to diverse tasks. Translational dynamic mechanistic modeling used to date in acute inflammation and other phenomena related to critical illness can be divided into two general types: continuous methods, generally employing differential equations (either ordinary or partial) and particularly useful in settings involving data that reflect the mean field approximations of behavior of a biological system, e.g., the concentrations of molecules in a biofluid [47–55]; and discrete methods, most notably agent-based modeling for settings in which spatial pattern/image data are involved or for prototyping initial computational models of a complex system [42, 56–59]. These various method have their respective strengths and weaknesses [24, 45, 60, 61] and have all been used in the setting of critical illness [15, 16, 44, 45, 60, 62, 63].

Dynamic computational modeling has improved our knowledge of the basic biology of inflammation, and, directly or indirectly led to translational applications in critical illness [7, 12–16, 24, 44, 63]. One key translational application, namely the in silico clinical trial, was pioneered in the arena of critical illness [48, 58]. The potential use of mechanistic computational modeling in the diagnostic arena is evidenced by studies showing the potential to predict the individual inflammatory and pathophysiologic outcomes of human subjects [64] and large, outbred animals [65]. Thus, it may be possible, in the not-too-distant future, to predict and impact the outcomes of individual critically ill patients [44, 63].

Given the multiscale complexity of the disease processes, we suggest that it is imperative to not only merely identify candidate molecules but also determine if the higher-order, system-level consequences of attempting to intervene in a particular pathway will lead to an ultimately beneficial or detrimental outcome. We have pointed out the need for a computational means of *dynamic knowledge representation* as a means of hypothesis instantiation and testing [41, 66]. In the context of

translating molecular-level mechanistic hypotheses up through the various steps of the healthcare delivery continuum, this process is envisioned as allowing one to determine if the assumptions regarding manipulating a given biological interaction at a given scale of organization (typically the molecular/cellular scale) is likely to behave as expected at another, typically higher scale (e.g., tissue, organ, or the entire organism). In this way, one may identify effects that would otherwise be considered "unanticipated." Dynamic knowledge representation may be augmented with insights derived from high-throughput/high-content data [41], along with appropriate data analysis and data-driven modeling [17, 44, 46] in order to generate and parameterize mechanistic computational models of disease, patient [44], or population [16, 24].

Dynamic Knowledge Representation in the Context of In Silico Clinical Trials

A key example of the in silico clinical trial as a form of dynamic knowledge representation can be seen in the simulated clinical trials of existing and hypothetical antimediator interventions for sepsis [48, 58]. Importantly, these simulated trials were based on the knowledge available at the time the actual clinical trials were performed. In one case, a simulation of neutralizing antibodies to proinflammatory cytokines was implemented in an agent-based model (ABM) [57, 58]. This dynamic computational model reproduced the general disease dynamics of sepsis and multiple organ failure and was used to generate a simulated population corresponding to the control group in a sepsis clinical trial. Highlighting the power of computational modeling as a high-throughput test bed for novel therapies, early in silico clinical trials simulated a series of existing [48, 58] and hypothetical [58] therapies targeting inflammatory mediator-based therapies. Importantly, these clinical trials were simulated in such a way that assumed that the proposed interventions behaved mechanistically exactly as had been hypothesized. Therefore, these in silico trials in the paper are a form of verification of the underlying hypotheses—either explicit or implicit—that formed the basis for such trials. The way in which these computational simulations were structured avoided the need to invoke factors such as heterogeneity of adjunctive therapy, different pharmacodynamics/kinetics, faulty randomization or other potentially confounding practical issues commonly used to explain negative outcomes of clinical trials. In line with actual outcomes, and not surprisingly for those studies that were purely hypothetical, none of the simulated interventions demonstrated a beneficial effect [48, 58]. The conclusion drawn from these findings is that, most likely, the underlying conceptual models that informed the development of these therapeutic strategies targeted at blocking individual mediators were flawed, precisely because the hypotheses underlying their selection as therapeutic modalities were flawed. That is not to say that—despite this flaw of universal therapeutic efficacy—these mediator-directed therapies would fail. Indeed, one of the studies, an in silico trial of anti-TNF-α therapy using an equation-based model of systemic inflammation, suggested that this type of therapy would

work on defined subsets of sepsis patients [48]. Thus, we suggest that flaws in the original hypotheses and assumptions underlying these failed clinical trials would have been exposed through the use of computational dynamic knowledge representation been available and used early and throughout the process of drug development.

As touched upon above, in silico clinical trials offer an unprecedented possibility to transcend the long list of practical limitations—including: relatively small cohort sizes, limited availability of measurements, finite study durations, and the presence of confounding factors—that affect real-world clinical trials. However, the interdisciplinary team of clinicians, biologists, and computational modelers who carry out these in silico clinical trials must assure that the base models and implementation of simulated populations represent both the biology and clinical setting.

In addition to providing a check of the plausibility of the underlying scientific basis of a proposed intervention, in silico trials can augment the current process of performing clinical trials in three significant ways:

1. **Enhancement of study group substratification**: Clermont et al. [48] demonstrate the use of an in silico trial to enhance subgroup stratification and candidate patient identification. The finer grained representation of each simulated patient, in terms of cytokine response trajectories, and how they respond to and without a proposed intervention allows the identification of potential biomarker-defined inclusion criteria for a clinical trial. In essence, this allows each simulated patient to act as his own control with respect to the proposed intervention. This type of analysis is functionally impossible to obtain in clinical trial cohorts that reflect the range of response that would arise in the general population. Furthermore, social or ethical factors that may limit the possible representation of specific groups (such as African-Americans, known to be generally underrepresented in many clinical trials, or women of child-bearing age, excluded for potential teratogenic risk). As a result, trials are very likely to miss important (positive or negative) effects in subgroups that are sampled inadequately. This missampling can lead to later discovery of adverse events following a promising clinical trial, or in the failure of truly useful treatments in clinical trials that were not properly targeted to the patients who would most benefit from them. By simulating massive virtual cohorts sampled from the space of potential patients, in silico clinical trials can achieve much more thorough sampling of possible patients. The acquisition and analysis of this simulation-generated data can in turn reveal clinical patient subgroups who merit particular attention and lead to better informed patient selection criteria and more effective clinical trials.
2. **Augmentation and optimization of protocol design**: Protocols for modern interventions depend on multiple complex and often interacting parameters (e.g., dosage levels, timing and frequency of administration, etc.). Attempting to determine these parameters experimentally over a wide range of individuals is functionally impossible, and therefore the optimal intervention strategy for an individual patient cannot pragmatically be determined. The inability to anticipate and account for this degree of interindividual heterogeneity will doom a

clinical trial to failure at the outset. In silico trials allow a more rigorous computational optimization of these parameters, both on massive populations and for individual patients and will increase the precision with which protocols can be designed, and therapeutic endpoints defined.

3. **Enhanced characterization of the control group**: Clinical trials rely on control groups against which the effect of a proposed intervention is compared. However, given the vagaries of clinical practice, many control groups may actually compare poorly to the intervention group. Interindividual variability in both underlying biology and clinical practice leads to a situation where the definition of "similarity" between control and intervention patients is often quite crude and imprecise. This situation confounds the ability to actually define the effect of the proposed intervention. In silico trials, however, offer the ideal control group: each simulated patient can be simulated with and without the intervention. Comparison of results against these "perfect" controls thus removes a source of uncertainty that is unavoidable in real trials.

An example of the potential insights obtained from carrying out in silico trials can be seen in a very early in silico trial based on an anti-TNF-α therapy [48]. These simulations recapitulated the general lack of efficacy of the intervention; however, the researchers used the power of computational modeling to evaluate what would have happened in the absence of intervention or in the setting of different doses of the drug. In essence, the placebo group was "cloned" into multiple treatment arms or the placebo arm. Consequently, this in silico analysis suggested specific characteristics of the simulated patients who had been helped by the intervention, had been harmed by the intervention, or had not been affected by the drug, thereby suggesting the possibility of using this in silico approach for deciding on inclusion and exclusion criteria for eventual clinical trials. Thus, the key take-home lesson of this study was that a failed randomized, placebo-controlled clinical trial could possibly have been successful through the use of in silico modeling.

Dynamic Knowledge Representation at the Individual Level: Optimization of Diagnosis and Therapy

It may be argued that the ultimate test of dynamic knowledge representation is that of characterizing the drivers of dynamic patient state to a degree sufficient to identify and treat the individual patient [12, 44]. To do so, a robust, mechanistic computational model (presumably the same one used for in silico clinical trial) must be adapted to reflect the temporal dynamics of inflammation and organ damage/dysfunction in the individual patient. From a practical standpoint, model parameters that alter the patient's dynamics (e.g., comorbidities, prior health history, relevant genetic traits, etc.) are modified over known or presumed ranges in accordance with known biology [44]. The applications of this approach are myriad. Of most direct connection to the in silico clinical trial, individual-specific models could be used to

generate much larger cohorts of virtual patients, which in turn could be used to make in silico clinical trials more realistic.

As an example of this approach, we constructed a multicompartment, equation-based model, consisting of the "tissue" (in which physical injury could take place), the "lungs" (which can experience dysfunction) and the "blood" (as a surrogate for the rest of the body). This model was initially calibrated with data on approximately 30 individual trauma patients, all survivors of moderate blunt trauma. Based on these individual trajectories of both inflammatory and physiological variables, normal and uniform distributions were created. These distributions were sampled repeatedly to create a population of 10,000 virtual trauma patients, where each patient is defined by his/her parameter values in the mathematical model. Each patient was then subjected to simulated low, moderate, and severe trauma. These virtual populations of trauma patients exhibited realistic and partially overlapping distributions of "damage" recovery times (which we equated with intensive care unit [ICU] lengths of stay) and total "damage" (which we equate with degree of multiple organ dysfunction). These virtual patients were queried as to the parameters driving the above distributions and found that for patients with a low Injury Severity Score (ISS), parameters related to IL-1β were the predominant drivers, while IL-6 was the main driver of outcome in patients with moderate or severe ISS. Principal Component Analysis of the circulating inflammatory mediators from the original trauma patients suggested that IL-1β was the principal driver of inflammation in these patients, in line with the results of the analysis of the equation-based virtual trauma patients. These results suggest the possibility of determining novel basic mechanisms in trauma, of individualized outcome prediction for trauma patients, and of virtual clinical trials based on a small number of actual patients.

These studies highlight some of the particular advantages that mechanistic models afford: virtual cohorts can be generated of any required size, and each individual patient's disease state can be tracked at an extremely high level of resolution (limited only by the resolution of the model) for as long as required. When information is available about the approximate distribution of these characteristics in real populations, this information can be used in the generation of a virtual patient population to ensure that the composition of simulated cohorts mirrors reality.

Another application of this approach involves in silico "testing" of multiple therapeutic modalities on individuals. As an example of this application of dynamic mechanistic modeling, an ABM of vocal fold inflammation and healing was calibrated to the early levels of inflammatory mediators present in the laryngeal secretions of individual humans subjected to experimental phonotrauma and could predict the later levels of these mediators in an individual-specific fashion [64]. Importantly, these individualized ABMs were utilized to predict the likely efficacy of a "rehabilitative" treatment, namely resonant voice exercises, both in patients who had in fact received this treatment and in patients who did not [64]. A similar process could be employed to evaluate the specific efficacy of a drug modulating an aspect of inflammation or healing, thereby forming the basis of a much more realistic in silico clinical trial.

Conclusions

What is clear now is that the biocomplexity of pathophysiological processes underlying the systems-level diseases that represent the greatest health risk today, such as cancer, diabetes, atherosclerosis, Alzheimer's, sepsis and wound healing, confounds the use of traditional experimental methods. These reductionist experiments and data-oriented descriptive methods are unable to evaluate and test multiscale causality, an essential and critical step in the design and development of therapeutic interventions for systems-level diseases. The complexity and dimensionality (in terms of multiple factors and variables) of these biomedical issues, particularly in terms of translating mechanisms across scales of organization, essentially precludes this approach. Reliance on only these traditional methods can produce, at best, "one-off" products based on fortuitous discovery but does not provide a robust and sustainable strategy. The Scientific Method mandates that it is the ability to evaluate mechanisms and causality sufficiently in a multidimensional, high-throughput world—as is potentially possible with dynamic computational modeling and the application of principles from Translational Systems Biology—that forms the crux of the translational dilemma. The use of dynamic computational modeling can provide a framework that allows the introduction of "theories" into biomedicine, to facilitate the translation of robust conceptual structures and architectures across experimental platforms as well as into the differences among individual patients [67]. Specifically, we assert that the computational approaches described in this chapter, with an explicit goal of addressing the challenges of implementing the last stage of getting a therapy to the bedside, represents a necessary step in the future of obtaining and implementing effective therapeutics for the complex diseases that challenge us today and in the future.

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Part III
Translational Modeling of Wound Healing

Chapter 10
Disorder of Localized Inflammation in Wound Healing: A Systems Perspective

Sashwati Roy, Amitava Das, and Chandan K. Sen

Chronic wounds represent a significant burden to patients, healthcare professionals, and the US healthcare system, affecting 5.7 million patients and costing an estimated 20 billion dollars annually [1]. Wound healing is a well-synchronized reparative process composed of a large number of concerted biological events harmonized in a sequential manner [2–4]. In order for perfect healing to occur, it is important to appreciate the underlying mechanisms that come together to orchestrate such harmony. Dysregulation of just one of the components can result in malfunctioning of the entire system. One such example is diabetic wound healing where dysfunction in the inflammatory response results in the chronicity of these wounds. The current review focuses on factors that regulate wound inflammation response and the dysfunction of these responses in diabetic wound healing.

Physiological Phases of Wound Healing

Wounds can be classified into two categories depending on the time of closure: an acute wound (which follows the healing process in a stepwise manner and thereby closes within days) and chronic wounds (which are disrupted from the normal physiological healing cascade and remains open for over 4 weeks). The process of wound healing is functionally divided into four sequential phases, viz. hemostasis,

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inflammation, proliferation, and remodeling, all of which occur in an overlapping series of events. Following an injury the process of hemostasis sets in, the main function of which is to put an end to blood loss and initiate the formation of fibrin plug, which also facilitates consequent inflammation and healing processes [5]. Initiation of an acute phase inflammatory response prepares the wound site for subsequent closure. The inflammatory phase is characterized by the four cardinal signs namely rubor (redness), tumor (swelling), calor (heat), and dolor (pain). The proliferative/remodeling phase takes place in conjunction with the inflammatory phase during which new blood vessels are formed and fibroblasts arrive and lay down the extracellular matrix. Remodeling represents the final phase of wound healing and is well synchronized by the balance between formation and degradation of the extracellular matrix components and the wound acquires tensile strength. This stage often continues even after months of wound closure and influences on the scar outcomes of the healed wound.

Diabetic Wound Healing: Inflammatory Response

Inflammation is a protective response of the body to infection or injury designed for removal of the causative agent and restoration of tissue structure and function [6]. Inflammation normally resolves following the reinstatement of normal tissue homeostasis. Prolonged inflammation can lead to damage and loss of function of tissues [6]. Wound inflammation is driven by a variety of mediators that are spatially as well as temporally tightly controlled [4, 7]. A dysfunctional immunological system results in the failure to switch from inflammation to resolution phase leading into chronic inflammatory non-healing wounds [8].

Over 23 million people or 7.8 % of the US population suffer from diabetes. It is estimated that up to 25 % of all diabetics will develop a diabetic foot ulcer. Sixty-seven percent of all lower extremity amputation patients have diabetes [9, 10]. Dysregulated inflammatory phase is a major factor that contributes to the impairment of diabetic wound healing [11, 12]. Using an excisional wound healing model in diabetic (db/db) mice, persistent (day 13 postwounding) expression of the inflammatory cytokines Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) was detected, indicative of a sustained inflammatory phase [11].

Recruitment and Chemotaxis of Inflammatory Cells

A small proportion of leukocytes reside in resting tissues under normal conditions, which increases multifold following an injury. This increase in the number of cells is due to the recruitment from the circulation following the inflammatory response at the site of injury [13]. The first to appear at the site of injury from the injured

vessels are **platelets**, which initiate the coagulation process. Platelets accumulate near the damaged blood vessels and through the coagulation cascade convert fibrinogen to fibrin thereby preventing further blood loss [4]. Though the platelets perform the crucial job of limiting the blood loss through clot formation, platelet activity is increased in patients with diabetes mellitus leading to intensified adhesion, activation, and aggregation, which results in high risk of atherosclerosis in these patients [14].

Neutrophils start arriving at the site within minutes of injury. The principal role of these cells is to clear microbes invading the open wound by generating copious amounts of reactive oxygen species (ROS) via respiratory burst. The excess of generated ROS has been suggested to be deleterious for the regenerating host tissue, especially in chronic wounds [15]. Because neutrophils arrive early at the wound site as compared to macrophages, it was hypothesized that neutrophils help in the recruitment of macrophages [16]. However, a study by Dovi et al. in 2003 reported that depletion of neutrophils during wound healing had no effect on number of macrophages recruited at the wound site [15]. Depletion of neutrophils improved the rate of reepithelialization thereby suggesting that neutrophils may impede wound healing [15]. Wound neutrophils might mediate innate immune responses through modulation of macrophage phenotype [17]. Neutrophils are also known to activate local fibroblasts and keratinocytes by releasing proinflammatory cytokines. Incidentally, neutrophils of diabetic patients produce increased levels of proinflammatory cytokines, such as Interleukin-8 (IL-8), IL-1β, TNF-α, and Interleukin-1 receptor antagonist (IL-1ra), compared to healthy individuals [18]. This excessive production of cytokines may lead to inappropriate activation of inflammation and tissue injury and may even increase susceptibility to invasive microorganisms [18]. Diabetic patients are at increased risk of infections of diabetic foot ulcerations (DFU) [19]. Diabetic mice subjected to Staphylococcal infection exhibited decreased neutrophil apoptosis resulting in extended TNF-α production and impaired neutrophil clearance by macrophages [20]. Impaired chemotactic, phagocytic, and microbicidal functions of the neutrophils under diabetic conditions were improved by insulin treatment [21].

Wound macrophages appear at the site of injury after neutrophils. These cells function as voracious phagocytes clearing the wound of all matrix and cell debris, which includes fibrin and apoptotic neutrophils. Apart from their scavenging activity, macrophages also secrete a wide array of cytokines, growth and angiogenic factors that has a major role in the regulation of fibroblast proliferation and angiogenesis [16, 22, 23]. Antimacrophage serum combined with hydrocortisone was found to reduce the accumulation of macrophages in healing skin wounds of adult guinea pigs, which eventually resulted in dysregulated disposal of damaged tissue and provisional matrix, decreased fibroblast count and impaired healing [24]. Several studies using macrophage-specific gene knock-out animal models have also provided critical information about the role of wound macrophages in the incorporation of inflammation and cellular movements at the site of injury to facilitate efficient skin repair in healing and inflammation [25, 26].

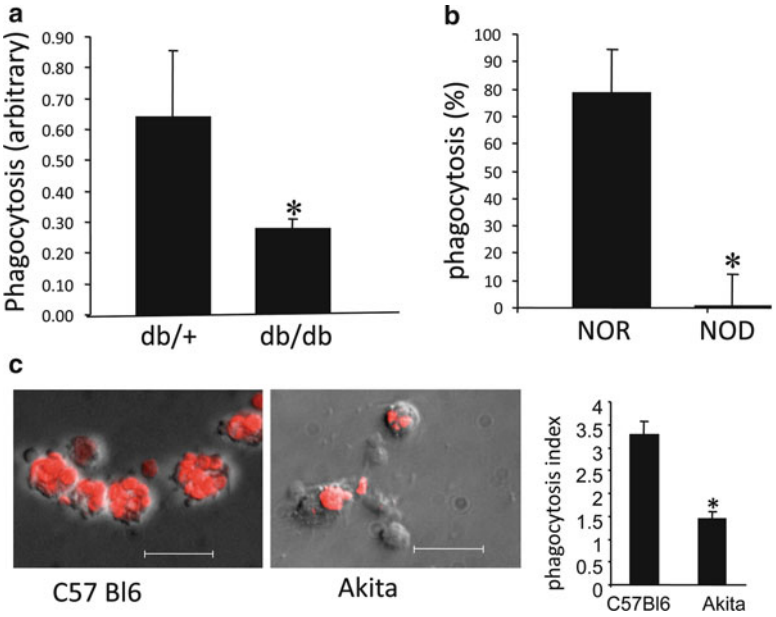

Fig. 10.1 Dead cell clearance is impaired in wound macrophages harvested from diabetic mice. PVA sponges were implanted subcutaneously on the back of mice. Wound macrophages were harvested and dead cell clearance activity assay was performed by coculturing macrophages with apoptotic or nonapoptotic cells. (**a**) obese (db/db, type II) and their matched control lean nondiabetic (heterozygotes db/+) mice. (**b**) Type I nonobese diabetes (NOD/LtJ) mice and matched control nondiabetic (NOR/LtJ) mice. (**c**) Akita (Japan SLC, Inc) and matched control (C57BL/6) mice. *Left*, representative images showing wound macrophage (phase contrast image) cocultured with apoptotic cells (*red*) in Akita versus BL6 mice *Right*, % phagocytosis. Data are mean ± SD ($n = 3$) *$p < 0.05$ compared to nondiabetic [27]

Wound Macrophage Dysfunction

Diabetes is known to impair macrophage function [27]. Immune function was found to be dysregulated in alloxan-induced diabetic mice [28], while type 1 diabetic patients were reported to have impaired monocyte "lectin-like" receptor activity [29]. The scavenging activity of macrophages is also altered negatively in a diabetic condition, which is evident from a study where the apoptotic cell clearance of beta cells is found to be impaired in neonatal autoimmune diabetes-prone rats [30]. We provided first evidence that macrophages from diabetic wounds suffer from impairment in dead cell clearance activity (efferocytosis) as one of the key factors resulting in increased apoptotic cell burden at the wound site (Fig. 10.1). This burden, in turn, prolongs the inflammatory phase and complicates the healing process and compromises resolution of inflammation (Fig. 10.2). Correction of impaired efferocytosis in diabetic wounds and strategies to intercept the adverse effects of impaired efferocytosis emerge as novel targets for the management of chronic inflammation commonly noted in diabetic wounds [27].

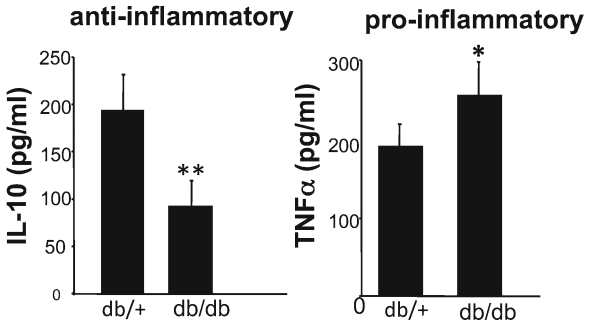

Fig. 10.2 Wound fluid from diabetic mice contain lower levels of anti-inflammatory cytokine IL-10 and higher level of proinflammatory cytokine TNF-α. Wire-mesh cylinders were implanted on the back of diabetic (db/db, type II) or control (db/+, nondiabetic) mice. Wound fluid was collected on day 5 after implantation to determine IL-10 and TNF-α levels using ELISA. Data are mean ± SD ($n=4$) *$p>0.05$, compared to control animals [27]

Macrophage Phenotypes

Macrophages are dynamic and heterogeneous cells. Since the introduction of the concept of alternative activation of macrophages in 1992 [31], these cells have been broadly assigned to two broad groups (1) classically activated or type I macrophages (M1), which are proinflammatory effectors and (2) alternatively activated or type II macrophages (M2) that possess anti-inflammatory properties [32]. Recently, characterization of distinct circulating monocyte populations that migrate into wounds was performed. Intriguingly, the phenotype of macrophages isolated from murine wounds partially reflected those of their precursor monocytes, changed with time and did not conform to current macrophage classifications, i.e., M1 or M2 [33]. Macrophage differentiation of peripheral blood mononuclear cells was found to be impaired in obese subjects and the monocytes of these subjects were found to have decreased susceptibility for differentiation towards the reparative phenotype thus retarding wound healing [34].

Chemical and Other Mediators of Inflammation

Cytokines and Chemokines

Cytokines represent the universal category of messenger molecules, while chemokines are cytokines specialized in directing and regulating the migration of white blood cells to infected or damaged tissues [35]. Proinflammatory cytokines, like Interleukin-1α (IL-1α), IL-1β, Interleukin-6 (IL-6) and TNF-α, are significantly

upregulated during the inflammatory phase [35]. Wound healing in IL-6 KO animals is three times longer than those of wild-type controls [36]. **TNF-α** is a potent proinflammatory cytokine produced by activated macrophages known to drive the inflammatory response to wounding. Depending on the concentration, length of exposure, and presence of other cytokines, the effect of TNF-α can be beneficial or deleterious. TNF-α is involved in tissue remodeling, mounting and sustenance of inflammation, cachexia, shock, and cell death [37]. Collagen deposition and wound disruption strength (WDS) are increased in adriamycin-treated animals subcutaneously injected with TNF-α [38]. Anti-TNF-α therapy to decrease the level of TNF-α secreted by activated macrophages restored the normal physiological healing in obese (ob/ob) mice [39]. On the other hand, mice lacking TNF-α strengthened Sma and Mad related proteins (SMAD) mediated fibrogenic reaction in the healing dermis, resulting in fibrosis and organ dysfunction [40]. **IL-10** is recognized as one of the key anti-inflammatory cytokines, which help resolve the wound inflammatory response [41]. It does so by downregulating the expression of proinflammatory genes such as TNF-α [41]. Diabetic macrophages secrete elevated levels of proinflammatory cytokines [39, 42]. Current evidence show that insufficiency of Interleukin-10 (!L-10) is a key factor underlying the exaggerated and sustained inflammatory response commonly noted in diabetic wounds [27]. Increased levels of the proinflammatory cytokines TNF-α and IL-6 and a decreased level of IL-10, an anti-inflammatory cytokine, were reported in excisional wound healing model in diabetic (db/db) mice tissue compared to nondiabetic healing wound tissue [11, 27] (Fig. 10.2). Finally, in diabetic wounds, prolonged neutrophils and macrophage infiltration leads to sustained expression of MIP-1 and MCP-1 [11].

Lipid Mediators

Stress, injury, or inflammatory stimuli results in the rapid release of arachidonic acid from membranes [43]. The released arachidonic acid undergoes metabolism by the cyclooxygenase (COX) pathway, involving COX-1 and COX-2, along with terminal synthases, to generate prostaglandins (PG), prostacyclin (PC), thromboxanes (TX), or by the lipoxygenase pathway to produce several classes of leukotrienes and lipoxins [44]. Eicosanoids thus generated act via G-protein-coupled receptors to initiate, amplify, and bring about inflammation in both acute as well as chronic wounds [45]. Similar to arachidonic acid the ω-3 PUFAs eicosapentaenoic (EPA; i.e., ω-3, C20:5) and docosahexaenoic acid (DHA; i.e., ω-3, C22:6) are metabolized by COX-2 and LOX enzymes producing a novel classes of endogenous anti-inflammatory lipid autacoids [43]. Important among them are **lipoxins**, which are generated through transcellular biosynthesis and hinder PMN chemotaxis [46]. They are also responsible for attracting monocytes thereby stimulating monocyte adherence to vascular endothelium [47] without releasing ROS [48]. **Cyclopentenone**

prostaglandins are formed by the dehydration of PGD2, reduces TNF-α-induced expression of vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) in human endothelial cells. **Resolvins and protectins** are a novel class of anti-inflammatory lipid mediators that have been implicated in resolution of inflammation [48, 49]. Resolvin E1 obstructed human neutrophil transendothelial migration thereby minimizing inflammation in vivo [49]. **Maresins** exhibit potent anti-inflammatory and proresolving activity [50]. The pathways that produce these lipid mediators are found to play regulatory roles in obesity-induced diabetes. In experimental obesity, the leukotriene pathway was found to regulate the development of adipose tissue inflammation [51]. Current evidences indicate that dietary intake of omega-3-PUFAs inhibits the formation of omega-6-PUFA-derived eicosanoids, while triggering the formation of omega-3-PUFA-derived resolvins and protectins. These pro-resolution lipid mediators, namely resolvin E1 and protectin D1, may mimic the insulin-sensitizing effects to a similar extent that of rosiglitazone, a member of the thiazolidinedione family of antidiabetic drugs [52]. Furthermore, these pro-resolution lipid mediators improved metabolic parameters in diabetes including blunted systemic inflammation, restored defective macrophage phagocytosis, and accelerated wound healing in animal models of obesity and diabetes [53].

MicroRNAs

Wound healing is highly dependent on injury-inducible protein-coding genes, which act as modulators of an intrinsic tissue repair program in order to restore structural and functional integrity of the injured tissue [54]. Work carried out during the recent years suggest that both transcription and translation are subject to regulation by microRNAs (miRNAs; 19–22-nucleotide long) that are noncoding RNAs found in all eukaryotic cells. miRNAs carry out posttranscriptional gene silencing (PTGS) through mRNA stabilization as well as translational repression. These miRNAs may control more than a third of all protein-coding genes and virtually all biological processes and wound healing is no exception. miRNAs are emerging as a key regulatory of the overall wound healing process [55–58]. Disruption of miRNA biogenesis has a profound impact on the overall immune system. Emerging studies indicate that miRNAs, especially miR-21, miR-146a/b, and miR-155, play an important role in regulating several phases that orchestrate the inflammatory process [59]. Specific miRNAs were shown to be regulated by lipid mediator miRNA–NF-κB axis was suggested as a key component in the RvD1-GPCR downstream signaling pathways [60]. miR-146a was found to be downregulated in diabetic mice wounds, which eventually increased gene expression of its proinflammatory target genes [61]. miR-21 expression in diabetic mice was increased in the skin but decreased during diabetic wound healing [62].

Conclusions

Reductionism, as a guiding principle, is of great value where a quick and effective solution is required for an isolated problem. Reductionism is less helpful for complex systems such as the process of wound healing where interactions between components dominate the components themselves in shaping the outcome and behavior of the system. A holistic or systems approach is essential to understand dynamics and interactions of various components with each other to dictate the fate of the outcome towards a healing or a chronic wound. This chapter highlights the multidimensional nature of the deregulation of diabetic wound inflammation, making it essential to understand the problems from systems perspective and not in a reductionist approach.

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Chapter 11
Equation-Based Models of Wound Healing and Collective Cell Migration

Julia Arciero and David Swigon

Introduction

Wound healing is a physiological process of repair of a tissue that has been structurally damaged. The most common wounds disrupt one of the epithelial tissues that protect the internal and external surfaces of the body and act as barriers against invasion by microorganisms. Such tissues include skin, corneal tissue, and the epithelial lining of the digestive tract (including the mouth and esophagus), respiratory tract (including the alveoli), urinary tract, and reproductive organs. Any disruption of these tissues can lead to serious health conditions or developmental abnormalities; for example, a wound in the gut epithelium can lead to necrotizing enterocolitis, which is the leading cause of death from gastrointestinal disease in preterm infants. Internal wounds may also arise due to physical overexertion or blunt force trauma. The biology of wound healing is reviewed in Chapter 10.

The ability to heal wounds is closely related to the regenerative ability of the organism to restore the function of many organs. Wound healing generally proceeds in four stages, although these stages differ in details depending on the location of the wound [1–4]. The **first stage** is hemostasis, characterized by the leakage of fluids out of broken blood and lymphatic vessels and the delivery of inflammatory cells and platelets to the wound. The platelets trigger vasoconstriction to reduce blood loss and form a blood clot to fill the wound. The clot contains fibrin molecules that provide an extracellular matrix (ECM) for the migration of leukocytes and fibroblasts, which are cells responsible for eliminating pathogens and repairing the

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tissue, respectively. The **second stage** (2–3 days for skin wounds) is an inflammatory reaction marked by neutrophil recruitment followed by macrophage infiltration. Neutrophils phagocytose necrotic tissue, kill bacteria that enter the wound, and produce chemoattractants to recruit macrophages. Macrophages secrete pro- and anti-inflammatory cytokines that regulate inflammation and trigger the phagocytosis of pathogens and cell debris. Macrophages also secrete growth factors necessary for the third stage of wound healing. The degree of inflammation that ensues is directly related to scar formation [5]; for instance, the lack of inflammation in embryos leads to scarless wound healing. The **third stage** of the process (3–10 days for skin wounds) is the recovery of the tissue via cell migration and proliferation. The wound is also infiltrated by fibroblasts, which initiate the formation of the collagen matrix to provide mechanical strength for the disrupted tissue and keratinocytes to regulate the reepithelialization process. In addition, new capillaries are grown by extension and sprouting in a process called angiogenesis, and the development of acute granulation tissue is initiated. This transitional granulation tissue replaces the provisional wound matrix and is characterized by a high density of fibroblasts, granulocytes, and macrophages. The **fourth stage** of the wound healing process is tissue remodeling, which can take anywhere from 21 days to 1 year for skin wounds. During this process, the formation of granulation tissue ceases, and collagen III, which forms a basket weave-like structure in the ECM, is replaced by collagen I, which is stronger and oriented in parallel bundles. Furthermore, the wound contracts and decreases the surface of the developing scar.

These four stages have been observed, measured, and assessed in a wide range of experimental and clinical scenarios. In some cases, shallow wounds are studied using in vitro experiments known as scratch-wound assays (depicted in Fig. 11.1), which are designed to track the migration and proliferation of a monolayer of cells. In a scratch-wound assay, cells are cultured (typically on a glass coverslip), grown to confluence, and then scraped with the tip of a pipette to create a gap that represents a wound in the tissue layer. Medium is continuously perfused across the cells, and the motion and deformation of cells in the layer is analyzed. Other in vitro assays include three-dimensional organ explant cultures or three-dimensional sprouting invasion assays from mesenchymal cells overlaid onto a three-dimensional ECM or implanted as a multicellular spheroid [6]. Using such assays, numerous studies have evaluated how extracellular stimuli, geometric anisotropy of substrates, or intracellular processes activate cell migration and trigger cell proliferation [7–11]. Measurements of the physical forces driving cell migration indicate that traction forces arise many cell rows behind the leading wound edge and extend across large distances [9]. Trepat et al. [9] demonstrated that individual cells at both the leading wound edge and inside the sheet engage in a “tug-of-war” that integrates local force generation into a global state of tensile stress. Mechanical forces exerted by epithelial cells were measured by du Roure et al. [11] using a technique that combines microfabrication of flexible substrates and multiple-particle tracking microscopy. Because each micropillar deflection is independent of its neighbors, the measured traction forces under the cells are uniquely determined.

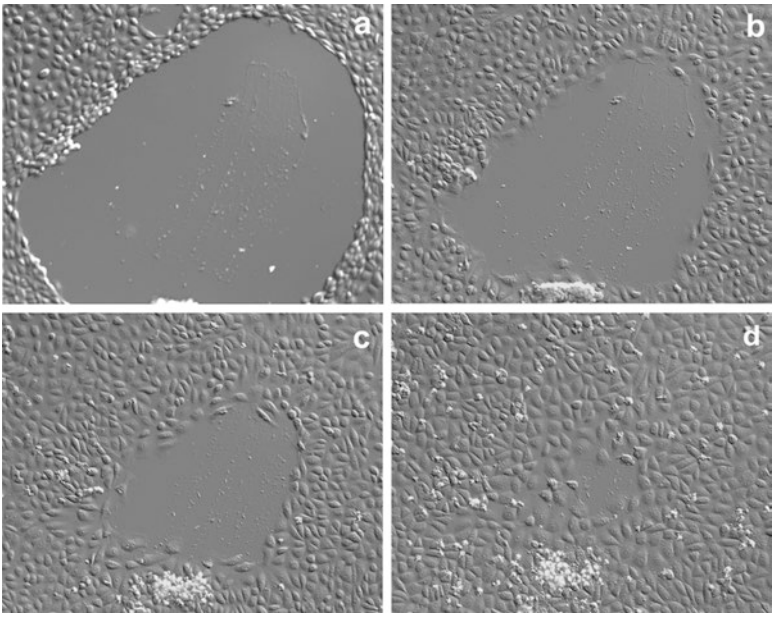

Fig. 11.1 Scratch-wound assay of intestinal epithelial cells. Large space void of cells denotes wound; surrounding region is the epithelial layer, which remains connected during closure. Panels **a**–**d** show the progression of wound closure after 0, 125, 250, and 500 min, respectively

In clinical settings, the progression of wounds, such as skin wounds or diabetic foot ulcers, are quantified according to measures such as the absolute wound area remaining, percentage of initial wound area remaining, wound volume remaining, or wound perimeter remaining. Some of these measurements can be difficult to obtain given the location of the wound or type of data needed, and, as a result, currently there is no universally accepted measure of wound healing [12]. In addition, although wound area is an obvious measure of wound closure, predicting healing time based on the percent of wound area healed tends to bias small wounds, and predicting healing time based on the absolute wound area healed tends to bias large wounds [13]. A reliable measure of wound healing time is of particular interest to both physicians and patients in order to determine effective treatment methods for various wounds.

Ultimately, a wound is considered healed once tissue functionality has been fully restored via the migration of cells into the wounded region and the proliferation of new cells to restore the original density of the tissue. Observations from multiple wound scenarios have shown that cell migration and proliferation as well as the overall healing time for a wound are affected by factors such as wound geometry, tissue type, cell–cell interactions, and the stage of the healing process (epithelialization, contraction, or proliferation). Combining these observations with mathematical modeling techniques may help to unravel the key aspects governing wound healing.

Modeling

As in the setting of sepsis, trauma, and other acute inflammatory conditions (see Chapter 2), mathematical modeling of wound healing is used to aid in the understanding of the complex processes involved in wound healing by providing a platform for testing various hypotheses regarding the interaction of wound healing components. Equation-based models describe biological processes by formulating interactions of individual biological components using a system of differential equations for variables that measure the concentrations of cells and chemicals in time and space. The equations are constructed using knowledge deduced from experiments and are calibrated using experimental data (for example, data could be used that describes the dependency of cell migration speed on the concentration of a chemoattractant). The equations are solved using a variety of analytical and numerical techniques and are used to predict the dynamics of cell populations within a wound. There are two classes of equation-based models in common practice—models based on ordinary differential equations (ODEs) and models based on partial differential equations (PDEs). The main difference is that ODEs can describe the time dependence of the wound healing process but not its spatial variability, while PDEs can describe both. In addition, there are also stochastic models that can include fluctuations in chemical concentrations and other random effects [14, 15]. A summary of these different types of models, many of which are described in more detail in this chapter, is provided in Table 11.1.

Equation-based models are beneficial in situations in which it is reasonable to assume that the components of the system are either of the same kind and respond identically to stimuli or are of different types, but their response depends only on the number of components at a given spatial location at a given time. For example, when fibroblasts are rebuilding the collagen matrix, the speed of rebuilding depends on how many fibroblasts are present, but not on where precisely each fibroblast is and how it moves about. In such a case, one may invoke the *continuum hypothesis* and assume that there is a density function $f(x, t)$ that depends on space and time, which describes the mass (or molar) density of the cellular component (cell or a chemical). The density can be understood in a statistical or probabilistic sense. In the first case, the quantity f is equal to the average number of components per unit volume centered at position x and measured at time t for a series of identical experiments; in the second case, f represents the probability of finding a component in that volume at time t. The use of the continuum hypothesis implies that we can only predict the behavior (motion and state changes) of population averaged properties, not of individual molecules or cells. In contrast to ODE models, models satisfying the continuum hypothesis preserve the possibility of properties varying in space.

By choosing a continuum formulation, we substantially reduce the amount of information needed to specify the state of the system. Imagine a 1-cm wound with 10^8 fibroblasts. In order to specify the fibroblasts' positions in 3-space, we would need 3×10^8 numbers. However, to define their spatial distribution, it may suffice to use a grid of spacing $100 \times 100 \times 100$ nodes (10 nodes per mm), which results in 10^6 values for the density and a 300-fold reduction in the amount of data needed to characterize the system. A coarser grid would result in even higher simplification

Table 11.1 Summary of equation-based and agent-based wound healing models

Model	Type	Examples	Phenomenon modeled	Tissue type
ODE		Cukjati et al. [20]	Wound area healing	Endothelium, etc.
		Johnson [17]	Wound area healing	Arteries, veins
		Bardsley et al. [5]	Wound area healing	General
		Baker et al. [18]	Wound area healing	Diabetic ulcers
		Jercinovic et al. [19]	Wound area healing	Pressure ulcers
		Menke et al. [21]	Fibroblasts; oxygenation	Dermis
PDE	Reaction–diffusion	Maini et al. [24, 25]	Migration; proliferation	Peritoneal
		Sherratt et al. [26, 27]	Migration; proliferation	Epidermis
		Sheardown et al. [29]	Migration; proliferation	Cornea
		Dale et al. [30]	Migration; proliferation	Cornea
		Tremel et al. [31]	Migration; proliferation	Fibroblast cells
		Gaffney et al. [33]	Migration; proliferation	Cornea
		Chen et al. [34]	Migration analysis	Tumor
		Dale et al. [36]	Migration analysis	Scar tissue
		Wearing et al. [37]	Migration; proliferation	Dermis
		Javierre et al. [4]	Migration; proliferation	General
	Continuum mechanical	Vitorino et al. [51]	Migration; proliferation	Endothelium
		Lee et al. [56]	Migration	Kidney cells
		Xue et al. [57]	Migration, oxygenation	Cutaneous
		Qi et al. [58]	Migration	Epithelium
		Arciero et al. [59]	Migration	Epithelium
	Cell signaling	Posta et al. [62]	MAPK activity	Epithelium
		Dale et al. [63, 64]	Fibroblasts; collagen	Scar tissue
		Murray et al. [66]	Wound contraction	General
		Palecek et al. [54]	Cytoskeleton-integrin	General
		Gaffney et al. [32]	Diffusion	Cornea
		Tranquillo et al. [67]	Migration; proliferation	Fibroblasts, ECM
		Olsen et al. [68]	Fibroblasts; proliferation	Scar tissue
		Sherratt et al. [69]	Wound contraction	Epithelium
		Murray et al. [70]	Morphogenesis	ECM
	Angiogenesis	Chaplain et al. [71]	New capillary formation	Tumor
		Anderson et al. [72]	New capillary formation	Cornea tumor
		Pettet et al. [75]	New capillary formation	Soft tissue
	Chemotaxis	Hillen et al. [77]	Chemical migration	General
		Schugart et al. [78]	Fibroblasts; oxygenation	Cutaneous
ABM		Dallon et al. [65]	Collagen deposition	Dermis
		DiMilla et al. [79]	Receptor-ligand binding	General
		Walker et al. [80, 81]	Migration; proliferation	Epithelium
		Bindschadler et al. [83]	Migration; proliferation	Scratch wound
		Ouaknin et al. [84]	Migration; chemotaxis	General
		Fozard et al. [86]	Collective cell migration	Epithelium
		Byrne et al. [87]	Cell expansion	General

and speedup in computation of the dynamical behavior of the system. Even more important is that we need not be concerned with the details of motion of individual cells; instead, the motion can be described in one of several standard ways (diffusion, chemotaxis, or convection) that we describe below.

The time rate of change of the density variable is expressed using an equation that involves partial derivatives of f with respect to the independent variables $\mathbf{x}$ and t. The most basic partial differential equation one can construct is the law of conservation of the number of cellular components in a given volume:

$$\frac{\partial f}{\partial t} = -\nabla \cdot \mathbf{J} + \sigma$$

Here $\mathbf{J} = (J_x, J_y, J_z)$ is a vector-valued variable that represents the flux of the component (migration or transport of the component away from the position $\mathbf{x}$), σ represents the local source or decay of the component, and $\nabla \cdot \mathbf{J} = \partial J_x / \partial x + \partial J_y / \partial y + \partial J_z / \partial z$ is the divergence of $\mathbf{J}$.

By using different relations between $\mathbf{J}$ and f, one can account for different types of transport such as (1) the *convection* of cells or molecules in the direction of velocity $\mathbf{v}$, defined by $\mathbf{J} = f\mathbf{v}$, and (2) the *diffusion* of cells or molecules, resulting from random motion of cells or molecules in all directions and defined by $\mathbf{J} = \nabla f$. A special case of convection is the *chemotaxis* of cells in which the direction of motion is defined by a gradient of a chemoattractant molecule with concentration c, i.e., $\mathbf{J} = f \nabla c$. The source σ is a function of f (expressing self-regulation of the component) and possibly other components (expressing the influence of components on each other). For example, in models describing the mechanics of a tissue, the variables are the mass density of the tissue, ρ, and the momentum of the tissue, ρ$\mathbf{v}$; the flux of the momentum is the stress tensor $\mathbf{T}$.

The system of PDEs formulated from known properties and interactions of cellular components forms the core of a PDE model. The remaining parts of the model are the definitions of the *domain* in which the equations are valid, the *initial values* of all variables across the domain, and the *boundary conditions* imposed at the boundary of the domain. The boundary conditions are typically defined as one of two types (1) Dirichlet conditions, representing a constant source or sink of the quantity and prescribed as a fixed value of the quantity at the boundary and (2) Neumann conditions, representing the flux of a quantity across the boundary and prescribed as the derivative of the quantity along the normal to the boundary.

The analysis of a PDE model starts with an examination of the existence and uniqueness of solutions. Contrary to strong results in the theory of ODEs, there are no generic results apart from the Cauchy–Kowalevski theorem guaranteeing local existence of a solution for a Cauchy problem. Global existence can be proved for a diffusion equation and systems of diffusion equations with the same diffusion constant. But in general, every system must be analyzed individually using techniques such as maximum principle, weak solutions, variational formulation, etc. (see, e.g., [16]). The next step in the analysis may be to search for special solutions that enable one to reduce the dimension of the equation. Such special solutions can be (1) a *steady state solution* in which f is independent of t, (2) a *self-similar solution*, which is invariant under a rescaling of the spatial and time variables, or (3) a *traveling wave solution*, which represents solutions invariant under the transformation $\mathbf{x} \to (\mathbf{x} - \mathbf{v}t)$. These special solutions are then analyzed for stability, i.e., invariance under a small perturbation. Stable solutions are of particular interest to various

applications since they describe the observed behavior of the system. If solutions do not converge to steady state solutions or traveling waves, they may approach singularities or blowups.

Once all information is extracted using analytical tools, numerical solutions of the system can be obtained. Numerical solutions of ODEs can be found using standard integration packages such as CVODE (available in C or FORTRAN) or MATLAB suite of integrators. A convenient free standalone program that allows the user to explore solutions of ODE systems is XPP. Numerical solutions of PDEs are obtained by converting the PDE system into a system of algebraic equations by transforming the derivatives into finite differences (finite difference methods) or by transforming the equations into a variational form formulated as integrals over appropriate test functions with simplicial support (finite element methods). User-friendly software packages have been developed that enable researchers with little knowledge of numerical mathematics to solve various types of PDE problems—see, e.g., the FEMLAB or Matlab Partial Differential Equation Toolbox.

ODE Models

The simplest ODE models of wound healing are phenomenological: they try to capture the time-dependent closure of the wound by formulating an equation for the wound area or circumference as a function of time and fitting the constants in the equation to observed data [5]. The majority of such models is based on linear or exponential functions that involve two parameters [17–19]. However, these are not sufficient to describe the initial delay of the healing process that is noticed in wound healing experiments. Cukjati et al. [20] formulated several ODE models by considering two, three, and four parameter functions of chronic wound healing and assessed their goodness of fit to 226 chronic wounds. They used a set of five criteria to qualitatively and quantitatively assess the model (statistical analysis of goodness of fit) and concluded that a delayed exponential model with three parameters is the most adequate for representing the healing process.

Mechanistic ODE models are based on formulation of equations describing the concentration of various components of the wound healing process. An example is the model of Menke et al. [21] which focuses on the second stage of the process (inflammation) by using an extension of an ODE model of inflammation [22, 23] to include tissue damage, pathogen level, inflammation, fibroblast concentration, and tissue oxygenation. The model is used to simulate impaired wound healing in hypoxic skin wounds with varied levels of contamination. Pathogen growth is assumed to depend on tissue oxygenation levels. The skin is assumed to have a baseline level of circulating fibroblasts, which increases in response to tissue damage and inflammation. The authors classify wounds as healed, nonhealing, or chronic and find parameter ranges for each type of outcome. Impaired wound healing is simulated in hypoxic wounds with varying levels of contamination, and the model is used to suggest possible components to target in therapies such as the fibroblast death rate and the rate of fibroblast recruitment.

PDE Models

PDE models of wound healing describe the spatial dependence of various components involved in the healing process and can predict the shape of the wound. Most existing PDE models focus on the third and fourth stages of wound healing process, i.e., on the repair of the epithelial layer and the remodeling of the scar tissue.

Reaction–Diffusion Models Repair of the epithelial layer is a combination of two processes migration of epithelial cells into the wound and cell proliferation. The simplest PDE model of wound closure that can be constructed is one that consists of a single equation for cell concentration with wound closure interpreted as a traveling wave of cell concentration. The equation commonly used in that interpretation is the Fisher–Kolmogorov equation, which is a reaction–diffusion equation with proliferation given by a logistic term. Maini et al. [24, 25] verified the validity of using the Fisher–Kolmogorov equation in a medical context by using a scratch-wound assay (for an example of a scratch-wound assay, see Fig. 11.1) and comparing model predictions with multiple experiments.

Both the migration and the proliferation of epithelial cells are regulated by growth factors. The first model to account for such chemical control was developed by Sherratt and Murray [26]. The model consists of two nonlinear reaction–diffusion equations that track epithelial cell density and a chemical regulating mitosis [the epidermal growth factor (EGF)] in the context of epidermal wound healing. The epidermis is assumed sufficiently thin and thus the tissue is modeled as two dimensional. The mitosis chemical is modeled as both an activator and inhibitor, and the effect of these two chemical behaviors on cell migration is investigated. The model was further analyzed in [27] by providing details for traveling wave solutions for cell density and chemical concentration. The results for wound radius as a function of time were shown to be consistent with experimental measurements. Clinical implications of the model were studied in [28], in particular the dependence of healing time on wound shape (e.g., cusped, ovate, and rectangular) and the dependence of predicted wound contours on the character of the growth factor.

The Sherratt–Murray model was extended to corneal epithelial wounds by Sheardown and Cheng [29] and by Dale et al. [30] who used the model to study the impact of increased mitotic and migratory activity due to an EGF. They also predicted the wound healing rate if the growth factor was applied only topically to the wound and found that the factors affecting migration include cell migration, cell-to-substrate adhesion, and cell mitosis. They noted that the model predicted a lag time immediately after wounding.

Tremel et al. [31] modified the Fisher–Kolmogorov equation to include the effects of cell density-dependent diffusion on wound healing. They assumed that diffusivity decreases with increasing cell density in order to capture contact-inhibited movement between cells so that cells slow, stop, or change direction when they encounter another cell in their path. In their study, cell tracking was performed on groups of cells in a wound healing experiment; in the images, it was observed that the cells initially located close to the wavefront traveled significantly greater

distances than cells starting farther behind the wavefront. Also, while the overall cell movement was directed, a significant amount of random motion was observed.

Several studies have modeled the influence of physiological electric fields on wound closure. In those studies, the PDE problem was formulated with a *free boundary*, i.e., a boundary whose position is changing in time. This change of position is governed by an additional equation. For example, Gaffney et al. [32] described the evolution of the free boundary problem for a system of two reaction–diffusion equations for cell density and chemical stimulus in the context of corneal wound healing. The formulation predicts a linear relation between the wound healing speed and the physiological electric field strengths over a physiologically large range of electric field strength. Spatial and temporal data on mitotic rates measured during corneal epithelial wound healing in a rat was studied by Gaffney et al. [33] who argued that earlier models were not adequate for the study of cell kinetics. Chen and Friedman [34] analyzed the Gaffney model [32] and applied a similar approach to predicting tumor growth [35].

In a subsequent paper, Dale et al. [36] presented a complex model for scar tissue formation in deep wounds and focused on the role of key chemicals in determining the quality of healing. The authors described wound healing as a traveling wave and investigated the factors controlling the speed of the wave. A more complex model accounting for the effect of the keratinocyte growth factor (KGF) was proposed by Wearing and Sherratt [37] who found that high KGF levels decreased the speed of healing but increased the cell division rate at a greater distance away from the wound edge. A comprehensive review of wound healing models of Sherratt and collaborators is given in [38] and [39].

Javierre et al. [4] also modeled the reepithelialization of the basal membrane of the epidermis by cell mitosis and migration in the presence of a generic EGF. The diffusion, depletion, and production of the concentration of the growth factor in the model are determined by a reaction–diffusion equation. The model assumes that cells become motile if the accumulated growth factor concentration exceeds a threshold value. A sigmoid function is used to relate cell mitosis and the growth factor concentration. Since cell migration is interrupted when the growth factor concentration drops below a threshold, cell motility is dose dependent in this model. Moreover, the wound closure rate is assumed proportional to the local curvature of the wound edge. Javierre et al. [4] analyzed the roles of diffusion, closure rate, and wound geometry on healing kinetics and concluded that healing is always initiated at regions with high curvatures, and that the evolution of the wound is sensitive to multiple physiological model parameters.

Continuum-Mechanical Models For the success of wound closure during the third stage of wound healing it is essential that the epithelial cells migrate collectively, in synchrony, so that the coverage of the wound is continuous without the formation of any holes in the remaining sheet. Cell migration at the single-cell level has been studied extensively over many decades [40–44]. In brief, each cell moves by a cyclic mechanism that proceeds through stages involving the formation of a lamellipodium, translocation of the nucleus in the direction of motion, and detachment of the

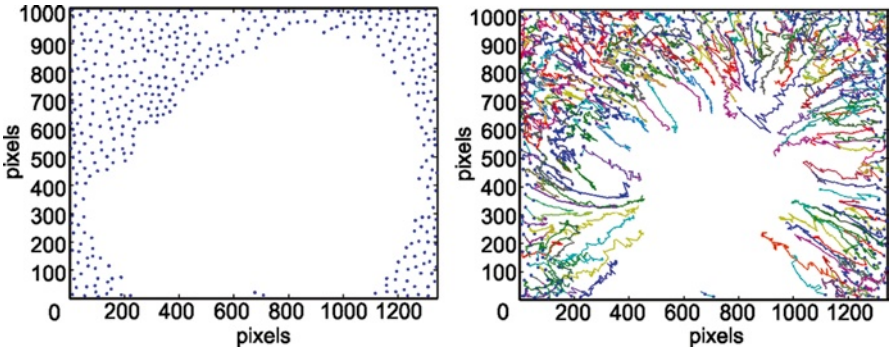

Fig. 11.2 *Left panel*: initial position of all cells in scratch-wound assay is indicated by *blue dots*. *Right panel*: cell trajectories of every initial cell position over the course of several hours

trailing edge [42, 45]. This mechanism is regulated by a complex signaling and regulatory network responsible for the underlying processes of actin polymerization and depolymerization, motor protein activation, and integrin formation and release.

Although the study of individual cell migration has been pursued vigorously, there is less understanding of the interactions that drive and synchronize collective cell migration in wound closure. Several mechanisms of closure have been proposed (1) a leader cell mechanism, (2) cooperative traction force mechanism, (3) steered migration mechanism, (4) differential adhesion hypothesis, and (5) differential interface tension hypothesis. In the leader cell mechanism [6], the cells at the edge of the wound are believed to change their phenotype and direct the migration of other cells towards the wound. In the cooperative traction force mechanism, cells near the edge of the layer exert coordinated forces that result in a cumulative stress within the layer and motion of cells towards the wound [9]. In the steered migration mechanism, the direction of autonomously migrating cells is changed in a gradual fashion by forces exerted on them by neighboring cells [46]. The differential adhesion [47] and differential interface tension [48] hypotheses stipulate that the cell layer evolves to minimize either the adhesion energy or surface tension of the constituent cells, which leads to the eventual wound closure.

As described earlier in this chapter, a typical experimental method used to study collective cell migration is the scratch-wound assay (Fig. 11.1). Farooqui and Fenteany [49] studied wound closure in Madin–Darby canine kidney (MDCK) epithelial cell layers and established that submarginal cells exhibit protrusive and migratory behavior similar to that of marginal cells. They found that the general direction of the coordinated cell movement was toward the center of the wound and the cell velocity within a sheet was found to be inversely proportional to the distance from the wound edge. Wound closure was shown to occur even if the motility of edge cells was inhibited, but it occurred at a slower rate [50]. Coordinated cell movement toward the center of a scratch-wound assay is depicted in Fig. 11.2. In the left panel, the starting positions of all cells of the scratch assay are denoted by

blue dots. In the right panel, colored lines define the trajectories of all the cells, with the blue dots indicating the starting point of the cell as in the left panel. The trajectories indicate the tendency of the cells to migrate towards the center of the wound.

Vitorino and Meyer [51] studied growth factor-induced migration of endothelial cell monolayers and proposed that the fibroblast growth factor (FGF) led to directed migration of leader cells but did not control cell migration and coordination of the follower cells. Mechanically robust and dynamic coupling of cells to one another and to the substrate is accomplished via adherens junction proteins, desmosomal proteins, and integrins [6, 52]. The cells in the interior are connected to the cells at the boundary by tight junctions, which prevent separation of the cells in the layer [53]. The level of adhesion between the cell and the substrate, moderated by integrins, was found to control the speed of wound closure [54]. The effects of substrate stiffness on cell traction forces were quantified for epithelial cells and fibroblasts, and it was shown that cell movement could be modulated by changing the stiffness of the substrate [8]. Trepat et al. [9] found that traction forces, applied by moving MDCK cells on the substrate, were smallest in the center of a cell colony and largest at the edge of the colony of cells moving radially outward. They estimated that tension in the cell layer increased with distance from the edge of the cell colony and argued that accumulated traction stresses were balanced by the forces within the cell sheet; the interplay of these two stresses was described using a tug-of-war model. In several studies, a release of tension was observed within the cell layer once a wound was induced [7, 55]. Block et al. [55] compared cell-sheet migration in wounds induced by different methods and hypothesized that the release of spatial constraints initiates a healing response. However, this hypothesis is difficult to verify experimentally since it is hard to eliminate all possible methods (such as biochemical communication) that may contribute to collective cell migration.

All the models described so far represented migrating cells using reaction–diffusion equations for cell density. Such equations are based on the diffusion mechanism for cell migration, which provides no guarantee of continuity of the cell layer. The process of collective cell migration is complex and requires fundamentally different, mechanics-based models. Lee and Wogelmuth [56] developed a model in which an MDCK cell layer was represented as a viscous liquid with orientation, similar to a liquid crystal; the layer orientation was equated with the direction in which the cell exerts a crawling force. They formulated equations of balance of forces on the cells and, using numerical solutions, were able to reproduce not only wound closure dynamics but also the irregular, undulating, progression of the edge of the layer typical for scratch-wound assays, without the need to specify leader cells. Xue et al. [57] developed a continuum model of ischemic dermal wounds with the wound boundary represented as a free boundary that moves with the velocity of the ECM at the wound edge. The model was used to predict how ischemic conditions may impair wound closure.

Mi et al. [58] recently developed a one-dimensional continuum mechanical model of a migrating IEC-6 enterocyte cell sheet to study the influence of lipopolysaccharide (a protein found in the coat of Gram-negative bacteria) and integrin

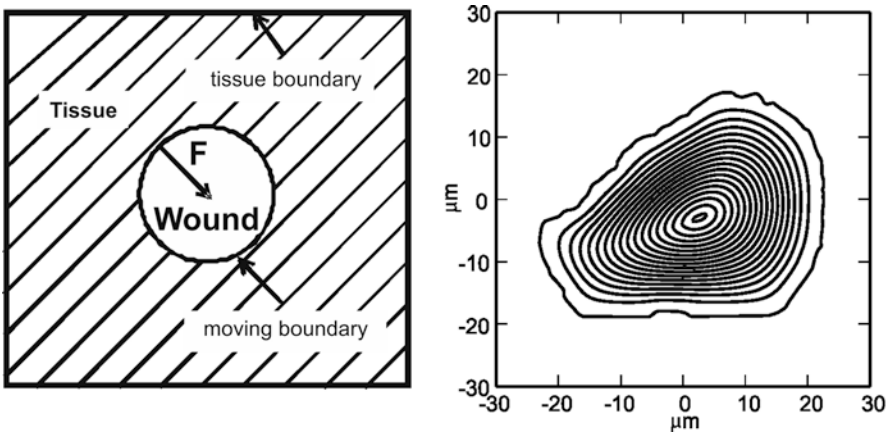

Fig. 11.3 *Left*: schematic of a circular wound surrounded by tissue. The force of the lamellipodia at the edge of the wound is denoted by F. *Right*: model-calculated contours of wound edge (initial position is outermost contour) every 30 min until wound closure

concentration on wound closure during experimental necrotizing enterocolitis. The model predicts low migration speed at high and low integrin concentrations and high velocity at medium concentrations, in agreement with experimental observations [54]. It also predicts that the edge velocity decreases with time, in accord with our experimental observations but contrary to the behavior of reaction–diffusion models. However, the model is only appropriate in situations in which the wound has a simple geometry with two long parallel wound edges. In a follow-up study, Arciero et al. [59] designed a two-dimensional model of cell layer migration that captures the same primary interactions driving the motion of the cell sheet, namely, the elastic coupling between cells in the layer, the adhesion of cells to the substrate, the force generated by lamellipodia both in the interior and at the wound edge, and the proliferation of cells within the layer, but has the additional benefit of being applicable to an arbitrary wound geometry. Figure 11.3 shows a model schematic of a wounded region and the model predicted contours for the closure of an experimental scratch wound at 30-min intervals until the wound is completely closed.

In Arciero et al. [59], the cell sheet is represented as a compressible inviscid fluid, and therefore individual cells are not distinguishable. The leader and follower cells are accounted for in an average manner by including a focused traction force applied by the lamellipodia at the edge of the sheet. The two-dimensional character of the problem requires the use of Eulerian-independent variables. The physical laws governing the mechanics of the layer then yield a partial differential equation problem with moving boundary that is known as the Stefan problem in other contexts [60, 61]. The problem is solved numerically using a level set method, and the basic properties of solutions are analyzed. The model is calibrated for two scenarios: the closure of a wound and the expansion of a cell colony. Parameter values in the model are fit to data from a scratch-wound assay as well as to data from a cell

colony expanding radially outward [9]. Cell proliferation is neglected in wound closure simulations but is included in colony expansion simulations. The model successfully reproduces cell density and edge migration velocity data from both types of experiments.

Cell Signaling Models Models that are developed to understand both the mechanical and biochemical aspects of cell migration can help to determine which phenomena are primarily responsible for initiating cell motility following an injury and what factors regulate the speed and direction of cell migration. In general, the regulation of wound healing by biochemical signals and feedback pathways remains poorly understood. Posta and Chou [62] developed a mathematical representation of ligand-mediated intercellular signaling mechanisms related to the cell migration of epithelial monolayers. Experiments have indicated the need for mitogen-activated protein kinase (MAPK) activation for coordinated cell movement following an injury. The model reproduces two waves of MAPK activity that have been observed experimentally and that may depend on reactive oxygen species (ROS) and competition between a ligand (such as a growth factor) and ROS for the activation of the epithelial growth factor (EGF) receptor. The resulting traveling wave solutions of the model are consistent with MAPK patterns observed experimentally.

Models of the fourth stage of wound healing, i.e., the remodeling of the scar tissue, are primarily concerned with the factors that determine the final size of the scar. Two key features of the scar tissue attract attention: details of collagen composition (relative proportion of type I and type III collagen) and orientation of the fibers. The balance between the two types is regulated by different isoforms of transforming growth factor (TGF)-β protein and was studied by Dale et al. [63] who developed a reaction–diffusion model. The model predicted that different ratios for fetus and adult tissues depend on the secretion of the different isoforms of TGF-β. In a follow-up paper [64], Dale et al. used the model to determine whether fibroblast cells enter the wound area from the surrounding unwounded dermis or from the underlying subcutaneous tissue and gave reasons favoring the latter. The orientation of fibers in the wound tissue was analyzed in a series of papers by Dallon et al. [65] who employ agent-based, as opposed to equation-based, models. In particular, fibroblasts were modeled as discrete entities and the ECM was assumed to be a continuous entity composed of collagen and a fibrin-based blood clot. The following interactions were captured by the model: fibroblasts orient the collagen matrix, fibroblasts produce and degrade collagen, and fibrin and the matrix direct the fibroblasts and determine the speed of the cells. The model was used to predict how multiple cellular phenomena play a role in collagen alignment during wound repair.

Wound contraction is also an important component of wound closure, especially in animals. Contraction is primarily caused by myofibroblasts that exert traction forces on their environment. Experimentally this process has been studied on collagen gels. Contraction was first studied mathematically by Murray et al. [66] who adapted a general model of tissue biomechanics to a wound healing situation. Subsequently, Tranquillo and Murray [67] investigated the interplay between cellular, biochemical, and biomechanical phenomena, which result in wound contraction.

They modeled fibroblast migration and proliferation as well as the deformation of the ECM and formulated an extended model that accounts for the influence of an inflammation-derived mediator on traction, growth, and chemotactic properties of fibroblasts in order to predict the qualitative features of a contracting wound. A similar model was also used by Olsen et al. [68] to study failures in wound closure due to fibroproliferative disorders such as keloid and hypertrophic scars. All of these models describe tissue as a linear viscoelastic material. For embryonic epidermal wound healing, Sherratt [69] developed a model involving actin filament network formation and wound contraction, based on a mechanochemical model for the deformation of epithelial sheets proposed by Murray and Oster [70].

Angiogenesis Models Angiogenesis in a growing tissue has been studied in the context of wound healing or tumor growth. The process of capillary ingrowth is essential to healing since it helps to maintain high levels of metabolic activity by increasing blood supply. The biology of angiogenesis has been studied mostly in the context of cancer growth, but the biology applies equally well to wound healing. Tumor angiogenesis has been modeled by Chaplain and Sleeman [71] and continued by Anderson and Chaplain [72]. Chaplain and Byrne [73] reviewed the similarities of wound healing and tumor growth and Olsen et al. [74] studied the interactions between endothelial cells and soluble regulators (such as growth factors), as well as the insoluble ECM substrate, which consists primarily of collagen. Pettet et al. [75] developed a model of angiogenesis during wound healing that includes contributions of capillary tips, capillary sprouts, fibroblasts, macrophage-derived chemical attractants, oxygen, and ECM. The model reflects the dependence of macrophage activity on local oxygen concentration, which is the major difference between the process in wounds and tumors, and is able to reproduce the failure of wounds to heal when the proliferation rate of endothelial cells is too low. A new version of the model was compared with experimental data by Byrne et al. [76].

Chemotaxis Models The directed movement of cells and organisms in response to chemical gradients, known as chemotaxis, plays an important role in several aspects of physiology, including embryonic development, inflammatory cell migration, wound healing, new vessel formation, and tumor growth. The deterministic Keller–Segel continuum model is a well-established method for representing chemotactic behavior of cell populations since it is able to capture key phenomena that are often lost on discrete or single-cell level models. Hillen et al. [77] analyze ten models that are variations of the Keller–Segel model in order to determine which model components relate most directly to biological observations of chemotaxis. Their analyses include the determination of the existence of model solutions and the identification of long-time behavior of solutions and the form of steady state patterns.

As an example of a chemotaxis model in the context of wound healing, Schugart et al. [78] presented a PDE model of wound healing that focuses on the release of angiogenic growth factors (e.g., VEGF) by inflammatory cells. In particular, the growth factors are assumed to interact with fibroblasts to produce collagen and other components of the ECM, which in turn facilitates the migration of cells into the wound. A circular wound is considered in this theoretical study, and thus the

model is solved over a radial cross section of the wound. Model results suggest that a hypoxic wound environment cannot sustain vascular growth, that hyperoxia promotes wound angiogenesis and healing, and that there is an optimal level of hyperoxia beyond which the beneficial effects of oxygen may be reversed.

Agent-Based Models of Cell Migration

Various types of agent-based models (ABM) have been used to test wound healing hypotheses and to isolate factors that may direct cell sheet migration. Since a detailed description of wound healing ABM is presented in a different chapter of this book, here we focus on ABMs of collective cell migration, as such models are often used as a basis for the development of equation-based models of wound healing.

A detailed model of the dependence of cell speed on adhesion-receptor/ligand binding was proposed by DiMilla et al. [79]. Walker et al. [80, 81] used an agent-based model to simulate the wounded epithelial cell monolayers and suggested that simple rules are sufficient to qualitatively predict the calcium-dependent pattern of wound closure observed in vitro. Khain et al. [82] built upon the work in [80, 81] and considered a simple discrete model, which focused on the effects of three key processes, cell–cell adhesion, diffusion, and proliferation, on wound healing in the context of a scratch-wound assay. Different cell behavior was predicted by the model depending on the adhesion strength and the proliferation rate. In general, the model is defined by a list of rules that dictate the conditions under which cells can proliferate or migrate, depending on the number of nearest neighbors to the cell.

Bindschadler and McGrath [83] used an ABM to simulate cell migration in which cells responded to crowded conditions by decreasing their cell division rates and moving to less crowded areas. The model predictions were consistent with experimental rates of closure. Ouaknin and Bar-Yoseph [84] used the Glazier–Graner–Hogeweg (GGH) model to simulate the collective movement of cells, taking into account adhesion energy, deformation energy, and stochastic behavior of the system. The model results were similar to experimental behavior obtained by Poujade et al. [85], in which leader cells progressed faster than the rest of the cell layer and a fingering morphology emerged. Fozard et al. [86] developed an ABM for epithelial monolayers and used it to derive an equation-based continuum model in the limit of a large number of cells. Relating agent-based and continuum models may help to estimate model parameters and justify model assumptions. Fozard et al. [86] assumed that the energy dissipation of individual cells was due to the drag between the cell and substrate, as well as due to the internal viscosity of the cells (which was not accounted for in the model presented here). Active cell migration and cell division were not included in their model, and a more complex formulation of cell–cell and cell–substrate adhesion could provide additional mechanical insight. The continuum model yielded results consistent with the ABM for even a moderate number of cells. Byrne and Drasdo [87] also derived a continuum model from their ABM for

the growth of cell aggregates on compact monolayers. Growth was assumed to be governed by contact inhibition, and cells were assumed to proliferate. The continuum model agreed with the ABM in the prediction of initial and asymptotic growth regimes for the radius of the colony and the cell population size. A detailed description of agent-based models of wound healing is provided in the next chapter.

Applications of Wound Healing Models

Both equation-based and agent-based theoretical models of wound healing have important applications that extend beyond the context of wound healing. The mechanisms and techniques used to describe migration and proliferation of a cell layer can be used to predict wound closure time as well as to describe the mechanical processes governing morphogenesis, tumor growth, and colony expansion.

Predicting Wound Healing Time Three commonly used methods for estimating wound closure time in clinical practice are the Absolute Area Reduction method, Percent Area Reduction method, and Linear Parameter method [13]. The Absolute Area Reduction method estimates the time rate of change in wound area as the ratio of the difference between the current wound area and original wound area to the total change in time. The Percent Area Reduction method estimates the rate of change in wound area as the difference in wound areas between two consecutive time points. The Linear Parameter method assumes that the average velocity of the wound edge over the wound contour is constant in time and uses the value of a linear healing parameter, which is defined as the ratio of the difference in wound areas to the average perimeter for two consecutive time points, to predict overall closure time for wounds. Recently, Arciero et al. [92] introduced two additional methods for calculating healing time, in which the time rate of change of wound area is not constant but is proportional to the square root (Square Root Method) or the first power (Proportional Area Method) of area. These methods were shown to provide better estimates of closure time than the three previously established methods since they both converge to the correct closure time as more data is available and they provide relatively accurate predictions at early stages of the closure process. While these two methods were shown to be useful for predicting a range of wound healing times for superficial epithelial wounds, other clinical aspects may be required to obtain accurate closure time predictions for wounds of various types and sizes. A comparison of the predicted healing times of these five methods is provided in Fig. 11.4.

Morphogenesis Cell and tissue mechanics are important components dictating embryonic development and organ shape within a body. In particular, at the tissue level, force production and viscoelastic material properties of tissues determine the direction and speed of tissue movements as structures are sculpted. Integrating intracellular force generation with the local micromechanical environment directs

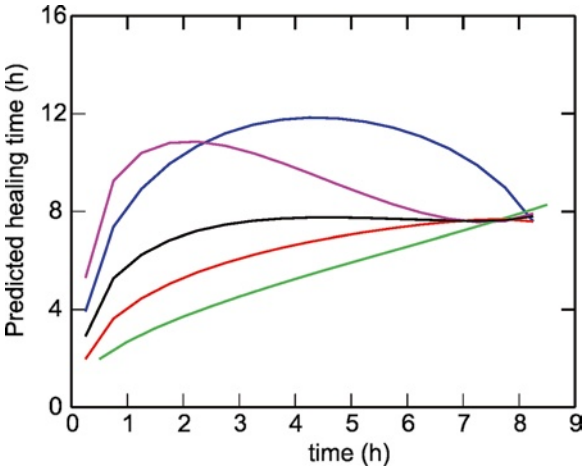

Fig. 11.4 Comparison of predicted healing times for a scratch-wound assay using five different methods: Absolute area reduction method (*green*), percent area reduction (*red*), linear parameter method (*blue*), square root method (*black*), and proportional area method (*magenta*). The predicted healing time is shown as a function of time

molecular–mechanical processes and cell differentiation [88]. Significant advances have been made in morphogenesis experiments, and the use of mechanical and theoretical analyses in this field is beginning to gain momentum. The combination of these experimental and theoretical techniques may help to answer three important questions in the field of morphogenesis outlined by Davidson et al. [89]: (1) are mechanical properties of the embryo important to morphogenesis? (2) at what scale are mechanical properties shaped? and (3) can the processes that generate force be separated from the processes that make tissues stiff?

Tracheal branching morphogenesis and mammary gland development are two examples in which morphogenesis of branched tubular organs or terminal end buds can be studied. Tracheogenesis occurs without mitosis, and thus collective cell migration can be studied in this context without interference from cell proliferation. It has been concluded that the pattern of tracheal branching emerges from the interplay between an extracellular chemoattractant and collective decision making that uses a negative-feedback loop to restrict the number of cells that respond to this chemoattractant [6]. Mammary gland development occurs via the branching morphogenesis of terminal end buds; this branching is unique from most other systems due to the absence of leader cells at the tip of the bud. Instead, the cells at the bud tip for a blunt-shaped multilayered bulb with cells continually exchanging positions [6].

Cancer Several models originally developed for wound healing have been employed to simulate expansive growth and cell migration of tumors [73, 87, 90]. Both discrete and continuous approaches have been used that consider the effects of mitotic inhibitors, nutrient depletion, cell cycle, and new capillary formation on tumor growth [90]. For example, Tracqui [90] developed models that relate cell motility and traction forces and that are used to simulate the transition from a homogeneous distribution of cells on a tumor surface to a nonhomogeneous density pattern that may correspond to a preinvasive stage of the tumor.

Colony Expansion Models for wound healing can be also transformed to simulate the process of cell colony expansion [59]. Trepat et al. [9] recorded the cell density of a canine kidney cell population as a function of distance from the leading edge of the cell layer at 24-h time intervals. Growth of the layer plays a prominent role in the context of colony expansion, and Poujade et al. [85] observed that cell proliferation by a colony of cells occurred almost exclusively within the band where cells were originally seeded, potentially due to the longer presence of cells in the originally seeded region or modifications made by cells to the underlying substrate. When applied to a cell colony scenario, the model in [59] predicts an increase in cell density when approaching the center of the cell colony. The results also suggest that in the experiments of Trepat et al. [9], as in those of Poujade et al. [85], the cells proliferate only in the region originally seeded by the cells.

Conclusions

A multitude of mathematical models of wound healing have been developed in the attempt to understand the qualitative and quantitative aspects of the process. Although many of the models differ substantially in scope, the mechanical and mathematical principles underlying all of the models are related and can be applied to multiple biological systems. The choice of model type depends on the information desired. Certain models are appropriate at a cellular level (e.g., to simulate individual cell motion), while other models are more beneficial on a tissue level (e.g., to represent collective migration).

The study by Stolarska et al. [91] provides a perfect example of differentiating among model types while also highlighting model similarities. In the study, three different cell and tissue mechanics models are presented: a continuous model of an arbitrarily deformable single cell, a discrete model of the onset of tumor growth, and a hybrid continuum–discrete model of later stages of tumor growth. Three essential processes involved with cell migration are captured in the single cell model: the controlled spatiotemporal remodeling of the actin network, the generation of traction forces to move the cell body, and the construction and destruction of focal complexes or focal adhesions. Cell-level details are incorporated into their tissue-level model, including how an individual cell reacts to forces on it, how cells interact mechanically with their surroundings, how growth and division are described, and how stress affects growth. And thus, predictions obtained across multiple levels of mathematical modeling can be used to gain insight into wound healing processes.

Byrne and Drasdo [87] compare the benefits of using a biophysical agent-based or a continuum mechanical model to track the expansion and migration of cells in a dense monolayer. Single-cell-based models permit a higher degree of spatial resolution than models composed of locally averaged quantities; however, large cell population sizes are not amenable to investigation using agent-based models. Ultimately, conditions under which spatiotemporal behavior of the different models agreed were identified in order to determine how to relate the parameters in the different

models. The same growth pattern for dense and sparse cell aggregates was obtained using both models.

Khain et al. [82] commented that most theoretical models of wound healing employ reaction–diffusion equations for the cell density and a growth factor. However, in their study, they demonstrated that simple discrete models can be applied to wound healing and yield the results obtained from reaction–diffusion equations when proliferation is small. Since biologically reasonable rates of perfusion are small compared to rates of diffusion, both continuum and discrete models provide good predictions of the velocity of a wound edge.

Whether an ODE, PDE, or ABM wound healing model is used to describe the migration of cells in response to an injury, all three model types aim to accomplish three main objectives: to track the cell response and position following the induction of a wound, to understand the role of tissue growth factors in the healing process, and to predict the time required for a wound to heal. As described in this chapter, the particular choice of theoretical wound healing model dictates the specific phenomena or elements that are most likely to be understood and uncovered.

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Chapter 12
Agent-Based Models of Wound Healing

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Introduction

Wounds, whether developed in hospital or present on admission, pose a great threat to a patient's health. Wounds provide an opportunity for pathogens to invade and also divert resources that the body could be using to restore health elsewhere. Furthermore, the inflammatory response incited as a consequence of tissue trauma can lead to extreme complications, especially when this inflammatory response becomes dysregulated.

Breakdown of the wound healing process at any level, leading to both acute and chronic failure of healing, is of interest across medical specialties. In patients with significant comorbid conditions including diabetes, obesity, or steroid use, these problems are compounded. Not only do wound healing problems cause morbidity to the individual patient but there is also a significant cost to the healthcare system as a whole [1]. Accordingly, there has been significant interest and research effort directed at understanding how wounds heal, with the goal of improved strategies and resources for prevention and treatment of wound-related complications.

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However, the overwhelming complexity of the molecular and cellular healing machinery defies study using traditional experimental methods. Much information has been elucidated regarding the roles of various individual components, but these components are generally studied using in vitro systems that are only abstractions of their actual biological reference systems. Adding to the difficulty in obtaining a systems-level view of the healing process is the disparate and often ambiguous information present in the literature. Moreover, while wound healing is well studied in animal systems [2, 3], only recently have experimental methodologies emerged that may allow for the study of the time courses of wound healing in humans [3]. Even in these settings, it is difficult to collect time courses of primary samples from humans suffering from chronic wound healing diseases without possibly disturbing the very process being measured. Perhaps more importantly from a translational standpoint, it is essentially impossible to modulate all possible mechanisms of inflammation and wound healing in an attempt to find novel therapies.

Wound Healing and Inflammation

Wound healing involves multiple cell types, intertwined signaling pathways, and numerous control and regulatory mechanisms [4, 5]. In general, the process can be divided into three phases: inflammatory, proliferative, and remodeling. The inflammatory phase of wound healing begins immediately after injury and primarily involves release of mediators to invoke both hemostatic and inflammatory responses [4]. The clotting cascade is initiated first, and the hemostatic plug is assembled on exposed collagen within the wound [4]. In addition to their role in controlling hemorrhage, platelets within the plug release mediators such as platelet-derived growth factor, which set the inflammatory cascade in motion. These factors act as chemotactic agents to recruit inflammatory cells, primarily neutrophils, as well as local vasodilators to allow passage of the cells into the damaged tissue [6]. The presence of infectious agents such as bacteria prolongs and exacerbates the inflammatory response, which prevents progression to the proliferative phase and can lead to nonhealing. The inflammatory phase of wound healing will be described in more detail below.

The proliferative phase consists of several key steps to form a temporary yet durable wound closure, which can later be remodeled into the final scar. One of the earliest steps is reepithelialization, which occurs via several mechanisms. Reestablishment of the epithelium restores its immunologic and barrier function, which is critical to the overall healing process by preventing further infection and propagation of the inflammatory response [7]. Thereafter, angiogenesis occurs and allows for sufficient nutrient delivery to enable deposition of granulation tissue into the wound bed [4].

Remodeling begins once primary wound closure has been achieved and is a much more prolonged process. Collagen is deposited into organized networks by fibroblasts, and the wound is gradually contracted by the action of myofibroblasts to form the mature wound. Abnormalities of remodeling lead to chronic wound problems

such as keloid and hypertrophic scar formation. Although clearly clinically important, this phase of wound healing will be underemphasized in this chapter, as the focus is more on understanding and modulating the acute wound healing environment.

As the initial phase of wound healing, inflammation is critical to successful wound healing. However, inflammation can also cause chronic tissue injury via a positive-feedback loop incited by incidental cell damage [4, 5, 8], and in extreme cases can even lead to distal organ dysfunction and death [9]. As a well-coordinated communication network, inflammation allows organisms to deal with a rapidly changing and often hostile environment. The mechanisms that have evolved to carry out these complex tasks are redundant, robust, and highly context dependent [10]; for example, the same cytokine may have opposite effects depending on other factors present in its local environment. Inflammatory mediators such as interleukin (IL)-6 [11], IL-10 [12], transforming growth factor-β1 (TGF-β1) [13], and nitric oxide [14, 15] all modulate the wound healing response in a highly context-dependent manner. Conversely, while the functions may remain constant, biological redundancy allows for variation in the players that pass individual messages that comprise those macrofunctions. For example, not only does the proinflammatory response consist of complex signaling involving many mediators, but any given mediator participating in that chain may also change from organism to organism or situation to situation.

These intracellular signaling networks and their products, including diffusible molecular mediators, are in essence the carriers of information in the network of inflammatory communication, and, therefore, possible targets for intervention. The problem, as mentioned above, is that any given pathway or mediator may exert either beneficial or detrimental effects in a dynamically varying fashion based on the nature of the wound and the particular aspects of the individual patient. Selecting likely therapeutic targets for wound healing—in a rational fashion that takes into consideration this complex system as a whole—is thus a tremendously difficult task.

Agent-Based Computational Modeling

Computational techniques are useful for amalgamating data and generating hypotheses in the study of complex biological phenomena. A mechanistic computational model based on literature knowledge could be validated experimentally or clinically and in turn may have applications in diagnosing/predicting the wound healing trajectories of individuals or possibly in the design of novel therapeutic modalities for wound healing. Differential equations are the classical method for modeling biological processes and have been used since the late 1980s to explore all phases of wound healing from inflammation [16, 17] to wound closure [18, 19] to tissue remodeling [20], incorporating terms for mechanical stress, population dynamics, and biochemical signaling [21]. This compendium of equation-based models has resulted in important insights into the wound healing response and has advanced our understanding of wound healing as it is supposed to work as well as suggesting

underlying pathological mechanisms and testing therapies in silico [17, 20]. However, while extremely useful, these continuum models cannot be readily used to create tissue-realistic simulations that involve stochastic biological effects, a capability of growing importance given the increased availability of spatial, noninvasive data (such as clinical photographs and real-time dynamic microscopy) along with physicomechanical information. We suggest that this need can be met, in a complementary fashion, through the use of agent-based modeling (ABM).

ABM is an object-oriented, rule-based, discrete event computational modeling technique that is well suited for integrating and synthesizing such data, thereby providing a useful, translational tool for examining the greater wound healing picture. In an ABM, agents representing cellular or molecular components of a system populate a "virtual world," in which their simulated behaviors are governed by rules extrapolated from known knowledge regarding their biological behaviors. This is called dynamic knowledge representation and can be used for integrating such disparate and scattered information, bridging gaps in the current knowledge base, and generating and instantiating novel hypotheses [22–24]. A significant amount of component-level mechanistic detail can be included in a set of agent rules, and qualitative overall system behaviors examined using a visual interface.

Because the rules in an ABM define local, concurrent interactions and are frequently probabilistic, simulation experiments with an ABM can often lead to nonintuitive and paradoxical behaviors. Such simulated outcomes mirror the translational gulf between basic science and clinical therapeutics [25]. However, as opposed to the seemingly insurmountable translational hurdle facing the traditional biomedical community, the ability of ABMs to cross this translation divide provides an opportunity for researchers to augment their ability to evaluate (and potentially discard) hypotheses that do not pass at least an initial "eye test" in terms of the generated system level output.

ABMs offer another advantage in this role of dynamic knowledge representation intended to increase the efficiency of integrative and translational research. Compared to more traditional mathematical models, ABMs can be more intuitive to nonmathematicians and therefore be more accessible to the general community of biomedical researchers. This makes ABM a particularly attractive tool to the biologist or clinician for dynamically representing their hypotheses and allowing them to carry out "thought experiments" [26]. This process can lead to the generation of novel, clinically relevant hypotheses that can then be examined in an iterative investigatory loop.

Further reducing the threshold for adoption of ABM is the fact that the development of a biomedical ABM very often involves the use of an existing software toolkit/development environment. This allows biologists to focus on the implementation of their biological concepts as opposed to the detailed issues related to software design. ABM toolkits that have been used by biomedical researchers include NetLogo [27], Repast [28], and Multiagent Simulation of Networks (MASON) [29]. More recently, the ABM software Simple Platform for Agent-based Representation of Knowledge (SPARK) [30, 31] was developed to facilitate tissue-realistic modeling in biological contexts. This type of software may be particularly useful as the goal of ABM expands beyond dynamic knowledge representation to augment basic research to more translational applications.

Agent-Based Modeling of Wound Healing

The ability of ABMs to represent spatial relationships and tissue patterning effects makes this class of models an appealing approach for modeling the biology of wound healing. As with other applications of biomedical agent-based modeling, simulations generated by an ABM concerning wound formation and evolution are marked by global, system-level morphological outputs, i.e., spatial patterns with identifiable temporal trajectories. Tracking these morphological features—along with numerical data concerning both the temporal trajectories of individual components (mediators and cell populations) and "experimental/epidemiologic" output derived from performing a series of simulation runs—provide a rich space of output features to which hypotheses can be examined, evaluated, and falsified.

In general, ABMs are developed by following a consistent series of steps. This process typically involves the initial integration of various sources of knowledge, guided by the expertise and intuition of the researcher into a putative hypothesis structure that is then instantiated into the ABM. The ABM must then be calibrated and validated, a process in which the spectrum of behaviors of the ABM is evaluated following iterative manipulation of its parameters (calibration). A separate step (validation) involves determining if the ABM behaves plausibly when compared against data not used in its construction. Finally, simulation experiments must be carried out in order to see if the ABM exhibits properties not previously described in the reference system. This process involves the generating perturbations (the addition of external factors or simulations of knockouts/knockdowns) to the model.

There are several examples of this multistep process in the context of ABMs of wound healing. In general, these ABMs can be divided into (1) those intended to provide greater insight into how the system works (as an adjunct to translating and integrating knowledge in a basic science setting), mostly involving the examination of intracellular signaling and gene regulation [32–35] and (2) those focused on characterizing the global system level properties arising from generative mechanisms and what might be done to potentially control them (as an adjunct to translational science and the rational development of clinical therapeutics) [23, 25, 36–41]. In the sections below, we highlight examples of both use cases.

Agent-Based Modeling for Basic Science Knowledge Integration: An ABM of Epithelial Restitution

A wound damages the epithelial tissue layer and exposes underlying tissues to potentially detrimental factors in the external environment [42]. Restitution (reapposition of opposing sides of the damaged epithelium) is the initial phase in the reestablishment of the epithelial barrier and occurs quickly and independently of cell proliferation [7]. Understanding the dynamics of this complex process requires integrating the disparate knowledge present concerning these pathways. Specifically, a substantial amount of study of epithelial restitution and healing has focused on

two pathways: Transforming growth factor-β1 (TGF-β1) and epidermal growth factor receptor (EGFR). However, despite extensive experimental work on both of these signaling pathways, there is a startling paucity of work concerning the intersection and integration of these two canonical systems. In order to integrate these two areas of study by identifying putative points of cross talk, as well as investigating the consequent dynamics of epithelial wound healing arising from this synthesis, Stern et al. [33] developed an ABM in NetLogo [27] that is an in silico analog of an in vitro scratch assay. A scratch assay involves scratching a confluent epithelial monolayer with a pipette tip or other instrument to create a reproducible linear defect and evaluating the subsequent healing dynamics time-lapse microscopy with or without fluorescent staining [43]. This ABM was termed the in vitro scratch agent-based model (IVSABM) and consisted of agents representing individual epithelial cells (IECs) existing within a simulated extracellular matrix (SECM) to generate an in silico analog of an in vitro cell culture. Such in vitro systems are a mainstay of basic science research, but to date most computational/mathematical models developed on the information generated from these cell culture systems are not able to capture the spatial patterning present, thereby losing a rich source of vital information contained in cell culture experiments. Since ABM is particularly well suited to reproduce exactly this type of output, the visual output of the IVSABM constituted a major advantage in its use as a means of integrating basic science-derived mechanistic knowledge (see screenshots below). Rules governing IEC agent and SECM behaviors were extracted from information present in the literature, and based on this information putative points of cross talk were hypothesized and instantiated in the IVSABM. This would allow the IVSABM's overall system dynamics to be examined against data from traditional experiments and allow the falsification of nonplausible hypothesis structures.

Model Construction and Overall Architecture

The population of IEC agents represents a single confluent epithelial cell layer, with each agent occupying a distinct space (patch) on the grid. Intracellular proteins and cell surface receptors were assigned as agent state variables, and diffusible factors, such as secreted mediators, were assigned as patch variables. IECs interact with their environment via surface receptors and secrete factors that then become patch variables in the SECM. For an overview schematic of the components and interactions in the IVSABM see Fig. 12.1. At baseline all IECs have intact tight junctions with all of their immediate neighboring cells. When a "scratch" is introduced, this results in a linear defect of cells and matrix across the center of the grid. IECs at the wound edge were considered to be "damaged" and able to elaborate damage signals, reactive oxygen species (ROS), damage associated molecular pattern proteins (DAMPs), and extracellular adenosine triphosphate (ATP), into the media as stimulatory variables for IECs. During each simulation run, IECs signal through their surface receptors in response to values of available stimulatory ligand on the patch

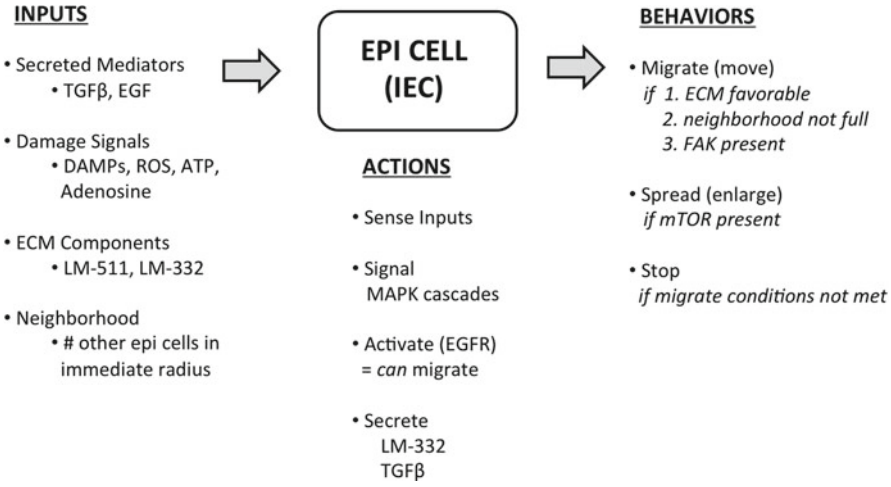

Fig. 12.1 State diagram for IEC agent in the IVSABM. This schematic depicts the inputs, outputs, and actions of an IEC agent. Reprinted with permission from [33]

they occupy. Bound receptors are no longer available to interact with additional ligands, but after signal transduction to intracellular components they are reconstituted to allow reactivation of the pathway if further ligand is available. Binding of a ligand to a receptor also removes the ligand from the patch it is on. Intracellular signaling cascades were modeled without signal amplification as 1:1 interactions, in which downstream molecules are assigned a value equal to their corresponding upstream molecule with each iteration of the simulation. Levels of intracellular molecules decremented at a set rate in order to simulate the actions of phosphatases and other degradative pathways. "Migrating" IECs move onto adjacent open patches in a semirandom manner, which leads to repopulation of the damaged area of the grid. As IECs move away from their neighboring IECs tight junctions are broken; these tight junctions are reformed when migrating IECs come back in contact with other IECs. Migration pauses when migrating IECs reach a distance of greater than 1 patch from any neighbor and resumes once the trailing cells have closed the gap. This mimics the "sheet-like" movement behavior of cell monolayers undergoing restitution as observed in vitro. The IVSABM is considered to be "healed" when IECs from one side of the scratched monolayer become apposed with cells from the opposite side and the stimuli for signaling mechanisms cease.

As noted above, epithelial reapposition has largely been studied, as have most biological processes, on an individual, pathway-by-pathway basis, with particular attention paid to signaling from TGF-β and activation of EGFR. These two canonical pathways are each recognized as critical and necessary components of epithelial cell restitution; however, cross-pathway interactions between the two have been poorly characterized. Following a review of the literature, both of these pathways

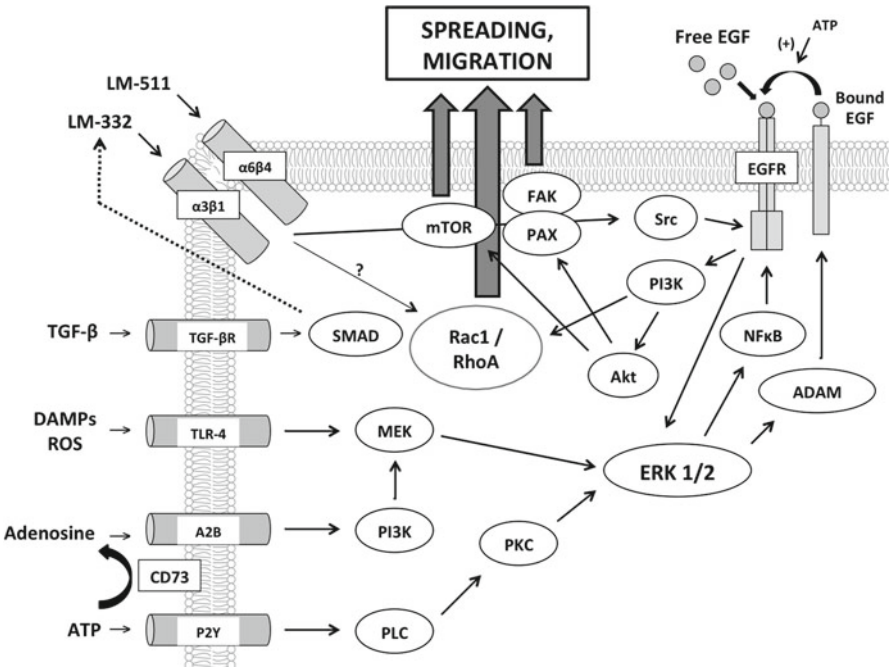

Fig. 12.2 EGFR and TGF-β pathways instantiated in the IVSABM. A depiction of the pathway components and interactions incorporated into the IVSABM. Reprinted with permission from [33]

were instantiated in the IVSABM (see Fig. 12.2 for a depiction of the components of the TGF-β1 and EGFR pathways included in the IVSABM). Based on this knowledge, three hypothetical mechanisms for interaction and control between the two pathways were proposed at the level of integrin–EGFR cross-phosphorylation and activation (see Fig. 12.3 for a depiction of these potential mechanisms):

1. *Mechanism 1*: Laminin-332 (LM-332), an extracellular matrix molecule secreted at the wound edge by IECs, binding to integrin α3β1 leading to intracellular signaling through Src.
2. *Mechanism 2*: Direct signaling from LM-332 binding to an as-yet unidentified receptor to Ras-related C3 botulinum toxin substrate (Rac1) independent of EGFR activity.
3. *Mechanism 3*: Extracellular binding of an EGF-like domain of LM-332 directly to EGFR.

These mechanisms were instantiated into the IVSABM code to examine their respective plausibility (see section "Simulation Experiments" below). Rac1, a small GTPase known to be an end-effector of epithelial migration and restitution [44], was used as a quantitative marker for healing capacity among IEC agents.

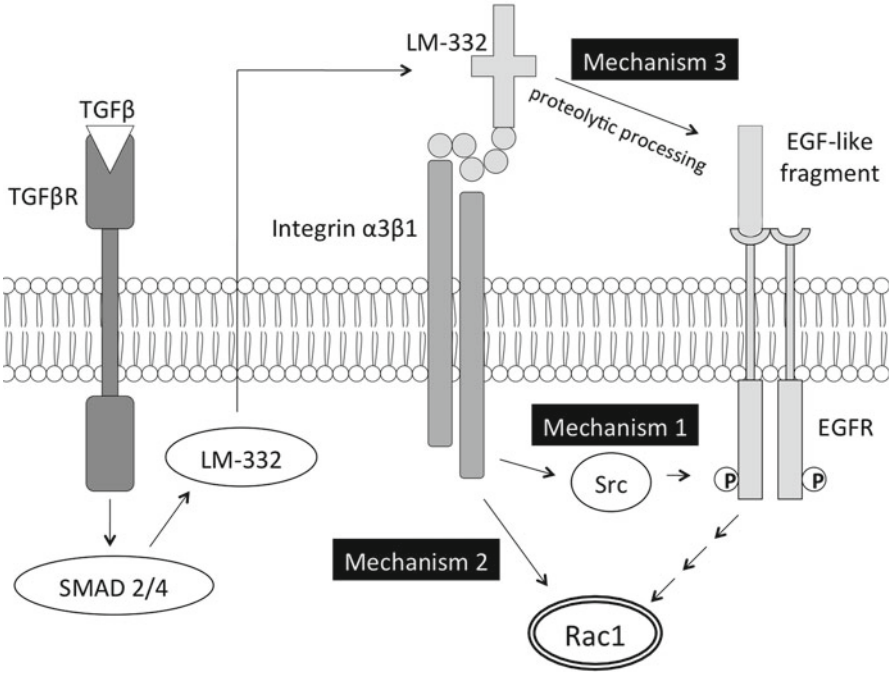

Fig. 12.3 Proposed mechanisms of cross talk between EGFR and TGF-β pathways. *Mechanism 1*: Laminin-332 (LM-332), an extracellular matrix molecule secreted at the wound edge by IECs, binding to integrin α3β1 leading to intracellular signaling through Src. *Mechanism 2*: Direct signaling from LM-332 binding to an as-yet unidentified receptor to Rac1 independent of EGFR activity. *Mechanism 3*: Extracellular binding of an EGF-like domain of LM-332 directly to EGFR. Reprinted with permission from [33]

Model Calibration: System-Level Dynamics

Baseline wound healing was examined in a series of calibration/validation simulations. IECs interacted with the extracellular environment and produced effector molecules leading to a migratory phenotype. DAMPs, ROS, and ATP produced by the initial scratch injury initiated stimulation of IECs, leading to an initial surge of EGFR signaling. Subsequently, EGFR activity was maintained at a relatively constant level by a signaling loop from laminin–integrin interactions. IECs from opposing edges of the monolayer migrate in a sheet-like pattern inwards through the described intracellular, cell–cell and cell–matrix interactions. When the IECs reattain "confluence," migration ceases the wound is considered healed. The quantitative time scale of the IVSABM is such that one iteration of the simulation corresponds to approximately 1 min. The IVSABM was successfully calibrated such that the condition using serum-based media, which takes approximately 24 h (1,440 min) to

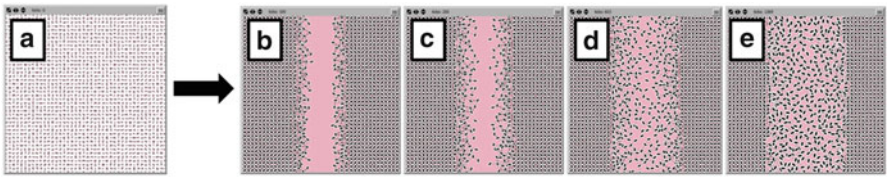

Fig. 12.4 Screenshots of IVSABM demonstrating successful healing of the scratch wound. The intact simulated monolayer prior to induction of the scratch wound is represented in panel **a**. Panel **b** depicts the scratch wound as a linear defect across the center of the IEC monolayer. During the course of the simulation run IECs migrate inwards to close the wound space until the two sides are reapproximated (**c**–**e**), resulting in a healed monolayer. Reprinted with permission from [33]

heal [45], heals in approximately 1,300–1,400 ticks. Figure 12.4 demonstrate successive screenshots of successfully healing simulations.

Additionally, since EGFR activation is absolutely required for epithelial healing in vitro [46], a simulated knockout of EGFR in the IVSABM should similarly lead to a lack of restitution. When a functional EGFR knockout IVSABM was studied, the healing capacity was indeed completely abolished. In the computational code, signals must go through EGFR in order to reach the effector molecules for migration (Rac1) and spreading mammalian target of rapamycin (mTOR). This must in fact be the case in vitro as well; otherwise, there would be some minimal level of healing seen through pathways, which circumvent EGFR. As such, the ABM represents at least a minimally sufficient overall mechanism for wound healing, and a plausible integrative construction of the major signaling pathways involved.

Simulation Experiments

As noted above, a primary benefit of computational dynamic knowledge representation is the ability to falsify clearly implausible or incorrect hypotheses; doing so automatically increases the efficiency of the experimental workflow by directing resources to more fruitful investigations. Therefore, the simulation experiments with the IVSABM involved introducing perturbations known to generate a particular biological outcome and then evaluating the model's consequent behavior to see if it matched what is observed in the real world. Toward this end, simulation experiments were designed to determine if simulated knockouts could meet the necessary/sufficient criteria for EGFR and TGF-β1 in terms of overall system behavior and healing for each of the three putative points of cross talk between TGF-β1 and EGFR: *Mechanism 1*: LM-332 binding to integrin α3β1 leading to intracellular signaling through Src; *Mechanism 2*: Direct signaling from LM-332 binding to an as-yet unidentified receptor to Rac1 independent of EGFR activity; and *Mechanism 3*: Extracellular binding of an EGF-like domain of LM-332 directly to EGFR. As noted above, simulated

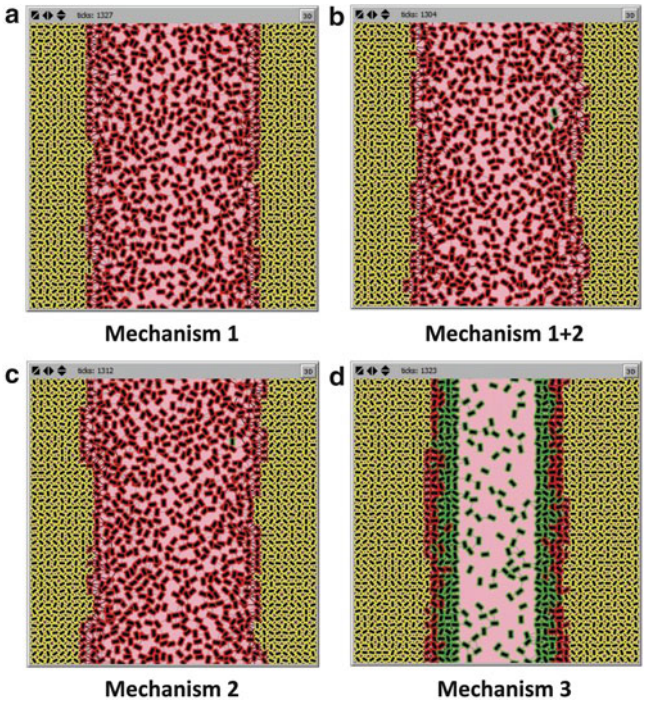

Fig. 12.5 Screenshots of IVSABM simulation experiments evaluating different hypothesized mechanisms for cross talk between EGFR and TGF-β pathways. Due to insufficient healing of the monolayer Mechanism 3 can be rendered implausible and therefore discarded

EGFR knockouts did not heal, therefore simulation experiments were performed with simulated TGF-β1 knockouts, looking at wound closure rates as well as levels of Rac1. The advantages of an ABM's ability to produce a visual/spatial output are immediately evident upon examining the visual output of the IVSABM at the end of the simulation runs. Figure 12.5 demonstrates IVSABM screenshots of simulations run with Mechanism 1, Mechanism 1 + 2, Mechanism 2, and Mechanism 3. Of these, the failure of Mechanism 3 to heal is readily apparent, and therefore that hypothesis can be discarded. More information about the outcomes seen in these simulations can be obtained by plotting the tabular data generated by the IVSABM, specifically levels of Rac1. In these simulations, Mechanism 1 produces accurate wound healing dynamics, suggesting that produced levels of Rac1 are sufficient for healing. Furthermore, this satisfies the condition that EGFR must be absolutely necessary since there is no way for these cells to signal directly from integrins to Rac1. Mechanism 2, where integrin signals lead directly to Rac1, also produces plausible healing dynamics, but occurring with substantially higher levels of Rac1. However, Mechanism 2 does not satisfy the necessity of EGFR, which suggests that while integrin signaling leads to promotility effectors, there must be an intermediate step

involving EGFR. Therefore Mechanism 2 is deemed implausible by not fitting with known observations and can be discarded. Mechanism 3, where a fragment of LM-332 binds directly to EGFR, does not lead to complete wound healing in the ABM as is seen in Fig. 12.5. Analysis of the visual output in conjunction with the generated Rac1 levels provides more information about why Mechanism 3 fails: this mechanism satisfies the EGFR requirement, but at levels of LM-332 that are normally sufficient for healing there is insufficient activation of EGFR downstream pathways. Given these findings, Mechanism 1 seems to be the most consistent with current knowledge and can therefore serve as a guide for additional experimentation to further refine mechanistic knowledge of restitution and suggest potential failure points associated with disease phenotypes.

Agent-Based Modeling as a Clinical-Translational Aid: An ABM of Pressure Ulcer Formation in Spinal Cord Injury Patients

Pressure ulcers are a common complication of hospitalization and are especially common in spinal cord injury (SCI) patients [47]. In acute care in the USA, pressure ulcers affect 2.5 million patients per year, costing up to $1.5 billion. 60,000 of these patients die each year from complications due to pressure ulcers. There are several accepted theories about how ulcers form, but the current standard of care is labor intensive and patients still develop ulcers daily. Serial, noninvasive wound imaging combined with ABMs could, in theory, allow for the investigation of both space- and time-dependent dynamics via visually realistic, mechanistic simulations.

Accordingly, an ABM was created in which pressure ulcer formation was simulated as arising from alternating pressure, as a patient might experience when being turned into and out of a position where pressure over a bony prominence reduced local perfusion. In the model, repeated cycles of ischemia followed by reperfusion cause tissue damage, inducing inflammation, which leads to additional damage caused by proinflammatory positive-feedback mechanisms in a cyclical fashion (unpublished observations). The ABM was calibrated against serial images of post-SCI pressure ulcers obtained from patients following Institutional Review Board approval and informed consent. Cell-level behaviors encoded in the ABM led to tissue-level phenotypes described in the literature [48, 49]. The model also recapitulated visual patterns of ulcer formation in SCI patients, while it was only calibrated on mechanistic, not visual data.

Model Architecture

This ABM was built using knowledge from the literature as an extension of an existing ABM of pressure ulcer formation [50]. The agents that comprise this model are cells and tissues (neutrophils, macrophages, epithelial cells, and blood vessels), with each cell type represented by its own class of agent. Data layers are employed to

simulate diffusible cytokines, free radicals, oxygen, oxidase enzymes, and sometimes drugs. The model architecture consists of a layer of tissue cells, fed by blood vessels throughout the tissue, carrying oxygen and inflammatory cells to the field. The basic model depicts a tissue region composed initially of healthy cells. In the absence of perturbation, the tissue remains intact for a reasonably long time (decays on a timescale much slower than the processes we are simulating). Pressure is simulated by a constriction of the blood vessels, decreasing the amount of material that can flow through them. Without oxygen, tissue cells are compromised and their health begins to decline. This stress leads them to release diffusible "danger signals" [damage-associated molecular patterns (DAMPs)], mediators that stimulate the inflammatory response [51].

I/R Mechanism: Implementation and Validation

In addition to ischemic injury (lack of oxygen reduces the health of epithelial cells), the model incorporates an additional method of tissue injury on pressure release: reperfusion injury. Ischemic cells build up the capacity to produce damaging free radicals upon reintroduction of oxygen. This is modeled by the accumulation of oxidase enzymes inside ischemic cells. When pressure is released and oxygen again perfuses these cells, oxygen free radicals will be formed in proportion with the concentration of oxidase present in that cell. Free radicals cause damage to the immediate cell and those they encounter via diffusion, and they do so in a stepwise manner: epithelial cells show no sign of damage from radicals until they have accumulated a certain threshold of insults. At that time, their health is drastically reduced. Varying the length of pressure cycles (turning rate) and measuring total tissue damage after a fixed period of ischemia revealed that all else being equal, a period of ischemia will cause less tissue damage than the same period of ischemia followed by a reperfusion event. These results agree with in vivo studies carried out in rats, wherein ischemia reperfusion was simulated using a compression via a magnet and a steel plate surgically implanted under the epidermis [48].

Inflammation Mechanism: Implementation and Validation

Three diffusible mediators represent the canonical early proinflammatory response, the canonical slower proinflammatory response, and the canonical anti-inflammatory response, each of which is secreted by activated neutrophils or macrophages (type I or II). This version of the model contains both neutrophils and macrophages, which are initially in a resting state. They are activated by local concentrations of mediators in a threshold-dependent manner. DAMPs, another data layer, above a certain local concentration will activate nearby neutrophils to produce early proinflammatory mediators (called TNF-α hereafter). At a certain threshold of local TNF-α

concentration, resting macrophages will be activated to a type I phenotype and begin secreting longer-acting proinflammatory mediators (called IL-1β hereafter). TNF-α also causes damage to nearby epithelial cells, thus restimulating the proinflammatory response. Local concentration of IL-1β above a threshold activates macrophages to type I (proinflammatory) phenotype and above a higher threshold, IL-1β induces macrophages to adopt a type II (anti-inflammatory) phenotype. Active type II macrophages produce anti-inflammatory mediators (called TGF-β1), which above a threshold cause further activation of type II macrophages.

The rules governing the inflammatory mechanisms in the model are based on dynamics of acute inflammation, so these dynamics were tested in a simulated acute wound, without repeated pressure cycles. In a successful incorporation of these mechanisms, tracking activation of neutrophils and macrophages would reveal cellular dynamics similar to those found in settings of acute inflammation. Since the mediators in the simulation represent amalgams of several mediators, it makes more sense to validate dynamics at the cellular level. An initial injury was created in the center of the tissue, pressure removed, and the dynamics of inflammatory cells were tracked in the field. Because the model is calibrated to real clock time, these results are directly comparable to measurements from published sources, and qualitative agreement was sufficient. The relative timing of peaks of cell populations was as expected. Interestingly, a single set of initial conditions and parameter values was able to give rise to two distinct outcomes. In all simulations, the initial injury was sufficient to incite the inflammatory response. In approximately 70 % of cases, this inflammatory response became self-sustaining and led to an ulcer. In the remaining 30 % of simulations, however, the inflammatory response resolved early enough that the tissue suffered minimal damage beyond the initial injury. This variation mimics variation in a population of patients who may present with the same intensity of disease receive the same treatment but experience very different disease progressions.

Sensitivity Analysis and In Silico Trials

Sensitivity analysis was used to explore the range of behaviors possible from the model. This group of methods gives the modeler a measure of which parameters account for the greatest amount of the variance in the model's output. For this model, parameters were partitioned according to which mechanistic cause of damage they could be attributed (ischemia/reperfusion (I/R) injury or proinflammatory ancillary damage). These experiments revealed that either increasing oxygen availability or the rate at which pressure is applied and released (simulating a patient being turned) led to predictions of improved outcomes, but that changing inflammatory parameters only led to modest improvement (unpublished observations).

The ABM was then used as a platform to investigate potential treatments in silico. To examine the effects of suppressing acute inflammation in manner more severe than tweaking parameters, a trial of corticosteroid application was simulated in silico. While this might be a controversial treatment plan in the clinic because

corticosteroids are broadly considered to put one at risk for chronic, nonhealing wounds [52], simulating this treatment strategy virtually allowed us to assess potential benefits without any negative consequences. In this in silico clinical trial of steroid treatment, steroids applied at early enough time points and at a high enough dose were effective in stemming the local inflammatory response, leading to predictions of improved outcomes in the early stages of pressure ulcer formation and progression. However, as suggested by the sensitivity analysis mentioned above, suppressing inflammatory damage was not sufficient to prevent an ulcer from forming. The I/R injury was eventually enough to cause an ulcer in the tissue.

In this ABM, DAMP molecules were key signals that led to inflammation following tissue injury. The DAMP high-mobility group protein-B1 (HMGB1) has emerged as a therapeutic target for inflammatory diseases [53–55]. Accordingly, an in silico trial of a putative, neutralizing anti-HMGB1 antibody therapy was implemented. In these simulations, this strategy was not successful in stemming the inflammatory response, whether incited by repeated ischemia/reperfusion injury or an initial acute tissue injury. Mechanisms encoded in the ABM allow activation of the inflammatory response via cell damage, without an explicit diffusible signal (Ziraldo et al. submitted), which we hypothesize may account for the apparent noneffect of the anti-HMGB1 antibody treatment. Together, these in silico trials and sensitivity analyses led to the conclusion that while inflammation is definitely an aggravating factor in pressure ulcer formation, ischemia is the most prominent cause of tissue injury.

Conclusions

In silico mechanistic models provide unique opportunities to study inflammation and wound healing dynamics, which is of obvious clinical importance. Recent ABMs have allowed not only mechanistic insight but also provide an inexpensive and accessible platform for hypothesis testing, simulating clinical trials, designing patient-specific therapies, and developing diagnostic tools. By providing a means of synthesizing disparate aspects of biomedical knowledge, mechanistic computational modeling provides a plethora of opportunities to continue exploring, make new insights, and ultimately help patients [5, 23, 33, 41, 56–59].

These systems have been extensively studied and are indeed well characterized, but in proceeding beyond the understanding of simple mechanistic detail to examination of whole system dynamics, computational models appear to have a useful role. The models presented here represent different levels of resolution of knowledge: molecular detail leading to cellular level dynamics in the epithelial restitution model, and cellular detail leading to tissue level dynamics in the pressure ulcer model. Furthermore, the models represented an even wider range of resolution of outputs. The decision of what resolution to model each system is determined by the available literature and how it relates to the modeling goals of the individual modeler. For example, epithelial restitution has primarily been studied in the context of

the relative importance and contributions of various signaling pathways to epithelial behavior. Hence, the goal of this model was to integrate known data at this level of resolution and provide information regarding amalgamated cellular behavior—exactly what the primary literature has been unable to achieve via traditional studies. Similarly, with regard to the pressure ulcer model, the impact of the early inflammatory response on ulcer progression was the goal of the modeler and this was achieved by integrating cellular and tissue-level detail. Both of these represent significantly important contributions, in that they fit into "knowledge gaps" in their respective knowledge bases.

Incorporating physically and physiologically relevant information into ABMs, such as blood flow in tissues and forces between cells, allowed for new mechanistic predictions. In the case of the former, a simple approximation of tissue blood flow differences between noninjured subjects and SCI patients, when juxtaposed on a stochastic model of inflammation and tissue injury, led to the prediction of higher propensity to ulcerate in SCI patients vs. controls. Even visual information can be deceiving, as illustrated by agent-based simulations of restenosis. Static cross sections of arterial restenosis suggested that neointima entered through a break in the external elastic lamina of a coronary artery, and therefore the degree of hyperplasia was thought to be proportional to the size of the rupture [60, 61]. However, time courses yielded by dynamic simulations revealed an alternative possibility. Infiltrating cells appeared to be actually pushing on the edges of the ruptured external elastic lamina, forcing it wider. This insight might impact the analysis of histological samples of balloon-injured arteries and may also affect the design of novel therapies aimed at mitigating these physical forces.

Incorporating tissue realism into ABMs of pressure ulcer formation has raised the possibility of noninvasive diagnostics and possibly therapy based on serial macroscopic images of nonhealing wounds. Due to the individualized and context-dependent nature of the inflammatory response, a tool that could help clinicians decide the best course of treatment based only on visual information could be revolutionary. Such an in silico diagnostic could improve patient care. This model has also been used to simulate a variety of treatment strategies, paving the way for fast, inexpensive early stage trials of newly designed treatments.

There are, of course, limitations to this approach. Simulations of biological processes are necessarily approximations. In order to balance computational cost with a model's utility, models must be parsimonious, including the smallest number of elements that will still yield a model that is useful for gaining mechanistic insights, clinical applications, or both. Often, modeling choices can be driven by the availability of data rather than which elements might be most critical to or informative of a process. These choices are subjective, and one can always find a justification to include additional elements in a given model, as long as those elements increase the validity or utility of the model. An implication of this simplified version of reality is that a model can only be useful to a certain level of resolution. It is important to keep in mind the scope a model can reach when probing it for insights.

Each of the models here represents a framework upon which further work can be added. The modular nature of ABMs allows more detail to be added as

more information is gathered—for example, when a new pathway is elucidated and published in the scientific literature this can be easily incorporated into an established model. If the model has been well validated, then changes seen in the model output with new information blocks can be enlightening with regard to both the plausibility of the new discoveries as well as the accuracy of the model itself. In fact, model outputs that are incongruent with expectations are often the most useful, as they provide the equivalent of scientific falsification and allow more accurate model calibration going forward.

The next frontier for tissue-realistic mechanistic computational modeling is really to form bridges between visual simulation outputs and clinical images. Image analysis methods could allow comparisons of simulations of pressure ulcers to photos of patients' wounds, extracting pertinent details that cause some simulations/subjects to resolve their wounds, while others progress to deranged inflammation and inappropriate healing. At the molecular level, models that can use concentrations of mediators as inputs to or outputs from equation-based models will have an advantage. Eventually, the goal is to link together local dynamics with the larger context of a patient's body and thus truly allow for rational, patient-specific diagnosis and therapy.

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Part IV
Translational Modeling of Host-Pathogen Interactions

Chapter 13
Modeling Host–Pathogen Interactions in Necrotizing Enterocolitis

Julia Arciero, Jared Barber, and Moses Kim

Introduction

Necrotizing enterocolitis (NEC) is a severe disease of the gastrointestinal (GI) tract that is characterized by increased permeability of the intestine and necrosis of the bowel wall. Although 5–25% of the cases seen occur in full-term term infants [1], the majority of NEC cases occur in very low birth weight (<1,500 g) premature infants; in fact, NEC affects up to 10% of low birth weight infants and is the leading cause of morbidity and mortality in the neonatal intensive care unit [2]. The onset of NEC is unpredictable but typically occurs within 7–14 days of birth [3]. NEC is categorized into three stages based on severity, and the symptoms of NEC include gastrointestinal dysfunction, abdominal distension, feeding intolerance, and cardiovascular compromise.

Pathogenesis Although its pathophysiology is not entirely understood, NEC is thought to be related to the interaction of a number of complex factors, including the physiological immaturity of the GI tract, abnormal bacterial colonization of the gut, and disordered inflammatory signaling. A mature intestine utilizes many defense mechanisms, including intestinal mucus, gastric acid, peristalsis, cell surface glycoconjugates, intestinal macrophages, and antimicrobial peptides, to prevent

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the translocation of bacteria across the intestinal barrier [2–6]. In preterm infants, the immaturity of these mechanisms (e.g., immaturity of GI motility, digestive ability, barrier function, immune defense, and circulatory regulation) can lead to increased bacterial translocation across the intestinal barrier. As a result, bacteria that are normally confined to the intestinal lumen are able to reach systemic organs and tissues, possibly leading to bacterial sepsis.

An abnormal pattern of bacterial colonization in preterm infants may also contribute to the pathogenesis of NEC. NEC does not occur in utero when the gut is sterile, implying that bacteria are involved in its pathogenesis [2]. Immediately following birth, the intestinal lumen is colonized with a diverse population of both harmful and helpful bacteria. Commensal bacteria (or normal flora) are helpful bacterial species, such as *Lactobacillus* or *Bifidobacterium*, that play a very beneficial role in the host by maturing the GI tract [7], enhancing digestion efficiency, decreasing the permeability of the intestinal wall [8, 9], and limiting pathogenic bacterial colonization by competing with pathogenic bacteria for binding sites and nutrients. Since preterm infants are susceptible to an abnormal composition of gut microflora, they do not experience as many of the benefits conferred by commensal bacteria and often lack the necessary mechanisms to prevent uncontrolled inflammatory responses or to respond appropriately to normal bacterial colonization [2].

Disordered inflammatory signaling is another factor thought to contribute to NEC and is often associated with disordered Toll-like receptor 4 (TLR4), Toll-like receptor 9 (TLR9), platelet activating factor (PAF), or nitric oxide (NO) signaling. Activation of TLR4 by lipopolysaccharide (LPS) can result in a widespread proinflammatory response [10]. The signaling cascade that follows TLR4 activation triggers the secretion of antimicrobial factors and IgAs [11, 12] as well as the production and release of various proinflammatory cytokines [13]. Sustained TLR4 activation leading to sustained inflammation can cause increased damage and bacterial translocation, contributing to the severity of NEC. TLR4 activation also causes increased apoptosis of intestinal epithelial cells and reduced intestinal healing.

It is important to note that all infants express TLR4 but most do not develop NEC. Thus, the means by which TLR4 responsiveness is maintained at an appropriate level to maintain homeostasis and protect the host is an important topic for investigation. TLR9 is a Toll-like receptor that recognizes DNA sequences that contain several CpG motifs, which are sequences that are characteristic of the bacterial genome [14]. Although TLR9 is known to be proinflammatory in many contexts, studies have shown that TLR9 activation with CpG DNA can limit TLR4 signaling in enterocytes and reduce intestinal inflammation in NEC [15, 16].

PAF is a potent phospholipid mediator that is secreted by multiple cell types. PAF release is triggered by several stimuli including hypoxia, infection, or local injury. PAF is hypothesized to contribute to the development of NEC since it has been shown to upregulate TLR4 on the intestinal epithelium [17], which in turn augments intestinal inflammation. PAF concentrations have been observed to be significantly higher in NEC patients, likely due to increased PAF production once LPS is introduced into the system and/or suppressed PAF catabolism [18].

Nitric oxide (NO) is a free radical that reacts with multiple substances, resulting in local and systemic effects that alter tissue inflammation. One of the most relevant reactions of NO is with superoxide to produce peroxynitrite [19]. Peroxynitrite is generated at inflammatory sites and is responsible for mediating tissue injury. Several studies have confirmed that NO causes epithelial injury by enhancing enterocyte apoptosis and disrupting the ability of the epithelial tissue to repair itself by inhibiting enterocyte migration [19]. In addition, NO has been shown to destroy tight junction proteins between enterocytes, thereby allowing bacteria to more readily penetrate the epithelial layer [20, 21].

In addition to the effects of TLR4, TLR9, PAF, and NO, Nanthakumar et al. [22] provided evidence that the excessive inflammatory response of the immature intestine may be due to inappropriate expression of innate immune response genes in immature tissue. For example, the study identifies developmental changes that coordinate the downregulation of TLR4 expression and their signaling molecules and upregulation of negative regulators that are likely important in establishing postnatal intestinal colonization [22]. It is therefore hypothesized that if an infant is born before those developmental changes have taken place, they may respond inappropriately to bacterial colonization by triggering excessive inflammation.

Together, immaturity of the GI tract, abnormal intestinal bacterial colonization, and altered inflammatory signaling at least partly account for the increased risk for preterm babies to develop NEC. Inflammation is an essential process that ideally results in complete healing and reconstruction of the injured tissue; however, inflammation can also cause substantial damage to surrounding tissue [23]. Under normal circumstances, the amount of damage caused to surrounding tissue during the inflammatory process is moderate and poses minimal threat to the individual. A problem arises, however, if the inflammation becomes excessive or persistent, as in NEC. The immune system is then faced with a complex challenge of responding sufficiently to the imposed threat without producing too much collateral damage [24]. We hypothesize that an imbalance in inflammation and immune mechanisms may occur in individuals with immature immune systems and may contribute to the progression of diseases such as NEC.

Intervention The American Academy of Pediatrics has acknowledged the benefits of human milk for all infants and particularly those born prematurely [25]. Beneficial effects of breast milk include improved host immune defenses, digestion, nutrient absorption, gastrointestinal function, and neurodevelopment [26]. These effects likely result from the combination of beneficial substances found in breast milk including anti-inflammatory cytokines that help suppress overactive immune responses, disease-specific antibodies, certain probiotic or beneficial bacteria, antimicrobial peptides, and immune cells from the mother. Heat shock protein 70 (Hsp 70), which has been shown to protect the intestinal epithelium in adults, was found in mother's milk [27]. Hsp 70 helps to maintain the intestinal barrier function by stabilizing the tight junctions between epithelial cells. It is not too surprising from this evidence, then, that NEC was 20 times more common in infants fed formula than in those whose diet included breast milk [28].

Currently, the majority of infants that present symptoms of NEC are managed with fluid resuscitation, total parenteral nutrition, bowel rest, and intravenous antibiotics [29]. There is some debate if the use of antibiotics is beneficial or harmful in the case of NEC. Some studies have related treatment with antibiotics with an increased incidence of NEC since antibiotics result in decreased diversity of intestinal flora and a predominance of less desirable bacteria [30].

Severe cases of NEC result in surgical intervention, which is often a challenging and insufficient option due to the fragility of the patients and rapid progression of the disease. Mortality from NEC is nearly 30–50% for infants with surgical intervention [31]. Moreover, infants who recover from such surgeries may experience complications and other disorders later in life, including short bowel syndrome, intestinal strictures, and neurodevelopmental delays due to poor nutritional status [2, 4, 29, 32–35].

Although significant experimental and clinical progress has been made to understand and identify factors that contribute to NEC [15, 19, 36–41], there is still a great unmet need to develop effective and noninvasive treatment strategies that can bolster the integrity of the epithelial wall, prevent excessive inflammation, and limit the colonization of pathogenic bacteria in the intestinal lumen. Probiotics, which are nonpathogenic bacteria species that are beneficial to the host, have been proposed as a possible treatment for NEC [41, 42]. The species of probiotics that are typically used are *Bifidobacterium* [43] and *Lactobacillus* [44], since these are the species of bacteria that constitute the normal flora of term infants and that occur naturally in breast milk. In theory, the administered probiotic bacteria will compete with the pathogenic bacteria while also stimulating host defense mechanisms and enhancing intestinal maturation [8, 9, 45, 46]. Recent findings suggest that the protective effects of probiotics are in part due to their ability to activate TLR9, which is known to inhibit TLR4 [16, 47].

Contradictory results have been obtained in studies implementing probiotic treatment for NEC. In many cases, neonates treated with probiotics have shown a reduced incidence and severity of NEC [1, 38, 40, 42, 48–50]. Hoyos et al. [49] noted an almost threefold reduction in the incidence of NEC after the administration of probiotics *Lactobacillus acidophilus* and *Bifidobacterium infantis*. However, other studies have shown sepsis resulting in infants that were treated with probiotics [39, 51]. Understanding when the administration of probiotics can be expected to produce positive outcomes is important if probiotics are to be used consistently as an effective treatment. After this is understood, the optimal treatment strategy, in terms of timing and dosing, can be determined.

A Systems Biology Approach The dynamics of NEC cross multiple scales from gene expression and inflammatory signaling cascades to organ failure. In addition, numerous factors have been implicated in the pathogenesis of NEC. The complexity of NEC coupled with its severity and lack of effective therapy has motivated the use of systems biology approaches, such as computational modeling, to gain fuller insight into the important mechanisms that affect NEC dynamics and possible effective therapies for the disease.

Mathematical modeling, an important tool for systems biologists, can be used to isolate the effects of particular mechanisms and treatments by including or excluding certain terms from model equations. In this way, nuances may be uncovered from theoretical studies that may not otherwise be observed or predicted using experimental or clinical studies. A symbiotic relationship between mathematical models and experiments should exist in which experiments help to inspire, refine, and correct mathematical models while insight gained from mathematical models helps to create, design, and direct experiments. Such a relationship maximizes progress towards the goal of effectively treating diseases.

For NEC, mathematical models have included both equation-based and agent-based models [52–56]. They have been used to understand the elements that contribute to NEC and to predict the effects of probiotics, TLR4, TLR9, NO, and breast milk on the development or progression of the disease. Since the association between the inflammatory response and NEC is strong, many of these models are rooted in models previously developed to study aspects of the inflammatory response [57–60] and adapted accordingly for the mechanisms of NEC and the structure of the intestine.

Computational Modeling Approaches

Three types of models have been used to investigate the dynamics that take place during NEC. The simplest approach uses a system of ordinary differential equations (ODEs) to simulate bacteria and immune system interactions in the intestine in the context of NEC. Partial differential equation (PDE) and agent-based models (ABMs) are two complex modeling approaches that have also been used to study NEC. ODE models are particularly useful for obtaining basic insight into NEC dynamics since the simulations are relatively straightforward and numerically inexpensive. PDE and agent-based models are used to answer many questions that cannot be addressed by ODE models such as the effects of spatial variability and dynamics. In this section, the approaches of each model are discussed and the strengths and shortcomings of each type of model are compared. Often a combination of the three approaches is most beneficial for obtaining the optimal amount of insight into NEC.

Ordinary Differential Equation Modeling

ODEs are used to study changes in quantities that depend on one variable [61]. In order to develop a mathematical model of biological phenomena, the important factors involved in the process must be identified as well as the ways in which these factors can be increased or decreased. Then, ODEs for each factor can be defined and the system of ODEs can be solved in order to predict the behavior of the system.

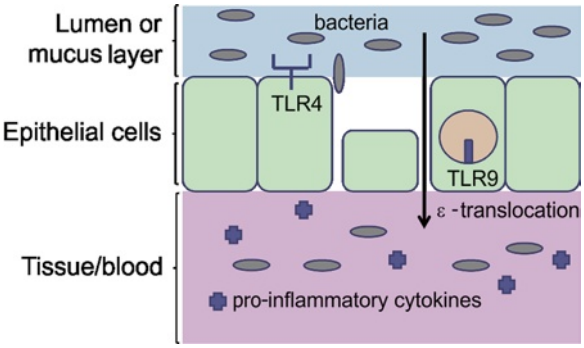

Fig. 13.1 Schematic of compartments of the small intestine that are used for the ODE models of NEC (not to scale). The compartments are divided into the lumen or mucus layer (*blue*), epithelial cell lining (*green*), and intestinal tissue with blood supply (*purple*). Bacteria (*gray ovals*), enterocyte receptors (TLR4 and TLR9), the rate of bacterial translocation across the epithelial barrier (ε), and immune system components (*plusses* denoting proinflammatory cytokines) are depicted within the various compartments

ODE models typically describe the time dependence of a particular phenomenon but not the spatial variability. Thus, the ODE modeling approach is appropriate when it is reasonable to assume that information about a system does not vary widely at different points in space. In that case, the domain is assumed to be well mixed, and the solutions describe the change in the average value of the variable in the region with respect to time. A spatial element can be included in an ODE model by using a compartmental ODE modeling approach in which the average values of the variables are tracked in multiple regions or compartments.

ODE Models of NEC ODE models that analyze some of the key governing mechanisms of NEC have been developed previously [52, 55]. Experimental and clinical evidence form the basis for these models, and the overall goals of the studies are to determine the protective potential of probiotics in infants with NEC and to identify the impact of TLR4 signaling on intestinal health. In both models, the intestinal system is divided into three compartments: the intestinal lumen (or mucus layer), the epithelial lining of the intestine, and the combined intestinal tissue and blood supply. In Fig. 13.1, the compartments are depicted as well as the main players or variables that are tracked in each compartment: bacteria, epithelial permeability, cytokines, and Toll-like receptors.

Bacteria The intestine is colonized immediately after birth by multiple bacteria types, including both pathogenic and commensal bacteria. The total bacteria population in the intestinal lumen can be grouped as a single population, or the bacteria can be divided into beneficial (commensal or probiotic) and harmful (pathogenic) bacteria, depending on the overall goal of the model. In most cases, bacteria are assumed to grow logistically, which indicates there is a maximum carrying capacity within the intestinal lumen. Competition between bacterial species for space and nutrient should be modeled explicitly if multiple bacterial species are tracked in the lumen.

The details of bacterial transport in and out of the lumen depend on the ability of bacteria to breach the barrier and the number of agents released into the lumen to eliminate foreign bacteria. In [55], a mucus layer compartment is included to model the layer of mucus that lines the apical side of the epithelium and that prevents direct contact of luminal components with the epithelial lining [62]. The mucus layer serves as one of the first barriers that bacteria encounter when trying to reach and enter the intestinal tissue. The composition of the mucus inhibits effective or rapid movement of bacteria [63], and multiple antimicrobial proteins are released into the mucus layer to target and eliminate bacteria [64, 65]. Bacteria thus exit the lumen and enter the mucus layer where they may remain, be eliminated by antimicrobial proteins, or breach the epithelial barrier and enter the intestinal tissue. It is not until the bacteria enter the tissue compartment that they invoke a significant immune response. Although a mucus layer was not specifically included in [52], similar growth dynamics and exit dynamics (via translocation across the epithelium) were assumed for bacteria in the lumen compartment.

Epithelial Permeability The intestine is lined with a single layer of epithelial cells that serve as a barrier between the outside world (lumen) and the intestinal tissue. A primary function of this epithelial layer is to regulate the passage of materials across this barrier, allowing for absorption of necessary materials while preventing the incorporation of harmful materials such as pathogenic bacteria.

Since preterm infants with NEC tend to have an injured epithelial cell lining due to immaturity or exaggerated inflammation that damages the epithelium, tracking the rate of translocation across this barrier is an important element in quantifying NEC. If the epithelium is not intact, bacteria are able to breach the barrier more easily [66], which can trigger an inflammatory response that can cause additional injury to the barrier. An increased rate of bacterial translocation is thus thought to contribute to the development of NEC.

It is important to note that since a large number of commensal bacteria are necessary for the maintenance of homeostasis, the host can identify and allow commensal bacteria to remain in the intestinal lumen or mucus layer [67]. A significantly different number or type of luminal bacteria from normal indicates the presence of a threat to the system, often resulting in bacterial translocation across the epithelial barrier into the intestinal tissue and bloodstream [68]. To interpret this phenomenon mathematically, Arciero et al. [52] introduced a threshold value, T, which corresponds to the resistance provided by the intestinal epithelium to bacterial translocation. If the product of bacteria and the rate of translocation is greater than the intestinal wall threshold, then bacteria are assumed to cross the barrier; otherwise, no bacteria are able to pass through the barrier. The following function is used to capture this idea: $[\varepsilon(B_L + kB_{PB,L}) - T]_+ = \max\left\{ \varepsilon(B_L + kB_{PB,L}) - T,\ 0 \right\}$. In this function, ε is the rate of bacterial translocation, B_L denotes pathogenic bacteria in the lumen, and $B_{PB,L}$ denotes the probiotic bacteria in the lumen. In models that distinguish between pathogenic and probiotic bacteria, as in [52], it is reasonable to hypothesize that probiotic bacteria are not as effective at breaching the barrier as pathogenic bacteria. To incorporate this hypothesis, a parameter k that varies between 0 and 1 is used to scale the contribution of probiotic bacteria to exceeding the threshold.

Probiotic bacteria have also been shown to enhance the viability of the intestinal barrier [3, 8, 9, 69]. Thus, beneficial effects of probiotics in NEC can be included in the model as a term that reduces the rate of bacterial translocation.

Immune System Components If the mucus layer is not successful at preventing bacterial penetration of the epithelium [70], bacteria may enter epithelial cells or pass through the gaps between them. In many cases, intestinal macrophages, which are immune cells that phagocytose and kill microorganisms but do not release proinflammatory cytokines like macrophages in other tissues [71–73], eliminate the bacterial invaders before the bacteria are able to invoke an overly robust inflammatory response.

However, if the mucus layer, intestinal macrophages, and dendritic cells (another type of immune cell that extends protrusions into the mucus layer to sample for bacteria and release antimicrobial proteins to eliminate the bacteria [74]) are all unable to prevent bacteria from entering the blood and tissue, an inflammatory response ensues, which includes the activation of blood macrophages and the systemic secretion of proinflammatory cytokines. Cytokines generate inflammation to defeat the invaders. Although the inflammatory response is necessary to destroy harmful bacteria, it also causes injury to the intestinal barrier and inhibits epithelial cell proliferation and migration, thereby propagating the epithelial injury to a large extent. As expected, blood cytokine levels are elevated in infants suffering from NEC since bacteria are able to breach the epithelial barrier and evoke an overwhelming inflammatory response. Studying the pattern of cytokine expression may yield important insights into the pathophysiology of NEC [21, 28].

The models in [52, 55] do not include explicit terms for the anti-inflammatory response. Instead, the idea of negative feedback in those models is captured via parameter values and inhibitory Toll-like receptor signaling. Nevertheless, the immune system also produces anti-inflammatory cytokines, which work to downregulate the initial proinflammatory response, and the contributions of this phenomenon should be included in subsequent studies of NEC.

Toll-Like Receptors The activation of TLR4 on enterocytes facilitates bacterial translocation across the intestinal barrier [75] and triggers a robust inflammatory response. TLR4 activation also increases the adhesion of enterocytes to the underlying matrix, which in turn restricts normal cell migration and prevents the efficient migration of epithelial cells into damaged regions of the epithelium. It has been observed that TLR4 expression is significantly elevated in experimentally induced NEC relative to control conditions [15]. When TLR4 is blocked, bacterial translocation is reduced significantly [36]. These observations provide evidence that TLR4 may be a key player in the disease and that its promotion of inflammation and inhibition of cell migration should be included in a theoretical model of NEC. Methods that limit TLR4 activation may have important application in the treatment of NEC.

Studies have shown that TLR9 activation can limit TLR4 signaling in enterocytes and reduce intestinal inflammation in NEC [15, 16], unlike the role of TLR9 activation in other contexts. This suggests that promoting TLR9 activation may be a strategy for treating infants suffering from NEC. It is hypothesized that probiotics

are a successful treatment for NEC because probiotic DNA can activate TLR9 which in turn inhibits the activation of TLR4 [76]. The dynamics of TLR4 and TLR9 can be predicted by the model by including the mechanisms of interaction between bacteria, the immune response, and these receptors.

Main Findings: The Role of Probiotics The model in [52] provides a preliminary tool for exploring the effects of probiotic treatment in NEC. The action of probiotics is represented by three key components in the model: competition with pathogenic bacteria in the lumen, maturation and restoration of the epithelial lining (i.e., reduction of the rate of bacterial translocation), and inhibition of the inflammatory response. The model is used to assess the impact of each of these factors by predicting the overall behavior of the system as the relative strengths of these three mechanisms are varied. To simulate infections of variable severity, the initial amount of pathogenic bacteria in the lumen or the growth rate of the pathogenic bacteria in the lumen is increased. A healthy outcome is predicted if bacteria are absent from the blood and the rate of bacterial translocation across the epithelium is at a baseline (normal) value. Aseptic death is predicted when the rate of translocation is elevated even though no bacteria enters the blood, indicative of a highly inflamed system. In septic death, both the translocation rate of the epithelium is elevated and bacteria are present in the blood/tissue compartment.

Bistability is predicted to occur between health and septic death states over a range of pathogenic growth rates wherein bacteria can reach one of two steady states depending on the initial number of bacteria in the system. This possibility for bistability results from the threshold that governs passage across the epithelial barrier. The barrier prevents activation of the inflammatory response when the number of luminal bacteria is below a threshold [21]; a transient increase in the number of pathogenic bacteria in the lumen, however, can lead to bacterial translocation and increased inflammation, resulting in a disease state. Interestingly, since the presence of probiotics in the lumen contributes to the total amount of bacteria in the lumen, probiotics may contribute to these transient elevations in the total luminal amount of bacteria and thereby have a paradoxically negative impact by lowering the level of pathogenic bacteria needed to induce disease.

Clinical studies have shown contradictory results when probiotics were administered to preterm babies [38–40, 49, 51, 77, 78] as treatment for NEC. In a meta-analysis of 20 randomized, controlled trials, preterm infants treated with a probiotic supplement had a significantly decreased risk of NEC and death [78]. Yet, in other cases, there was no reduction in the risk of NEC with probiotic treatment [77]. The model predictions also show contradictory results. For example, in most instances, the model predicts that the introduction of probiotics improves health (i.e., the inclusion of probiotics increases the regions in parameter space where health outcomes are predicted); however, there exist some cases in which health would have been predicted in the absence of probiotics, but disease is predicted in the presence of probiotics. An example of such a case is highlighted in Fig. 13.2. The figure depicts health and disease predictions in the presence or absence of probiotics as the pathogen growth rate and initial level of pathogen in

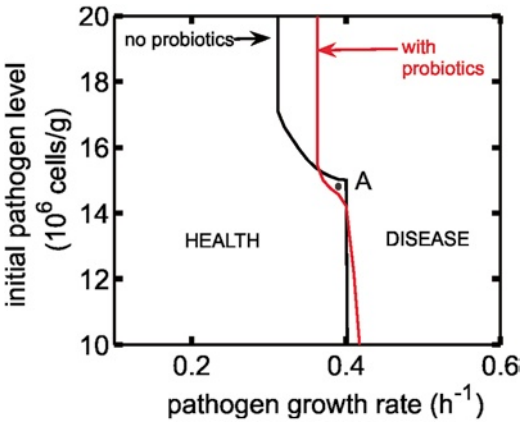

Fig. 13.2 Effect of the initial number of pathogenic bacteria on predictions of health and disease is shown as the pathogen growth rate is varied. *Black curve*: separates regions of health and disease in the absence of probiotics. *Red curve*: separates regions of health and disease in the presence of probiotics. Point A highlights a combination of parameters for which health is predicted without probiotics, but disease is predicted when probiotics are included

the lumen is varied. In the parameter region, points (corresponding to a pair of parameter values) located below or to the left of the solid lines yield predicted healthy states, and points located above or to the right of the solid lines yield disease (septic death) states. The point labeled "A" in the figure gives an example of a case that is healthy without probiotics (i.e., to the left of the black curve) but is predicted to result in septic death following probiotic treatment (i.e., to the right of the red curve). Additional work is needed to determine how these model predictions correspond with the clinical observations.

Since the effectiveness of probiotic treatment on the incidence and severity of NEC may also depend on other factors [79], including feeding type [80], delivery type [73], or other existing health disorders of the infant, certain model parameters should be adjusted to test the impact of these factors in future studies. Moreover, it has been suggested that some of the positive clinical results associated with NEC be reconfirmed in order to verify that the original results were not affected by inadequate sample size, lack of adequate quality control of the products, differences in outcomes with different probiotic strains and lack of uniformity, questionable biostatistical methodology, or the potential for adverse events [81].

Main Findings: The Role of TLR4 and TLR9 The model in [55] extends the probiotic model developed in [52] to study the effects of enterocyte TLR4 and TLR9 in NEC. The effects of TLRs are investigated by performing simulations where no TLRs are active and comparing those results to simulations in which only TLR4 is active and in which both TLR4 and TLR9 are active. Performing these three sets of simulations allowed the contributions of each receptor to be isolated. The model predicts a

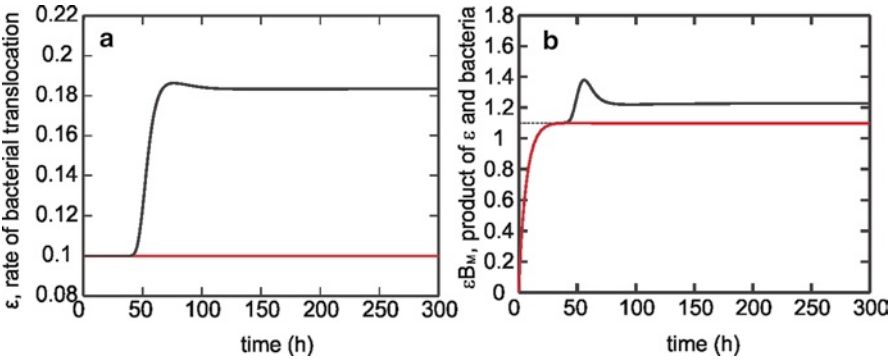

Fig. 13.3 (**a**) Comparison of the time dynamics for the rate of bacterial translocation (ε) for the model case with TLR4 only (*red*) and the model case with both TLR4 and TLR9 present (*black*). ε = 0.1 corresponds to the baseline (*normal*) rate of translocation. In the absence of TLR9, ε is at the baseline level. In the presence of TLR9, ε is elevated, corresponding to an inflamed state. (**b**) Comparison of the time dynamics for the product of the rate of bacterial translocation and bacteria in the mucus layer (B_M) for the two model cases described in (**a**). The *dotted line* corresponds to the threshold value of the intestinal wall. If the product εB_M is greater than the threshold, bacteria enter the tissue. If the product is less than threshold, no bacteria enter the tissue. In the case depicted here, bacteria enter the tissue in the presence of TLR9 but do not cross the barrier in the absence of TLR9

sensitive interplay among mucus layer dynamics, the severity of infection, and the degree of TLR4 activation. Most model results indicated relative promotion of health and disease that were expected from the combined TLR effects. However, the model also uncovered parameter regimes exhibiting unexpected outcomes, such as the direct promotion of health by TLR4 alone in some circumstances.

Including TLR9 often not only promoted health in place of disease but also converted healthy states to disease states, depending on the balance of other system effects. For example, there are parameter values that define the strength of TLR4 activation for which the presence of TLR4 alone can expand the health region and the inclusion of TLR9 further expands the predicted region of health. However, for other values of these parameters, the inclusion of TLR9 changes otherwise healthy outcomes with TLR4 alone into outcomes involving aseptic death despite the inhibitory effects of TLR9 on TLR4. Figure 13.3 provides an example of this unexpected situation of TLR9 causing harm in the system. The rate of bacterial translocation (panel a) is at baseline and the product of the translocation rate and bacteria in the mucus layer (panel b) is below threshold for TLR4 alone, corresponding to a health state prediction (red curves). Yet, when TLR9 is included, the translocation rate is elevated above baseline and the threshold value is exceeded, corresponding to a prediction of septic death (black curves). Arciero et al. [55] hypothesized that this sensitivity to parameter values is evidence that final maturation in the womb likely brings out a balance of parameters that yields a healthy outcome and that preterm babies may be unhealthy if this balance of parameters has not been established.

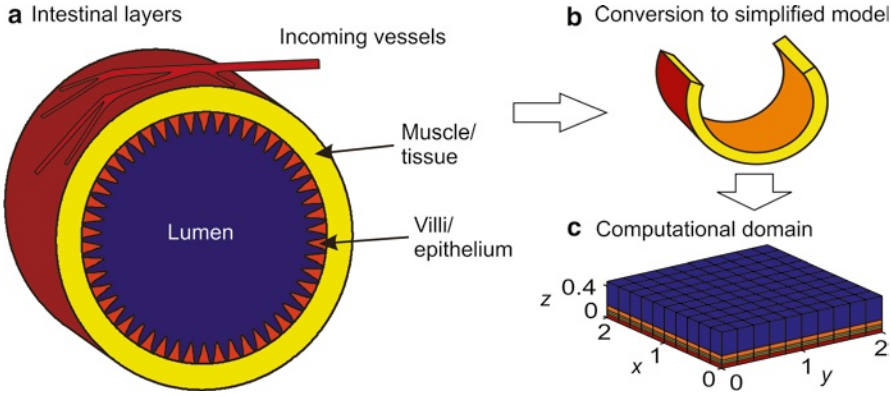

Fig. 13.4 (**a**) General intestinal structure with regions represented in the PDE model labeled. From inside to outside there is the lumen, the usual home of the bacteria (*blue*), the villous region lined with epithelial cells (epithelium) that prevents bacteria from leaving the lumen (epithelial region in the model, *orange*), the muscle and tissue that help with intestinal function and structure (tissue region in the model, *yellow*), and the incoming blood vessels (blood region in the model, *red*). (**b**) To arrive at the corresponding computational model, a short section of the intestine is sliced longitudinally and laid out flat as seen in (**c**). (**c**) The corresponding computational domain with, from *top* to *bottom*, a lumen, epithelial, tissue, and blood region. The computational domain is shown to scale with unit being in centimeters

Partial Differential Equation Modeling

The previous ODE model included, to some extent, the effects of the spatial structure of the intestine by utilizing separate compartments for different regions of the intestine. While such compartmentalization could be further refined to better model the effects of spatial structure of the intestine using ODEs, another approach is to transition from ODE models to PDE models. While the computational time for PDE model simulations is longer than for ODE model simulations, PDE models allow more complete tracking of spatial dynamics than compartmental ODE models. In addition, theory and methods for the numerical and analytical solutions of PDE models are more developed than for ABM models (see section "Agent-Based Modeling"). Nonetheless, PDE models inherently assume that model players can be modeled by differentiable functions, an assumption not shared by ABM models and an assumption that can break down at smaller length scales.

PDE Model of NEC Barber et al. [56] presented the first PDE model of NEC. This model expands on the four variable ODE model of Reynolds et al. [57] by adding variables specific to NEC and including the spatial structure specific to the intestine. These additions introduce complexity into the system, and thus a few approximations and simplifications were made. In particular, the spatial structure of the intestine and the corresponding domain on which the PDEs were solved (Fig. 13.4) were simplified

and multiple key players in the immune response were lumped into single variables. For example, the multiple proteins identified as proinflammatory cytokines were lumped into one variable, c_p (proinflammatory cytokines). It is important to note that, because of these simplifications, the model has inherent limitations in terms of its accuracy, predictive capabilities, and scope of applicability.

Intestinal Physiology A depiction of the intestine and the corresponding computational model is provided in Fig. 13.4. This PDE model extends the three-compartment ODE models in [52, 55] to include intestinal dynamics in four regions. Bacteria are located in the intestinal lumen (blue) and are considered the primary source of external pathogens. The epithelial cell region (orange) regulates the translocation of pathogenic bacteria from the lumen into the intestinal tissue. The tissue region (yellow) consists of intestinal cells that can be damaged and destroyed by both bacteria and an excessive inflammatory response. The blood region (red) reflects the blood supply to the intestine and acts as a source of neutrophils. As the figure suggests, the regions have been converted into rectilinear regions and homogenized so that spatial structures, such as the undulating villi and blood vessels, do not explicitly appear in the model. Absorption of nutrients in the epithelial region and clotting of blood near damaged vessels are not included in this version of the model. Throughout the rest of the chapter, the phrase "vertical direction" will correspond to the z-direction in Fig. 13.4c and is the primary direction of bacterial movement from the lumen into the tissue.

Model Variables The model includes 11 key players in the intestinal inflammatory process:

- b: bacteria, which are pathogens that trigger the immune response
- m, m_a: resting and activated macrophages, which are immune cells that initiate the immune response once they are activated by a pathogen
- n, n_a: resting and activated neutrophils, which are immune cells that are recruited/activated by macrophages and assist in the immune response
- c_p, c_a: pro- and anti-inflammatory cytokines, which are proteins that enhance (pro-inflammatory) or inhibit (anti-inflammatory) the immune response
- d: Damage associated molecular pattern molecules (DAMPs), which are molecules that track tissue damage and upregulate the immune response
- e_c: epithelial cell integrity, which is a measure of health of the epithelial layer
- ZO1: zonula occludens-1, which are tight gap junction proteins connecting epithelial cells that help to limit bacterial translocation into intestinal tissue
- NO: nitric oxide, which is a molecule that destroys ZO1, thereby promoting bacterial translocation

The last three players, while not unique to the intestine, are particularly important in NEC because of the importance of the epithelial barrier in containing the large number of bacteria in the intestine. The model simulates the dynamics of all variables in all four regions except e_c and ZO1, which only need to be tracked in the epithelial region.

Units All units except bacterial units (10^6 bacteria/cm^3) are nonspecific units of the form x-units/cm^3 (e.g., c_p-units/cm^3). While using specific units allows model results to be readily compared with experimental data, it is reasonable to use nonspecific units when the goals of the model are qualitative in nature or if there is not sufficient experimental data available. The choice of nonspecific units is also appropriate when several model players have been lumped into one variable. For example, in the NEC PDE model, multiple types of proinflammatory cytokines have been lumped into one variable, c_p. It would be difficult to assign specific units to the variable since different types of proinflammatory cytokines have varying molecular weights and degrees of effectiveness. Instead, nonspecific units of c_p-units/cm^3 are used and are best interpreted as corresponding to the average proinflammatory cytokine *effectiveness* per cm^3.

Model Equations There are ten PDEs and one constraint (the number of resting neutrophils is approximately constant) that govern the dynamics of the 11 key players in the model. Four of the ten PDEs are described here. Details of the other six PDEs are similar and given in [56]. The values of all parameters are also given in [56].

The PDE governing bacteria is:

$$\frac{\partial b}{\partial t} = \nabla \cdot \left(D_b \nabla b \right) + k_{bg} b \left(1 - \frac{b}{b_{\max}} \right) - R\left(c_a \right) \left(k_{bm_a} m_a b + k_{bn_a} n_a b \right) - \frac{k_b b}{1 + \dfrac{b}{\varepsilon}} \tag{13.1}$$

The terms on the right hand side correspond, in order, to diffusion (i.e., random wandering of bacteria through space), logistic growth of bacteria, bacteria being killed by activated macrophages and neutrophils, and bacteria being killed by immune response players other than macrophages and neutrophils (e.g., antibodies). $R(c_a)$ is a special "retardation factor" given by

$$R\left(c_a \right) = \frac{1}{1 + \left(c_a / \overline{c}_a \right)^2} \tag{13.2}$$

This term represents the inhibitory effect of the anti-inflammatory cytokines on the immune response. In Eq. (13.1), $R(c_a)$ reduces the rate at which activated macrophages and neutrophils kill bacteria in the presence of high levels of anti-inflammatory cytokines.

The PDE governing macrophages is:

$$\begin{aligned} \frac{\partial m_a}{\partial t} = {} & \nabla \cdot \left(D_{m_a} \nabla m_a - \gamma_{m_a c_p} m_a \nabla c_p - \gamma_{m_a b} m_a \nabla b \right) - k_{m_a} m_a \\ & + R(c_a)\left(k_{mb} mb + k_{mc_p} mc_p + k_{md} md \right) \end{aligned} \tag{13.3}$$

The first term on the right hand side contains three parts: the diffusion of macrophages, taxis of macrophages towards regions of higher proinflammatory cytokine concentrations (i.e., macrophages move to areas of trouble), and taxis of macrophages

towards regions of higher bacterial concentrations. Reasonable reviews of chemotaxis and PDE models used to represent chemotaxis can be found in [82–84]. The second term of Eq. (13.3) corresponds to eventual natural death of activated macrophages. The third term corresponds to recruitment of activated macrophages from the available pool of resting macrophages by bacteria, proinflammatory cytokines, and DAMPs. The rate at which activated macrophages are recruited is reduced in the presence of high levels of anti-inflammatory cytokines. Activated neutrophils are governed by a similar PDE except that they are not activated directly by bacteria. Resting macrophages are also defined by a similar PDE except they do not diffuse or undergo taxis.

The PDE governing proinflammatory cytokines is:

$$\frac{\partial c_{\mathrm{p}}}{\partial t} = \nabla \cdot (D_{c_{\mathrm{p}}} \nabla c_{\mathrm{p}}) - k_{c_{\mathrm{p}}} c_{\mathrm{p}} + R(c_{\mathrm{a}})(k_{c_{\mathrm{p}} m_{\mathrm{a}}} m_{\mathrm{a}} + k_{c_{\mathrm{p}} n_{\mathrm{a}}} n_a) - R(c_{\mathrm{a}})(k_{nc_{\mathrm{p}}} c_{\mathrm{p}} n + k_{mc_{\mathrm{p}}} c_{\mathrm{p}} m) \tag{13.4}$$

The terms on the right hand side include the diffusion of proinflammatory cytokines, the natural decay or degradation of proinflammatory cytokines, the production of proinflammatory cytokines by activated immune cells, and the uptake of proinflammatory cytokines by resting immune cells. The equations for anti-inflammatory cytokines, damage, and nitric oxide take on similar forms.

All model PDEs are reaction–diffusion equations that include typical terms for interaction, recruitment, production, and decay. While most terms are straightforward, some of the more complex terms have been included in order to model relevant mechanisms for the biological system (e.g., taxis and retardation factor) to obtain physiologically realistic steady-state behavior. For instance, the fourth term in Eq. (13.1) was chosen so that the model can yield both a stable unhealthy and a stable healthy steady state, which corresponds physiologically to the fact that some patients die while others successfully recover.

While the other equations employ linear diffusion operators, the following PDE for epithelial integrity (e_{c}) includes nonlinear diffusion to incorporate the complex barrier function of the epithelial layer:

$$\frac{\partial e_{\mathrm{c}}}{\partial t} = \nabla \cdot \left(\alpha(b)\beta(e_{\mathrm{c}})\nabla e_{\mathrm{c}}\right) + k_{\mathrm{p}} e_{\mathrm{c}} \left(1 - \frac{e_{\mathrm{c}}}{e_{\mathrm{c,max}}}\right) - k_{\mathrm{a}}(n_{\mathrm{a}}, c_{\mathrm{p}}, b) e_{\mathrm{c}} \tag{13.5}$$

The first nonlinear diffusion term corresponds to changes in epithelial integrity resulting from the migration of healthy epithelial cells from regions of high epithelial integrity to low epithelial integrity. The second logistic growth term corresponds to increases in epithelial integrity resulting from the innate ability of epithelial cells in the epithelial layer to recover when in normal surroundings (i.e., adequate nutrition and no intruders). The third term corresponds to decreases in epithelial integrity due to death or injury of epithelial cells caused by activated neutrophils, proinflammatory cytokines, and bacteria. The destruction rate of epithelial cell integrity is given by $k_{\mathrm{a}}(n_{\mathrm{a}},c_{\mathrm{p}},b)$, which is an increasing function of activated neutrophils, proinflammatory cytokines, and bacteria.

The two functions appearing in the nonlinear diffusion term are given by:

$$\alpha(b) = D_{e_c} \frac{(b_{\max} - b)^{1/4}}{(b_{\max} - b)^{1/4} + b^{1/4}}; \quad \beta(e_c) = \frac{e_c^2}{e_c^2 + (e_{c,\max} - e_c)^2} \tag{13.6}$$

The product of these two functions corresponds to the effective diffusion rate of epithelial cell integrity or, alternatively, the migration rate of epithelial cells from regions of low epithelial cell integrity to high epithelial cell integrity. The choice for $\beta(e_c)$ employs the assumption that epithelial cells in regions of low epithelial cell integrity remain in their current location, while epithelial cells in regions of high epithelial cell integrity will readily migrate to assist in restoring epithelial cell integrity in other regions. The function for $\alpha(b)$ is chosen so that migration is inhibited in the presence of bacteria [85].

Epithelial Barrier The epithelial layer limits translocation of bacteria from the lumen into the surrounding tissue, corresponding to zero permeability of the epithelial region to bacteria. When the epithelial layer loses integrity, however, bacteria can more freely translocate. This alters the diffusion into and out of the epithelial region and is modeled by redefining the vertical diffusion coefficient for the variables as follows:

$$D^z_{\text{effective}} = D^z_{\text{baseline}} + \left(D^z{}_{\max} - D^z_{\text{baseline}}\right) \frac{(\text{ZO1}_{\max} - \text{ZO1})^{1.5}}{(\text{ZO1}_{\max} - \text{ZO1})^{1.5} + \text{ZO1}^{1.5}{}_{\max}} \tag{13.7}$$

Equation (13.7) is adopted so that ZO1, the concentration of tight junction protein in the epithelial region, determines the effective vertical diffusion rate in the epithelial region. When the tight junction protein concentration is at its maximal amount, the baseline diffusion rate is used in the model. For bacteria and the immune cells, the baseline diffusion rate is zero. For variables corresponding to smaller sized objects (cytokines and nitric oxide), the baseline diffusion rate is one-tenth of the maximal diffusion rate. When the tight junction protein concentration is zero, the maximal diffusion rate is used. In this way, ZO1 also serves as a measure of the epithelial layer permeability (see [56]).

Numerics The PDEs are solved using standard centered finite differences with the exceptions of the taxis and nonlinear diffusion of e_c, which use upwinding. Different diffusion constants are used in each region to more closely model reality, and thus the linear diffusion rates are discontinuous functions in the vertical direction. Harmonic averaging of the vertical diffusion constants at the boundaries between the regions has been performed in order to more appropriately and accurately treat these discontinuities (see [86]).

Model Initial Conditions and Scenarios The model considers NEC dynamics *after* an initial injury has formed. This corresponds to an initial condition for the PDE in which the epithelial integrity is less than 1 or 100% within the epithelial layer. For simplicity, it is assumed that at the start of a given simulation the system is completely healthy with the exception of lowered epithelial integrity and ZO1

values inside an injured area within the epithelial region. Typically, unless otherwise stated, we use a circular cylindrical injured area so that

$$e_c(x, y, z, t = 0) = e_{c,0} + (1 - e_{c,0})H(x^2 + y^2 > r_0) \tag{13.8}$$

where $e_{c,0}$ is the initial epithelial integrity in the injured area, r_0 is the initial radius of the injured area, and H is the Heaviside function. ZO1 is similarly initialized:

$$\mathrm{ZO1}(x, y, z, t = 0) = \mathrm{ZO1}_0 + (1 - \mathrm{ZO1}_0)H(x^2 + y^2 > r_0) \tag{13.9}$$

where $\mathrm{ZO1}_0$ is always chosen to be equal to $e_{c,0}$.

To test the effects of breastfeeding in NEC, the model was adjusted so that the variable c_a corresponds to only *exogenously derived* anti-inflammatory cytokines, in particular, anti-inflammatory cytokines coming from the mother's breast milk [87, 88]. In addition, breast milk contains antimicrobial peptides that are capable of killing multiple pathogenic organisms and modulating the immune system [87, 88]. To include these antimicrobial effects, an additional death term of the form $-k_{amp}b$ is included in the PDE for bacteria [Eq. (13.1)]. Individuals not fed by breast milk (henceforth called "formula fed" individuals) are assumed to have no additional antimicrobial peptides or corresponding additional bacterial death term and no exogenously derived anti-inflammatory cytokines (i.e., $c_a = 0$ and has no corresponding PDE).

Results The model is first used to depict a typical scenario for formula-fed infants. It is then used to investigate the effects of breast feeding and the severity and extent of the epithelial injury. Finally, the model is used to consider how the shape of an injured region may affect the outcome of a patient.

Formula-Fed Simulation Figure 13.5 shows typical temporal dynamics for the model by plotting the average values of the variables in each region for a given formula-fed simulation with an initial epithelial integrity of 0 in a circular injured area that occupies 20% of the epithelial region (see Fig. 13.6). The absence of epithelial integrity and gap junction proteins in this area allows bacteria to invade the epithelial and tissue regions. The invasion, however, is relatively short lived as the immune response quickly responds and kills off most bacteria. Unfortunately, in this simulation, the immune response is overactive and causes damage or DAMPS to form in the tissue, which further activates the immune response resulting in a positive feedback loop that ends with an elevated damaging immune response in both regions.

While the PDE model yields a lot of spatial information, the information presented here is limited to the dynamics of the epithelial integrity, since epithelial integrity is a good indicator of the general health of the infected intestinal region. The epithelial integrity at the z-value halfway through the epithelial region is plotted as a surface in Fig. 13.6. The figure shows the initial condition for epithelial integrity at $t = 0$ h as well as the spatial dynamics of epithelial integrity as time progresses. The plot shows epithelial integrity diffusing into the injured area, which corresponds to healthy epithelial cells migrating into the injured area. As such, the epithelial integrity inside the initial injured area improves with time. As epithelial cells move into that region, however, they also move away from the initially uninjured area. This causes the epithelial integrity outside the initial injured area to

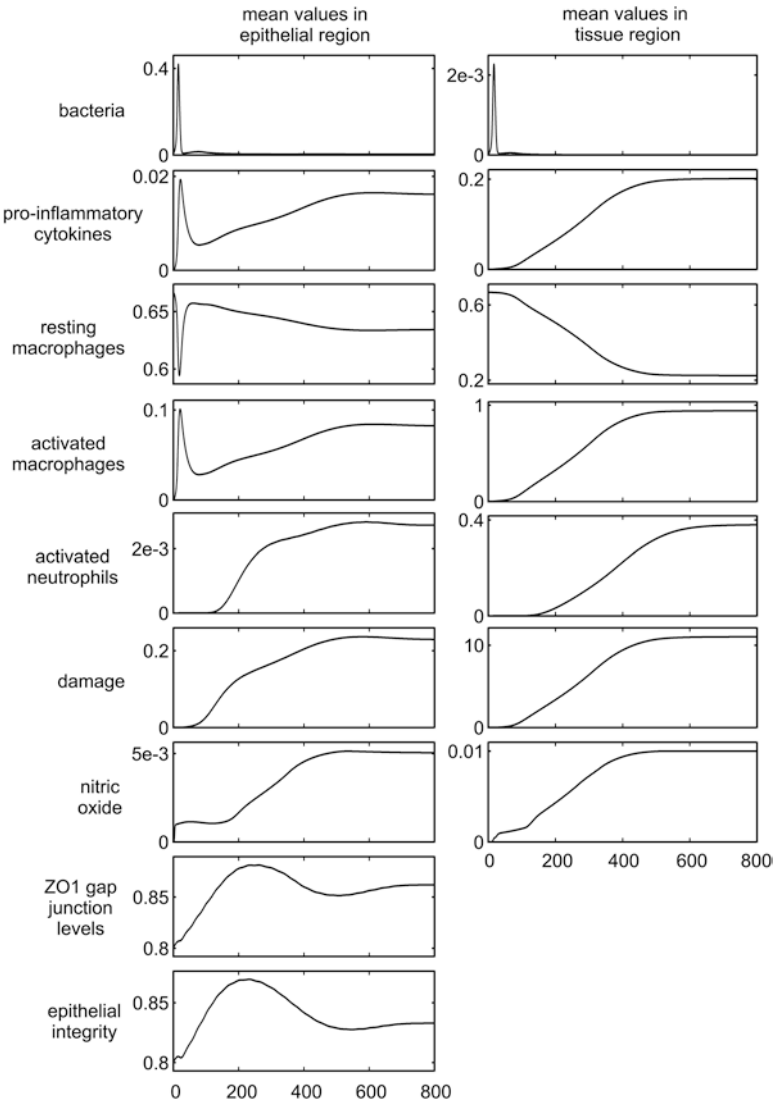

Fig. 13.5 Temporal dynamics of the system as measured by the average values of the variables in each layer. The plots show an initial bacterial invasion that is easily eliminated by an immune response that continues to grow well after the bacteria are gone because of the collateral damage being produced by too many activated immune cells

decrease as time progresses. The end result can be seen at the last time where epithelial cell integrity is approximately 80%. While the original injured area is healthier than when it was initially, it still has not fully recovered. In addition, the area around it has fallen from 100% epithelial integrity to 80% epithelial integrity. The reason for this failure in recovery despite lower bacterial levels (see Fig. 13.5) is because the overactive immune response is generating damage at a rate that does not allow the epithelial integrity to fully recover.

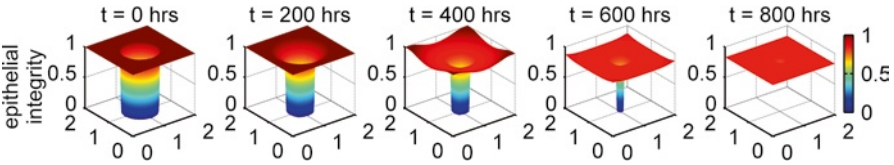

Fig. 13.6 Spatiotemporal dynamics of the system as measured by the epithelial cell integrity midway through (with respect to the z-direction) the epithelial region. The plots show the initial injured area is improved while the area outside the initial injured area is reduced from its initial 100% integrity. The end result is suboptimal with an intestinal region whose epithelial integrity lies below 100% at approximately 80%. This corresponds to an unhealthy outcome

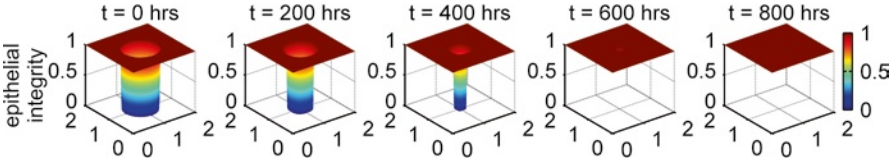

Fig. 13.7 Spatiotemporal dynamics of the system when exogenous anti-inflammatory cytokines and antimicrobial peptides are introduced. The initial injured area steadily increases back to 100% epithelial integrity, while the areas outside of the initial injured area stay at 100% epithelial integrity corresponding to a healthy outcome

Effects of Breastfeeding Figure 13.7 shows a simulation corresponding to a breast-fed individual where exogenously derived anti-inflammatory cytokines and antimicrobial peptides have been included. The same injured area size (20%) and initial epithelial integrity (0%) have been used in this simulation. In contrast to the previous simulation, the injured area recovers quickly to 100% epithelial integrity. Thus, the model confirms the benefits of breast milk and suggests its importance in cases of overactive immune responses, as in NEC.

Effect of Injury Size and Severity The PDE model was used to explore circular injured areas for all possible values of epithelial integrity levels (0–100%) and all possible values of initial injury sizes (0–78.5%; note that 78.5% corresponds to the largest circular injury that will fit within the computational domain). For each pair of initial epithelial integrity and initial injury sizes, the final average epithelial integrity level after 600 h was plotted in Fig. 13.8. Values closer to 0 or 0% correspond to failed recoveries, while values closer to 1 or 100% correspond to successful recoveries. The majority of the cases correspond to successful recoveries near 1, while a small minority of the cases correspond to failed recoveries. This is consistent with what is seen clinically where a majority of neonatal intestinal injuries resolve themselves.

Effect of the Shape of the Injured Area One interesting question that can be asked of a PDE model that cannot be easily ascertained from an ODE model is: What are the effects of the general shape of an injured area on the ability for the system to

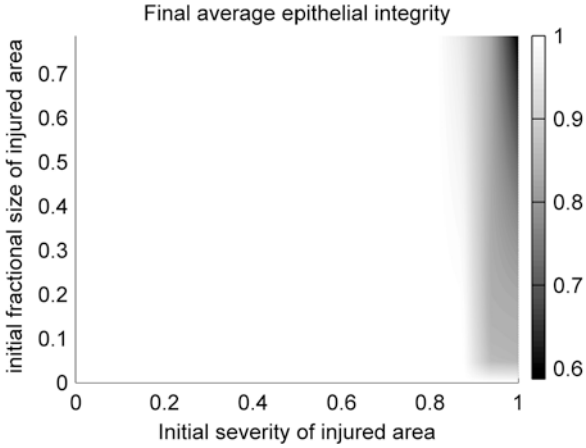

Fig. 13.8 Dependence of injury resolution outcome as measured by the final average epithelial integrity in the epithelial region on the initial severity (initial epithelial integrity in the injured area) and initial fractional size of the injured area. The model predicts most injuries naturally resolve themselves. It also suggests a strong dependence on the injury severity and a weak dependence on the injury size

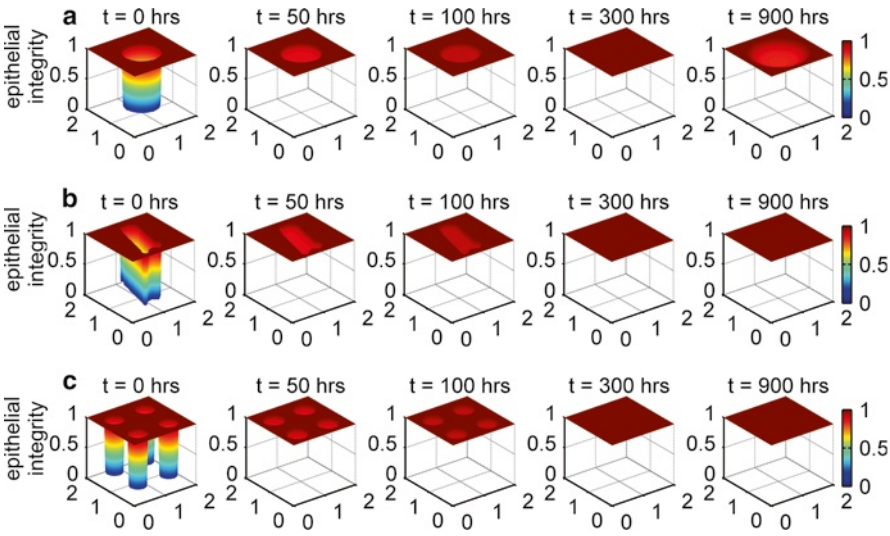

Fig. 13.9 Spatiotemporal dynamics of the system for three different initial shapes of the injured area. For the *circular shape* in (**a**), the wound does not resolve itself as too much damage accumulates in the tissue (see text). For the *shapes* in (**b**) and (**c**), however, the injuries do resolve themselves. This shows the outcome of a given simulation can depend on the shape of an injured area

recover? To investigate this question, three different initial shapes were considered: a circular shape (Fig. 13.9a), an irregular shape (Fig. 13.9b), and four small circular shapes (Fig. 13.9c). All initial shapes occupied 20% of the epithelial region. When using 0% initial epithelial integrity, all shapes gave unhealthy results with quantitative results differing minimally between the simulations. This motivated an investigation of whether or not there were certain initial epithelial integrity levels for which the shapes gave different results. For an initial epithelial integrity level of

12.7%, the circular shape did not fully heal while the other two shapes did fully heal (see Fig. 13.9). While there are other initial epithelial integrity levels for which shapes can cause qualitative differences in the eventual outcome of a simulation, those cases are relatively few (<1% of all epithelial integrity levels).

One important note is that in Fig. 13.9a, the injured area seems to heal and then become reinjured. This occurs because damage (in the form of DAMPs) is slowly increasing in the epithelial region due to an overactive immune system. In the model, if damage remains below a certain critical level, the system is able to recover without the immune response becoming overactive. This is the case for the simulations corresponding to Fig. 13.9b, c. If, however, DAMPs go above that critical level, as it does in Fig. 13.9a, then the high levels of DAMPs activate immune cells fast enough so that the rising numbers of immune cells produce DAMPs at a faster rate than they can decay. DAMPs often accumulate slowly in the tissue, however. This is the case in Fig. 13.9a. Before DAMPs have accumulated to excessive levels, the injured area has time to resolve itself. After that point, however, the DAMPs in the tissue finally accumulate to excessive levels and cause injury. To make sure this did not happen in Fig. 13.9b, c, the simulations were run until $t = 2{,}400$ h with all variables, including damage/DAMPs, returning to their healthy levels in all intestinal regions.

Summary of Findings The PDE model is able to produce physiologically realistic results that are consistent with the general effects of breast milk and the observation that most neonatal intestinal injuries resolve themselves. In addition, the model is able to show that spatial details (e.g., injured area shape) can alter the outcome of an injury resolution and that a PDE modeling approach can be used to investigate the effects of those spatial details.

Limitations and Extensions While the model has been developed with NEC specifically in mind, the model is still somewhat limited in its scope. It assumes the existence of an initial injured area when the simulation begins and does not consider how that initial injured area may have originally developed. In addition, the simulations can only be considered to be physiologically accurate for a limited amount of time since the model does not include effects of later stages of NEC such as the effects of microvessel breakage, blood clotting, and necrotic tissue. Adding in some of these effects to the PDE model would help extend the scope of the model.

The model has not yet been ideally calibrated as it is still only in its initial stages. Calibration includes finding not only one optimal set of parameters but also finding a distribution of parameters that can produce the range of physiological behaviors seen in patients. In fact, there are certain sets of parameters for which simulations can evolve into patterned or chaotic movement [89]. Structured parameter estimation techniques such as Markov chain Monte Carlo and Kalman filtering methods can be used to help quickly and thoroughly explore the parameter space for this model, which has over 50 parameters, many of which do not have well-determined values. In addition, those parameter estimation techniques can also be used to explore the possible physiologically realistic behaviors that the model can produce.

Other aspects of breast milk besides anti-inflammatory cytokines and antimicrobial peptides can be included in this model. Also, the simplified geometry of the model can be replaced with a more accurate geometry including the shape of the villi in the intestine as well as the microvasculature. Only ten variables have been used. Likely candidates for variables that can be added to the model in order to improve results include TLR4 and probiotics (see the previous section) as well as other immune response members like dendritic cells. It is important to mention that while all of these suggestions would help to improve the model, some will help more than others and it is important to choose adjustments wisely if one is to avoid producing a needlessly complicated model whose results are difficult to interpret. Nonetheless, by carefully calibrating and developing this model, the model can eventually be used to make useful predictions and treatment suggestions.

Agent-Based Modeling

Agent-based modeling is an object-oriented, rule-based, discrete-event computational modeling technique that employs a modular, scalable architecture to simulate biological systems [90–92]. Agent-based models (ABMs) are composed of virtual environments populated with objects (agents) that execute behaviors based on programmed rules that govern interactions with the local environment and other agents. Agent-based modeling has been used to dynamically represent complex biological processes such as inflammation [93–99], cancer [100–103], infectious diseases [104–108], and wound healing [109, 110].

In many biomedical applications of agent-based modeling, agents are used to represent individual cells within a system, with multiple classes of agents (cell types) sharing rules extrapolated from mechanistic knowledge obtained from in vitro experiments. The cell-as-agent is an intuitive level of resolution for biomedical agent-based modeling, since much basic research describes mechanistic processes that define cellular behavior such as signal transduction, gene regulation, protein synthesis, and compound secretion. ABM provides a useful platform for modeling the various cellular agents and molecular pathways involved in NEC and to view these complex interactions in real time to aid in hypothesis generation and evaluation.

ABM of NEC An et al. presented the first ABM of NEC in 2011 [54] using Netlogo, an agent-based modeling platform based on Java. The ABM was created to evaluate a minimally sufficient unifying hypothesis incorporating the following observations noted in the majority of cases of NEC:

- NEC occurs predominantly in premature infants
- Enteral feeding precedes the development of NEC in nearly all cases
- Microbes (bacterial or viral) are associated with NEC

The ABM incorporated the following cell types of the premature gut: neonatal gut epithelial cells (NGECs), goblet cells (GCs), submucosal cells (SCs),

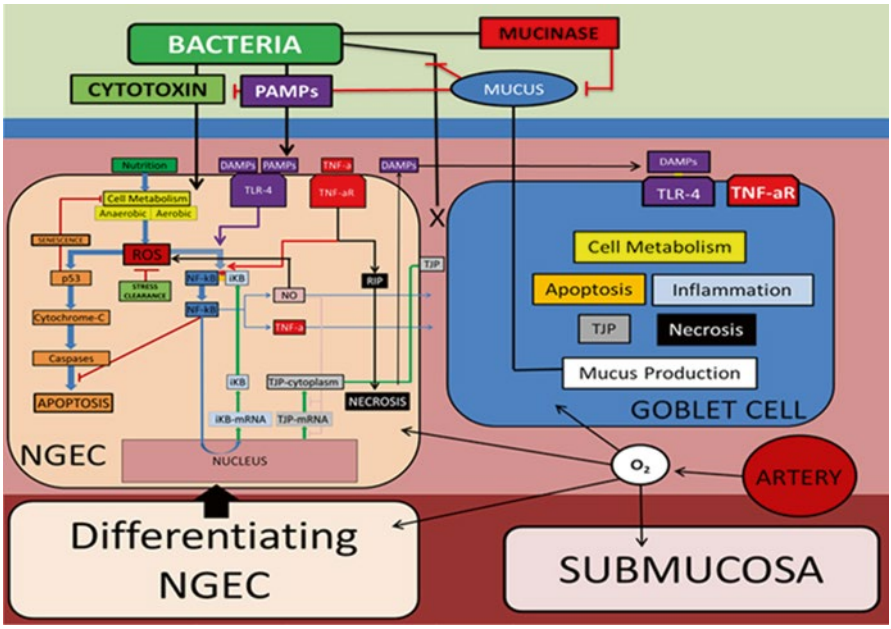

Fig. 13.10 Schematic of ABM depicting cellular agents and molecular pathways incorporated into model. Modeled neonatal gut epithelial cell (NGEC) pathways include cellular metabolism (*yellow*), ROS generation (*red*) and clearance (*green*), apoptosis (*orange*), inflammation (*blue*), necrosis (*black*), and tight junction metabolism (*gray*). Differentiating NGECs eventually become fully functional epithelial cells, and submucosal cells represent additional bowel wall layers. Oxygen is secreted and diffused by arteries for use by all agents in cell metabolism. Bacterial agents are able to interact with NGECs by direct contact (LPS to TLR-4) or induce cell death by a cytotoxic exotoxin

differentiating NGECs (DECs), and bacteria (Fig. 13.10), each with different molecular pathways modeling cell metabolism, inflammation, tight junction formation, and cell death by apoptosis or necrosis. The ABM was created to test the hypothesis that prematurity impairs the NGEC's ability to manage its redox state, resulting in inflammation with the initiation of enteral feeding. Adding virulent bacteria further enhances this propensity towards inflammation, resulting in a cascading system failure that causes widespread necrosis of the entire NGEC population.

The agent rules representing molecular processes, such as receptor activation, signal transduction, metabolism, and transcription factor effects, were expressed using a detailed, qualitative approach [111, 112]. This approach consists of relatively detailed component representation (i.e., enzymes, molecular species, and genes) with qualitative representation of biochemical kinetics using a logic-based, algebraic rule construction. As a result, molecular interaction rules are expressed as conditional statements of the form:

if Ligand A is present, then bind to and activate Receptor B
if Receptor B is activated, then increase Signal Transduction Enzyme C by 1
And so on…

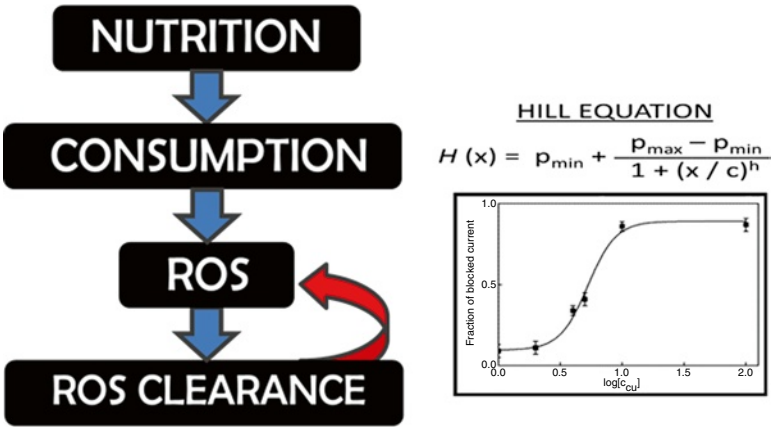

Fig. 13.11 Flow diagram (*left*) depicting a series of Hill equations (*right*) that model nutrient metabolism and ROS generation and clearance. Hill equations were chosen to demonstrate a graduated molecular response (depicted by *elliptical curve* in *bottom right*). Reprinted with permission from Mary-Anne Liebert, Inc. [54]

The exception to this type of rule construction in the NEC ABM is the use of Hill Functions to represent feedback control loops for metabolic stress homeostasis.

Modeling Oxidative Stress Management For each NGEC, a series of Hill equations were used to model the relationships between nutrients, cellular consumption, reactive oxygen species (ROS) generation, and ROS clearance (Fig. 13.11). Hill equations are often used to mathematically model the dose–response relationship between a receptor and ligand, producing a sigmoid or "S-shaped" curve. Hill equations were used in the NGECs to [1] approximate the kinetics of the cell's graduated generation of ROS secondary to metabolism and [2] represent the response of the cell's intrinsic oxidative stress-clearance machinery to clear the produced ROS. Each NGEC was randomly assigned a stress-clearance-capability (SCC) value prior to the start of each experiment based on a normal distribution, which represents its enzymatic capability to clear all forms of oxidative stress.

Consequences of Excess ROS Decreased ability of an NGEC to clear the ROS resulted in excess ROS, which would ultimately lead to several different inflammatory states:

1. Low stress, where metabolism and consumption are governed by the baseline Hill equations, and in this state the NGEC levels of p53 will decrease over time.
2. Mid-range stress, where p53 is produced and, as it rises, shifts the metabolism-governing Hill function to reduce consumption (i.e., produce senescence). This allows metabolism-derived oxygen species to decrease until the stress level drops into the Low Stress range, at which point p53 will then decrease.
3. High stress reached when levels of p53 continue to be produced until they cross a set threshold and will lead to the generation of variables for cytochrome-c and caspase proteins that subsequently activate apoptotic mechanisms. This level of stress also activates inflammatory pathways.

Table 13.1 Table depicting agent types and functions

Agent	Description	Functions
NGECs	Able to perform basic metabolic functions, secrete inflammatory mediators, and regulate cell death pathways	*Cellular respiration*: nutrient consumption, which leads to generation of ROS (via aerobic or anaerobic metabolism depending on oxygen content) *ROS clearance*: process to decrease total ROS within each agent *Tight junction formation*: prevents interaction of bacteria with NGECs *Apoptosis*: programmed cell death, with no spillover of cell contents *Inflammation*: activation leads to production of mediators (TNF-α and NO) *Necrosis*: cell death by excessive inflammatory signaling (via TNF-α or DAMPs) or reduced oxygen content
Goblet cell	Agents with ability to create protective mucus barrier for NGECs	Same cellular and metabolic processes as NGECs Secretion of mucus, which prevents interaction between NGECs and bacteria
Bacteria	Agents with ability to activate NGEC inflammatory pathways	Generation of PAMPs, which cause activation of NF-kB in NGECs via TLR-4 Interaction with NGECs inhibited by tight junctions and mucus
Differentiating epithelial cells	Precursor agents to NGECs	Differentiates into NGEC after set period of time Undergo necrosis with reduced oxygen content
Submucosal epithelial cells	Agents representing additional gut layers	Undergo necrosis with reduced oxygen content
Blood vessels	Agents that secrete oxygen	Provides oxygen to other agents

High levels of stress trigger the production of NF-kB, which leads to the secretion of tumor necrosis factor alpha (TNF-α) [113] and nitric oxide (NO) [114, 115]. Secreted TNF-α activates production of NF-kB by other NGECs, in addition to directly activating the RIP-kinase pathway, which ultimately leads to necrotic cell death [116]. Secreted NO disrupts tight junction formation [20, 21], which NGECs use to block interactions with bacterial agents and is also a source of exogenous ROS that affects the total stress level of an NGEC.

Other Gut Cells Other cell types were incorporated in the neonatal gut model (summarized in Table 13.1). Blood vessels were modeled to secrete a variable for oxygen, which all cellular agents utilized to perform aerobic or anaerobic metabolism. Goblet cells incorporate the same molecular pathways as NGECs, with the added ability to secrete a variable for mucus [117, 118]. The presence of mucus on the surface of NGEC prevents their interaction with bacterial agents. Differentiating epithelial cells (DECs) are created once an NGEC has undergone apoptosis. The DEC has limited metabolic activity until it becomes an NGEC after a set period of time [119]. Submucosal cells (SCs) represent additional cell layers beyond the NGEC. They undergo necrosis based upon ischemia, which propagates via microvascular thrombosis of blood vessels originating from the NGEC cell layer [120].

Bacterial Agents Bacterial agents were modeled with several different functions to interact with NGECs. In the absence of tight junctions and low levels of mucus, bacterial agents are able to attach and secrete a variable for LPS, which activates the inflammatory signaling of NGECs through TLR4 [15, 47, 121]. Bacteria retained this interaction at baseline and were given additional "virulent" characteristics such as the ability to secrete a mucinase or cytotoxic exotoxin that is able to induce necrotic cell death.

Experimental Method In an ABM, each agent function is defined in a particular section of the model code. One complete iteration of the ABM's code is defined as a "run," and an experiment is performed by repeating each "run" in succession until the experimental time frame or a particular outcome is reached. The outcomes measured in the NEC ABM were "necrosis" or "survival" depending on the percentage of NGEC undergoing necrosis.

Minimum and maximum SCC (SCC_{min} and SCC_{max}, respectively) values were defined for the entire population of NGECs at the beginning of each experiment. SCC_{min} and SCC_{max} values were altered for each experiment. Populations "susceptible" to necrosis were identified at lower SCC_{max} values, while "surviving" populations (that is, resistant to necrosis) were identified at higher SCC_{max} values. Parameter sweeps of the SCC_{max} were performed by increasing its value in small increments to determine the point at which a population became significantly susceptible to necrosis. Bacterial agents were then added with varying degrees of virulence factors to determine their effect upon susceptibility of the NGEC population to necrosis.

Results The addition of "control" bacteria, which were only able to interact with NGECs through secretion of LPS (but no other virulence factors), demonstrated an increase in necrosis outcomes, but a similar SCC_{max} threshold at which the necrosis outcome disappears (Fig. 13.12). The addition of bacterial agents with virulence factors significantly increased the rate of necrosis, particularly with bacterial agents that were able to secrete a cytotoxic exotoxin. NGEC populations with higher SCC_{max} values (hence better ROS management) steadily became resistant to necrosis, with the outcome disappearing outright at a threshold SCC_{max} value (Fig. 13.13).

The dynamic relationship between stress clearance and bacterial virulence demonstrates the complex interplay between host susceptibility and environmental factors that can influence the pathogenesis of NEC. Although evidence does not currently exist to suggest that impaired stress clearance is the most proximal event in the pathogenesis of NEC, the ABM demonstrates its plausibility as an initiating factor in NEC. In addition, the ABM further explores the idea of a NEC "disease space" in which the actual clinical phenotype of necrosis lies within some actual range of host susceptibility (i.e., degree of stress clearance impairment) and microbial virulence. The degree of host susceptibility secondary to prematurity likely exists on a spectrum as demonstrated by the SCC_{max} and it is important to note that the goal of this ABM is not to prove the hypothesis that impairment in stress-clearance is the proximal event in NEC, but that it is plausible. In reality, there are a number of other systems in the epithelial cell (which were also modeled in the ABM), which could be explored as potential causative factors in NEC.

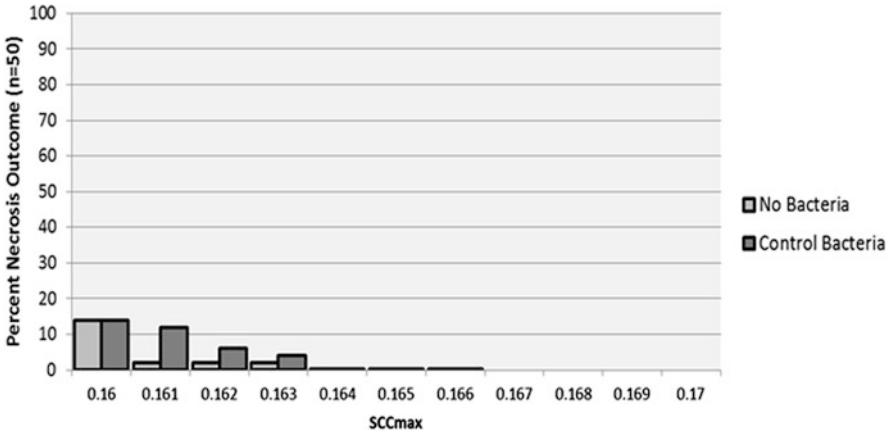

Fig. 13.12 Comparing rates of necrosis in NGEC populations (per 50 experimental runs and 10-day experimental time). Rates of necrosis are similar but increased in the group with control bacteria. It is important to note that the necrosis outcome disappears if the SCC_{max} of the population is adequate enough to clear the stress that is generated by cellular consumption and the presence of bacterial agents. Such a population that is completely resistant to necrosis represents a healthy, mature NGEC population akin to a fully mature infant

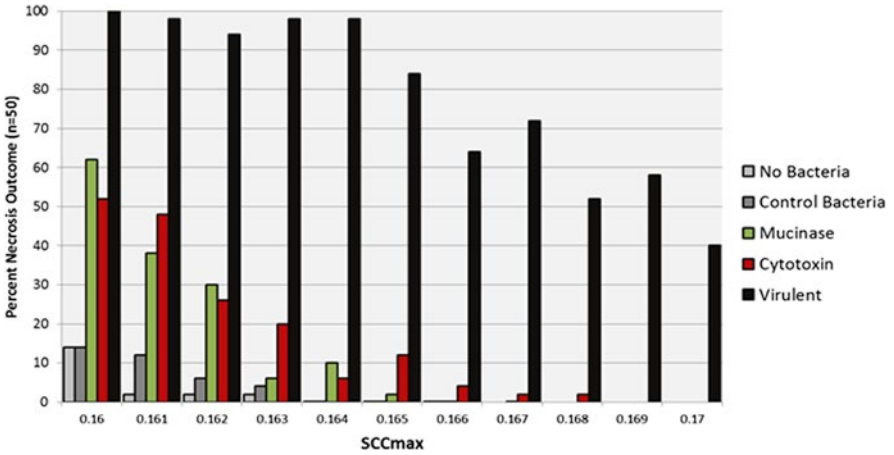

Fig. 13.13 Bacteria with virulence factors significantly increased the rates of necrosis across all SCC_{max} values, with eventual disappearance of the necrosis outcome at higher SCC_{max} values (not depicted). "Virulent" bacteria were able to secrete both mucinase and cytotoxin. The presence of necrosis at higher values of SCC_{max}, which previously did not demonstrate necrosis with control bacteria suggests that bacterial virulence plays an important role in precipitating necrosis. This suggests that more virulent strains of bacteria may be able to affect disease in premature infants that otherwise would have not underwent NEC

For instance, data from the model suggests that bacterial virulence may play a significant role in causing NEC. As no single species of bacteria has been implicated in the pathogenesis of NEC [122], this suggests that the context-dependent activation of bacterial virulence factors may be more important to identify rather than isolating a single type of pathogen.

Conclusions

The multifactorial nature of NEC and the questions that still remain regarding its pathogenesis provide an excellent opportunity to supplement traditional basic science with modeling approaches. Part of the difficulty in understanding the pathogenesis of NEC is due to its broad clinical presentation, ranging from "NEC scares" that are managed conservatively to complete bowel necrosis, which requires surgery. Animal models have been useful in studying NEC, albeit with extreme physiologic and environmental manipulation that are not necessarily present in the clinical disease [37].

Mathematical and computational modeling allow for a higher degree of temporal and spatial resolution, particularly to aid in understanding the proximal events that lead to the generation of NEC. ODE models have been developed to analyze the impact of probiotic administration and the interplay of TLR4 and TLR9 in NEC [52, 55]. A PDE model [56] was used to consider the possible effects of the size and shape of the epithelial layer injury in the process of healing in NEC. ABMs [54] have been used to investigate the complex interplay between host susceptibility and bacteria virulence in the pathogenesis of NEC. Through continued development and rigorous verification, these models can ultimately be used as effective tools to understand this complex disease, predict disease outcomes, and design successful therapies for clinical application.

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Chapter 14
Modeling Host–Vector–Pathogen Immuno-inflammatory Interactions in Malaria

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Introduction

Half of the global population is at risk for malaria, which results in nearly one million deaths annually, 86 % of which are in children [1]. *Plasmodium falciparum*, the most important human malaria parasite, is transmitted by female *Anopheles* mosquitoes. Parasite development in the mosquito begins with the ingestion of

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blood containing sexual-stage gametocytes. Mobile ookinetes penetrate the midgut epithelium 24–36 h later and transform into midgut-bound oocysts within the open circulatory system of the mosquito. Oocysts grow and develop for 10–12 days and then release thousands of sporozoites, which invade the salivary glands and are released during later blood feeding by the mosquito.

Infectious organisms, in general, have evolved alongside the host immune system and developed strategies for evasion and modulation of immunity in the host [2, 3]. In the case of diseases such malaria, the addition of an invertebrate vector host introduces a further layer of complexity in the disease process. In the setting of these infections, the blood compartment serves both as the site of immune system coordination within the host and as an interface for communication and interaction between the parasite, vector, and host [3, 4]. This complex ecology is being reassessed in light of the modern view of the vector as an organism that mounts an immune/inflammatory response in an attempt to control parasite growth, rather than as a willing partner in parasite transmission [3, 4]. Moreover, multiple studies have suggested that parasite killing capacity comes at a fitness cost to the mosquito, typically observed as reduced lifespan [5–7].

In malaria, multiple agents transit from the vector to the host and from the host to the vector. Blood feeding thus juxtaposes the mammalian and mosquito immune systems proximally at the midgut epithelium and distally due to the passage of some factors into the mosquito body. Many of the host-derived factors that are transferred to the mosquito are in fact immune/inflammatory mediators that are both retained selectively and drive signaling and biological effects in the mosquito (reviewed in [3, 4]). We initially established that the ingested mammalian cytokine transforming growth factor-β1 (TGF-β1) and the hormone insulin can function as signals to the midgut epithelium and can affect malaria parasite development in *Anopheles stephensi*, a major mosquito vector of malaria in India and parts of Asia [3, 8–13]. Our recent data suggest that multiple other mammalian blood-derived ligands may be selectively retained in the mosquito midgut (see below).

Based on these studies, we have hypothesized that this information transfer allows the mosquito vector to sample the immune/inflammatory state of the vertebrate host and thereby provide an early warning to the infection status of that host [14]. We have sought to use computational modeling and analysis in order to gain insights into how such information transfer affects the mosquito, the parasite, and the mammalian host, with the ultimate goal of developing new therapeutic strategies for malaria. We have suggested a systematic approach, starting with data-driven modeling and correlative studies that inform mechanistic models and analyses of the inflammatory response [15, 16], in order to generate a comprehensive understanding of the molecular and cellular mechanisms underlying the interspecies immune control of malaria parasites [17]. This approach is essential for identifying "master regulators" in the mosquito vector that could act as therapeutic targets for disease control via genetic modification [18–20].

Computational modeling has been utilized extensively in the past to help clarify various aspects of malaria transmission and immunobiology. However, most modeling work in malaria has been focused mainly on epidemiological aspects of the

disease or very coarse-grained mechanistic modeling of host–pathogen interactions [21], rather than models on the intra- and intercellular scale that involve the mosquito vector and that build directly from the genomic studies or other quantitative experimental data. In the decade since the publication of the genomes of the malaria parasite(s) and of *Anopheles gambiae*, the major African mosquito vector of malaria, there have been several functional and comparative genomic analyses that have helped uncover regulatory networks through correlative studies [22–27]. Some of these studies have focused on the interface between vector and parasite, identifying gene clusters/networks responsible for the mosquito's control of parasite growth [28].

Recent studies from our group have focused on identifying, at the genomic level, signal transduction pathways in *A. gambiae* [29] to complete analogous studies in *A. stephensi*, supported by a 2012 genome release (http://www.vectorbase.org/Anopheles_stephensi/Info/Index) and to integrate these data into computational models. As detailed below, we have identified multiple host blood-derived factors, including—in addition to TGF-β1 and insulin—insulin-like growth factor-1, insulin-like peptides (ILPs), and cytokines such as interleukin (IL)-10 [3] that can induce signaling in the mosquito midgut epithelium and modulate protein expression and activity to regulate the growth of *Plasmodium* parasites [30, 31].

Multiple Ligands and Signals Affecting *Anopheles* Mosquitoes: Integration via Computational Modeling

Our earliest work focused on immune cross talk driven by the ingestion of mammalian TGF-β1 by *Anopheles* mosquitoes during blood feeding. TGF-β1 has been identified as a central player in the immune response to parasite infection within the mammalian host [32]. However, much less is known about the converse, namely the possible role of TGF-β1 in mosquito immunity and physiology. In mosquitoes, ingested latent human TGF-β1 is rapidly activated in the midgut by factors commonly released during blood digestion such as NO and heme [9]. The latent form of TGF-β1 is detected in the circulation of healthy adults at concentrations as high as 5 ng/ml and is, therefore, ingested at levels that can be biologically active for arthropod cells and tissues [33]. Human TGF-β1 ingested by *A. stephensi* via a blood meal was shown to induce expression of the *A. stephensi* ortholog of inducible nitric oxide synthase, AsNOS [11]. Inducible NOS has been associated with human host responses to malaria [34–36], and our studies have shown that the mosquito also regulates parasite development through complex, multiphasic expression of AsNOS [37] with concomitant inflammatory synthesis of NO and reactive nitrogen species [38].

The regulation of the TGF-β1-AsNOS response in *A. stephensi* is complex. In particular, we found that TGF-β1 induces mosquito mitogen-activated protein kinase (MAPK) signaling [30]. There is evidence of specific feedback regulation of the actions of TGF-β1 in *Anopheles* by the extracellular signal-regulated kinase (ERK) and the upstream activating kinase MEK [30]. These pathways are networked extensively with canonical Smad-dependent signaling in mammalian

cells [39] and we suspect that the same is true in mosquito cells. Finally, there are dichotomous, dose-dependent effects of mammalian TGF-β1 on AsNOS induction and parasite growth [11].

Taken together, these studies suggested that computational modeling could facilitate identification of the mechanisms underlying these diverse interactions. Accordingly, various mechanistic computational models were created to examine both qualitative and quantitative features of this biological system [40]. An ordinary differential equation (ODE) model of the mosquito response predicted oscillations in AsNOS as well as MEK/ERK-dependent signaling, providing one possible mechanistic model that was consistent with experimental data. In addition, this model yielded qualitative predictions supported by experimental data, albeit with apparent phase shifting in the multiphasic behavior of *AsNOS* in different mosquito cohorts treated with several doses of TGF-β1 [40]. This model also suggested a need for the persistent presence of a TGF-β1-like signal to drive the multiphasic expression of AsNOS. However, experimental data had previously suggested a short half-life for TGF-β1 that would be insufficient to support the observed multiphasic time course of AsNOS. This discrepancy was reconciled by a model-generated hypothesis for an endogenous mosquito ortholog of TGF-β that could be induced by exogenous mammalian TGF-β1 to drive the long-term AsNOS response. Indeed, the mosquito TGF-β ortholog As60A [41] was shown to exhibit the dynamics that the model predicted for the hypothesized TGF-β1-like molecule in order to maintain the observed AsNOS response [40]. We note that ODE models are the classical mathematical modeling framework, and this methodology is discussed extensively elsewhere in this book.

β-Nicotinamide adenine dinucleotide (NAD^+) is a ubiquitous cellular constituent that is used by cells as an electron acceptor (or, in its reduced form, NADH, as an electron donor) in a wide variety of enzyme-catalyzed redox reactions. In addition, NAD^+ is now known to exert extracellular effects in multiple cell types secondary to the formation of cyclic adenosine dinucleotide ribose (cADPR) from NAD^+, with subsequent release of Ca^{2+} [42]. In *A. stephensi*, NAD^+ could be released as a consequence of tissue microtrauma and hemorrhage [43] during the act of blood feeding, and thereafter be sensed by *A. stephensi*. An evolving literature [42–57] suggests that NAD^+ can exert extracellular effects on various cell types. Although elevated NAD^+ levels have not been reported in malaria, perhaps due to the short half-life NAD^+, we suspect that inflammation associated with this infection could be modulated by NAD^+ synthesis. In most models, NAD^+ is converted to cyclic ADP ribose (cADPR) and nicotinamide by CD38/157 extracellular ADP-ribosyl cyclases [42, 43, 52]. cADPR then enters cells through either CD38-dependent [49] or -independent [50] mechanism(s), and subsequently binds to ryanodine-sensitive calcium channels to induce cellular calcium fluxes [52, 58]. Alternatively, NAD^+ can be converted to cADPR by intramolecular ADP ribosylation catalyzed by ADP-ribosyltransferases (ARTs [53, 54]). While orthologs of CD38/157 do not appear to be encoded in the existing insect genomes, the *A. gambiae* and *A. stephensi* genomes encode ART orthologs that could support NAD^+/cADPR physiology.

The effects of NAD^+/cADPR are wide ranging, including activation of latent TGF-β1 in mouse macrophages [59]. In particular, we showed that NAD^+

stimulation increased both active and latent cell-associated TGF-β1 in RAW 264.7 mouse macrophages as well as in primary peritoneal macrophages isolated from both C3H/HeJ (TLR4-mutant) and C3H/HeOuJ (wild-type controls for C3H/HeJ) mice [59]. The time and dose effects of NAD^+ on TGF-β1 were complex and biphasic. A statistical model suggested that the effects of NAD^+ on TGF-β1 were nonlinear and this model could predict not only the levels of active and latent TGF-β1 but also the biphasic dose effect of NAD^+ [59]. Based on these data-driven modeling studies, we inferred that the effects of NAD^+ on TGF-β1 were nonlinear. Accordingly, we created a nonlinear ODE model of interactions we considered the most parsimonious and yet still capable of recapitulating the complex biological phenomena observed experimentally. Model-predicted levels of TGF-β1 protein and mRNA were not only largely confirmed experimentally but also suggested the presence of other mechanisms of regulation of TGF-β1 by NAD^+ [59]. Given the analogous biology in *A. stephensi*, we hypothesize that mosquito cells can respond to NAD^+ in contexts that are may be relevant to *P. falciparum* infection.

Based on our understanding of the blood meal interface and the extreme conservation of responsive signaling pathways in the mosquito host, we hypothesized that *A. stephensi* could ingest and be stimulated by other host-derived inflammatory mediators. To test this hypothesis, *A. stephensi* were blood fed to repletion on normal mice. At multiple times postfeeding, blood-filled midguts were assayed for 20 mouse cytokines and chemokines using Luminex™. According to our data, a variety of mouse cytokines and chemokines were apparently selectively retained in the mosquito midgut. We hypothesized that this possible selective retention might establish a dynamic signaling network in the mosquito midgut. Accordingly, we carried out Dynamic Bayesian Network (DBN) inference, using modifications of previously published methods [60]. Briefly, the algorithm uses an inhomogeneous dynamic change-point Bayesian metric of Gaussian networks having score equivalence (BGe) model that allows the reconstruction of time-varying DBNs. In our studies, we chose to focus on a static network that describes the entire time course. For each node, a new set of parent nodes was sampled directly from the posterior distribution and the local scores computed using the BGe model. Each node was subject to a fan-in restriction of three parent nodes. The Gibbs sampling procedure was run for 100 steps to yield a final network structure. Individual networks were then averaged to obtain a consensus network for each condition according to the following rule: if a particular edge was present in more than 50 % of the individual networks in a particular condition, it was included in the consensus network, otherwise it was excluded.

This analysis suggested that the formation of a mediator network in the *A. stephensi* midgut was initiated by IL-1α and propagated by IL-10 and associated with the production of the chemokines monokine inducible by gamma-interferon (MIG/CXCL9) and gamma-interferon inducible protein of 10 kDa (IP-10/CXCL10). Interleukin-1α is a key proinflammatory cytokine that was reported to rise in parallel with malaria severity in humans [61], and certain single-nucleotide polymorphisms in the IL-1α gene have been associated with increased susceptibility to malaria [62]. Interleukin-10 has been established as a key anti-inflammatory cytokine associated with poor outcomes in malaria [63, 64]. Both MIG/CXCL-9 and IP-10/CXCL-10 are induced in the brains of mice undergoing cerebral malaria

induced by infection with *P. berghei* [65]. Interestingly, Dunachie et al. showed that the production of MIG/CXCL-9, IL-10, and TGF-β1 all correlated with the immunogenicity and efficacy of malaria vaccine, as assessed at the mRNA level in peripheral blood mononuclear cells from healthy volunteers given an experimental malaria vaccine [66]. Taken together with our prior work, these findings suggest that TGF-β1 and IL-10, cytokines associated with the outcome of many parasitic diseases along with malaria, along with key proinflammatory cytokines and chemokines, may be selectively retained by *A. stephensi* to mediate novel biology in the invertebrate host, with possible effects on the mammalian host as well.

Multiple additional ligands affect the biology of *Anopheles* mosquitoes and, consequently, also result in differential uptake or killing of *Plasmodium* parasites. Like TGF-β1, human insulin is ingested by *Anopheles* mosquitoes during blood feeding. Circulating insulin in the blood of healthy humans vary widely [67], and infection with malaria can induce a rise in blood insulin above normal levels [67, 68]. Ingested, intact insulin persists in the *A. stephensi* midgut for up to 24 h and in the hemolymph of the head and thorax for up to 18 h postblood feeding [69]. Importantly, the mosquito midgut is exquisitely responsive to ingested insulin [12, 31, 70]. Human insulin can activate both the phosphatidylinositol-3K (PI3K)/Akt and MAPK branches of the insulin/insulin-like growth factor signaling pathway in *A. stephensi* [7, 10, 12, 31] and thereby affect *P. falciparum* infection in the mosquito [7, 30]. Furthermore, we have shown that *A. stephensi* ILPs change in response to ingested human insulin and to *P. falciparum* infection, suggesting that endogenous ILP expression is affected by activation of insulin/insulin-like growth factor signaling. Thus, mosquito ILPs might amplify the response to ingested insulin [10, 71]. This effect might be amplified further by the ingestion of insulin-like growth factor-1 (IGF-1), which is highly similar in structure, utilizes the same receptors, and activates many of the same signaling pathways as insulin [72]. During malaria infection, serum levels of IGF-1 fall dramatically, correlating with increased parasitemia reviewed in [69]. Both insulin and IGF-1 can activate the PI3K/Akt branch of the mosquito IIS pathway [69], but ingested human IGF-1, in contrast to insulin, extends lifespan and enhances resistance of *A. stephensi* to *P. falciparum* [69].

These data indicate that TGF-β1, insulin, IGF-1, IL-10, MIG/CXCL-9, IP-10/CXCL-10, and IL-1α all may persist for sufficient time to stimulate multiple interacting signaling pathways in the mosquito midgut and, after crossing the midgut epithelium, likely in other body tissues as well. These pathways include the NF-κB pathway and the three MAPK pathways (JNK, ERK, and p38). Control of cross talk among the MAPK pathways has been attributed to MAPKKs [73, 74] as well as downstream DUSPs [75, 76]. In addition to intracascade cross talk, the MAPKs are integral components of a variety of other signaling cascades including those with relevance to the immunobiology of *A. stephensi*. Specifically, several kinases of the MAPK cascades are involved in the activation of NF-κB in mammalian cells (reviewed in [77]). Further, the MAPKKK TAK1 regulates NF-κB in *D. melanogaster* cells (reviewed in [78]). In addition to NF-κB activation, the canonical TGF-β/Smad signaling pathway is strongly integrated with the MAPK cascades [79]. Indeed, all three MAPKs can facilitate activation of the cytoplasmic Smads [79].

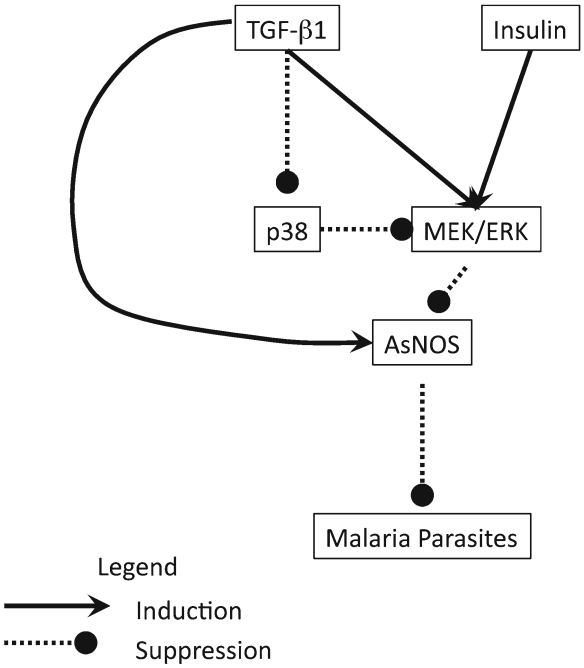

Fig. 14.1 A Markov chain network model of signaling in the mosquito midgut. A coarse-grained network model of TGF-β1- and insulin-induced signaling in the *Anopheles* midgut, incorporating hypothetical feedback loops that account for the activity of MAPK enzymes on AsNOS and, ultimately, malaria parasites. Each vertex (e.g., p38) of the network should be viewed as a pair of points, one representing the suppressed state and the other the active state of that vertex. An edge between two points carries a corresponding transition probability. A suppressed edge indicates that the overwhelming probability ends in the suppressed state of the target vertex through that edge; with complete analogy for inductive edges. Assignment of probabilities is then made by simulating to obtain network output that matches the observed data under various experimental conditions

To begin to define the connections among cytokines, chemokines, growth factors, and signaling pathways, we have created multiple computational models that capture the signaling network at some level of abstraction and include the ultimate effect on parasites in the mosquito midgut. In the first model, the presence of parasites within a mosquito was modeled as a geometric distribution in which the probability of failure represents the chance that the mosquito will host a parasite. This probability was itself viewed as a function of the internal mechanisms of the mosquito, including the signaling pathways triggered, and the presence of certain key mediators relevant to the sustainability or destruction of the parasite. We simulated the modulation of TGF-β1, MAPKs, and ROS and conclude that inhibitors of the p38 MAPK were likely to have a significant impact on reduction of parasite burden in the mosquito. An initial, simplified formulation of the mosquito signaling pathways was modeled as a Markov chain process (Fig. 14.1). Transition probabilities, leading ultimately to the probability of the mosquito killing a parasite, were

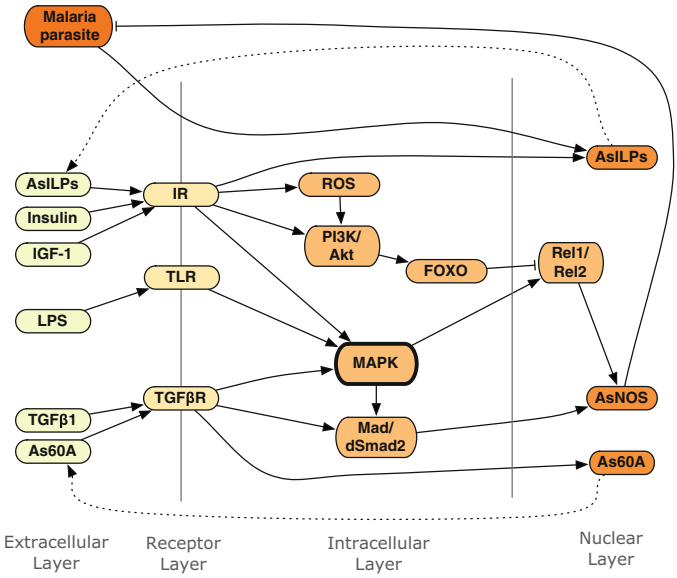

Fig. 14.2 Boolean network model of signaling in the mosquito midgut. Solids lines ending with triangular arrowheads indicate activating influences between elements, whereas lines ending with flat arrowheads (e.g., FOXO to Rel1/Rel2) indicate inhibitory influences. Shaded elements in the nuclear layer - AsILPs, AsNOS, and As60A - represent mRNA forms. Dotted lines represent expression and secretion of the corresponding protein product

estimated through Monte-Carlo simulations calibrated to best fit the observed data under various experimental conditions. For a fixed Markov chain network, the simulations show how transition probabilities change as a function of the initial inputs.

The most complex model includes several layers, starting with receptor signaling on the surface of the cell, through signal propagation and intertwined feedback loops inside the cell, to gene transcription and secretion of several proteins outside of the cell, and ultimately to effects on the *Plasmodium* parasite. At the receptor layer, our model incorporates the activation of the insulin receptor, Toll and related receptors, the TGF-β receptor, as well as signals for which receptors are not yet well characterized. The signal from the insulin receptor is propagated through PI3K- and MAPK-dependent pathways, which activate JNK1/2, ERK, and p38 MAPK and are regulated by feedback loops involving DUSP/MAPK-dependent phosphatase (DUSP/MKP) regulation. Toll signaling regulates JNK1/2, p38 MAPK, and ERK, while TGF-β receptor activation results in MEK/ERK- as well as Smad-dependent signaling. In the nuclear layer, we model regulation of genes encoding As60A and AsNOS as well as a variety of other antiparasite effectors, which ultimately affect the number of parasites in the mosquito midgut.

The described signaling network is implemented as a discrete logical network, which is a generalization of the Boolean modeling approach that has become

popular in recent years [80, 81]. Nodes in the regulatory graph (Fig. 14.2) are modeled as discrete variables, meaning that they take on a finite set of values, usually two or three. Boolean models are those in which all variables are restricted to two values. Directed edges between nodes represent regulatory relationships, which are implemented formally as rules. These rules are used to update values of variables from current-state value to next-state value. Logical operators such as "AND," "OR," and "NOT" are combined with the regulatory variables to form logical expressions, which may become complex in the case of nodes that have multiple regulators such as AsNOS. In our model, most variables have three possible values—(0,1,2)—representing inactivity, low activity, and high activity, respectively. Several elements also have one additional level, which represents regulation by permanent inactivation by overoxidation (as would occur in settings where the production of reactive oxygen and nitrogen species is elevated).

The computational methodology that allows for efficient design of discrete models [82] includes several steps. The first step consists of collecting knowledge about the system, and defining elements of the network and their interactions (activation or inhibition). Next, the interaction network is combined with existing experimental results to design influence tables for network elements. Each element has one such table associated with it, which defines values of the element resulting from combination of values of its regulators. Finally, logic update rules for each element can be derived from influence tables in an automated fashion using existing techniques from electronic design automation [82]. This approach allows for developing rules that implement multiple discrete variable values and complex logic functions that are otherwise difficult to derive manually.

Simulation is performed starting from some initial set of variable values and then sequentially updating the system for a specified number of update rounds or until a steady state is reached. There are two different update procedures, synchronous and asynchronous. The synchronous procedure is deterministic, since in each round of updates all elements are updated synchronously using previous element values, and thus each network state has only one possible next state. The asynchronous procedure on the other hand updates one element at a time in a random order within a round, therefore often leading to multiple possible next states. Typically, the initial state in the simulation represents the resting state of a cell before stimulus is introduced. There are two basic forms of steady states—point attractors and cyclic attractors. If the system reaches one specific state and does not leave that state unless there is an external stimulus, it is said that the system is in a point attractor. If the system cycles through a set of states and cannot leave the cycle without an external stimulus, then the system is said to have reached a cyclic attractor. Both kinds of behavior may be observed in our mosquito system response model. We primarily use asynchronous updates to perform simulations of our model because we are interested in determining the range of behaviors that can occur. Furthermore, there is significant heterogeneity in this system from the number and virulence of the incoming parasites to the complexity and networked behavior of components of the host immune response. In addition to software simulation of logical models described above, and commonly used in the past [80], one can

emulate models in hardware to obtain orders of magnitude speedup in simulating models and collecting results [83, 84].

The logical modeling approach allows for qualitative analysis of the system, which arises from the system topology and logical interactions, as well as to some extent, quantitative characterization enabled by multiple levels of activity of individual elements and by modeling relative timing of different pathways [82]. Since the order of element updates is asynchronous and random, multiple simulations of the model for the same initial conditions must be analyzed to effectively sample the range of behaviors that can occur and to generate accurate statistics on their frequency. The random order of updates most often affects the timing of events, leading to the transient behavior of elements being shifted across different trajectories. In addition to this time shift in transients, larger differences between trajectories can also occur, such as reaching different steady states, which represent different cell fates. An alternative approach to analyzing the dynamics that is based on static analysis is model checking [85], which determines possible outcomes given a specified topology [82, 86]. Model checking allows the modeler to find out if a given model structure is or is not capable of a particular behavior. These behaviors are specified using temporal logic [85].

In our discrete, logical model, we implement a coarse-grained version of several MAPK cascades along with cross talk between them. We define variables for a large number of elements including multiple signaling mediators within the MAPK pathway. Modeling multiple levels of activity of these signaling mediators allows the model to account for different strengths of interaction among model elements. This approach also facilitates modeling of memory (that is, taking into account previous element values when computing new values), which is essential for modeling systems with multiple possible outcomes (cell fates). Preliminary simulation results show that the complexity of the network and its intertwined feedback loops give rise to oscillations in a number of model variables [82]. These oscillations eventually settle to a fixed value, but the duration of the transient behavior varies depending on the stimulation and the initial state of the system. Previous work by Prince et al. [40], who modeled the potential effects of negative feedback between ERK and NOS—a subset of the current model—has shown that such oscillations may be part of an immune strategy that attempts to balance between effective parasite killing and minimizing host damage.

Conclusions

The studies presented here begin to provide insight into some of the conserved, cross-species mechanisms of immune modulation that exist between the mammalian host and mosquito vector. Our studies show that cytokines, chemokines, and growth factors can be retained in the mosquito midgut and function as important signals for the regulation of malaria parasite development and transmission. Notably, these studies highlight a new role for the host blood as a medium for the interface

and biological communication between species, with particular implications for vector-borne diseases.

Ultimately, we seek to modulate the interspecies immune response to infection via the use of transgenic mosquitoes, ideally at the blood feeding interface. Genetically modified mosquito (GMM) vectors have become an attractive option for disease control in the past decade as efforts to eradicate mosquitoes or modulate human immunity to malaria infection have been met with reduced efficacy and other challenges [18–20]. Key to the success of a strategy involving GMMs is ensuring that the modification remains dominant and spreads throughout the population while maintaining the fitness of the mosquito. Recent studies, including our own, have generated mosquitoes with increased and even complete parasite killing but with detrimental effects on fitness [7, 87, 88]. These studies are more descriptive of the phenotype than the underlying mechanism driving it and much remains to be learned about the pathways driving the observed responses. Thus, a systems level understanding of the blood factor-modulated immune response of the mosquito is needed to account for the tradeoffs between parasite killing and mosquito metabolism and fitness. In this chapter, we have discussed our initial computational modeling studies aimed at gaining insight into the complex, cross-species biology that takes place when host factors are transferred to the *Anopheles* mosquito during blood feeding. Though much additional work remains, these initial strides will hopefully drive both novel insights and translational applications.

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Part V
Future Perspectives: Translation to Implementation

Chapter 15
The Rationale and Implementation of Translational Systems Biology as a New Paradigm for the Study of Inflammation

Gary An and Yoram Vodovotz

This book has been both a compendium and a snapshot of a particularly critical and difficult period in basic and applied research. Just as we have described "tipping points" in terms of the biology of inflammation, so too can this concept be applied to the socioeconomic dynamics of biomedical research. While the actual amount invested in funding biomedical research is very high, as we have noted throughout this book the successful translation of that research into clinical therapeutics is not keeping up with these expenditures. Furthermore, despite the increased aggregate amount allocated to biomedical research, for the individual researcher the likelihood of actually obtaining funding is near an all-time low [1].

The question in many minds (especially those of taxpayers and legislators faced with fiscal shortages) is: what are we getting for the money invested in research? This question is especially pertinent given that many recent breakthroughs have been met first with tremendous excitement and optimism, often presented in hyperbolic terms, only to have the reality of the translational dilemma result in the equivalent of a market crash in terms of reproducibility and applicability. Is it any surprise then that the current funders of biomedical research are increasingly challenged to choose what to fund and what to not? Adding to the forward feedback loop of uncertainty is the need (perceived and real) of researchers to "sell" their hypotheses with increasing vigor. This has led to the paradoxical situation in which investigators now must become *advocates* for their hypotheses in order to obtain the means to be able to carry out an investigatory process that is, fundamentally in terms of the philosophy of Science, predicated upon critically applied skepticism.

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We assert that the solution to this dilemma is to focus on *process* (which is, after all, what Science is) in such a way that allows us to be aware of the impermanence and fluidity of "facts." This requires returning the fundamentals of the Scientific Process, of the need to close the iterative loop between correlation and mechanistic causality, with the expressed goal of being able to exercise the skeptical underpinnings of Science and pursue hypothesis falsification.

We and others have suggested that mechanistic computational modeling is just this type of process, that modeling provides a means for researchers to express putative mechanistic hypotheses more formally and completely, and be able to set these mechanisms in action in order to evaluate whether these hypotheses produce plausible and clinically realistic behavior [2]. Used as an exploratory process, integrating researcher intuition and expertise with the increasingly broad data sets generated through new technologies, mechanistic computational modeling may be used to suggest fruitful biological pathways that may be targets for drug development; this is not just the identification of drug candidates, but whether the presumed basis for targeting a particular pathway is even a good idea given the know multiple interactions and feedbacks influencing overall system behavior [3]. Dynamic computational modeling may also augment the search for diagnostic biomarkers by providing the all-important temporal dimension into the characterization of disease/health states [4–7]. Critically, the dynamic component of mechanistic computational modeling may facilitate the identification of paths from health to disease, and (hopefully) back; not just in terms of a series of disconnected data snapshots, but rather in a process in which data snapshots are tied to each other by an actualized biological mechanism. Finally, dynamic computational modeling may serve as a "binding knowledge structure," to identify and represent what it is at a mechanistic and functional level, that ties populations of individuals together [8]. This is true not only for the ability to facilitate the translation of preclinical to clinical situations (i.e., what aspects of mammalian biology can we reliably trust to be similar enough to compare between and translate across species and experimental models) but also to drive personalized medicine, allowing the ability to generate the different characteristics of individuals within a cohort and between cohorts. We suggest that this approach is the only way to achieve the "$N=1$" nature of personalized medicine, where specific therapies and interventions are tailored to individual patient properties and disease dynamics [9].

This book is focused on the acute inflammatory response and its manifestations in sepsis, trauma, and wound healing. The contributors to this book have given their perspectives on systems approaches to these disease states and have shown specific examples of the translational utility of mechanistic computational modeling. Though this book covers over a decade of progress, these are still early days in this field. The central challenge remains integrating the multiscale, multisystem nature of acute inflammation into computational models that drive actionable outcomes. Translational Systems Biology must rise to the challenge of integrating inflammatory, neuroendocrine, and physiologic processes in order to unravel the multidimensional, multicompartment, and highly dynamic disease landscape.

How can these goals be met, especially in the broader context of a biomedical research enterprise that—despite calls for increased use of systems and computational biology—is still overwhelmingly focused on reductionist research focused

on single molecules, along with a clinical regulatory infrastructure that—despite similar call for the incorporation of computational modeling—is still focused on single therapeutic targets? We discuss how to implement Translational Systems Biology in the context of the work presented in this book.

An early entry point for Translational Systems Biology was the *in silico* clinical trial (see Chap. 9). This methodology, in some form or another, is beginning to inform the design of actual clinical trials by the pharmaceutical industry. However, the drugs being tested *in silico* continue to be developed by some variant or another of a painstaking, slow, and expensive process that has not, at the very least, facilitated the concept of "fail early, fail often, and fail cheaply" that underlies successful drug development [10]. In this context, the value of *in silico* clinical trials may well rise dramatically if mechanistic computational modeling could be employed at the earliest stages of drug development, ideally being part of the initial process of weeding out the large number of potentially druggable compounds obtained via high-throughput screens. Moreover, this approach—especially incorporating quasi-mechanistic data-driven modeling—has the potential to suggest disease biomarkers simultaneously with drug targets. For example (see Chap. 8), statistical analyses, hierarchical and *k*-means clustering, Principal Component Analysis, and Dynamic Network Analysis suggested the chemokine MCP-1/CCL2 and IL-1α as central coordinators of hypoxia-induced inflammation in mouse hepatocytes based on a screen of nearly 20 inflammatory mediators assessed over multiple time points. This finding led to the hypothesis the MCP-1 was a central coordinator of hepatic inflammation. In support of this hypothesis, hepatocytes from MCP-1-null mice had altered dynamic inflammatory networks. Importantly, circulating MCP-1 levels segregated blunt trauma survivors from nonsurvivors. Furthermore, patients with elevated early levels of MCP-1 postinjury had longer total lengths of stay, longer intensive care unit lengths of stay, and prolonged requirement for mechanical ventilation. This study identifies MCP-1 as a main driver of the response of hepatocytes in vitro and as a biomarker for organ damage in a clinical setting and suggests an experimental and computational framework for discovery of novel clinical biomarkers in inflammatory diseases (unpublished observations). We speculate that MCP-1 may serve as a therapeutic target in addition to being a potential diagnostic biomarker. In support of this hypothesis, MCP-1-null mice had lower levels of circulating damage markers following experimental trauma/hemorrhage (unpublished observations). Future mechanistic, equation-based computational models (see Chaps. 1, 8, and 11) that incorporate dynamic inflammation networks driven by MCP-1 may thus serve as a basis for *in silico* clinical trials of existing and novel compounds targeting MCP-1.

In silico trials and drug development may also be implemented using agent-based models (Chaps. 3 and 12). As in keeping with the progression of the use of modeling and simulation in other fields, there are successive tiers of validation targets, ranging from face validity and determination of plausibility to near-engineering grade simulations that can be used to augment individual trials. However, the concept of an *in silico* trial need not wait for such high-resolution modeling; as alluded to above the pervasive issue associated with planning control is whether, at a system

level, is a particular pathway even a good idea? As such, the use of agent-based modeling is particularly well suited for dynamic knowledge representation, determination of the sufficiency of existing knowledge structures, and to allow researchers to identify "holes" in their knowledge that can be filled with new experiments and new classes of therapies. The intuitive nature of agent-based modeling makes the use of dynamic computational modeling a bit more accessible to the average biomedical researcher and facilitates the ability of those researchers to visualize and manipulate these "instantiated thought experiments" on their way to greater translational efficiency [11].

Systems-level insights derived from the study of acute inflammation in sepsis and trauma (Chap. 6) are also likely to guide translational advances in settings such as wound healing (Chap. 10) as well as elucidating key host–pathogen interactions in various other diseases [e.g., necrotizing enterocolitis (Chap. 13) and malaria (Chap. 14)]. Ultimately, these developments will likely impact the study of chronic inflammatory diseases, settings in which the crucial dynamics of the disease processes are even more pronounced and manifest as altered dynamic equilibrium states that are even more complicated to reset and control [6, 7].

In conclusion, and as noted above, we believe that the state of biomedical research sits at a crucial tipping point where the continued credibility of scientific claims is at stake, driven by the manifest desires of an increasingly wishful public but in a form that may, at times, lead to disappointment [12]. We suggest that a future path predicated upon returning to scientific fundamentals, with an emphasis on inherently dynamic processes, is a means of backing away from the credibility cliff and onto the ground of rationally grounded expectations and clinically relevant progress.

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