Progress in Inflammation Research
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Paola Mina-Osorio *Editor*

Next-Generation Therapies and Technologies for Immune-Mediated Inflammatory Diseases

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Preface

As our understanding of immune-mediated chronic inflammatory diseases (IMIDs) grows, it becomes more and more clear that these conditions result from the convergence of a multitude of pathogenic mechanisms whose relative individual contribution is different in different patient subsets. As a result, patients suffering from these diseases present with diverse phenotypes that we artificially pool together for the investigation of therapeutic efficacy.

Basic researchers have concentrated their efforts in modeling complex human disease in vitro and in animals. Even though this practice has indeed led to important discoveries trailed by the development of breakthrough medicines that curve the inflammatory cascade responsible for some of the most important signs and symptoms of IMIDs, none of these medicines constitute a cure. This fact supports the prevailing idea that targeting multiple pathways while "resetting" the immune system is a requirement to achieve truly curative therapies.

Promising new technologies have been conceived that address the hypotheses that targeting multiple pathways simultaneously, selectively delivering therapeutics to areas of inflammation and/or resetting the immune system, could take efficacy to new levels. However, we have long waited for the arrival of some of these technologies to the bedside, or even far enough in the drug development process in spite of the initial enthusiasm. Some of the examples covered in this book include bispecific antibodies and genomic medicines, microparticles and targeted delivery of drugs to the inflammed vasculature.

Most published reviews and book chapters on novel therapies for inflammatory diseases describe positive attributes of molecules or technologies under investigation and the rationale for developing them into therapeutics. The originality and potential value of this book is not in the description of these targets or technologies from the point of view of their structure or mechanism of action exclusively, but rather, in making an effort to critically address the question of what is needed to move these technologies into the clinic. Has the technology not made it past the preclinical stage and why? Has it already been tested in humans and failed? What are the potential reasons behind those failures? What do experts in each field believe can be done better to increase the probabilities of success?

In addition, I have asked the authors to address the competitive landscape and to summarize clinical studies that have failed in the respective area. I have asked them to talk about the patient populations that would be required for the successful conduction of a clinical trial to test certain molecules, and to proactively share their views regarding both the potential and the drawbacks of targets or methodologies.

This book begins with an opinion piece on disease heterogeneity viewed from the lens of a physician and comparing it with that of a basic researcher. Next is a thorough review of available biomarkers for patient stratification including thoughts on what biomarker research could look like in the future, followed by a chapter exploring the potential of microparticles as biomarkers. The third section of this book contains a chapter on genomic medicine that explores its past, present, and future, evaluating potential costs and routes for success. This chapter is followed by a critical review of bispecific antibodies. Finally, a section exploring "cross-functional" drug development, in which targets and technologies currently used for diseases other than IMIDs are explored as potential targets. This section includes a chapter on selective drug delivery to areas of inflammation which is a concept being explored in oncology, one chapter on bioenergetics and metabolic targets in inflammation, and one chapter on the intestinal microbiome and its therapeutic potential in IMIDs. As a side note, in this section I would have liked to include a chapter about neurological targets, but the more we learn of the tremendous influence of the nervous system on the immune response, the more I realize that such topic would require an entire volume. The book ends by exploring the concept of immune system "resetting" with a detailed review of stem cell technologies in inflammation.

We have come very far since the discovery of the first few immune cell phenotypes a few decades ago. It is time to begin the discovery of the many IMID phenotypes aided by reliable clinical and molecular biomarkers to evolve the term "next-generation therapy" towards a true model of personalized medicine. It is important for the scientific and medical communities to work together in an effort to redefine disease and the way we measure and classify it. It is important to critically review and learn from our failures. I want to express my sincere appreciation to the authors for their contributions.

New York, NY, USA Paola Mina-Osorio

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About the Editor

Paola Mina-Osorio MD, PhD has 11 years of post-graduate experience in inflammation/immunology research. She has had prominent roles in several drug development programs in rheumatoid arthritis, psoriatic arthritis and lupus, including small molecule and biologic drug candidates. At the time of this submission, she was a Medical Advisor at Eli Lilly and Company. She is currently Immunology Medical Director at Regeneron Pharmaceuticals Inc. in Tarrytown, NY, USA.

Part I
Diagnosis, Patient Stratification and Biomarkers

A Tale of Two Worlds

Pharmaceutical Investigators and Clinicians Define "Diagnosis"

Michael D. Lockshin

1 Introduction

This book describes exciting opportunities for drug development for patients with autoimmune illnesses. The opportunities include targeted delivery, genome-based drugs, nanomedicine, microparticles, metabolic targets, microbiome, nervous system control, biomarkers, and stem cells. Answers in any one of these areas may lead to dramatic changes in the care of ill humans.

The chapters present the points of view of scientists engaged in this enterprise. Clinicians like me see the problems differently. Better outcomes—for the patient, scientist, and the clinician—will follow, if and when these viewpoints merge.

The starting point for all clinical research and treatment trials is correct diagnosis. To achieve this, investigators study only patients who meet well-defined inclusion and exclusion criteria—those with "pure" diagnoses—and they assume that information from the "pure" group will apply to excluded patients as well. Clinicians, however, treat patients who have "pure" and "impure" diagnoses. Neither investigators nor clinicians know whether the same mechanisms apply to both groups or whether the treatment algorithms should be the same.

Investigators and clinicians rarely ask: What is a correct diagnosis? How precise must it be? For how long does a diagnosis remain valid? While investigators require clear definitions of diagnoses, clinicians do not. For clinicians, disease symptoms and actionable mechanisms are important. External variables that influence a patient's outcome are important. Diagnosis names are not.

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2 The Many Faces of a Diagnosis

Diagnoses have clear definitions—in medical texts and in study protocols. Texts and protocols assume that any clinician, given the right tools and intellect, can make a correct diagnosis, even in patients with puzzling symptoms. An undiagnosed disease program of the US National Institutes of Health (NIH) is founded on this belief [1]. An Institute of Medicine (IOM) committee concluded that to make a wrong diagnosis is a mark of failure [2]. Correct, precise diagnosis leads to a comprehended mechanism, which leads to a treatment target, the goal of the reigning paradigm. The research priorities announced by granting agencies are to identify the targets [3]. The pharmaceutical industry concurs.

But what if the clinicians are correct? What if precise diagnosis is unimportant? Can research focus on a disease process if a diagnosis is unclear?

Clinicians know that many patients do not have "pure" diagnoses. Yet for administrative purposes—billing, authorization for treatment, and public health requirements—they must assign putatively clear diagnoses to patients, usually by using International Classification of Diseases (ICD) codes. Although investigators believe that a diagnosis name assigned by a clinician has a narrow definition, a clinician knows that it does not. Indeed, the clinician may use the same diagnosis name or code in at least six different situations, only the first of which meets an investigator's needs: when the diagnosis is typical, when it is atypical, when it is similar but better labeled with a different name, when the patient receives treatment thought inappropriate for the diagnosis, when the patient has an unexpected course, and when the patient has a bizarre response to appropriate therapy (Table 1). The investigator studies patients in the first scenario. The remaining five are what a clinician also sees.

These scenarios illustrate the clinical heterogeneity known to clinicians. Are they also known to others? How often are they seen?

3 Heterogeneity

3.1 Prevalence of Heterogeneity

Investigators do know that patients are clinically heterogeneous [5], not surprising when patients have multisystem disease. Theoretical scientists also know that patients are heterogeneous. Patients described by a single diagnosis name can have different genomes, microbiomes, and molecular pathways [6]. Clinicians ask whether each organ system requires a different hypothesis and treatment target [7]. Yet, for studies and for administrative purposes, we group patients by the clinician's clinical diagnoses, not by mechanisms a scientist might prefer.

How prevalent are atypical patients? We surveyed our electronic databases to identify patients who carried an assigned (ICD 9 or 10) diagnosis but did not fit established diagnostic or treatment guidelines. Patients were atypical because they had overlapping autoimmune diagnoses or because they did not fit diagnosis or treatment guidelines.

Table 1 Different scenarios to which a clinician may assign the same diagnosis. Only the first scenario is acceptable for inclusion of a patient in a clinical study or trial

Scenario	Definition
Typical diagnosis	Typical symptoms, signs, tests, and treatment algorithms; their disease evolves in expected ways; outcomes are predictable. These patients are eligible for, and are recruited into, clinical studies
Atypical diagnosis	Atypical symptoms, signs, or laboratory tests that do not "fit criteria," such as having anti-Smith or anti-Ro/SSA antibody instead of anti-DNA in lupus patients. They are excluded from trials
Different diagnosis	Patients who meet inclusion criteria for a diagnosis but have a second, overlapping disease that permits a different diagnosis, for example, patients with "rhupus" [4]. Whether to include these patients in clinical studies is a choice made by the investigator
Different treatment	Patients who have typical diagnoses (first scenario above) but cannot receive conventional treatment. They will not participate in clinical studies
Different course	Patients who have typical diagnoses but who evolve in unexpected ways. How these patients should be analyzed, and for how long one should consider a diagnosis valid, remains unknown
Bizarre response to therapy	Patients who have typical diagnoses but who do not respond to standard therapy or respond in bizarre ways. Patients often participate in trials because they have failed conventional therapy. We do not know whether they differ from those who responded well or from those whose treatment has not yet begun

Table 2 Different ways in which overlapping autoimmune rheumatic illnesses present

Type of overlap	Definition
Simultaneous	Two or more diagnoses that each fit diagnostic criteria, such as systemic lupus and rheumatoid arthritis ("rhupus")
Evolving	Patient has typical illness of one diagnosis that over the years evolves to typical illness of another, for instance, lupus that becomes rheumatoid arthritis
Non-rheumatic	Systemic rheumatic autoimmune disease in combination with a non-rheumatic autoimmune illness, such as multiple sclerosis, myasthenia gravis, Crohn's disease, and Hashimoto thyroiditis
Undefined	Objective autoimmune illness, such as polyarthritis and positive antinuclear antibody, without diagnostic features like rash, nephritis, or anti-DNA antibody

3.2 Heterogeneity Due to Overlap

Many patients have more than one autoimmune diagnosis (overlapping autoimmune diseases). A study of 1,321 consecutive patients seen in our center found that 424 (32%) with any autoimmune rheumatic disease diagnosis had other overlapping autoimmune diagnoses. These included 39% of patients with lupus, 30% of those with rheumatoid arthritis, and 51% of those with Sjögren's syndrome. Overlap occurred in four patterns: simultaneous, evolving, overlap with non-rheumatic autoimmune illness, and undefined autoimmune illness (Table 2).

3.2.1 Simultaneous

Simultaneous overlap occurs when two or more clinically typical rheumatic disease diagnoses are simultaneously present. The most common pattern is rheumatoid arthritis and lupus together, which is frequent enough to have an (unofficial) name, "rhupus." We diagnosed "rhupus" in 4% of patients who carried a diagnosis of lupus and 2% of those who carried a diagnosis of rheumatoid arthritis. Another pattern manifests simultaneous symptoms of lupus, scleroderma, and dermatomyositis, has a specific autoantibody (anti-U1 RNP), and is called mixed connective tissue disease (MCTD) [8].

3.2.2 Evolving Overlap

Patients who begin with one clear-cut diagnosis then change to another have evolving overlap. Examples are patients who first came to medical attention with clinically and serologically typical lupus, went into remission after several years, had recurrence as serologically and clinically typical rheumatoid arthritis, then remitted again, and had recurrence after another decade. This pattern occurred in 2% of patients in our study.

Though rare, these patients can teach us something important about mechanisms of disease, the durability of a diagnostic label, and the duration of time a mechanistic hypothesis is valid.

3.2.3 Overlap with Non-rheumatic Autoimmune Illness

Many patients with autoimmune rheumatic illnesses have an overlapping non-rheumatic autoimmune illness. Hashimoto's thyroiditis occurred in 10% of our patients; others had inflammatory bowel disease, multiple sclerosis, myasthenia gravis, and other diagnoses. Overall, 16% of patients with lupus, 16% of those with rheumatoid arthritis, and 22% of those with Sjögren's syndrome had a non-rheumatic overlapping autoimmune diagnosis

3.2.4 Overlap That Does Not Meet Criteria

Some patients have clear-cut autoimmune phenomena clinically and serologically but have no criteria-recognized diagnosis. Such patients have undifferentiated connective tissue disease (UCTD) [9]. These patients constituted 14% of all of our patients with systemic rheumatic autoimmune illness.

Table 3 518 consecutive patients with autoimmune diseases who can ("pure") and cannot ("atypical") be treated according to established treatment guidelines and the reasons why

Status	No.	%
"Pure" diagnosis, treatable by guidelines	299	57.7
Atypical diagnosis or cannot be treated by guidelines	219	42.3
Multiple diagnoses	77	14.8
Cancer	25	4.8
Not cancer	52	10.0
Pregnant	41	7.9
Rare diagnosis without guidelines	42	8.1
Uncertain diagnosis	23	4.4
Miscellaneous	17	3.3
Dialysis or transplant	9	1.8
Too disabled	5	1.0
Insurance does not cover treatment	3	0.6

3.3 Heterogeneity Due to Inapplicable Diagnosis and Treatment Guidelines

A different analysis included 518 consecutively seen patients with any named systemic rheumatic autoimmune illness. Of these, 42.3 % had comorbidities, pregnancies, or other complicating factors that would preclude their participating in a clinical study or treatment trial (Table 3).

4 Physician Inconsistency

4.1 Inconsistent Diagnoses

Doctors differ in their methods of taking histories and requesting laboratory tests; patients differ in the ways in which they explain symptoms; and laboratories differ in the methods by which they perform and report tests. Hence different doctors, looking at the same patients, can make different diagnoses and treatment decisions. Clinical studies and treatment trials rarely consider pre-study selection bias that may affect the population they recruit. Studies that identify patients by ICD codes ignore that physicians often upcode their chart records to preempt payment denials. Multicenter studies and central laboratories minimize bias; studies that reflect the recruitment choices of a small set of clinicians are suspect.

Table 4 Different scales of time and how they affect our understanding of the biology, treatment, and outcome of patients with chronic illnesses

Type	Example	Biology	Treatment	Outcome
Instant	Seizure	Electrical dysregulation	Anticonvulsant	Possible anoxic damage
Clock	Fever	Likely inflammatory; IL-1, IL-6, TNFα	Antipyretics	Likely none unless sustained, then cachexia
Calendar	Polyarthritis	Likely inflammatory; IL-1, IL-6, TNFα, and many others	Anti-inflammatories and DMARDs	Joint destruction
Generational	Heart valve disease	Inflammatory plus cicatricial? PDGF, TGFβ	Uncertain, antifibrotics? Tyrosine kinase inhibitors	Organ failure

4.2 Inconsistent Treatment

Because most studies are not inception studies, prior treatment may influence the outcome of interest, but physicians are inconsistent in their treatment choices. A recent paper found that only 27.3 % of expert rheumatologists agreed about treatment for serious thrombocytopenia in lupus patient, and only 36.4 % agreed about treatment for constitutional symptoms [10]. Another paper found inconsistency in clinical practice guidelines [11]. Pre-recruitment treatment differences are points rarely considered when patients are enrolled in studies.

5 Effect of Time

Physicians who treat patients with autoimmune or any chronic illness work simultaneously in four time scales: instant (minutes), clock (hours), calendar (weeks or months), and generational (decades) time (Table 4).

These scales apply to interpretation and prediction of symptoms, laboratory findings, damage, and outcome. The time scales may apply to individual patients simultaneously. For instance, at a single point in time, a lupus patient may suffer a seizure (instant time scale), fever (clock), destructive polyarthritis (calendar), and dementia (generational). The choice to use corticosteroid in an acute (clock) situation may result in osteonecrosis years later (generational).

Each time scale entails its own mechanisms, targets, treatment, and outcome, which may or may not agree. It is improbable that the same target will be valid for all the scales. Most clinical studies focus on only one time scale, while either the

Table 5 Proposed additional scalable variables for predicting outcomes in patients with SLE

Variable
Cytokine markers
Gene markers
Current and past medications
Comorbidities other than pregnancy
Pregnancy
Illness duration
Family history
Health habits, including diet, exercise, and sleep
Tobacco use
Substance abuse
Disabilities, such as being nonambulatory
"Compliance," including attitude to physicians and medical infrastructure, collaborative aspects of personality, medical beliefs (including alternative and non-Western medicine), and religion
Social strength, including wealth, insurance coverage, support systems, domicile arrangements, travel time to appointments, and country of birth including language spoken and traditions

patient or the physician may prefer another. The choice of scale will dictate what the clinician prescribes and what the outcome will be.

6 Variables Not Considered in Disease Activity, Severity, and Damage Scales

The SLEDAI, SELENA-SLEDAI, LAI, and BILAG are a few of the disease activity scales used for studies of lupus [12]. Clinically based, they score the symptoms, signs, and laboratory findings that are easily obtained in routine care. Some of the scales score variables dichotomously, present or absent; others use semiquantitative or quantitative scores. There are no rules for establishing disease activity scales; they only roughly agree among themselves [13]. All weigh clinical activity and damage scores. Some studies that use these scales also stratify patients by race, sex, age, and socioeconomic variables [14–18].

Other variables affect treatment response. They can be given weights. But they are never included in clinical studies and treatment trials (Table 5).

Every clinician is familiar with patients who disagree with and reject recommended treatment and with others whose personal circumstances (finances, comorbidities, fertility desires) preclude that treatment. Patients with new onset disease differ from those with long-standing disease, but only a few studies examine only inception cohorts. The items listed in the table affect outcome and can be quantitated in clinical trials. Clinicians know, but investigators often ignore, that treatment

Table 6 Some reasons why mouse experiments do not parallel human experience

The problem with the mouse
Mice are never illegal immigrants
A mouse bred to have a specific diagnosis will not also have comorbid illness as well
Most mice studied have not been ill for the mouse equivalent of 40 years, nor have they been treated with different and now obsolete treatments throughout that time
Mice that become ill are not treated to recovery and tested once again. In fact, a mouse that becomes ill is usually killed
A mouse does not have a problem being a single mom
Mice do not refuse to take a drug because it causes weight gain
Mice do not take birth control pills
They do not try herbal remedies that their cousins recommend
Most mice who are ill will not have just returned from a 3-week vacation in Thailand
Mice do not take three trains to clinic. In fact, doctors make house calls to them
Mice are not on food stamps. In fact, they seldom go hungry
Mice do not have insurers who refuse to pay. In fact, mice are never uninsured
Mice are never homeless

choices and outcomes reflect not only the biology but also the sociology/environment of a patient.

7 The Problem with Mice as Proxies for Human Disease Measures

The predictability of studies on experimental animals is a reason for the belief that the biology of illness is its primary determinant of outcome. But mice are not subject to the socioeconomic and environmental variables of humans and, hence, are poor proxies for outcome of human illness (Table 6).

8 What Is Precise Diagnosis?

A common way to classify illness segregates human ills into these scientific domains: genetic, infectious, neoplastic, autoimmune, degenerative, deficiency, and trauma/poisoning. The level of molecular understanding of disease mechanisms differs among these domains. If one assigns rank to these domains, such that prevention constitutes the highest level of understanding, some infectious and nutritional deficiency diseases have achieved the highest rank; genetics and oncology, in which one can envision cures, have achieved the next highest. These domains enjoy definitive, targeted interventions (Fig. 1).

The level of understanding is lower for autoimmune and degenerative diseases, which are still diagnosed by clinical history, physical examination, and laboratory

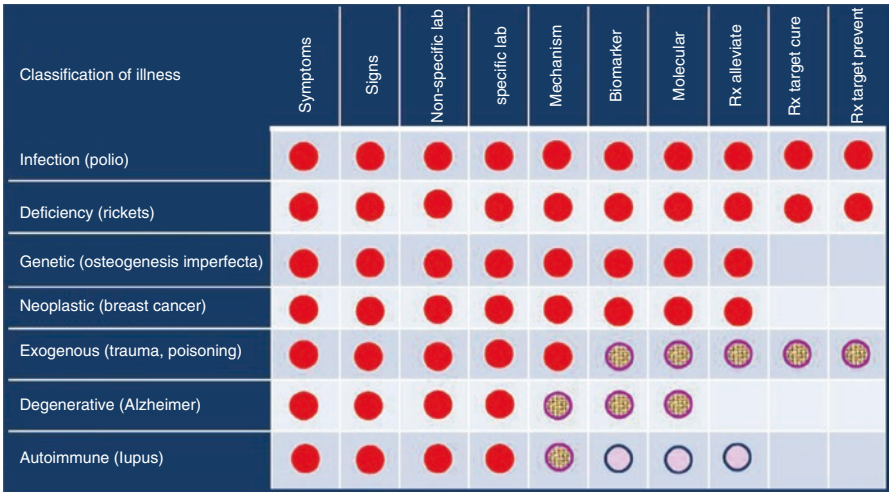

Fig. 1 Degree of advancement of science in different classifications of illness. *Red* indicates findings well advanced in the listed level of science; hatched indicates non-definitive progress in these areas. *Light pink* indicates initial studies began with no actionable findings to date. *Blank* means no active research

tests of inconstant specificity. The mechanisms and biomarkers currently under investigation remain speculative. Clinical management is dictated by disease activity indices, not by biomarkers.

9 Proposals

Clinical studies today began with a physician's *clinical* diagnosis: signs, symptoms, and serological tests. *Molecular* diagnosis—genes, cytokines, and cell surface markers—is possible in some fields, but not for autoimmune disease. Molecular diagnosis defines targets for therapies. The paradigm is: Interfere with the target's function and cure the disease.

So long as we diagnose autoimmune diseases by clinical criteria and define treatment success by clinical measures, we do not target molecules. Many patients' illnesses are not "pure"; such patients may be included or excluded from trials. The potential molecular targets that are available apply to subsets of patients; targeted therapeutic molecules block the targets' functions only partially; because the targets are functionally broad, unacceptable side effects ensue. Hence targeted therapy has limited use in systemic autoimmune illness. Regarding outcome, when we aggregate scales of organ system injury, we ignore the clinical heterogeneity, exogenous factors, and time scales that alter the results.

We can design our studies and treatments more effectively than we do now.

9.1 Stratify Patient Populations by Disease Process, Not by Diagnosis

Today physicians and the public, payers and administrators, and the pharmaceutical industry identify patients according to clinical diagnoses made by practicing physicians. A better way would be to identify patients by biomarkers or by understood disease mechanisms. A better way would be to target interventions to those biomarkers or mechanisms, in the subset of patients for whom the marker is valid, not apply them to all persons who carry a clinician's diagnosis.

Today we measure improvement by aggregated clinical activity and damage scores and consider "20 %" or "50 %" improvement to be a mark of success. It will be more efficient to measure short-term response of a biomarker as a proxy and to defer a final conclusion about the validity of that proxy to definitive, but future, clinical outcome measurements made in calendar or generational time. We should use simple, easily measured, outcome measures, such as organ failure, disability, or death. We should assess success by outcomes of single organ systems, not by scales that aggregate organ systems. In measuring outcomes, we should stratify patients according to the exogenous variables of Table 5 [19].

9.2 Seek Larger, Simpler Databases

Because autoimmune rheumatic diseases are uncommon and heterogeneous, clinical analyses require populations large enough to support complex multivariate analyses. Trials that are national or international in scope, with uniform and simple entry criteria, and equally simple outcome criteria, are required.

Assigning diagnoses by mechanism, adjusting for biases of patient recruitment, measuring outcome simply, and requiring large, less complex trials—these are concepts that constitute revolutions in physician collaboration and in study design; they require assent of the public, clinicians, scientists, pharmaceutical industry, and regulatory agencies, a difficult but necessary task [20].

Of course it is possible that an unanticipated cure will arise from today's science. More likely, because of the large number of clinical variables, because known biological targets apply to a subset of persons with autoimmune diagnoses, and because we measure amelioration rather than cure, our small-scale studies will remain incremental. We must not rely on serendipity. If we restructure our concepts of diagnosis, if we target interventions to subsets of patients likely to respond, and if we consider both the exogenous variables that affect outcome together with the endogenous, we will be able to move ahead.

References

1. Gahl WA, Wise AL, Ashley EA (2015) The undiagnosed diseases network of the National Institutes of Health. A national extension. JAMA 314:1797–1798

2. Balogh EP, Miller BT, Ball JR (eds) (2015) National Academies of Sciences, Engineering, and Medicine, Improving diagnosis in health care. The National Academies Press, Washington, DC, 369 pp
3. http://www.lupusresearch.org/lupus-research/for-researchers.html#.Vi-Iv6TTR_N. 17 Nov 2015
4. Li J, Wu HH, Huang X et al (2014) Clinical analysis of 56 patients with rhupus syndrome: manifestations and comparisons with systemic lupus erythematosus. A retrospective case–control study. Medicine (Baltimore) 93(10):e49. doi:10.1097/MD.0000000000000049, Published online 2014 Aug 22
5. Mina-Osorio P (2015) Basics of drug development in rheumatology. Arthritis Rheum 67:2581–2590. doi:10.1002/art.39253
6. Cho JH, Feldman M (2015) Heterogeneity of autoimmune diseases: pathophysiologic insights from genetics and implications for new therapies. Nat Med 21:730–738
7. Merrill JT (2015) Is SLE many single-organ diseases or an overlapping spectrum? Nat Rev Rheumatol 11. doi:10.1038/nrrheum2015.61
8. Lockshin MD, Levine AB, Erkan D (2015) Patients with overlap autoimmune disease differ from those with 'pure' disease. Lupus Sci Med 2(1):e000084. doi:10.1136/lupus-2015-000084, PubMed PMID: 25973214, PubMed Central PMCID: PMC4422903, eCollection 2015
9. Carpintero MF, Martinez L, Fernandez I et al (2015) Diagnosis and risk stratification in patients with anti-RNP autoimmunity. Lupus 24:1057–1066. doi:10.1177/0961203315575586
10. Mosca M, Tani C, Vagnani S et al (2014) The diagnosis and classification of undifferentiated connective tissue diseases. J Autoimmun 48–49:50–52. doi:10.1016/j.jaut.2014.01.019, PMID 24518855
11. Muangchang C, van Vollenhoven RF, Bernatsky SR et al (2015) Treatment algorithms in systemic lupus erythematosus. Arthritis Care Res 67:1237–1245. doi:10.1002/acr.22589
12. Tunnicliffe DJ, Singh-Grewal D, Kim S et al (2015) Diagnosis, monitoring, and treatment of systemic lupus erythematosus: a systematic review of clinical practice guidelines. Arthritis Care Res 67:1440–1452. doi:10.1002/acr.22591
13. Thanou A, Chakravarty E, James JA, Merrill JT (2014) Which outcome measures in SLE clinical trials best reflect medical judgment? Lupus Sci Med 1(1):e000005. doi:10.1136/lupus-2013-000005, eCollection 2014
14. Romero-Diaz J, Isenberg D, Ramsey-Goldman R (2011) Measures of adult systemic lupus erythematosus. Arthritis Care Res 63:s37–s46. doi:10.1002/acr.20572
15. Lockshin MD (2016) The prince at the ruined tower: time, uncertainty, & chronic illness (in preparation)
16. Katz PP, Barton J, Trupin L et al (2015) Poverty, depression, or lost in translation? Ethnic and language variation in patient-reported outcomes in rheumatoid arthritis. Arthritis Care Res (Hoboken). doi:10.1002/acr.22748 [Epub ahead of print]
17. Feldman CH, Yazdany J, Guan H, Solomon DH, Costenbader KH (2015) Medication nonadherence is associated with increased subsequent acute care utilization among medicaid beneficiaries with systemic lupus erythematosus. Arthritis Care Res (Hoboken). doi:10.1002/acr.22636 [Epub ahead of print]
18. Yazdany J, Trupin L, Schmajuk G, Katz PP, Yelin EH (2014) Quality of care in systemic lupus erythematosus: the association between process and outcome measures in the Lupus Outcomes Study. 15. BMJ Qual Saf 23(8):659–666. doi:10.1136/bmjqs-2013-002494, Epub 2014 Mar 10
19. Yelin E, Yazdany J, Tonner C et al (2015) Interactions between patients, providers, and health systems and technical quality of care. Arthritis Care Res (Hoboken) 67(3):417–424. doi:10.1002/acr.22427
20. Mikdashi J, Nived O (2015) Measuring disease activity in adults with systemic lupus erythematosus: the challenges of administrative burden and responsiveness to patient concerns in clinical research. Arthritis Res Ther 17:183. doi:10.1186/s13075-015-0702-6

Biomarkers in Clinical Trials for Rheumatoid Arthritis

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1 Introduction

Rheumatoid arthritis (RA) is a chronic multisystem disease that is characterized by variable inflammatory involvement of joints with the subsequent destruction of cartilage and bone. Because of advancements in biomedical technology, new treatments have been developed and have improved patient outcomes considerably in the last 20 years and now include targeted disease-modifying therapies. However, considerable heterogeneity exists between patients in their clinical manifestations, disease course, and response to newer agents. These differences have led some investigators to conclude that rheumatoid arthritis is comprised of a group of disorders with apparent differences in their clinical phenotype and genetic expression that may variably impact their clinical responses to medications [36].

Clinical trials for patients with rheumatoid arthritis also have evolved considerably since the first few US Food and Drug Administration (FDA) approvals of therapeutic agents for RA. The changes that have occurred may be due in part to the increasingly competitive clinical trial landscape, technological advances, and the requirements imposed by regulators over time [41]. Progress in RA clinical trials

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has resulted in more robust clinical trial designs, more appropriate characterization of target subpopulations and more clinically meaningful disease assessments. Nevertheless, evidence-based treatments available for patients with RA have yet to achieve sustainable remissions for the majority of patients [17]. Instead, novel treatments are needed.

Biopharmaceutical companies and others are interested in making go/no go development decisions sooner to enroll trials faster and make effective treatments more available to patients with unmet medical needs. Unfortunately, unless there is a shift in our current approach to research, greater numbers of RA patients will be needed to properly conduct all of the clinical trials currently being planned or underway. Given the complexities of RA diagnosis, assessment, and treatment, the need for sensitive and specific biomarkers is critical. Biomarkers that can help effectively diagnose disease are important as many patients are only diagnosed once permanent damage has started and the time for optimal treatment may have passed [23]. Biomarkers may be necessary to further advance drug development for RA to achieve sustained remissions in disease activity. Biomarkers identify more homogeneous RA populations and allow insight to be gained into individual patient responses. Current biomarkers in RA are diverse and include acute phase reactants, autoantibodies, cell subsets, synovial immunohistochemistry, genetic markers, gene expression markers, cytokines, and growth factors that might be used for diagnosis, prognosis, treatment response, determination of remission, and induction of tolerance. Herein, we will explore some of the biomarkers that have been identified for RA and their current use in clinical trials and discuss important considerations for advancing biomarker detection and utilization in the near future.

2 What Are Biomarkers?

A 2001 joint publication of the FDA and the National Institutes of Health (NIH) has clearly defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention” [5]. This canonical definition contains two critical components; the first is the biological parameter to be measured, and the second is the application of that measurement to a clinical decision or outcome. What is also implied here is that there is a sound method to measure the biomarker. An effective biomarker must be validated for both the robustness of the assay and the utility of the marker. Biomarker assays typically are developed in the lab and tested for assay robustness first in lab models followed by testing in relevant human populations. Testing the utility of the assay may have to be conducted in multiple clinical settings to ensure it will answer the question(s) posed.

Biomarkers can be used in a variety of ways. They may be used to confirm diagnosis of disease and disease stage (remission to severe RA) and to provide a prediction of response to therapy and disease prognosis. These types of markers can be used to stratify patients going into a clinical trial. Response biomarkers are used to monitor the treatment effect of either an approved drug or experimental treatment. Response biomarkers also can be used during clinical trials to help understand drug

Table 1 The definition and characteristics of stratification and response biomarkers

Biomarkers in clinical trials and clinical practice	
Stratification biomarkers	Response biomarkers
Clinical trials: measured before entry into a clinical trial and used to include or exclude patients and/or balance treatment arms	Clinical trials: typically measured at time zero and one or more times during the clinical treatment. Changes are compared to baseline
Patient care: used for patient diagnosis and initial treatment decisions	Patient care: used to monitor response to treatment and adjust treatment
Diagnostic—accurately diagnose disease and disease subclass	Pharmacodynamic (PD)—dynamically assess physiological/biochemical effect of treatment; includes understanding mechanism of action (MOA) and target engagement
Prognostic—predict natural course of disease	Theragnostic—monitor progression and/or response to therapy
Predictive—predict likely response to treatment(s)	Surrogate endpoint—substitute for a clinical efficacy endpoint

mechanism of action (MOA) or as a surrogate endpoint (Table 1). In some cases the same biomarkers are used as both stratification and response markers. Developing new biomarkers and taking them from the bench through clinical trials and into clinical practice can be long and challenging. However, the rewards for RA patients may be quite significant in that the clinician's treatment selection is likely to be more precise and overall patient outcomes better.

3 Biomarkers Currently Used in Rheumatoid Arthritis Clinical Trials

Precision medicine is an emerging approach for disease prevention and treatment that focuses on tailoring prognostic and therapeutic strategies to a patient's individual characteristics [9]. Precision medicine hopes to provide "the right dose of the right drug for the right indication for the right patient at the right time" [12] and is based on a full understanding of the patient's disease and the mechanism of different therapies, as well as empirical evidence linking the two to provide effective treatment guidelines. Due to the large degree of heterogeneity in RA, applying precision medicine will be challenging but potentially very rewarding. The disease heterogeneity in RA is a current limitation to the successful conduct of clinical trials because of the need for increased patient numbers to demonstrate benefit and as such can hinder the discovery of effective evidence-based treatments for use in clinical practice.

Many patients seen by rheumatologists, such as older patients and those with multiple comorbid conditions, are often excluded from clinical trials [6]. While broader inclusion criteria (IC) might help to ensure results are applicable to a larger percent of patients, thereby increasing patient heterogeneity in trials, they also may be more likely to produce inconclusive results [20] and are contrary to the endeavor of precision medicine.

3.1 Biomarkers as Inclusion Criteria for Clinical Trials

The use of biomarkers may allow the identification of more homogeneous subpopulations for enrollment into clinical trials. Understanding the basis of disease heterogeneity and stratifying patients based on effective biomarkers allows trials to enroll an enriched patient population and move toward more precise medicinal treatment. This should lead to increased treatment success rates by allowing trials to meet their endpoints with smaller populations, lower costs, and faster timelines [1, 3, 4]. A biological understanding of RA disease heterogeneity will help both the development of new targeted therapies and finding the correct patient subpopulation for the treatment [25] since homogeneous subpopulations in rheumatoid arthritis may be more responsive to particular therapies that target specific factors playing a role in the pathogenesis of disease. As such, having biomarkers that enable the identification and stratification of distinct RA subpopulations that are related by their underlying disease pathogenesis would likely result in clinical trials that are better designed to answer research questions posed and ultimately allow greater discrimination between treatment cohorts. Doing so will also increase the likelihood of identifying drugs that can induce a sustained remission of disease activity.

Classification criteria for the diagnosis of RA have included biomarkers for many years. The ACR/EULAR rheumatoid arthritis classification criteria include four different biomarkers for use as diagnostic criteria: rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and anti-cyclic citrullinated peptide (anti-CCP). As only a subset of RA patients express many of these biomarkers, their use as inclusion criteria risks the exclusion of some RA patients from trial participation due to the lack of a sufficiently sensitive biomarker profile, despite fulfilling the current guidelines for diagnosis of RA via other criteria, which may be both frustrating to investigators and exert a negative impact on recruitment.

A search of Citeline Trialtrove resulted in identification of 359 Phase I to Phase III trials enrolling RA patients that concluded or will conclude between 1 May 2012 and 2030 (3 years of data for ongoing and planned trials) for which details on the inclusion criteria (IC) were available. Of these, 151 (42.1 %) include at least one mandatory inclusion criterion related to biomarkers (see Fig. 1 and Table 2). The use of biomarkers to define the target patient population varies with study phase but is most frequent in Phase I/II and Phase II studies. Acute phase reactants (ESR and CRP) are the most common biomarkers used as inclusion criteria. Among studies using biomarkers, 135 of 148 studies specified a minimum value for at least one of the acute phase reactants. Although most studies provide acceptable ESR or CRP levels for eligibility, some base eligibility on CRP alone. Due to limitations inherent in the use of acute phase reactants as biomarkers, determination of eligibility based on the ESR or the CRP rather than to one or the other may increase the size of the available RA patient pool [46].

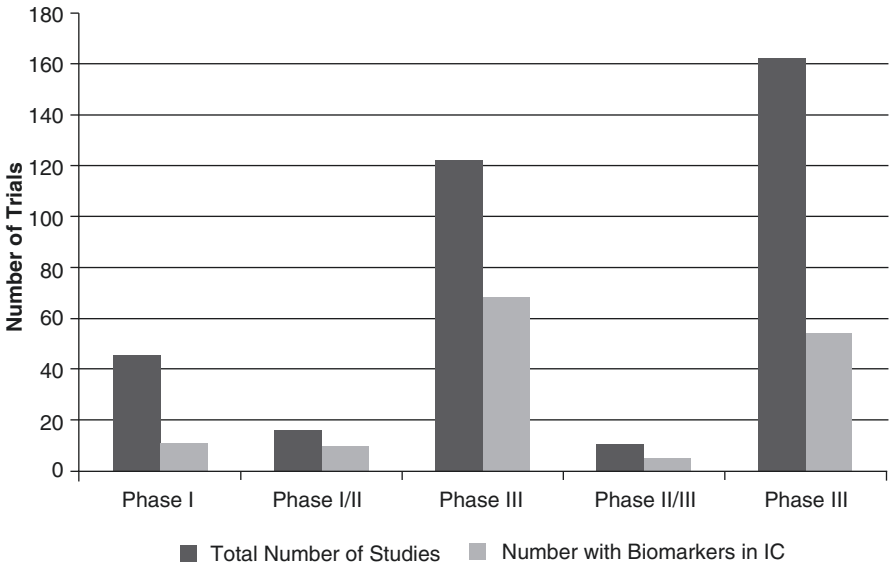

Fig. 1 135 of 359 studies of rheumatoid arthritis included a minimum value of the CRP or ESR as part of the inclusion criteria. *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *IC* inclusion criteria. Use of CRP or ESR in clinical trials for rheumatoid arthritis

Table 2 Summary of biomarkers in recent clinical trials in rheumatoid arthritis

Category	Number of trials	% of total trials
Total number of RA studies	359	–
Total with biomarkers in inclusion criteria	148	41
Total with acute phase reactants[a]	135	38
CRP or ESR	71	20
CRP only	47	13
ESR only	5	1
ESR or CRP or a non-biomarker measure	7	2
Total with anti-CCP and/or RF only	9	3
Anti-CCP[b] or RF	7	2
Anti-CCP only	2	<1
Total with anti-CCP and/or RF in combination with CRP and ESR	50	14

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *anti-CCP* anti-cyclic citrullinated peptide, *RF* rheumatoid factor

[a]Five additional studies had more complex inclusion criteria that did not fit into these categories

[b]Two studies required ACPA positivity

4 Use of CRP or ESR in Clinical Trials for Rheumatoid Arthritis

Evaluation of the inclusion criteria for these studies demonstrated the biomarkers used in study inclusion criteria which were primarily limited to ESR, CRP, RF, and anti-CCP. The frequency of individual biomarkers as a criterion in the dataset is presented in Table 2.

The most common biomarker used to determine patient eligibility was CRP, and the most common minimum required CRP value for eligibility was 1.0 mg/dL, although the range of acceptable CRP levels was wide (0.3–2.0 mg/dL). Approximately 20 % of studies defined acceptable CRP levels based on the upper limit of normal (ULN), rather than as an absolute value. Studies defining acceptable CRP levels in relation to ULN most commonly allowed subjects with a CRP >ULN or > 1.2× ULN. Figure 2 presents the frequency of each minimum CRP value for study eligibility and the upper limit of normal CRP is in Table 3.

Selection of an appropriate biomarker inclusion criterion is a challenge in RA. Inclusion criteria requiring comparatively high minimum CRP values are likely to result in increased screen failure rates, which may delay achievement of recruitment goals and cause frustration for investigators and potential participants. Low minimum values risk inclusion of patients with only low basal disease activity or who may have disease that cannot improve. On the other hand, since the presence of anti-citrullinated protein antibody (ACPA) and their concentration at baseline has been shown to be strongly predictive of radiographic progression, higher values as an inclusion criterion are important in the evaluation radiographic outcomes [18, 37].

Requirements for RF or anti-CCP antibody positivity were present in the inclusion criteria for fewer studies than CRP or ESR; only 59 studies required RF or anti-CCP antibody positivity, primarily in the Phase II and Phase III settings. Interestingly, studies requiring antibody positivity frequently also specified inclusion criteria related to ESR or CRP values (85 %).

5 Diagnostic Biomarkers

Multiple biomarkers have been shown to be useful in confirming the diagnosis of RA, both in clinical practice and in the clinical research setting. Recognition of the value of biomarkers in the diagnostic process is exemplified by the inclusion of both serology (RF and/or anti-CCP) and acute phase reactants (CRP and/or ESR) in the ACR/EULAR 2010 rheumatoid arthritis classification criteria [2]. A diagnosis of definite RA requires evaluation of at least one serological test and one acute phase reactant. Rheumatoid factor and anti-CCP, however, may perform better as diagnostic tests if they had greater sensitivity and specificity. Consequently, there remains a need for additional diagnostic biomarkers with greater sensitivity and specificity than those that have been used to date, as well as biomarkers that allow the identification

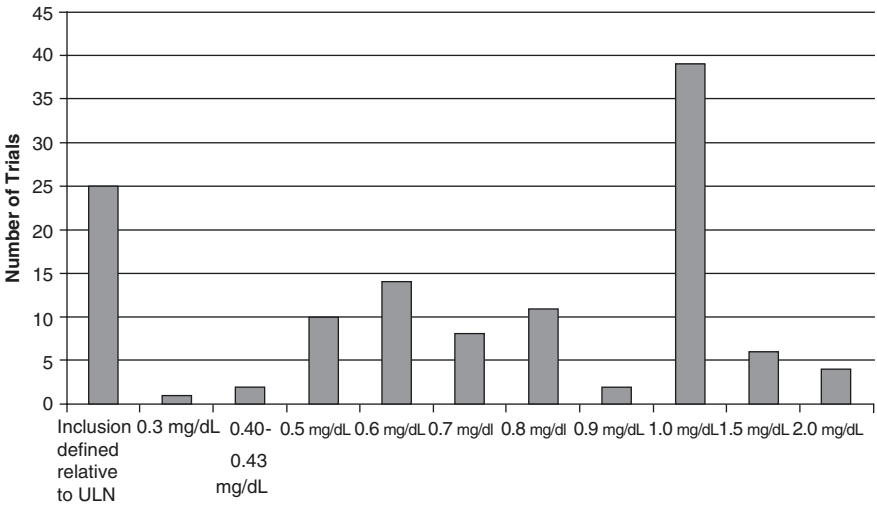

Fig. 2 Minimum CRP value for inclusion. Minimum CRP values required per trial inclusion criteria

Table 3 The C-reactive protein (CRP) upper limit of normal was specified in 25 of the clinical trials inclusion criteria based on the upper limit of normal (ULN)

Required CRP for eligibility relative to upper limit of normal (ULN)	Number of trials
> ULN	11
>1.2× ULN	11
>1.4× ULN	1
>1.5× ULN	2

of more homogeneous subpopulations of patients with rheumatoid arthritis to determine the benefit of therapies to which they are more likely to respond. Novel biomarkers or combinations of biomarkers with better operating characteristics that have been identified may allow research subject stratification within clinical trials, the diagnosis of patients with early disease, and the identification of patients in clinical practice that are more likely to achieve the goals of treatment. However, additional testing and validation in RA are needed.

6 Rheumatoid Factor

Rheumatoid factor, an autoantibody targeting the Fc region of IgG, is among the most widely used biomarkers in RA diagnosis. Although it is widely used and a valuable tool in diagnosis of RA, there are limitations to the use of RF as a diagnostic test.

Table 4 Sensitivity and specificity of RF and/or anti-CCP antibody for the diagnosis of RA

Biomarker	Sensitivity (%)	Specificity (%)
RF	91.7	74.4
Anti-CCP antibody	88.0	90.4
RF + anti-CCP antibody	90.2	83.3

RF rheumatoid factor, *anti-CCP* anti-citrullinated protein antibody

Notably, RF is not specific to RA and is elevated in other immune-inflammatory diseases as well as in certain infections. A meta-analysis that included IgM, IgA, and IgG RF isotypes to assess the diagnostic accuracy of anti-CCP and RF for RA demonstrated qualitative similarity between them [31]. The pooled sensitivity and specificity of IgM RF for RA were 69 % and 85 %, respectively. A small study [19] demonstrated that 70 % of RA patients positive for RF had elevation of two or more isotypes, compared to 16 % of RF positive patients with other rheumatic conditions, and that IgM and IgA RF antibodies in combination were significantly more common in RA patients than in patients with other rheumatic conditions, suggesting that determining the presence of multiple isotypes of RF antibodies may provide increased specificity for RA.

A recent prospective cohort study in Denmark demonstrated that individuals with elevated plasma IgM RF levels are at increased long-term risk of developing RA, and this risk increased with increasing RF levels—a finding that could be beneficial in identifying patients prior to the onset of clinically significant disease [30]. Notably, IgA RF may be present years before the onset of clinical symptoms, although specificity is comparatively low (Rantapää-Dahlqvist 2003).

In 2015, the value of RF with and without coexistent ACPA was assessed for the diagnosis of RA [35]. They evaluated 135 subjects with RA who were outpatients or inpatients over a 1 year period and compared their results to 50 healthy patients who underwent physical examinations in their hospital during the same period. The sensitivity and specificity of RF for the diagnosis of RA were 91.7 % and 74.4 %, respectively, while that for anti-CCP antibody were 88.0 % and 90.4 %, respectively. For the combined detection of RF and anti-CCP antibody, the sensitivity and specificity were 90.2 % and 83.3 %, respectively (Table 4).

7 Anti-citrullinated Protein Antibodies

Anti-citrullinated protein antibodies (ACPAs) are highly specific to RA, a distinct advantage over RF. Anti-CCP is the most common ACPA in the current use and may be present years before the onset of clinical symptoms and may increase in frequency closer to disease onset. Anti-CCP antibodies are present in a greater percentage of RA patients than RF in most settings, the exception being early RA (IgM RF present in 73 % vs. 70 %). Detection of both anti-CCP and RF antibodies prior to symptom

onset has resulted in specificities approaching 100 % [34]. On the other hand, sensitivities of these combinations prior to onset of symptoms remain low (range: 6–52 %), a known limitation of anti-CCP in the diagnostic setting (Rantapää-Dahlqvist 2003). Anti-CCP antibodies have been shown to have the greatest diagnostic performance and have been recommended for consideration as a first-line screening technique [31].

The presence of ACPAs has been associated with a more aggressive disease course than is observed in ACPA negative disease. A study of 454 patients with early RA in the Netherlands demonstrated that although patients had similar symptoms at inclusion, anti-CCP positive patients had significantly higher radiological scores, as well as a larger number of swollen joints after 4 years of follow-up, although the distribution of erosion scores, joint space narrowing, and inflamed joints in the hands was similar between the groups [44].

Although the presence of anti-CCP and RF typically is associated with aggressive disease, recent clinical evidence suggests that this outcome can be modulated in patients with early RA. Data from a randomized, placebo-controlled clinical study in Sweden demonstrated that in patients randomly assigned to receive low-dose prednisolone (7.5 mg/day) or placebo for 2 years, the presence of RF and anti-CCP antibody predicts radiographic progression in only the placebo group [14]. Similar findings were reported from an analysis of data from the Combination Anti-rheumatic Drugs in Early RA (CARDERA) trial, in which 467 patients with early, active disease were assigned to receive methotrexate, methotrexate + cyclosporine, methotrexate + prednisolone, or methotrexate + cyclosporine + prednisolone. Among subjects positive for ACPA, treatment with any of the study treatment options resulted in a statistically smaller change in Larsen score relative to ACPA negative subjects. In contrast, no statistically significant change in Larsen scores for any treatment arm was observed in the ACPA negative group relative to placebo, and, overall, the change in Larsen scores over the 2-year study period was smaller in the ACPA negative group compared to that observed for ACPA positive patients (Seegobin 2014). These studies provide evidence of the potential for diagnostic biomarkers to impact the disease state in patients with RA. In addition to anti-CCP, other citrullinated proteins that may be targeted by antibodies include perinuclear factor, keratin, vimentin, fibrinogen, histones, MBP, type II collagen, and α-enolase. Anti-carbamylated protein antibodies (anti-CarP), including those recognizing homocitrulline, carbamylated fibrinogen, or carbamylated vimentin, also serve as biomarkers for RA, although the sensitivity of these antibodies is lower than that of the ACPAs [10, 15, 29]. Approximately 43 % of patients with RA are positive for IgG anti-CarP antibodies and 45 % for IgA anti-CarP antibodies [39, 40]. Also of note, the presence of anti-CarP antibodies was noted in some patients who were negative for ACPA antibodies and appears to correlate with a more severe disease course [40]. Anti-CarP antibodies may be detectable prior to the diagnosis of RA [40] and, therefore, may have potential utility in identifying patients with early disease.

Table 5 Corresponding disease activity score (DAS)28-erythrocyte sedimentation rate, DAS28-C-reactive protein, sensitivity, and specificity values derived from the receiver operating characteristic curves for each criterion

Criteria	DAS28-ESR	DAS28-CRP	Sensitivity	Specificity
Remission	2.6	2.32	0.921	0.869
Low disease activity	3.2	2.67	0.908	0.893
High disease activity	5.1	4.09	0.925	0.970

Ann Rheum Dis 2007; 66:407–409
CRP C-reactive protein, *DAS* disease activity score, *ESR* erythrocyte sedimentation rate

8 14-3-3

The proteins 14-3-3 eta and gamma have been demonstrated to be elevated in synovial fluid and serum of patients with inflammatory joint disease relative to control subjects [21, 26]. Recent work by Maksymowych et al. [27] suggested a role for 14-3-3η as a potential diagnostic biomarker for rheumatoid arthritis. The authors demonstrated sensitivity of 63 % and specificity of 93 % for 14-3-3η alone as a diagnostic marker for early RA versus healthy controls and sensitivity of 77 % and specificity of 93 % in established RA via an ELISA-based assay. The combination of 14-3-3η, ACPA, and RF was found to have specificity of 78 % in early RA versus 71 % for ACPA and RF alone. However, the sensitivity of 14-3-3η, ACPA, and/or RF was 78 %, as compared to 84 % for RF and/or ACPA alone.

8.1 Disease Activity Biomarkers in RA

Measures of disease activity including the DAS28 (ESR, CRP) and the SDAI use a combination of tender and number of swollen joints and global assessments of disease activity and include the ESR or CRP to produce an overall disease activity score (Table 5).

In addition to the use of biomarkers for confirmation of diagnosis and assessment of disease activity, these and others have recently been used to predict the response to therapy. Understanding the operating characteristics of existing biomarkers and those being studied will enable their application and utilization in the most effective manner possible.

9 Vectra® DA

Recent investigators have evaluated panels of proteins in the assessment of rheumatoid arthritis disease activity. The Vectra® DA blood test integrates the concentrations of 12 distinct protein biomarkers consisting of vascular cell adhesion molecule-1, epidermal growth factor, interleukin-6, tumor necrosis factor receptor

type I, matrix metalloproteinase 1, matrix metalloproteinase 3, human cartilage glycoprotein-39, leptin, resistin, serum amyloid A, and CRP into a single score between 1 and 100 that indicates the current level of RA disease activity based on an algorithm [38]. The numerical score is reported along with a classification of the disease into low (<30), moderate (30–44), and high (>44) disease activity. Currently, the Vectra® DA score in Phase II and III clinical trials is increasingly being utilized as an independent inclusion criterion for disease activity and is being evaluated for response to novel disease-modifying antirheumatic drug (DMARD) therapy as a secondary or exploratory variable.

A recent study evaluating RA subjects with and without fibromyalgia demonstrated similar levels of disease activity between the CRP and a multi-biomarker disease activity score using the same reagents and algorithm as the Vectra® DA score (MBDA), whereas the patient global assessment and the DAS28-CRP were significantly greater [24], suggesting the possibility that it may be a better disease activity measure than some of the parameters currently being used in clinical trials. These findings, however, are not particularly surprising and are consistent with findings in previous studies in which radiographic progression was assessed in relation to DAS28-CRP and MBDA scores [45]. Among subjects who achieved a DAS28-CRP remission, those continuing to have a high MBDA score (>44) were more likely to have joint progression during the subsequent year as opposed to those with an MBDA score in the remission range (≤25). Similarly, another study evaluated the ability of an MBDA score using the same algorithm as the Vectra® DA score at baseline to predict progression in radiographic joint damage in DMARD-naïve early RA subjects in whom a treat to target strategy was being used [28]. The latter study further demonstrated that the MBDA score independently predicted progression in radiographic joint damage and that subjects with higher MBDA scores were more likely to have progression in radiographic joint damage.

10 Validation of Rheumatoid Arthritis Biomarkers

The Outcome Measures in Rheumatology (OMERACT) initiative has worked on validating tools for evaluating the effect of therapeutic interventions in rheumatic diseases since 1992. The OMERACT initiative identified important questions to address with respect to imaging and soluble biomarkers [11]: first, whether the outcome measure relates to the suspected pathophysiological change; second, whether the measure has an agreed and consistent procedure; and third, to what extent operator expertise is a prerequisite. Importantly, it was recognized that while the CRP has been demonstrated to be sensitive to change and to fulfill most of the aspects of truth for therapeutic purposes, insufficient data existed for other proposed soluble biomarkers, and further validation was needed for recommendations to be made.

Recent draft guidance from FDA states that, “Biomarkers can be used for a wide variety of purposes during drug development; therefore, a fit-for-purpose approach should be used when evaluating the extent of method validation that is appropriate.

When biomarker data will be used to support a regulatory action, such as the pivotal determination of safety and/or effectiveness or to support labeled dosing instructions, the assay should be fully validated" [43]. Requirements for validation involve measuring an assay with well-established performance characteristics and agreement on the physiologic, toxicologic, pharmacologic, or clinical significance of the results [13]. Once in the clinic, both analytical validation of the assay (accuracy of the measurement versus a gold standard) in patients and clinical validation (correlation with the clinical endpoint) must be completed.

11 Technological Advancements in Testing for Biomarkers

Several technological and scientific advancements are aiding in both the discovery and development of new therapies and biomarkers. These include sequencing of the human genome and access to next-generation sequencing (NGS), improved technologies for biomedical analysis, and new tools for using large datasets [8, 9]. These trends are affecting all disease areas, including biomarkers for RA.

Sequencing the human genome and NGS has revolutionized the field of genetics and genomics and provides virtually limitless data to investigate the genetic causes of diseases. As these technologies mature, rapidly decreasing costs further enhance their value to drug development. The cost of sequencing a single whole-human exome has dropped well below $5,000, and it continues to fall, although analysis and informatics costs are not figured into that number. Several trends have made the data more available as well, such as an increasing informed and proactive consumer and NGS being directly marketed to consumers. Based on NGS, several disease-associated genes have been linked for rheumatic diseases in both case-control and family-based studies [47], although much work will need to be done to explore whether they are causative variants. Future scientific advancements, including multiple technology platforms and multifactorial testing (multi-gene or multi-analyte signatures), will increase our ability to interrogate the molecular pathways involved in common and complex diseases.

12 Main Challenges in Biomarker Discovery

Due to the progressive nature of RA, an early diagnosis, prognosis, and treatment of the disease are needed, especially in patients without clear manifestation. Early-stage diagnostic and prognostic biomarkers will facilitate clinical practice decisions and selection of the appropriate populations in clinical trials. Strategies to improve the predictive value of biomarkers include combinations of biomarkers and the use of imaging techniques in combination with biomarkers.

Usefulness of biomarkers depends on biomarker discovery, their availability in practice, and their validation at the time of their need. While the use of biomarkers

to understand disease pathophysiology and for diagnostic and prognostic purposes is more direct, strict qualification and clinical validation are a must in order to support approval of medicines. Furthermore, few biomarkers are accepted as a surrogate endpoint. The validation of biomarkers in RA and their cut points is a major challenge and will need coordinated efforts from the regulatory authorities, academia, and industry consortia. The same collaborative approach is needed to demonstrate the translation of the use of a biomarker into actual clinical benefit for the patients. The relationship of biomarkers with relevant clinical outcomes requires large sample sizes and very meticulous observation. Clinical outcomes, patient-reported outcomes, and disability also should be considered to assess the value of a biomarker or a treatment strategy that employs biomarkers in decision-making. New technologies and statistical methodologies are facilitating the discovery of biomarkers at a much faster pace. Their rapid assessment to determine their operating characteristics will be important to advance clinical research. On the other hand, complexity (biomarker panels) may be a barrier to the application of biomarkers, especially if more wide-scale profiling aims to guide medical decision-making. The high costs of testing and limitations of access to new technologies will require the demonstration of significant cost-benefit before they are broadly accepted by multiple stakeholders.

13 Emerging Trends in Biomarkers

Well-organized and agile consortia from academia and industry will be essential to identify and validate new biomarkers. The rapid progress in the fields of biotechnology, genetics, and genomics and their integration in clinical practice and in product development require collaboration from a variety of different stakeholders and disciplines.

Biomarkers will be fundamental tools not only to demonstrate proof of concept but also for establishing the required dose and dose window and improving the effectiveness of classical dose-finding studies based on clinical efficacy and safety. A deep understanding of the molecular basis of disease and dynamics of response to treatment is needed to assess the relationship between pharmacodynamic (PD) effect and downstream clinical effect.

Innovative approaches to increase efficiency in clinical trials are currently being used [42]. Adaptive clinical trial designs aim to introduce flexibility and facilitate decision-making during the implementation of a trial. Practical examples that have been used in other disease indications (e.g., oncology) can be applied to rheumatoid arthritis. An umbrella protocol is designed to allow enrollment of patients into different treatment arms based on their specific biomarker profile within the same indication [22]. Randomization to different drugs, combinations, or dosing strategies can be stratified according to the subjects' biomarker profiles. Biomarkers are the essential instruments that allow a personalized medicine approach to the application of patient-specific profiles based in biomarkers and clinical factors to assess

individual risks and prognosis and to provide tailored prevention and disease-management strategies.

Understanding molecular medicine based on a single biomarker does not address the full picture of the connections and feedback between closely related pathways. As a consequence there is a need to integrate combinations of biomarkers from related pathways to increase the predictive value. The Vectra DA is an example, combining measurements of 12 serum proteins to calculate a multi-biomarker disease activity score. In addition, the integration of different technologies, for example, imaging techniques in combination with biomarkers, may improve early diagnosis of RA particularly in seronegative patients and the assessment of response to therapy.

The application of "big data" to biomarkers in rheumatoid arthritis may yield benefit at three levels: descriptive models to gain information and knowledge, predictive models to better understand what will happen in the future, and prescriptive models to provide recommendations for decision-making. Trial simulations, virtual trials, and strategy trials are additional examples of the potential utility of big data. The inclusion of different biomarkers in the database should facilitate estimation of their usefulness and potential.

Personalized medicine with a biomarker foundation will produce changes in the reimbursement policies. In a heterogeneous disease such as rheumatoid arthritis where numerous expensive drugs are available, personalized medicine would have an impact on drug budgets. Linking research and electronic health records can strategically optimize patient segmentation, clinical development, and health outcomes. Moreover, patient stratification in the real world may enable a medication to increase effectiveness and achieve the reimbursement that would not be achieved in a broader population. Consequently, a new dimension is now highly relevant for biomarkers: how the biomarkers behave across a large number of patients and their effectiveness in real-world personalized medicine.

14 Potential Investment Required for Use in Clinical Trials

Currently, biomarkers fall short of what is needed to change our approach to clinical trials for rheumatoid arthritis. The use of a combination of biomarkers, new technologies, and multidisciplinary approach requires heavy investment. DNA sequence data alone is not enough in complex diseases as rheumatoid arthritis and different data are now of interest beyond DNA sequence: DNA methylations, SNPs, protein-coding RNA, noncoding RNA, histone modifications, transcription factors and their DNA binding sites, transcription start sites, promoters, protein-protein interactions, protein modifications, and metabolites. Investment in these technologies is only the first step since the data they generate require the use of a systems biology approach to data integration.

Additional requirements include investments in tools and resources that allow merging of data from biomarker research with data from health care and clinical

trials as well as investments in informatics systems that enable the analysis of diseases and therapeutic interventions. One example of this is Project Data Sphere (www.projectdatasphere.org) [33], a database that allows researchers affiliated with life science companies, hospitals, and institutions, as well as independent researchers, to share, integrate, and analyze patient-level, comparator arm, Phase III cancer de-identified data.

15 Ethical and Legal Considerations

Respect for human dignity of all individuals voluntarily participating in human research and donating biological materials is mandatory and correct. The four conventional bioethical principles of autonomy, beneficence, non-maleficence, and justice should be ensured. In that respect, research based in biomarkers without careful consideration of the ethical principles may affect those principles when they have an effect on patient selection, access to clinical trials, access to medications, and data privacy. The use of biomarkers has an effect beyond the utility in product development. In the near future, new technologies and cost reductions will make available the whole-genome sequencing as a standard test. The consequences of the generalized use of biomarkers and genetic tests are clear. False positives or false negatives may have an impact in people's lives when it affects prognosis, access to treatments, stigmatization, insurance reimbursement, and work opportunities.

Genetic testing is heavily regulated, but research using nongenetic biomarkers should follow strict procedures as well. Local and international deontological codes, research guidelines, data protection laws, and regulatory directives should be followed in biomedical research. The use of stored biological materials of human origin is a powerful tool in biomarker research. The benefit of this secondary research goes beyond the individual and may improve human health and health-care systems. If stored samples were not used, the alternative prospective is the collection of new biological materials specifically for each project. Nevertheless, this effort would not be feasible in many cases or would be too costly and would take a long time, making unavailable the benefits of research to the health system or delaying those benefits for years. New knowledge brings new hypothesis and induces new uses and analyses of stored biological materials. Despite the controversies regarding the limitations for research, existing regulations regarding the use of stored material in full respect for private life should be considered. In order to find a balance, there are some guidelines in which the use of stored samples may be approved legally and ethically [7]. In cases where the consent for a further use of stored samples is lacking, reasonable efforts to contact the subject to obtain specific consent to use materials and personal data should be taken. If the person concerned cannot be contacted and there is no known objection from the subject, the use of the samples and data may be granted upon independent confirmation that the following conditions are met: the research is of important scientific interest and the expected scientific benefits support the proportionality principle between the rights of the person concerned and those expected

benefits; the objectives of the research cannot reasonably be achieved using other prospectively obtained biological material [7].

Anonymized, non-identifiable biological materials and data also may be used for secondary research use unless such use was not limited by the subject providing the materials and data and does not violate any law or restrictions placed by the person concerned. The objectives and methods of secondary research with non-identifiable data should be ethically evaluated as well.

Big data brings new legal and ethical issues that affect individuals and communities in different ways. Big data generates secondary uses of data from disparate sources, including research, clinical, registry, administrative, claims, and patient-generated data. Protected health information is an individually identifiable information relating to an individual's care or past, present, or future physical or mental health condition or payment for care. Individually identifiable information directly identifies a person or contains information that permits identification, and big data may increase the possibilities to identify individuals. Legal security and breach notification rules apply differently for regulated entities and public administration than for private users. Security measures should be applied to reasonably and appropriately protect electronic records at the administrative, physical, technical, and organizational levels. Information may be de-identified by different methods, including the removal of 18 specific identifiers ("Safe Harbor" method; [32]), or by expert determination that there is minimal risk that information could identify individuals ("Statistical" method; [16]). Nevertheless, de-identified data is not useful for all research and some biomarkers, as genetic information, are considered identifiable information. Disclosure and the use of identifiable patient data is allowed if there is patient consent. But there is a lack of a consistent framework for patient consent, and requirements vary depending on the type of information and intended use. New ways to get patient approvals are needed, and there is a major shift in public perceptions of privacy as social use of the Internet is spreading. Therefore, ethical and legal considerations are expected to change in the future and affect the way research based in biomarkers and share of data will be performed in complex diseases such as rheumatoid arthritis.

16 Conclusion

Rheumatoid arthritis is a heterogeneous, systemic, autoimmune disease that will likely require the identification of more homogeneous subpopulations to achieve the desired treatment goals. Biomarkers in RA may allow earlier diagnosis, the prediction of responses to therapies, and advancements in clinical trial design. Biomarkers should be an essential part of a precision medicine approach that focuses on tailoring prognostic and therapeutic strategies to a patient's unique underlying disease profile. Traditional RA and novel biomarkers offer the potential to advance care especially when combined with robust data linking biomarker signatures to successful outcomes.

References

1. Aleksey M, Natalya L, Polina O, Peter S, Christian H, Juliane C, Bogdanos DP, Lapin SV, Dirk R (2014) Anti-hnRNP B1 (RA33) autoantibodies are associated with the clinical phenotype in Russian patients with rheumatoid arthritis and systemic sclerosis. J Immunol Res 2014:516593. doi: 10.1155/2014/516593
2. Aletaha D, Neogi T, Silman AJ et al (2010) Rheumatoid arthritis classification criteria. An American College of Rheumatology/European league against rheumatism collaborative initiative. Arthritis Rheum 62(9):2569–2581
3. Allinson J, Brooks S (2004) Biomarkers in drug development – a CRO perspective. Curr Sep 21(1):15–19
4. Al-Mughales JA (2015) Immunodiagnostic significance of anti-RA33 autoantibodies in Saudi patients with rheumatoid arthritis. J Immunol Res 2015:604305. doi: 10.1155/2015/604305
5. Biomarkers Definition Working Group (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69:89–95
6. Cameron HJ, Williams BO (1996) Clinical trials in the elderly should we do more? Drugs Aging 9:307–310
7. Casteleyn L, Dumez B, Jamers A, Van Damme K. Ethics and data protection in human biomarker studies. In: Environmental cancer risk, nutrition and individual susceptibility. ECNIS, 2010. Published by Nofer Institute of Occupational Medicine, Lodz, Poland. Website: http://www.ecnis.org/images/stories/ecnis/documents/reports/Ethics_and_data_protection/ethicsand_data_protection.pdf
8. Cheng Y, Chen Y, Sun X, Li Y, Huang C, Deng H, Li Z (2014) Identification of potential serum biomarkers for RA by high resolution quantitative proteomic analysis. Inflammation 37(5):1459–1467. doi: 10.1007/s10753-014-9871-8
9. Collins FS, Varmus (2015) A new initiative on precision medicine. N Engl J Med 372:793–795
10. Conrad K, Roggenbuck D, Reinhold D, Dörner T (2010) Profiling of rheumatoid arthritis associated autoantibodies. Autoimmun Rev 9(6):431–435. doi: 10.1016/j.autrev.2009.11.017
11. D'Agostino M, Boers M, Kirwan J et al (2014) Updating the OMERACT filter: implications for imaging and soluble biomarkers. J Rheumatol 41(5):1016–1024
12. Frueh FW. Personalized medicine: what is it? How will it affect health care? 11th Annual FDA Science Forum. 26 Apr 2005; Washington, DC. Available at: http://www.fda.gov/downloads/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm085716.pdf
13. Goodsaid F, Frueh F (2007) Biomarker qualification pilot process at the US Food and Drug Administration. AAPS J 9(1):E105–E108
14. Hafström I, Engvall I-L, Rönnelid J, Boonen A, van der Heijde D, Svensson B (2014) Rheumatoid factor and anti-CCP do not predict progressive joint damage in patients with early rheumatoid arthritis treated with prednisolone: a randomized study. BMJ Open 4:7e005246
15. Hassfeld W, Steiner G, Hartmuth K, Kolarz G, Scherak O, Graninger W, Thumb N, Smolen J (1989) Demonstration of a new antinuclear antibody (anti-RA 33) that is highly specific for rheumatoid arthritis. Arthritis Rheum 32:1515–1520. PMID:2597207
16. HIPAA Privacy Rule. Other requirements relating to uses and disclosures of protected health information. 45 CFR § 164.514, 2013.
17. Isaacs J, Ferraccioli C (2011) The need for personalized medicine for rheumatoid arthritis. Ann Rheum Dis 70:4–7
18. Jilani AA, Mackworth-Young CG (2015) The role of citrullinated protein antibodies in predicting erosive disease in rheumatoid arthritis: a systematic literature review and meta-analysis. Int J Rheumatol 2015:728610
19. Jónsson T, Steinsson K, Jónsson H, Geirsson AJ, Thorsteinsson J, Valdimarsson H (1998) Combined elevation of IgM and IgA rheumatoid factor has high diagnostic specificity for rheumatoid arthritis. Rheumatol Int 18(3):119–122

20. Kent D, Alsheikh-Ali A, Hayward R (2008) Competing risk and heterogeneity of treatment effect in clinical trials. Trials 22:9–30
21. Kilani RT, Maksymowych WP, Aitken A et al (2007) Detection of high levels of 2 specific isoforms of 14-3-3 proteins in synovial fluid from patients with joint inflammation. J Rheumatol 34(8):1650–1657
22. Kim ES, Herbst RS, Wistuba II, Lee JJ, Blumenschein GR, Tsao A, Stewart DJ, Hicks ME, Erasmus J, Gupta S (2011) The BATTLE trial: personalizing therapy for lung cancer. Cancer Disc 1:44–53
23. Landolt-Marticorena C (2015) The need for preclinical biomarkers in systemic autoimmune rheumatic diseases. J Rheumatol 42(2):152–154
24. Lee Y, Haney D, Alexander C et al (2013) Application of a multi-biomarker disease activity (Vectra® DA) score for assessing rheumatoid arthritis patients with low CRP or fibromyalgia. Ann Rheum Dis 72(suppl 3):A612–A613
25. Lindstrom T, Robinson W (2010) Biomarkers for rheumatoid arthritis: making it personal. Scand J Clin Lab Invest Suppl 242:79–84. doi: "//dx.doi.org/10.3109/00365513.2010.493406"10.3109/00365513.2010.493406
26. Maksymowych WP, Fitzgerald O, Wells GA et al (2009) Proposal for levels of evidence schema for validation of a soluble biomarker reflecting damage endpoints in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis, and recommendations for study design. J Rheumatol 36:1792–1799. doi: 10.3899/jrheum090347
27. Maksymowych WP1, Marotta A (2014) 14-3-3η: a novel biomarker platform for rheumatoid arthritis. Clin Exp Rheumatol 32(5 Suppl 85):S-35–S-39
28. Markusse IM, Dirven L, van den Broek M et al (2014) A multibiomarker disease activity score for rheumatoid arthritis predicts radiographic joint damage in the BeSt study. J Rheumatol 41
29. Nell VPK, Machold KP, Stam TA et al (2005) Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. Ann Rheum Dis 64:1731–1736. doi: "//dx.doi.org/10.1136/ard.2005.035691"10.1136/ard.2005.035691
30. Nielsen SF, Bojesen SE, Schnohr P, Nordestgaard BG (2012) Elevated rheumatoid factor and long term risk of RA: a prospective cohort study. BMJ 345:e5244
31. Nishimura K, Sugiyama D, Kogata Y et al (2007) Meta-analysis: diagnostic accuracy of anti-CCP antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med 146(11):816–817
32. Peddicord D, Waldo AB, Boutin M, Grande T, Gutierrez L Jr (2010) A proposal to protect privacy of health information while accelerating comparative effectiveness research. Health Aff (Millwood) 29(11):2082–2090
33. Project Data Sphere Initiative. www.projectdatasphere.org. doi: 10.1634/theoncologist.2014-0431
34. Rantapää-Dahlqvist S et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48(10):2741–2749. doi: dx.doi.org/10.1002/art.11223"10.1002/art.11223
35. Rongchun Shen BM, Xiaojuan Ren BM, Rongrong Jing MM et al (2015) Rheumatoid factor, anti-cyclic citrullinated peptide antibody, C-reactive protein, and erythrocyte sedimentation rate for the clinical diagnosis of rheumatoid arthritis. Lab Med Summer 46:226–22
36. Romão et al. Old drugs, old problems: where do we stand in prediction of rheumatoid arthritis responsiveness to methotrexate and other synthetic DMARDs?, BMC Medicine 2013;11:17. doi: "//dx.doi.org/10.1186/1741-7015-11-17"10.1186/1741-7015-11-17
37. Seegobin SD, Ma MHY, Dahanayake C et al (2014) ACPA-positive and ACPA-negative rheumatoid arthritis differ in their requirements for combination DMARDs and corticosteroids: secondary analysis of a randomized controlled trial. Arthritis Res Ther 16(1):R13. doi: 10.1186/ar4439
38. Segurado OG, Sasso EH (2014) Vectra DA for the objective measurement of disease activity in patients with rheumatoid arthritis. Clin Exp Rheumatol 32(suppl 85):S29–S34
39. Senolt L, Grassi W, Szodoray P (2014) Laboratory biomarkers or imaging in the diagnostics of Rheumatoid arthritis. BMC Med 12:49. doi: 10.1186/1741-7015-12-49

40. Shi J, Knevel R, Suwannalai P et al (2011) Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci 108:17372–17377
41. Strand, Sokolove (2009) Randomized controlled trial design in rheumatoid arthritis: the past decade. Arthritis Res Ther 11:205
42. Tufts Center for the Study of Drug Development. The adoption and impact of adaptive trial designs. http://csdd.tufts.edu/reports/white_papers, May 2013
43. U.S. Food and Drug Administration. Guidance for industry: bioanalytical method validation. Draft. Sept 2013. Available at: Http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf
44. van der Helm-van Mil AHM, Verpoort KN, Breedveld FC et al (2005) Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. Arthritis Res Ther 7:R949–R958
45. van der Helm-van Mil AHM, Knevel R, Cavet G et al (2013) An evaluation of molecular and clinical remission in rheumatoid arthritis by assessing radiographic progression. Rheumatology 52:839–846
46. Wahab AA, Mohammad M, Rahma M, Said M (2013) Anti-cyclic citrullinated peptide antibody is a good indicator for the diagnosis of rheumatoid arthritis. Pak J Med Sci 29(3):773–777
47. Wiley GB, Kelly JA, Gaffney PM (2014) Use of next-generation DNA sequencing to analyze genetic variants in rheumatic disease. Arthritis Res Ther 16(6):490

The Role of Microparticles as Biomarkers in the Development of Therapy for Autoimmune Disease

David S. Pisetsky

1 Introduction

Autoimmune diseases are a diverse group of conditions that result from abnormalities in immune cell function that culminate in tissue inflammation, destruction, or dysfunction [1, 2]. These conditions can cause highly discrete tissue involvement as exemplified by organ-specific autoimmune diseases such as multiple sclerosis or type 1 diabetes or more generalized tissue involvement as exemplified by systemic inflammatory diseases such as systemic lupus erythematosus (SLE). At present, whatever the pattern of tissue involvement, the treatment of the immune component of these diseases utilizes similar agents to either curtail inflammation or to attenuate T and/or B cell reactivity [3]. If damage is irreversible, however, then treatment involves agents that restore to the extent possible the functional impairment that results from unopposed autoimmune attack.

While the etiology and clinical manifestations of autoimmune diseases may vary, the development of new therapies confronts many similar challenges especially in the setting of clinical trials. A particularly serious and vexing challenge relates to biomarkers. For most autoimmune diseases, studies on both patients and animal models have documented a host of phenotypic and functional immune cell abnormalities [4]. Translating these observations into the creation of reliable and actionable markers for use in clinical trials has been difficult, however. Furthermore, as "big data" approaches

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become more common, the number of biomarkers will undoubtedly rise dramatically; the complexity of these markers will also grow as each marker becomes a composite of thousands or even millions of data points [5–7].

The gap between the biomarkers of today (e.g., C-reactive protein, erythrocyte sedimentation rate, cytokine levels) and those projected for the future (e.g., RNA-seq of individual cells at the site of tissue injury) is enormous, and it will take years before the promise of big data is realized. During the transition from little to big data, the development of new therapies may benefit by some intermediate approaches which can provide a more granular picture of the immune system disturbances associated with autoimmune disease. The value of such approaches will be increased if they can employ instrumentation readily available in most clinical laboratories.

Among these approaches, the analysis of microparticles holds great promise as a platform for the development of new biomarkers to assess the operation of the immune system in autoimmunity. Microparticles (MPs), also called microvesicles (MVs), are small membrane-bound vesicles that are released from activated and dying cells [8]. Following release from cells, MPs enter the blood where their component molecules populate the many "omes" (e.g., proteome, nucleome) that can be identified and quantified by big data. Often these molecules are considered to be free and soluble, whereas, in reality, they are embedded in a particle matrix. In view of the origin of MPs during activation and cell death, particle release is a prominent feature of the pathogenesis of many autoimmune and inflammatory diseases. This chapter will review the structure of particles, their release from cells, and the various ways in which they can be assessed as biomarkers and conceptualized as targets for new therapy.

2 Generation of Microparticles

During the normal function of cells as well as the special circumstances of activation and death, cells emit a variety of particle types into the extracellular space. These particles differ in size, composition, and function. As a group, such particles can be termed extracellular vesicles (EVs) [9–11]. The smallest EVs are called exosomes and emanate from the multivesicular bodies on the cell interior. Exosomes are approximately 50–100 nm in diameter. On the other end of the size spectrum, apoptotic bodies are the largest EVs. Apoptotic bodies are the collapsed remnants of dying cells or large fragments that have broken off as death. Given their size and origin, apoptotic bodies can contain a panoply of intracellular constituents albeit in a degraded or rearranged form. Apoptotic bodies can approximate several microns in diameter.

The middle size range of EVs is occupied by MPs. Microparticles range in size from approximately 0.2–1.0 μm and contain a large collection of cellular constituents, including proteins, lipids, and nucleic acids. Importantly, MPs can display bioactive molecules; while the contribution on these molecules to the overall mass may be small, they may contribute importantly to the functional properties of the MPs. MPs result from two seemingly disparate processes: activation and cell death

[8–10]. While this situation may seem paradoxical, in the immune system, activation can lead to cell death, likely as a regulatory strategy to limit or attenuate responses that depend on cellular proliferation.

While the origin of MPs is not fully understood, at least some of these structures may correspond to blebs which arise during apoptosis. Blebs are bubble-like structures that form on or near the cell surface as cells die; blebs may also form during processes such as locomotion as an extension of the cell structure [12, 13]. In the setting of apoptosis, blebs may occur during cell shrinkage to adjust the surface to volume ratio as the cell collapses and dwindles in size. Blebbing may not be simply a physical-chemical response to decreasing volume, however, since there is strong evidence for the role of the ROCK enzyme in their generation [14, 15]. Blebs can occur during early and late apoptosis, and, while the size of blebs and MPs is similar, it is not clear that all MPs come from blebs [16, 17]. Looking at the phenomenon from the perspective of the blebs, it is not clear which blebs leave the cell to enter the extracellular cell space.

A striking feature of blebbing is the translocation of nuclear molecules into these structures as cell death processes proceed [18–22]. Thus, cellular demise during apoptosis involves a regulated process by which nuclear contents are reconfigured and rearranged in a way that fundamentally changes the dying cell's potential interactions with the immune system. The basis of this translocation is speculative although it may facilitate immune clearance of the remnants of dying cells since they are present in a smaller and more "appetizing" form to promote uptake by macrophages. Alternatively, the translocation and associated proteolytic and nucleolytic events may impede processes such as viral or bacterial replication and spread in cases where infection is the proximate cause of the apoptosis.

Whatever the cause of the nuclear translocation, the resulting particles become a rich source of extracellular nuclear molecules. This material includes DNA, RNA, histones, and nonhistone proteins. Indeed, particles are an important source of extracellular nuclear molecules, with their inclusion within the protective space of a membrane-bound structure shielding them from the degradative enzymes present in the blood [23, 24]. Since DNA and RNA are informational macromolecules, their presence in particles points to an important function of particles in the transmission of information from one cell to the next, with microRNA, for example, providing a mechanism for directly modulating cell function. Practically, mining the array of nucleic acids present in MPs as well as exosomes represents a powerful biomarker platform to measure the types of cells that have died and their functional or metabolic state.

3 The Assay of Microparticles

Particles are small, with their detection and enumeration presenting significant challenges when these parameters are analyzed by flow cytometry, the current mainstay for these determinations. Flow cytometry performs well for cells but encounters technical difficulties when applied to the submicron size of most particles. Depending

on the instrumentation, particles below 200–500 nm are variably detected. Even this assessment is subject to uncertainty since the size range for detection has been determined on the basis of rigid beads, whereas particles are flexible and indeterminate in shape. Using light scattering for particle detection and logarithmic gain for amplification, flow cytometry can detect many but unlikely not all MPs in a fluid sample. Furthermore, differences in the composition and size array of particles in different biofluids may limit direct comparisons of the properties of particles. For MP analysis, plasma rather than sera is used since blood clotting can lead to particle generation [25–28].

In addition to light scattering, analysis of the "particulome" can utilize many of the same approaches and reagents that are used to analyze cells, recognizing that the small size of particles means that all signals are small and that staining which would make a cell bright will at best lead to staining which is dim. Since MPs have the membrane components from the cell of origin, they bear the characteristic differentiation markers that distinguish lineages. Thus, it is possible to enumerate from the same plasma sample the number of particles from different cell types such as platelets, lymphocytes, or endothelium, all of which are highly relevant to autoimmune disease.

Although measuring events in a particular size range can suffice for counting MPs, some approaches incorporate a further element of staining to assure that MPs are in fact being detected. MPs can arise from apoptotic cells; as such, their membranes have "flipped" and exposed phosphatidylserine (PS) on the surface. Exposed PS is a hallmark of apoptosis and allows identification of apoptotic cells by staining with a fluoresceinated annexin V reagent. While many particles stain positively for annexin V, such positivity is not an invariable feature of particles likely because of the different origins of particles; it is also possible that particles, even from the same cellular source, are heterogeneous in composition and molecular structure [29–31].

Nucleic acids and associated nuclear molecules are important constituents of particles and can be measured by two main methods. Dyes that bind DNA and RNA, such as propidium iodide and SYTO13, can stain particles for enumeration by flow cytometry; the limited amount of material in a small particle and consequent weak signal intensity can challenge this method of detection [32]. In addition, the presence of DNA and other nuclear molecules can be assessed by antibody binding, either a monospecific serum from an autoimmune patient or a monoclonal antibody preparation with a well-defined autoantigen binding [22]. As these nuclear molecules are the targets of antinuclear antibodies (ANAs) in diseases such as lupus, particle assessment is an important element in determining the type and amount of autoreactive material in the blood, recognizing that, in some instances, particles are only one component of this material.

While advances in instrumentation will undoubtedly improve and refine this analysis, at present, flow cytometry can provide information on several aspects of the blood "particulome" that makes this assessment valuable in developing new therapy for autoimmunity, including particle elimination. Table 1 lists these assays and the features which, in some instances, can indicate functional activity of MPs and their putative role in autoimmunity. In comparison to information provided by

Table 1 Determination of microparticles by functional and cytometric assays

Light scattering to assess particle number
Binding of annexin V to measure exposed phosphatidylserine
Characterization of cell surface markers to define cell of origin
Functional assay for tissue factor
Molecular approaches for enumeration of informational nucleic acids

other biomarkers, even a simple enumeration of MPs can point to the nature of the cells involved in a disease and their physiologic or pathologic state (i.e., activated or dying). Furthermore, the presence in blood of MPs from otherwise inaccessible tissues (e.g., blood vessels) can indicate their possible role in pathogenesis.

4 The Functions of Microparticles

The functional activities of MPs are highly varied and consistent with the expression of so many different molecules in these structures, including their surface decoration. As signaling elements, MPs display activities associated with the full size range of immune mediators from the small (e.g., cytokines) to the large (e.g., cell-cell interaction). In general, MPs are pro-inflammatory and pro-thrombotic and can impact on multiple cell types, most prominently, lymphoid and myeloid cells of the immune system and endothelial cells of the vascular system [33–37]. In many respects, MPs can mediate essentially all of the activities that have been considered key to the underlying immune disturbances of autoimmune and inflammatory disease. This possibility should not be surprising since MPs have so many components which are immunologically relevant. The presence of tissue factor is important for the ability of particles to promote thrombosis.

Operationally, distinguishing the functional role of MPs in pathogenesis is difficult since there are few ways at present to either block specifically the activity of MPs or block their production; at this time, inhibition of particle release entails agents that are very broad in activity and likely to affect many other processes (e.g., inhibition of apoptosis, inhibition of activation). While delineating the role of MPs is likely to remain challenging, nevertheless, both in vivo and in vitro studies clearly demonstrate that MPs can provoke inflammation and thrombosis and therefore can contribute to pathogenesis of autoimmune diseases that are characterized by both of these features [37]. Certainly, the presence of so many bioactive molecules on one structure suggests that MPs can amplify responses by multi-receptor interactions; furthermore, the physical attachment of MPs to another cell type may increase the response of component molecules by increasing the local concentration, causing their transfer or producing repetitive stimulation.

In considering how the functional properties of MPs can impact on autoimmunity, the assay of both MPs and their constituent molecules can strongly influence this assessment. As noted, the assay of MPs requires the use of plasma. In contrast, the assay of cytokines can be accomplished with either plasma or serum. These

fluids differ in composition and the representation of particles. Few studies have directly addressed the differences in levels of various analytes in biofluids. As a result, the contribution of the particle component of an overall cytokine response may be missed. In this regard, certain cytokines (i.e., IL-1β) can be a component on MPs, with assay of sera possibly missing this important component of the overall response [38]. As the activity of cytokines may be enhanced by its representation on MPs, information of cytokine localization can provide a more complete picture of the potential contribution of a cytokine to disease in comparison to assay of just a biofluid. On the other hand, the contribution of MPs may be missed if particles are bound to cells and are therefore not counted when the cells in plasma are removed during centrifugation. Knowledge of the function of particles is just emerging.

The following sections will focus on two aspects of particle biology that are relevant to autoimmunity.

5 MPs as a Source of DAMPs and Alarmins

Microparticles have potentially two important roles in autoimmunity related to their content of nuclear molecules. The first is as a source of alarmins. The second is as a source of nuclear molecules which are the targets of antinuclear antibodies (ANAs) in the context of SLE and related autoimmune disease. As discussed previously, during apoptosis, nuclear molecules undergo translocation as the death process proceeds, with many ultimately residing in blebs. As blebs develop into particles that detach from cells, nuclear molecules can enter into the extracellular space where the particle structure provides a protected environment that may be at least partially resistant to degradation. As some nuclear molecules have immunological activity, MPs have the potential to be important players in pathogenesis.

As shown in many in vivo and in vitro studies, nuclear molecules can act as damage-associated molecular patterns or DAMPs when they leave their usual intracellular location. A DAMP is an intracellular molecule that can be released from injured, dying, or dead cells and, when in the extracellular space, can exert immunological activity [39, 40]. DAMPs can be large or small molecules and can stimulate immunity by utilizing the same receptors such as the toll-like receptors (TLRs) that are activated by bacterial or viral molecules. These molecules are termed pathogen-associated molecular patterns or PAMPs, while their cognate receptors are called pattern recognition receptors (PRRs). The term alarmin can be applied to certain DAMPs because they alarm the immune system, inducing chemotactic and adjuvant activity. Another term for this group of molecules is danger molecule since they can signal “danger” which represents threats to the organism, including infection or injury, that can induce cell injury.

Among the alarmins, HMGB1 or high-mobility group box 1 protein has potent immune activity that suggests a key role in autoimmunity [41, 42]. HMGB1 is a nonhistone nuclear protein that can bind DNA, mediating processes as a transcription and chromosomal structure. HMGB1 is 215 amino acids long and is comprised

of two DNA binding boxes (A box and B box) as well as a C-terminal tail. This protein is widely expressed in all cells and, while predominantly nuclear in location, can also have cytoplasmic expression. Since all cells can suffer injury and die, HMGB1 has the potential to serve as a uniform danger signal.

HMGB1 can leave cells during activation as well as cell death, with the functional activity resulting from posttranslational modifications (PTMs) that occur during these processes. During activation by TLR agonists such as LPS (endotoxin), or by cytokines, HMGB1 undergoes acetylation; this PTM allows translocation to the cytoplasm for eventual secretion. The presence of acetylation marks the origin of extracellular HMGB1 as activation. Once released from the cell, HMGB1 can serve as a late mediator of endotoxin shock; levels of HMGB1 are increased in conditions such as sepsis, trauma, and malignancy [41–44].

In addition to the occurrence during cell activation, HMGB1 release can occur during different death processes such as apoptosis, necrosis, and pyroptosis. Each of these forms of cell death is distinct in terms of inducing stimulus, a downstream pathway, and ultimately HMGB1 PTMs. Among these PTMs, the redox state is key because of the influence on three sulfhydryl groups at positions 23, 45, and 104. Fully reduced HMGB1 can bind to the chemokine CXCL12 and stimulate chemotaxis. Partially reduced HMGB1, with a disulfhydryl bond between cysteines 23 and 45, can bind to TLR4 and can activate processes in much the same way as does LPS. Fully oxidized HMGB1, as may occur during apoptosis, is inactive. Depending on its biochemistry, HMGB1 can also stimulate cells during TLR2 and RAGE (receptor for advanced glycation end products) [41–44].

As these considerations suggest, HMGB1 release can occur in many of the same situations as does MP release, with both emanating from cells in the seemingly disparate processes of activation and death. Given similarities in the release of HMGB1 and MPs, studies have investigated the presence of HMGB1 on MPs. These studies have assessed HMGB1 content by both flow cytometry and immunoblotting of MPs purified by differential centrifugation; for these studies, particles have come from both blood and cell cultures. Together, these studies demonstrate clearly that HMGB1 can be an important constituent of MPs [45–47]. This finding suggests that at least some of the activities of MPs may result from the presence of a potent alarmin like HMGB1. Because of the importance of PTMs, the presence of HMGB1 on particles does not in and of itself mean that either the HMGB1 is active or that the activity of MPs results from the presence of HMGB1. Determination of the PTMs is necessary to define the activity profile of particle HMGB1.

While HMGB1 can be a component of particles, it can also appear in the blood in a more free or soluble form. Soluble is a relative term that can be defined operationally in terms of behavior during differential sedimentation or size by gel exclusion chromatography. The structure of the more “soluble” form of HMGB1 is not as yet known. Nevertheless, it is possible to assay separately soluble and particle HMGB1 using enzyme-linked immunosorbent assays (ELISA). In this case, the particle and soluble forms can be separated by centrifugation, assaying three sources of HMGB1: uncentrifuged plasma, particle-free or soluble plasma, and sedimented

particles reconstituted to the starting sample volume. Flow cytometry can complement the immunochemical assay of these preparations.

Analysis of HMGB1 by these approaches shows a number of important features relevant to the role of HMGB1 as a disease mediator as well as a biomarker in autoimmunity. First, HMGB1 in the blood can exist in a free- and particle-bound component. Second, changes in the expression of HMGB1 may be detected by assay of HMGB1 on particles by flow cytometry that may not be apparent in the ELISA. In a study of normal volunteers receiving a low dose of LPS systemically, changes in the number of particles positive for HMGB1 were observed by flow cytometry, whereas the levels by ELISA were unchanged [48]. These findings suggest that analysis of MP levels of alarmins may provide more sensitive detection of HMGB1 in time-course studies than the overall protein levels in unfractionated or uncentrifuged plasma.

Another finding that emerges from analysis of MP levels relates to quantitation of the overall magnitude of the response. As shown in preliminary experiments, the amount of HMGB1 measured in the combination of the soluble and particle components can be greater than the amount measured in the uncentrifuged plasma (unpublished observations). While the explanation for this finding is not known, it is possible that the physical process of separation can reveal or unmask HMGB1 that is ordinarily present on the interior of the particle and thus unavailable for detection in an immunoassay. Sample handling, especially the mechanical forces that occur with high-speed centrifugation, may disrupt particle structure or cause fragmentation to increase the availability of this interior component.

Many molecules in the blood are putatively "soluble" and assayed as biomarkers for damage or death of cells (e.g., ALT and AST for the liver and troponin for the heart) or activation of cells (e.g., soluble IL-2 receptor). Serum is the usual source of blood for assay and the existence of a particle component is not generally considered. With the precedent of particle-bound HMGB1 in mind, subsequent studies explored the representation of soluble CD40 ligand (sCD40L) in a particle and free form. sCD40L is a transmembrane protein found on T cells and platelets, with its soluble form assayed as a biomarker for inflammatory and thrombotic disorders. Using blood from the same population of volunteers given LPS, studies demonstrated that sCD40L also exists on particles, with assay of the particle-bound form providing information not apparent with assay of the soluble form [49]. Thus, these studies indicate that solubility in the context of the biomarker studies on blood does not signify an intrinsic physical-chemical property but rather an operational property dependent on the handling of specimens.

6 Microparticles as a Source of Immune Complexes

The formation of immune complexes (ICs) is a central event in the pathogenesis of many autoimmune and inflammatory diseases. These ICs can occur in blood as in the case of SLE or can occur in local spaces such as the joint in rheumatoid arthritis

(RA). ICs have several distinct roles in the pathogenesis: they can deposit in the tissue to activate complement and promote tissue injury (i.e., lupus nephritis); they can activate complement to induce local inflammation (i.e., rheumatoid synovium); and, depending on the target antigens in the ICs, they can induce cytokine production by cells of the innate immune system, most prominently, the plasmacytoid dendritic cells (PDCs) [50–54]. The latter mechanism is particularly important in SLE since the ICs contain nuclear molecules. While nuclear molecules like HMGB1 appear active when alone, DNA does not induce immune response unless bound by a protein such as an autoantibody.

The enhanced activity of DNA in immune complexes likely results from the uptake of complexes into the cells in a manner that exposes the DNA to internal nucleic acid sensors [55]. These sensors include molecules such as TLR9 and cGAS and represent an internal defense system which likely has evolved to meet the challenge of intracellular infection, whether bacterial or viral. Incubation of cells such as macrophages with DNA does not allow access to the subcellular compartment in the cytoplasm where these receptors are located. In contrast, an IC can essentially transfect DNA into the cytoplasm where it can mimic intracellular DNA from an infection and trigger cell activation. This mechanism can lead to the production of cytokines such as type 1 interferon whose molecular signature is a hallmark of lupus pathogenesis.

The evidence for the role of ICs in lupus pathogenesis is strong, with depression of complement levels and increases in complement split products demonstrating the existence of complexes somewhere in the body. While blood is the obvious place to look for ICs, they have been in fact difficult to demonstrate by biochemical and immunochemical techniques in lupus. Two main explanations have been invoked to explain this difficulty: the formation of ICs in situ in tissue (e.g., kidney) rather than blood and rapid clearance or deposition of ICs such that their presence in blood is ephemeral or otherwise undetectable [54]. Given the centrality of ICs to the pathogenesis of lupus nephritis and interferon production, the absence of a direct measure of their presence has deprived the field of a critical biomarker.

As in the case of molecules that are putatively soluble, complexes, which are also generally considered soluble, may in fact be particulate. Furthermore, in view of evidence that MPs can contain the nuclear antigens targeted by ANAs, a role of particles as a source of ICs becomes very plausible. Studies have therefore investigated MPs as a source of ICs critical to lupus, and several lines of evidence are very consistent with this possibility. Thus, ANAs, either sera from patients or lupus mice as well as murine monoclonal antibodies, can all bind to particles that have been generated in vitro from cell lines treated with agents that cause activation or cell death [22, 56–58]. The binding is not invariable, however, reflecting either the fine specificity of the antibodies as well as the amount and the extent of surface expression of the target antigens.

Most importantly, particles from the blood of patients with lupus and certain strains of autoimmune mice contain bound IgG at levels that far exceed those of control particles from healthy individuals or mice. The presence of such IgG can be demonstrated by flow cytometric techniques as well as proteomic analysis. Since levels of

Table 2 Role of MPs in the pathogenesis of autoimmunity

Stimulate inflammation via constituent cytokines
Stimulate inflammation via constituent alarmins
Promote thrombosis
Form immune complexes with tissue deposition
Transfer information via constituent nucleic acids

IgG on particles can be related to levels of anti-DNA, these findings suggest that particle DNA can be an important source of antigen for the formation of ICs [59–61]. Indeed, the finding of IgG on MPs provides some of the most decisive evidence for the presence of circulating ICs in the blood of lupus patients and points to the utility of this assessment for biomarker purposes. The demonstration of a microparticle component in renal biopsies of patients with lupus nephritis supports this view [61].

Similar studies point to a role of MPs in IC formation in rheumatoid arthritis although these studies suggest that local factors may determine this process [62, 63]. Interestingly, particles in synovial fluid of patients with RA bear IgG as well as complement components demonstrating directly that MPs can be IC components. In contrast, the plasmas of the same patients did not show increased numbers of IgG positive particles. These observations could suggest differences in the antigenic content of MPs that either form in the joint space or localize there. Alternatively, autoantibody synthesis in the synovium may lead to sufficient amounts to coat particles, whereas dilution of these antibodies in the blood may prevent appreciable particle binding. Antibodies to citrullinated proteins (ACPAs) are the likely specificity contributing to the formation of these complexes, perhaps by binding proteins that have undergone citrullination.

Elucidation of the role of MP complexes in other autoimmune diseases is just beginning although, given the content of self-molecules in MPs, these structures could represent a common nidus for IC formation. Particles, whether coated with autoantibodies and complement, could also act in many other ways in the disease setting. Table 2 summarizes potential roles of MPs in the pathogenesis of autoimmunity.

7 Implications for New Therapies

MPs are newly recognized players in the pathogenesis of autoimmunity and therefore can serve as both biomarkers and targets of therapy. The biomarker potential of MP assessment is high since their analysis in blood can provide a window to observe events in the periphery including activation and death of cells in locations such as the vasculature. Since assays involve relatively small amounts of blood, analysis of changes over time to monitor disease activity or the response to treatment can be readily accomplished. Such assessment can be quantitative although current technology may not provide a full and completely accurate picture of the number and array of particles present. Nevertheless, the detail captured in this picture can exceed that currently available from other approaches [64].

The analysis of MP ICs found in plasma represents an entirely new approach for characterizing the vasculature in autoimmune disease. Studies in the context of atherosclerosis, diabetes, and metabolic syndrome have clearly demonstrated the value of particle assessment in developing predictive markers for events such as cardiac ischemia and stroke [65–70]. Importantly, the presence of endothelial MPs in blood allows analysis of the state of the endothelium in these conditions, characterizing particles using different cell surface markers associated with their physiologic state [71]. Since atherosclerosis involves localized inflammation of the plaque in the vessel wall, analysis of immune cell properties of circulating MPs can augment any information provided by nonspecific markers such as C-reactive protein.

Since many autoimmune diseases have an increased frequency of atherosclerosis, analysis of MPs can provide a simultaneous assessment of the immune system and vascular system. In this regard, particles have pro-thrombotic properties, with analysis of their number and properties potentially providing predictive information on thrombotic events which can involve a variety of organ systems in autoimmune disease. Studies in oncology have explored the value of this type of assessment since thrombosis is an important complication of many malignancies; as in the case of cardiovascular disease, cancer is a setting for high levels of particles in the blood [72].

At present, the link between inflammation and vascular disease is not well understood nor are the effects of current treatments on the risk for cardiac events. As the armamentarium of new immunomodulatory agents grows along with the number of combinations between new and existing agents, it will be important to have markers that could be useful in distinguishing effects on cardiac risk compared to other inflammatory disease manifestations such as synovitis or glomerulonephritis. The sensitivity of MP assessment in comparison to noninvasive tests of cardiovascular disease (e.g., flow-mediated dilatation) is an exciting area of future research that could provide unique biomarker information to sort out the effects of treatment on different target tissues [73].

The assessment of MPs occurs at the junction of little and big data. The little data aspect involves a simple count of particle types. The big data aspect involves a detailed analysis of the constituent molecules of the particles-proteins, lipids, nucleic acids-by array or omics techniques. In particular, MPs can provide a unique source of RNA for analysis of both messenger and microRNA species. Such an analysis would clearly place MP studies in the big data arena although the advantage of MP assessment in comparison to that of total blood comes from knowledge of the cell of origin of any RNA in the blood. Since MP can be separated by flow cytometric techniques on the basis of phenotype, an analysis of their macromolecular composition may allow determination of events in even uncommon or rare cell populations, possibly including those critically involved in pathogenesis.

At present, the main limitation in the study of MPs as biomarkers is technical and relates to the small size of particles. Even with the best instruments, the total counting of particles is uncertain given their size [74]. Furthermore, as particles are small, detection of certain cell populations on the basis of their differentiation markers may be insensitive especially if the density of the marker is low or the detecting antibody produces a weak binding or a weak signal. Development of more sensitive

Table 3 Advantages of MPs as biomarkers

Information on a wide range of cell types from a single sample
Reveal events in rare or inaccessible cell populations (e.g., endothelium)
Delineate pathogenic processes (e.g., activation or cell death)
Provide a source of material from a single cell type for omics assay
Reveal changes of diverse cell types with treatment and disease activity

particle assays will therefore be important in exploiting more fully the potential of MP assessment as a platform for novel biomarkers. Table 3 lists advantages of MP assessment for biomarker purposes.

The targeting of MPs for therapy would represent a fundamentally new direction in the treatment of autoimmune disease although, as noted, agents such as anti-cytokines and anti-HMGB1 may in fact work by interdicting molecules on particles. Similarly, agents designed to block IC formation or promote IC dissolution could be explored whether the IC is soluble or particulate [75]. On the other hand, strategies to prevent particle release by specific blockade of the steps in particle formation could have therapeutic applicability although the development of such approaches requires understanding of not only the actual processes of particle formation and release but also their physiological consequences [76, 77]. Studies in a number of disease settings have demonstrated reduction of MP levels with a variety of treatment; whether these effects are primary or secondary is not known, however.

If particles are simple by-products of other processes, their production could at least be theoretically blocked without interfering with cell function. If, however, particle release is integral to some critical process (e.g., detoxification or removal of damaged subcellular organelles), then its blockade could have adverse effects. In this regard, if particle release is essential to the response to danger, inhibition of this process could impair host defense and increase susceptibility to infection. At present, these considerations are speculative and point to the many unknown aspects of particle biology.

8 Conclusions

Microparticles are small membrane-bound vesicles that carry intracellular molecules into the extracellular space and exert many important biological activities. The potential role of these structures in the pathogenesis of autoimmune disease is very high since MPs can promote both inflammation and thrombosis. At present, MPs represent novel biomarkers to measure disease activity and the functional status of diverse cell populations, expanding the perspective currently available for noninvasive assessment of steps essential for pathogenesis. Future studies will determine whether MPs can also be a target of therapy, with their elimination or functional inactivation a promising avenue for next-generation treatments.

References

1. Davidson A, Diamond B (2001) Autoimmune diseases. N Engl J Med 345:340–350
2. Rosenblum MD, Remedios KA, Abbas AK (2015) Mechanisms of human autoimmunity. J Clin Investig 125:2228–2233
3. Thanou A, Merrill JT (2014) Treatment of systemic lupus erythematosus: new therapeutic avenues and blind alleys. Nat Rev Rheumatol 10:23–34
4. Mohan C, Assassi S (2015) Biomarkers in rheumatic diseases: how can they facilitate diagnosis and assessment of disease activity? BMJ 351:h5079
5. Chiche L, Jourde-Chiche N, Whalen E, Presnell S, Gersuk V et al (2014) Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. Arthritis Rheumatol 66:1583–1595
6. Dennis G Jr, Holweg CT, Kummerfeld SK, Choy DF, Setiadi AF et al (2014) Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. Arthritis Res Ther 16:R90
7. Housley WJ, Fernandez SD, Vera K, Murikinati SR, Grutzendler J et al (2015) Genetic variants associated with autoimmunity drive NFkappaB signaling and responses to inflammatory stimuli. Sci Transl Med 7:291ra293
8. Beyer C, Pisetsky DS (2010) The role of microparticles in the pathogenesis of rheumatic diseases. Nat Rev Rheumatol 6:21–29
9. Gould SJ, Raposo G (2013) As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles 2: 20389
10. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S (2014) Emerging role of extracellular vesicles in inflammatory diseases. Nat Rev Rheumatol 10:356–364
11. Minciacchi VR, Freeman MR, Di Vizio D (2015) Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. Semin Cell Dev Biol 40:41–51
12. Charras GT (2008) A short history of blebbing. J Microsc 231:466–478
13. Charras GT, Coughlin M, Mitchison TJ, Mahadevan L (2008) Life and times of a cellular bleb. Biophys J 94:1836–1853
14. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A et al (2001) Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol 3:339–345
15. Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J et al (2001) Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat Cell Biol 3:346–352
16. Wickman GR, Julian L, Mardilovich K, Schumacher S, Munro J et al (2013) Blebs produced by actin-myosin contraction during apoptosis release damage-associated molecular pattern proteins before secondary necrosis occurs. Cell Death Differ 20:1293–1305
17. Zirngibl M, Furnrohr BG, Janko C, Munoz LE, Voll RE et al (2015) Loading of nuclear autoantigens prototypically recognized by systemic lupus erythematosus sera into late apoptotic vesicles requires intact microtubules and myosin light chain kinase activity. Clin Exp Immunol 179:39–49
18. Casciola-Rosen LA, Anhalt G, Rosen A (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 179:1317–1330
19. Halicka HD, Bedner E, Darzynkiewicz Z (2000) Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 260:248–256
20. Lane JD, Allan VJ, Woodman PG (2005) Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. J Cell Sci 118:4059–4071
21. Schiller M, Bekeredjian-Ding I, Heyder P, Blank N, Ho AD et al (2008) Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. Cell Death Differ 15:183–191
22. Reich CF 3rd, Pisetsky DS (2009) The content of DNA and RNA in microparticles released by Jurkat and HL-60 cells undergoing in vitro apoptosis. Exp Cell Res 315:760–768

23. Pisetsky DS, Gauley J, Ullal AJ (2011) Microparticles as a source of extracellular DNA. Immunol Res 49:227–234
24. Dye JR, Ullal AJ, Pisetsky DS (2013) The role of microparticles in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. Scand J Immunol 78:140–148
25. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ et al (2011) Sizing and phenotyping of cellular vesicles using nanoparticle tracking analysis. Nanomedicine 7:780–788
26. Robert S, Poncelet P, Lacroix R, Raoult D, Dignat-George F (2011) More on: calibration for the measurement of microparticles: value of calibrated polystyrene beads for flow cytometry-based sizing of biological microparticles. J Thromb Haemost 9:1676–1678; author reply 1681–1672
27. van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL et al (2014) Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. J Thromb Haemost 12:1182–1192
28. Poncelet P, Robert S, Bailly N, Garnache-Ottou F, Bouriche T et al (2015) Tips and tricks for flow cytometry-based analysis and counting of microparticles. Transfus Apher Sci 53:110–126
29. Connor DE, Exner T, Ma DD, Joseph JE (2010) The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemost 103:1044–1052
30. Frey B, Gaipl US (2011) The immune functions of phosphatidylserine in membranes of dying cells and microvesicles. Semin Immunopathol 33:497–516
31. Niccolai E, Squatrito D, Emmi G, Silvestri E, Emmi L et al (2015) A new cytofluorimetric approach to evaluate the circulating microparticles in subjects with antiphospholipid antibodies. Thromb Res 136:1252–1258
32. Ullal AJ, Pisetsky DS, Reich CF 3rd (2010) Use of SYTO 13, a fluorescent dye binding nucleic acids, for the detection of microparticles in in vitro systems. Cytom A 77:294–301
33. Aras O, Shet A, Bach RR, Hysjulien JL, Slungaard A et al (2004) Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. Blood 103:4545–4553
34. Morel O, Toti F, Hugel B, Freyssinet JM (2004) Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. Curr Opin Hematol 11:156–164
35. Angelillo-Scherrer A (2012) Leukocyte-derived microparticles in vascular homeostasis. Circ Res 110:356–369
36. van Es N, Bleker S, Sturk A, Nieuwland R (2015) Clinical significance of tissue factor-exposing microparticles in arterial and venous thrombosis. Semin Thromb Hemost 41: 718–727
37. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE et al (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066
38. Prada I, Furlan R, Matteoli M, Verderio C (2013) Classical and unconventional pathways of vesicular release in microglia. Glia 61:1003–1017
39. Pisetsky D (2011) Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and DAMP-PAMP complexes. Swiss Med Wkly 141:w13256
40. Venereau E, Ceriotti C, Bianchi ME (2015) DAMPs from cell death to new life. Front Immunol 6:422
41. Harris HE, Andersson U, Pisetsky DS (2012) HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. Nat Rev Rheumatol 8:195–202
42. Andersson U, Antoine DJ, Tracey KJ (2014) The functions of HMGB1 depend on molecular localization and post-translational modifications. J Intern Med 276:420–424
43. Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A et al (2012) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med 209:1519–1528
44. Yang H, Antoine DJ, Andersson U, Tracey KJ (2013) The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. J Leukoc Biol 93: 865–873

45. Maugeri N, Rovere-Querini P, Baldini M, Baldissera E, Sabbadini MG et al (2014) Oxidative stress elicits platelet/leukocyte inflammatory interactions via HMGB1: a candidate for microvessel injury in sytemic sclerosis. Antioxid Redox Signal 20:1060–1074
46. Pisetsky DS (2014) The expression of HMGB1 on microparticles released during cell activation and cell death in vitro and in vivo. Mol Med 20:158–163
47. Spencer DM, Mobarrez F, Wallen H, Pisetsky DS (2014) The expression of HMGB1 on microparticles from Jurkat and HL-60 cells undergoing apoptosis in vitro. Scand J Immunol 80:101–110
48. Soop A, Hallstrom L, Frostell C, Wallen H, Mobarrez F et al (2013) Effect of lipopolysaccharide administration on the number, phenotype and content of nuclear molecules in blood microparticles of normal human subjects. Scand J Immunol 78:205–213
49. Mobarrez F, Sjovik C, Soop A, Hallstrom L, Frostell C, et al. (2015) CD40L expression in plasma of volunteers following LPS administration: A comparison between assay of CD40L on platelet microvesicles and soluble CD40L. Platelets: 26:486–490
50. Koffler D, Agnello V, Thoburn R, Kunkel HG (1971) Systemic lupus erythematosus: prototype of immune complex nephritis in man. J Exp Med 134:169s–179s
51. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ et al (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and toll-like receptors. Nature 416:603–607
52. Lovgren T, Eloranta ML, Bave U, Alm GV, Ronnblom L (2004) Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. Arthritis Rheum 50:1861–1872
53. Tian J, Avalos AM, Mao SY, Chen B, Senthil K et al (2007) Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol 8:487–496
54. Seredkina N, Van Der Vlag J, Berden J, Mortensen E, Rekvig OP (2013) Lupus nephritis: enigmas, conflicting models and an emerging concept. Mol Med 19:161–169
55. Kawasaki T, Kawai T, Akira S (2011) Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity. Immunol Rev 243:61–73
56. Ullal AJ, Reich CF 3rd, Clowse M, Criscione-Schreiber LG, Tochacek M et al (2011) Microparticles as antigenic targets of antibodies to DNA and nucleosomes in systemic lupus erythematosus. J Autoimmun 36:173–180
57. Ullal AJ, Pisetsky DS (2013) The role of microparticles in the generation of immune complexes in murine lupus. Clin Immunol 146:1–9
58. Ullal AJ, Marion TN, Pisetsky DS (2014) The role of antigen specificity in the binding of murine monoclonal anti-DNA antibodies to microparticles from apoptotic cells. Clin Immunol 154:178–187
59. Nielsen CT, Ostergaard O, Stener L, Iversen LV, Truedsson L et al (2012) Increased IgG on cell-derived plasma microparticles in systemic lupus erythematosus is associated with autoantibodies and complement activation. Arthritis Rheum 64:1227–1236
60. Ostergaard O, Nielsen CT, Iversen LV, Tanassi JT, Knudsen S et al (2013) Unique protein signature of circulating microparticles in systemic lupus erythematosus. Arthritis Rheum 65:2680–2690
61. Nielsen CT, Ostergaard O, Rekvig OP, Sturfelt G, Jacobsen S et al (2015) Galectin-3 binding protein links circulating microparticles with electron dense glomerular deposits in lupus nephritis. Lupus 24:1150–1160
62. Biro E, Nieuwland R, Tak PP, Pronk LM, Schaap MC et al (2007) Activated complement components and complement activator molecules on the surface of cell-derived microparticles in patients with rheumatoid arthritis and healthy individuals. Ann Rheum Dis 66:1085–1092
63. Cloutier N, Tan S, Boudreau LH, Cramb C, Subbaiah R et al (2013) The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: the microparticle-associated immune complexes. EMBO Mol Med 5:235–249
64. Franca CN, Izar MC, Amaral JB, Tegani DM, Fonseca FA (2015) Microparticles as potential biomarkers of cardiovascular disease. Arq Bras Cardiol 104:169–174

65. Omoto S, Nomura S, Shouzu A, Nishikawa M, Fukuhara S et al (2002) Detection of monocyte-derived microparticles in patients with Type II diabetes mellitus. Diabetologia 45:550–555
66. Nomura S, Shouzu A, Omoto S, Nishikawa M, Iwasaka T (2005) Long-term treatment with nifedipine modulates procoagulant marker and C-C chemokine in hypertensive patients with type 2 diabetes mellitus. Thromb Res 115:277–285
67. Nozaki T, Sugiyama S, Koga H, Sugamura K, Ohba K et al (2009) Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. J Am Coll Cardiol 54:601–608
68. Lee ST, Chu K, Jung KH, Kim JM, Moon HJ et al (2012) Circulating CD62E+ microparticles and cardiovascular outcomes. PLoS One 7:e35713
69. Sarlon-Bartoli G, Bennis Y, Lacroix R, Piercecchi-Marti MD, Bartoli MA et al (2013) Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis. J Am Coll Cardiol 62:1436–1441
70. Wekesa AL, Cross KS, O'Donovan O, Dowdall JF, O'Brien O et al (2014) Predicting carotid artery disease and plaque instability from cell-derived microparticles. Eur J Vasc Endovasc Surg 48:489–495
71. Dignat-George F, Boulanger CM (2011) The many faces of endothelial microparticles. Arterioscler Thromb Vasc Biol 31:27–33
72. Pabinger I, Thaler J, Ay C (2013) Biomarkers for prediction of venous thromboembolism in cancer. Blood 122:2011–2018
73. Augustine D, Ayers LV, Lima E, Newton L, Lewandowski AJ et al (2014) Dynamic release and clearance of circulating microparticles during cardiac stress. Circ Res 114:109–113
74. Robert S, Lacroix R, Poncelet P, Harhouri K, Bouriche T et al (2012) High-sensitivity flow cytometry provides access to standardized measurement of small-size microparticles – brief report. Arterioscler Thromb Vasc Biol 32:1054–1058
75. Stearns NA, Lee J, Leong KW, Sullenger BA, Pisetsky DS (2012) The inhibition of anti-DNA binding to DNA by nucleic acid binding polymers. PLoS One 7:e40862
76. Morel O, Jesel L, Freyssinet JM, Toti F (2011) Cellular mechanisms underlying the formation of circulating microparticles. Arterioscler Thromb Vasc Biol 31:15–26
77. Roseblade A, Luk F, Rawling T, Ung A, Grau GE et al (2013) Cell-derived microparticles: new targets in the therapeutic management of disease. J Pharm Pharm Sci 16:238–253

Part II
Genomic Medicine

The Future for Genomic Medicine in Inflammatory Diseases

Chris Kitson

1 Expanding the Druggable Target Universe

Sequencing of the human genome [1, 2] and the advent of mammalian RNA interference [3] at the beginning of the twenty-first century opened the eyes of many to the real possibilities of genomic medicine. The National Human Genome Research Institute (https://www.genome.gov/) defines genomic medicine as follows:

> An emerging medical discipline that involves using genomic information about an individual as part of their clinical care (e.g. for diagnostic or therapeutic decision-making) and the other implications of that clinical use.

In the following chapter, we will use genomic medicine to define a treatment that may manipulate DNA or RNA in such a way as to elicit a therapeutic effect. The advent of this discipline began approximately a quarter of a century after the structure of DNA was solved [4] and approximately a quarter of a century before the sequencing of the human genome. In 1978 Zamecnik and Stephenson [5] published a paper demonstrating that DNA complementary to *Rous sarcoma virus* prevented viral production in infected chick embryonic fibroblasts. From this the field of antisense grew and ultimately gene therapy and RNA interference, which will be discussed in later sections.

Drug discovery has followed an ever more complex path over the last century, culminating in a highly automated, high throughput industry encompassing more and more technology and knowledge in the hopes of gaining an edge over the competition. The traditional pharmaceutical company still exists but is widely supplemented with biotech, contract research, and academic institutions, all striving toward drug discovery. Prior to the completion of the genome, drug targets were

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limited to approximately 500 [6] made up mainly of receptors and enzymes with a few secreted proteins and ion channels. Many successful drugs were developed against these relatively few targets, including anti-inflammatory, as an example celecoxib was approved in 1998 (for review [7]). The human genome sequence uncovered new avenues for drug discovery including expanded targets within already mined classes and the opportunity to consider every gene a potential drug target. From the relatively small number of genes, ~30,000, it was considered there were maybe up to 1500 traditionally druggable genes, if normal pharmaceutical rules applied [8]. Genome sequencing only uncovered more proteins to which pharmaceutical companies could apply their vast resources including but not limited to scaffolding, chaperoning, translating, transcriptional, and other key functions. Novel nonprotein targets were identified such as microRNAs (miRNA), the number of which increased quickly following the adoption of rapid sequencing technologies [9]. Advances in genetic and genomic technologies have really culminated in an expanded target set, better and faster ways to validate, and ultimately newer ways to interfere clinically with targets causative for disease.

2 A Brief History of Genomic Modalities

2.1 Antisense

As mentioned, antisense technology was really conceived in the 1970s but was limited by a lack of sequence information and the required scale to synthesize oligonucleotides [10]. The definition of antisense here will be the delivery of a single-stranded oligonucleotide to cells eliciting an effect on RNA translation. Many advances were made in the modification of the phosphate backbone including the very important phosphorothioate [11, 12] providing nuclease resistance and supporting in vitro activity. Isis pharmaceuticals (www.isispharm.com) were a driving force in the use of antisense oligonucleotides for clinical use and were able to secure regulatory approval for Vitravene™ (fomivirsen) in 1998 (http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20961_Vitravene.cfm). This drug, a 21-nucleotide "thioate, was designed to treat CMV retinitis in AIDS patients and was locally delivered." The advancement of effective HIV drug cocktails essentially ended the value of the drug which had demonstrated proof of concept for this new class [13, 14] and it was discontinued. Further modifications of this class were developed over the next decades including gapmer oligonucleotides where a 6–10-nucleotide thioate core was inserted between ends of modified RNA nucleotides of three to five residues [15]. These modified RNA nucleotides such as the Isis developed MOE (methoxyethyl) reagents increased affinity, nuclease resistance, and pharmacology, yet still engaged the favored mechanism where the hybridization of the antisense oligonucleotide to its cognate mRNA molecule recruited RNase H to elicit cleavage and subsequent degradation [16]. Other chemistries and modifications elicit mechanisms distinct from RNase H cleavage as well as imparting greater affinity and

stability. These chemistries include morpholino, peptide nucleic acid, locked nucleic acid, and other modifications of the sugar molecule. Additional mechanisms to impart activity include forming a triplex directly with DNA, translation arrest through hybridization as well as oligonucleotides which block certain splicing events [17]. As with all new technologies, the initial successes, in vitro activity, design of effective reagents, and better and better chemistry, were tempered by in vivo toxicity including immune activation [18]. The two disciplines of antisense technology with increased understanding of innate pattern recognition uncovered the ability of CpG duplexes as well as four contiguous guanosines to elicit unfavorable responses for this field. On the flip side, so-called immunostimulatory oligonucleotides were designed and clinically tested in the context of vaccines, allergy, and cancer [19, 20]. Removing the offending sequences has enabled the field to continue its evolution, and 15 years after the first approval, Isis was able to deliver a second molecule to market with their partner Genzyme who licensed the drug after phase 2 [21]. The drug (mipomersen/Kynamro) is designed against apolipoprotein B-100 and indicated in patients with homozygous familial hypercholesterolemia. An interesting note in the field of antisense is the disparity between delivery of oligonucleotides in vitro and in vivo. For cell culture oligonucleotides are delivered via transfection reagents of electroporation, whereas in vivo the naked oligonucleotides are successfully delivered to cells albeit in a fairly well-understood organ hierarchy [22]. Today there are still ongoing antisense clinical trials and the field has helped more recent genomic technologies to learn from both success and failure, as well as the required development path for market approval.

2.2 RNA Interference

RNA interference was a somewhat serendipitous finding, when scientists were trying to deepen the purple coloring of petunias by overexpression of an enzyme in the pathway. Unexpectedly they produced white or less pigmented petals, which they termed co-suppression [23]. The next big step in RNAi was the publication by Fire and Mello demonstrating that double-stranded RNA generated potent and stable gene suppression in *C. elegans* [24]. They also confirmed this was a gene-specific effect and not the known dsRNA-dependent protein kinase response. The next two discoveries ensured the technology leapt to mammals and were the determination of long double-stranded cleavage into 21–23 nucleotide fragments [25] and the seminal Tuschl paper [3]. This demonstrated mammalian RNA interference in HeLa and Hek293 after transfection of short interfering RNA (siRNA) and suppression of lamin A/C. Additional discoveries such as being able to express short hairpin RNA (shRNA) which were then cleaved to siRNA molecules enabled packaging in viruses for broader in vitro and in vivo delivery [26]. This new technology was not without problems and it was soon discovered that shorter sequences, with imperfect complementarity, could induce “off-target” effects. Although microRNA-induced translational repression from 3′ UTR binding was beginning to be understood, these

new findings demonstrated translational repression from within coding regions [27]. Better understanding of how siRNA duplexes engaged the cellular RISC complex and elicited RNA degradation [28] supported a more rational design [29] along with a number of companies providing reagents for in vitro study, for example, Dharmacon (http://dharmacon.gelifesciences.com/) and Ambion (http://www.lifetechnologies.com/us/en/home.html). RNAi oligonucleotides underwent a similar revolution to antisense where modified bases and linkages were evaluated to reduce off-target effects as well as to impart stability in vivo [30]. From the beginnings of RNAi, biotechnology companies (originally focused on genomic modalities such as antisense or ribozymes) were closely watching the field. Soon enough companies were evolving to focus on this newer technology, and company names such as Sirna and Alnylam were becoming familiar and are now a single entity (http://www.alnylam.com/home-page-content/alnylam-acquires-sirna-therapeutics/). Alnylam are the world leaders in RNAi therapeutics and in November 2013 began a phase 3 trial targeting knockdown of transthyretin (TTR) and preventing the buildup of amyloid deposits from misfolded, mutant TTR. This study follows successful phase 1 and 2 studies demonstrating protein knockdown and clinical effects (http://www.alnylam.com/product-pipeline/ttr-amyloidosis-fap/). One of the major challenges with in vivo delivery of siRNA is the requirement to wrap the dsRNA in a carrier particle to allow for tissue penetration.

2.3 MicroRNA

MicroRNAs (miRNAs) are small noncoding RNAs of similar length and structure to shRNAs and processed from longer primary miRNAs into pre-miRNA and finally the mature miRNA. Their role is to regulate gene expression often through binding in the 3′ UTR and negatively impacting translation. The primary miRNA may consist of more than one miRNA and forms a series of stem-loop structures that are processed into the shRNA-like structure. From this stage the mature miRNA is processed through the same dicer machinery as the RNAi molecules and either the sense or the antisense strand or both may be functional. The mature miRNA often binds with imperfect complementarity and in this way each molecule may suppress multiple targets [31]. Almost 1,900 human miRNAs have been reported (http://www.mirbase.org/) since the initial identification of lin-4 in *C. elegans* [32]. Since these first studies, miRNAs have been identified as actively supporting translation and being able to bind to promoters to support expression and have been identified as having targets within coding regions of genes to destabilize mRNA [33]. Due to their large numbers of potential targets, miRNAs are thought to coordinate gene expression in part by acting as a brake to developmental pathways, for example. In 2002 it was discovered that miR-15 and miR-16 were downregulated, through mutation, in a large percentage of human CLL suggesting a tumor suppressor role [34]. Further studies demonstrated miRNAs could also act as oncogenes and were regulated by well-known tumor genes such as myc [35] and p53 [36]. In other

therapeutic areas such as cardiovascular, neurodegenerative, and autoimmune diseases, miRNAs are shown to play key roles [37]. Two approaches to therapeutic targeting of miRNA were sought. First is to inhibit overexpressed miRNAs such as those identified as driving tumorigenesis. This could be achieved in a manner analogous to the approach taken by the antisense field. Deliver a chemically stable single-stranded oligonucleotide complementary to the active miRNA sequence. Silencing miRNAs with antagomirs was demonstrated in mice, with broad tissue distribution and long-lasting effects [38]. The first clinical delivery of anti-miR was targeting miR-122 and was conducted as a proof of concept to suppress HCV (for review of the discovery of this drug [39]). To overexpress a downregulated miRNA is more analogous to the delivery of siRNA, where a double-stranded, usually hairpin, pre-miR molecule is complexed within some form of delivery reagent to support both stability and tissue penetration [40]. A number of miRNA companies such as Miragen (http://miragentherapeutics.com/), Mirna Therapeutics (http://www.mirnarx.com/), Santaris Pharma acquired by Roche in 2014 (http://www.roche.com/media/store/releases/med-cor-2014-08-04.htm), and Regulus Therapeutics (http://www.regulusrx.com/) are evaluating therapeutic modulation with these molecules.

2.4 Gene Therapy

Gene therapy is classically thought of as compensating for a defective gene or protein through delivery of a vector encoding a wild-type version. The concept of gene therapy began at a similar time to that of antisense though a lack of basic molecular biology at that time saw a slow start to this potentially therapeutic approach. Small breakthroughs occurred including the calcium phosphate transfection method [41] which enabled a slightly more efficient delivery of DNA into cells in vitro. The ability to engineer retroviral genomes provoked a sense that gene therapy would become a reality, and in the mid-1980s, correction of the adenosine deaminase deficiency in isolated T cells from human severe combined immunodeficiency (SCID), patients further enhanced this belief [42]. This led to the first approved clinical trial where two SCID children had gene replacement therapy; however responses were modest [43]. Virally delivered replacement therapy was considered more efficient and also (dependent on the virus) allowed for integration of the gene into the genome for stable expression. There was a large effort based on nonviral-mediated gene therapy and the development of cationic liposomes [44] bolstered these efforts. Both concepts were not without problems, and for virally delivered genes, the unfortunate death of one patient from the high titer of virus administered [45] tainted the field for a significant time. Both methods also generated immune responses to either viral proteins or pattern recognition sequences such as CpG within the plasmid [46]. Overcoming these hurdles has been a long road and limited successes have been observed to date in clinical trials. Worldwide there are a small number of approved therapies including the first which was achieved in 2003 in China [47]. This approved therapy uses recombinant adenovirus expressing p53 for head and neck

squamous cell carcinoma. In a continuation of the ADA early trial, successes have been observed in patients with related conditions such as beta-thalassemia [48], hemophilia [49], Wiskott-Aldrich syndrome [50], and metachromatic leukodystrophy [51]. In Europe Glybera, in 2012, became the first gene therapy to be approved for the treatment of the ultra-rare inherited disorder lipoprotein lipase (LPL) deficiency. This treatment utilizes a recombinant AAV coding for LPL and is injected intramuscularly (http://www.uniqure.com/news/167/182/). As yet there are no FDA-approved gene therapy products.

2.5 Genome Editing

As noted successful gene therapy, antisense, and RNA interference at the clinical stage are becoming a reality. These techniques rely on either overexpression of a wild-type gene to compensate for a mutant protein (gene therapy) or a random integration event if delivered using an integrating viral vector. Antisense and RNAi rely on knocking down a protein either aberrantly overexpressed or to try and tip the balance of a pathologic condition to a more benign phenotype. Neither technique gets to the root cause of the problem, which may be a mutation in the genome itself. Genome editing technologies may now be able to fill this gap to target and repair the defective gene. These technologies include zinc fingers (ZFN), transcription activator-like effector nucleases (TALENs), and clustered, regularly interspaced short palindromic repeats (CRISPRs) [52] based on targeting endonucleases to a specific sequence, eliciting a double-strand break and then repair, either nonhomologous end joining (to knock out) or homology directed to repair a mutation. Ex vivo gene knockout or repair is a favored approach due to a more controlled environment to manipulate the cells in question. Studies in mouse models were conducted to determine if zinc finger nucleases targeting CCR5 would be effective in reducing HIV viral load. Primary CD4+ T cells were transduced with adenoviral-encoded ZFN constructs to target CCR5, expanded and adoptively transferred to immunodeficient NOG mice with HIV-1-infected or HIV-1-noninfected PBMC. The CCR5 knockout cells conferred resistance to HIV-1 infection, preferential expansion, and decreased viral load [53]. Following this a phase 1 study was conducted in 12 HIV-infected patients and deemed safe as well as demonstrating some proof of concept [54]. As with other modalities, in vivo targeting requires a system which ideally localizes to the organ of choice. For the first in vivo gene correction success, factor IX was chosen as it is produced in the liver, one of the easier organs to effectively target. Adeno-associated virus (AAV) was used to first create deletions in humanized F9 gene mice (where the murine gene was already knocked out). Donor AAV vectors were used containing homology flanking arms to correct the mutation. After 10 weeks homologous recombination was detected and circulating factor IX detected, albeit at low levels. This work did demonstrate that the correction of mutated genes in vivo was feasible [55]. CRISPR (clustered, regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) and TALENs

(transcription activator-like effector nucleases) are more recent gene editing methods which may overcome some inefficiencies of ZFNs through simpler targeting. CRISPRs have been used in many in vitro settings as well as to generate engineered preclinical animal models including a recent publication of a gene-modified cynomolgus monkey [56]. Injection of the Cas9 mRNA and short guide (sg) RNA into single-cell embryos enabled precise gene targeting and the birth of founder animals. Excitement in the field has led to companies forming in the hope of exploiting these newer technologies for clinical applications and includes Editas Medicine (http://editasmedicine.com/index.php) and CRISPR Therapeutics (http://crisprtx.com/).

3 State of the Art in Genomic Medicine

There are those drugs that make it all the way to registration but also those that fall at different stages of the process for different reasons, toxicity, lack of efficacy, and noncompetitiveness; and genome medicine is no exception. From phase 1 only about 10 % of drugs make it to approval [57] and this ignores the attrition that has already taken place leading to phase 1. Taking a new type of medicine into the clinic, in all likelihood, increased the chances of failure as unforeseen issues arise.

In the antisense field, we've already heard about the registration of fomivirsen and then the 15-year gap before the registration of Kynamro; however the pipeline for antisense oligonucleotides is still rich [58]. One other approval to mention is that of Macugen, a RNA aptamer, and although it is an oligonucleotide, its mechanism of action is more like a traditional antagonist (binding VEGF). The approval in 2004 (https://www.macular.org/macugen) for wet age-related macular degeneration highlighted again that local delivery, intravitreal injection, enabled successful targeting of drug and a higher chance of success.

Clinical development of RNA interference is being led by Alnylam, who currently boasts a relatively large pipeline and some clinical successes. The focus of the company is on liver-targeted strategies within three defined areas, genetic medicines, cardiometabolic, and hepatic infectious diseases. This builds on their expertise in targeting siRNA molecules to the liver through the use of lipid nanoparticles (LNP). These LNP-siRNA complexes recently demonstrated successful targeting of transthyretin (TTR) gene in an extension of a phase 2 open-label trial in patients with familial amyloidotic polyneuropathy (FAP). This is caused by mutations in the TTR gene resulting in abnormal amyloid protein accumulation in various regions of the body, results in neural and cardiomyopathy, and is often fatal. The trial outcome showed sustained serum TTR reductions of 80 % as well as potential stabilization of neuropathy. This was intravenously (iv) delivered, with 7–11 doses per patient, no serious adverse events and only mild infusion reactions. Moving away from iv delivery is an important consideration with chronic diseases, and therefore Alnylam has developed a subcutaneous (sc) approach using a GalNAc-siRNA conjugate delivery system. Here the *N*-scetylgalactosamine (GalNAc) binds to asialoglycoprotein receptors on hepatocytes for a more targeted uptake. A sc formulation, ALN-PCSsc,

is currently recruiting for a phase 1 trial targeting PCSK9 to lower LDL. A completed phase 1 study with an iv formulation resulted in a 70% reduction of the targeted protein and 40% lower LDL [59]. Tekmira Pharmaceuticals (http://www.tekmira.com/) also have an ongoing phase 1/2 clinical trial, targeting PLK1 in adrenocortical carcinoma and neuroendocrine tumors. Results from this study are expected in 2015. Silence Therapeutics (http://silence-therapeutics.com/) lead molecule, Atu-027, targets a protein, PKN3, in the vascular endothelium and is addressing pancreatic tumors. Recent phase 2a data suggested some positive outcome when six doses of drug were compared to eight doses of drug over 8 weeks, median PFS of 1.81 months (six doses) versus PFS of 5.33 months (eight doses). A number of phase 1 studies are being conducted by other companies with the majority targeting the liver or oncology indications.

Progress in microRNA therapy is also notable, with most success delivering anti-miR reagents, and therefore could use lessons learned from the antisense field. MiR-122 was identified as highly abundant in the liver and was a proof of concept that antagonizing it could have functional consequence. Antagomir delivery to mice resulted in modulation of cholesterol levels, as predicted from the recognition motifs for miR-122 in biosynthesis genes for the pathway [38]. A second novel finding for miR-122 was that HCV utilized this miR to support propagation [60]. A mir-122 LNA-based anti-miR demonstrated impressive suppression of HCV, a large reduction in genome titer, and no safety issues in nonhuman primates [61] suggesting translatability to human. Santaris Pharma (now Roche) has taken miravirsen as far as phase 2 with recently reported data. Results showed an enduring dose-dependent reduction in HCV RNA levels, no dose-limiting adverse events, and no escape mutations in 36 patients with chronic HCV genotype 1 infection [62]. Regulus Therapeutics also has an anti-miR-122 program; RG-101 is a GalNAc-conjugated molecule for the treatment of HCV and has completed phase 1 clinical trials. A total of 58 healthy volunteers and 32 HCV patients (multiple genotypes), liver fibrosis status, and treatment histories were enrolled in the study. Treatment resulted in significant, sustained viral load reductions in all treated HCV patients, a favorable safety profile with no serious adverse events or discontinuations reported in the treated HCV patients (http://ir.regulusrx.com/releasedetail.cfm?ReleaseID=895314). Phase 2 trials are expected to commence in 2015. Regulus also expects to initiate a phase 1 clinical trial in 2015 targeting miR-21 for the treatment of Alport syndrome, an orphan kidney disease resulting in fibrosis and organ failure. Another trial to note is that of miR-34 as this miRNA is frequently silenced in variety of tumors and has a tight relationship with p53. Overexpression of miR-34 inhibits proliferation, epithelial to mesenchymal transition, migration, invasion, and metastasis of various cancer cells [63]. Therefore the approach Mirna Therapeutics is taking is to add this miRNA as a mimic with a liposomal delivery formulation in patients with unresectable primary liver cancer or solid cancers with liver involvement. The phase 1 open-label trial is ongoing and expected to complete in 2015 (http://www.mirnarx.com/pipeline/mirna-MRX34.html).

Gene therapy approvals are limited to China and Europe although a wide range of trials have been conducted over the past 25 years and continue today. As we have

seen, the ability to localize delivery, e.g., to the eye or liver or even to modify cells ex vivo, has proven to be successful in this and other areas. The approval of Glybera also demonstrated the value of a protein factory approach, using muscle to produce the deficient protein. The lung was also seen as a local delivery opportunity and a long history of gene replacement therapy will be discussed later. Spark Therapeutics (http://www.sparktx.com/pipeline) is in phase 3 for the treatment of inherited retinal dystrophies (IRDs) caused by mutations in the RPE65 gene delivered via an AAV vector. Bluebird Bio (http://www.bluebirdbio.com/product-overview.php) is in phase 2/3 with their Lenti-D candidate, a potential one-time treatment to stabilize and prevent progression of childhood cerebral adrenoleukodystrophy. This approach involves ex vivo transduction of the ABCD1 gene into the patient's hematopoietic stem cells. Celladon (http://www.celladon.com/mydicar/) is currently in phase 2/3 trials with Mydicar for heart failure. This therapy delivers an AAV vector by catheterization and encodes for serca2a a key enzyme deficient in heart failure patients. In the CUPID 1 phase 2 trial 12 months after receiving a single infusion of Mydicar, patients treated with the highest dose versus placebo had an 88 % risk reduction of major cardiovascular events. Voyager Therapeutics (http://www.voyagertherapeutics.com/programs.php), in phase 1 for Parkinson's disease, also uses AAV gene replacement therapy but additionally image-guided neurosurgical methods for precise infusion into the brain.

4 Barriers to Successful Gene Therapy: Cystic Fibrosis as a Case Study

The cloning of the gene which when mutated resulted in cystic fibrosis, the CFTR gene, was a key trigger for a 25-year effort at gene replacement therapy, which is still ongoing. Cystic fibrosis, resulting from a defect in the CFTR gene, a chloride channel with additional regulatory roles, results in impaired clearance of lung pathogens, persistent infection, decline in gas exchange, and at best a life span of around 40 years [64]. The persistent efforts of researchers attempting gene therapy for this still largely unmet disease did uncover multiple roadblocks which has supported the genomic therapy fields discussed. Both viral and nonviral vectors were evaluated, with a first barrier determined to be the lung itself, an organ designed to limit topical insults. In CF patients, the thick infected mucus or sputum was determined to be a significant barrier to the delivery of gene therapy reagents (for review of extracellular barriers [65]). One issue with early viral gene transfer (using Ad and AAV) was the requirement to repeatedly administer, and this resulted in immune responses to the viruses and therefore inefficient gene transfer. More recent use of lentiviral vectors suggests persistence of gene transfer and lack of immune responses and therefore may be a way forward [66]. Nonviral vectors are inherently less efficient due to the lack of evolutionary design which viruses have employed. The encoded plasmid needs to be optimized for non-immunogenicity and high expression, while the delivery vehicle needs to lack immunogenicity and toxicity and avoid extracellular barriers. Once the complex is internalized, additional barriers

remain including endosomal entrapment (and avoiding triggering of pattern recognition receptors), nucleases, nuclear entry, and stability of expression [67]. Development of the nonviral vector component has been limited with no real step changes, although developments did support the nascent RNAi field where the siRNA required a vehicle for successful in vivo delivery, and nonviral gene delivery had proven safe in both nasal and lung trials for CF [68]. Optimization of the plasmid expressing the CFTR gene was also stepwise with modifications to the promoter, from CMV to a human CMV enhancer and the human elongation factor 1a promoter hybrid. Identifying and removing CpG motifs to prolong expression and prevent TLR9 engagement also moved this and other programs forward [69]. In 2013 a phase IIb, randomized, double-blind, placebo-controlled trial was announced consisting of the originally identified, optimal lipid termed GL67 and the fully optimized plasmid termed pGM169 [70]. Results from the study have recently been published [71] and culminate the 25 years of effort so far in this field. These efforts may serve to support the field of genomic medicine, while they may not benefit CF patients if small molecule drugs such as ivacaftor and lumacaftor from Vertex Pharmaceuticals fulfill their phase 3 promise at market.

5 The Rise and Fall and Rise of Genomic Medicine in the Pharma and Biotech Sectors

The history of genomic medicine is no different to many new inventions; there are ups and downs along the way. We've seen how gene therapy and other new technologies have developed over the past 30 years, and investment within the pharma and biotech sectors has mirrored the hype, the failures, and the gradual resurrection. In the 1990s a number of small companies, some headed by respected gene therapy researchers, had been formed or realigned as the promise of commercialization loomed. These included Targeted Genetics, Genetic Therapy Inc., Oxford Biomedica, Vical, and Benitec Biopharma, to name a few. Large pharma companies all invested either internally or in some cases with smaller companies to ensure opportunities were evaluated. The initial excitement was very much curtailed by the unfortunate aforementioned death in 1999, and large companies in particular started to downsize efforts or exit gene therapy altogether. Pharma companies then, as now, needed to generate blockbuster drugs, and in the early 2000s, it appeared that gene therapy may only be applicable to targeted ex vivo approaches in patients with rare diseases such as immunodeficiencies. Together with increased regulatory oversight and unclear paths to registration, most large companies sat back and waited for the next big thing. Fortunately this was already on the horizon and RNAi saw a burst of high-value deals in the mid-2000s. The first big deal arose between Novartis and Alnylam in 2005 (http://investors.alnylam.com/releasedetail.cfm?ReleaseID=446283) described as a major alliance, initially costing close to $60 million with the potential to exceed $700 million. A year later Merck announced the acquisition of SIRNA Therapeutics for $1.1 billion signaling a huge gamble in the RNAi field [72]. Pfizer

and Quark made a much smaller deal in 2006, although not fully disclosed but in excess of $100 million. In 2007 AstraZeneca and Silence Therapeutics jumped into a deal worth ~$400 million suggested to be in the respiratory space. Another huge deal in 2007 saw Roche partner with Alnylam (http://www.roche.com/media/store/releases/med-cor-2007-07-09.htm) valued at ~$1 billion and with Roche acquiring a former Alnylam site in Germany. Around 2010 Major Pharma started to get cold feet in the RNAi field with Novartis failing to extend a deal with Alnylam and finally appearing to stop all RNAi work in 2014. Roche announced plans to stop all RNAi work at its German and US sites in 2010. Merck closed SIRNA Therapeutics in 2011 and eventually sold its patent portfolio to Alnylam, at a significant loss. Once again, Pharma was investigating the next big thing, miRNA Therapeutics, as RNAi therapeutics excitement was waning. The miRNA field, having evolved in the academic world, exploded after sequencing the human genome and had learned considerably from the antisense field in terms of progressing single-stranded oligonucleotides into the clinic. In 2008 GlaxoSmithKline and Regulus Therapeutics announced a collaboration for inflammatory disease indications with the deal worth a potential $600 million if drugs were developed. A second deal in 2010 was struck to focus on miR-122 for HCV treatment (RG-101) but now being developed solely by Regulus suggesting a waning of GSK interest. Regulus and Sanofi-Aventis penned a multiyear deal to collaborate on up to four microRNA targets in the area of fibrosis, including the lead program targeting microRNA-21. A third deal was signed with AstraZeneca in 2012 to work on cardiovascular targets. In 2014 Roche acquired Santaris Pharma allowing access to both antisense and anti-miR opportunities. The antisense field has seen small deals and one off drug discovery programs, including a deal between ISIS and Bristol-Myers Squibb in 2007 to target PCSK9. A phase 1 trial was prematurely stopped in 2010, although the reasons were not disclosed. Interestingly Santaris Pharma also stopped a PCSK9 antisense phase 1 trial (in 2011) for undisclosed findings. One further antisense success story so far has been Celgene's agreement with Nogra Pharma Limited, to develop and commercialize GED-0301, an oral antisense DNA oligonucleotide targeting Smad7 (mongersen) for the treatment of Crohn's disease (CD) and potentially additional indications. Recent data from a phase 2 trial showed clinical remission in CD patients after 2 weeks of dosing and maintained out to 4 weeks. No serious drug-specific adverse events were noted [73]. In January 2015 Isis and Janssen announced a collaboration to discover and develop antisense drugs that through formulation could be locally administered (including orally), to treat autoimmune disorders in the GI tract. The agreement covers three programs potentially worth up to $800 million to Isis (http://ir.isispharm.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2002820). Gene therapy has now come full circle within the pharma industry with a number of high-profile deals as well as smaller biotechs coming to the fore. The larger companies seem to have learned from the failures and are now focusing more on orphan indications such as GSK program focusing on ADA-SCID (http://us.gsk.com/en-us/media/press-releases/2010/gsk-fondazione-telethon-and-fondazione-san-raffaele-to-collaborate-on-gene-therapy-for-rare-diseases/) or ex vivo manipulation of T cells to fight tumors [74]. Novartis and U Penn entered into a collaboration in 2012

to investigate this new form of immunotherapy (http://www.novartis.com/newsroom/media-releases/en/2012/1631944.shtml) with impressive results being presented [75]. A number of smaller biotechs are also back in the field including Bluebird, Juno, and Spark Therapeutics. The latter with former BMS R&D head Elliot Sigal on the board. The latest in large deals involves BMS and collaboration with UniQure, the only company with an approved gene therapy outside China. The initial program aims to deliver S100A1 via AAV for congestive heart failure, with potential for nine additional programs, for a potential $1 billion deal (http://news.bms.com/press-release/rd-news/bristol-myers-squibb-and-uniqure-enter-exclusive-strategic-collaboration-devel). A word of caution, however, with a recent announcement from Celladon of the failure of their phase 2b advanced heart failure trial (http://ir.celladon.net/releasedetail.cfm?ReleaseID=908592) delivering SERCA2a via AAV.

6 Genomic Medicine and Inflammatory Diseases

Rheumatoid arthritis is probably the most studied inflammatory disease with respect to genomic medicine, and this is related in part to the better understanding of disease pathogenesis, a longer history of successful treatment with biologics, and somewhat localized pathogenesis. There is still the need for better treatment and longer-lasting remission and genomic medicine may be part of the answer. Translating the discovery of novel targets to therapeutic modalities that derive efficacy in clinical populations is still a huge hurdle. The basic thought process of RA gene therapy was to transduce cells in the joint or ex vivo, which would then sustain production of a therapeutic protein agonist or antagonist. The first phase 1 trial transduced autologous fibroblasts with a retrovirus expressing IL1Ra and delivered some encouraging data [76]. This overcomes the potential inefficiencies of systemic delivery where drug load may be limited (narrowing therapeutic indices) as drug will accumulate in tissues other than the joint. A second study also delivering cells to finger joints, in two patients, suggested some clinical benefits [77]. Further clinical phase 1 and 2 studies delivered a soluble TNF antagonist (analogous to Enbrel) from an AAV vector injected in large (ankle, knee, elbow) and small (wrist, finger) joints. The data from the study demonstrated that gene transfer was generally safe and feasible, although antibodies were generated to the vector, and one fatal adverse event was noted although deemed not related to the gene transfer [78]. One other anti-TNF strategy was run by Isis Pharmaceuticals and engaged an antisense oligonucleotide in a phase 2 trial designed to assess safety and efficacy of ISIS 1048383 by subcutaneous injection. It was administered for 3 months versus placebo with three different dosing regimens, in approximately 160 TNF-alpha inhibitor-naive patients (www.clinicaltrials.gov studyNCT00048321). Results posted by Isis stated “Patients receiving the once- and twice- weekly doses experienced similar responses to treatment, with 41 % of evaluable patients achieving a 20 % decrease in disease activity. In comparison, 23 % of placebo-treated patients achieved a 20 % decrease ($p = 0.04$)” (http://ir.isispharm.

com/phoenix.zhtml?c=222170&p=irol-newsArticle_pf&ID=1289726&highlight=). However no further development of the molecule is apparent.

For inflammatory bowel diseases, there are one partial success and a second greater success with antisense molecules targeting ICAM1 and SMAD7, respectively. Alicaforsen targets ICAM1 and has been run in a number of phase 2 studies and two phase 3 studies in patients suffering with ulcerative colitis (UC) or Crohn's disease (CD). The phase 3 studies in CD were not superior to placebo and therefore Isis stopped further development. In a phase 2 study delivering the drug by enema formulation to UC patients, some superior efficacy was observed [79]. Development of this molecule has been taken on by Atlantic Healthcare. Mongersen, as mentioned earlier, has demonstrated efficacy in a phase 2 trial and is noted for its oral bioavailability designed into the formulation to be released in the terminal ileum and right colon through a pH-mediated capsule degradation. This strategy, although high risk, has paid off so far and utilizes a local delivery approach, which has been at the forefront of recent clinical successes in genomic medicine. Targeting SMAD7 increases the signaling of TGFB, supporting an anti-inflammatory role, although this molecule is pro-fibrotic which might suggest long-term adverse effects. The localized delivery limits any systemic delivery so should limit a more widespread adverse effect potential. RNA interference is yet to make a clinical splash in the RA pool although preclinical data supports proof of concept; a short review highlights some studies [80]. Gene therapy and RNAi approaches in preclinical models have shown potential with oral delivery strategies. IL-10 gene delivery in microspheres (gelatin nanoparticles) demonstrated reduced inflammatory mediators, weight gain, and favorable clinical activity scores in a murine colitis model [81]. IL-10 has been widely tested in a number of inflammatory conditions by administration of recombinant protein but with largely unsatisfying clinical data [82], possibly due to relatively short half-life and low local concentration, particularly with respect to inflammatory bowel diseases. A more recent study used RNAi targeting CD98 in intestinal epithelial cells to dampen colitis in a murine model [83]. This oral delivery system used polylactic acid (PLA) nanoparticles loaded with CD98 siRNA/polyethyleneimine (PEI) complexes for colonic targeting.

ATL1102 is a second-generation antisense oligonucleotide licensed to Antisense Therapeutics from Isis targeting CD49d RNA, the alpha chain of VLA-4 and indicated for multiple sclerosis. In a phase 2 trial, approximately 80 patients with relapsing-remitting MS were treated with drug or placebo for 8 weeks, twice weekly, with an 8-week follow-up. The drug met its primary efficacy end point of reducing new active lesions with an acceptable safety profile [84]. This target is the same as the approved drug, Tysabri (http://www.tysabri.com/), a monoclonal antibody binding VLA-4. This approved drug carried with it a risk of fatal PML, and identifying a differential (safer) profile of ATL1102 versus Tysabri might be key to taking it into phase 3 and approval. In the publication, the authors discuss how ATL1102 may be differentiating through reducing pre-B cells compared to Tysabri which increases circulating B cells, although further studies are warranted to establish the hypothesis.

7 Converting the Promise of Genomic Medicine into Reality for Inflammatory Diseases

In the past four decades, we have seen a revolution in genetics and genomics, culminating in small but hugely important successes for the progress of genomic medicine, from very limited sequence information to full genome, uncovering RNA interference and microRNA regulation, manipulating viruses to deliver genes, and successful launch of gene therapy products. It is expected that the FDA will approve a gene therapy product in the next year or two, likely for a genetically defined or monogenic disease. It is the lessons learned from these preceding decades which should become a platform for inflammatory disease genomic medicine approval. The Celgene antisense molecule is already looking very promising and has encouraged the likes of Janssen to commit to the area. But what have we learned and what do we need to be aware of as we watch the continued development of the field?

7.1 Delivery

Access to the tissue or cell of choice has been a hurdle for the whole area and remains a significant obstacle for inflammatory diseases. Oral delivery for IBD appears to be bearing fruit. For RA, MS, and SLE, questions remain as to which the appropriate cell might be to target and how selective targeting might be achieved. Direct injection into the joint for RA may not be tolerated unless long-term benefit could be achieved? Ex vivo delivery has proved successful, as well as the liver-targeted approaches. Identifying inflammatory diseases where either may be appropriate remains a challenge.

7.2 Target

Identifying pathologic targets is a mainstay of the pharmaceutical industry and is the first step on the path toward drug development. A large proportion of early programs fail due to lack of validation or efficacy in preclinical models. A smaller number probably fail due to lack of tractability with conventional modalities such as small molecules or antibodies. These are the likely targets that genomic medicine can take into the portfolio. Yet a high attrition is still likely and would require a constant target flow and commitment; something that has been lacking. The best target in the genome can only become the best drug target if there is a path to developing that drug. This includes the standard drug discovery processes of molecule optimization, efficacy, and safety. For genomic drug targets, there are added components to consider.

7.3 Development Path

Isis delivered a single-stranded oligonucleotide to the eye to gain approval for the first antisense drug, encompassing local delivery of a single chemical entity. Gene therapy and RNAi protocols are using nanoparticles or viral vectors, adding to the complexity of the eventual drug. Although small molecules may have off-target effects, these may be uncovered post approval if the drug is safe and effective. RNAi, antisense, and particularly miRNA drugs could have much more damaging off-target effects and are something that is taken into account very early on in the discovery process. Formulation and drug stability may also be an issue if there are multicomponents or if specific storage conditions are required. Each drug may be slightly different, requiring alternate development paths. Again, this is something large pharmas are not used to, being more comfortable developing standard drugs for large patient populations.

7.4 Target Regulation

Small and large molecule PKPD relationships are established preclinically and projected to human doses aimed at inhibiting the target (usually) completely or activating a target for an established time period before re-dosing. Many challenges exist for genomic medicine not least the different modalities employed such as gene therapy to overexpress a defective or missing protein, RNAi or antisense aiming to knock down a protein, or miRNA regulation of potentially multiple genes. Each is challenged by how much can be delivered/knocked down and is dependent on design of the plasmid/siRNA/antisense/miRNA as well as the delivery vehicle and the ability to target the delivery as required. Ensuring cross-species activity can also be a challenge as well as having the correct preclinical models to interrogate the target. Translational challenges exist for small molecules and biologics, with many failures, but are an accepted part of the process. New technologies take time to be accepted, with early failures often a reason for large pharma to step out until a more established platform is developed.

7.5 Control of Expression

Gene therapy or gene editing may be expected to elicit a permanent “cure”; therefore being able to control the amount of expression could be important. Gene dosage effects can be problematic as in the extreme case of trisomy 21. Generating sufficient expression to elicit a biological effect has been the biggest problem to date with gene therapy, but these issues are more of a concern compared to small molecule or biologic administration where the PK properties are generally well understood. The knockdown of a gene could also lead to unwanted side effects, with

again no good way to immediately reverse effects. Toxicities so far appear more related to, for example, oligonucleotide chemistry or indeed an immune response to a vector component. If genomic medicine continues to evolve, better control of expression may become a desired parameter. Cell-specific promoters may be one way in which a more selective expression is achieved. Other ways to control expression may incorporate tissue stress, such that the desired effect is only seen, for example, under hypoxia. Incorporating some measure of control may be a niche that small molecules and biologics struggle to fill, although antibody-drug conjugates are being approved for certain oncology indications to selectively target tumors.

7.6 Superior Efficacy Versus Standard of Care

For genomic medicine to be successful, the treatment has to deliver superior efficacy to current standard of care. This requires the identification of disease-changing paradigms and the delivery of safe, transformative medicines. These are big challenges for small molecule and biologic research, and as we have seen, progress in the genomic medicine field has really focused on rare diseases where there are no other options to treat. In these cases the treatment can be lifesaving, as in the case of ADA-SCID, with GSK filing for European approval in mid-2015. For inflammatory diseases equally transformative treatments will have to be pursued to enable genomic medicine to become more accepted.

7.7 Costs

Glybera® was announced in 2014 as costing close to $1.5 million essentially for a cure. Although a high price to pay, the small patient pool equates to a relatively small health cost burden compared to chronic, prevalent diseases. Equally for other rare diseases requiring enzyme replacement, the costs per year can be anywhere between $200 and $500 K and requiring many years of treatment. Hepatitis C cures (Sovaldi®) and melanoma cures (Yervoy®) costs approach $100 K. Life-changing treatments cost significantly to develop and transform patients' lives. Pharmaceutical companies are willing to invest billions to develop small molecules and biologics and are beginning to invest in genomic medicines, knowing that there can be a return on investment if transformative medicines make it to market.

8 Summary

The reinvestment in the genomic medicine field is gaining pace with some recent major deals and collaborations from large pharma. Academic groups and biotech companies can take a lot of credit for this change given the investments they were

willing to make to pursue these new treatment modalities. A combination of this, together with the willingness of pharma companies to invest in rare diseases, often as stepping stones into larger diseases has further supported this change. It is hoped that mechanisms, pathways, and targets identified in rare inflammatory diseases, targeted with genomic medicines, may cross over into the larger inflammatory disease populations and deliver transformative benefit to millions of patients.

References

1. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
2. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al (2001) The sequence of the human genome. Science 291(5507):1304–1351
3. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411(6836):494–498
4. Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 171(4356):737–738
5. Zamecnik PC, Stephenson ML (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A 75(1):280–284
6. Drews J (2000) Drug discovery: a historical perspective. Science 287(5460):1960–1964
7. McCormack PL (2011) Celecoxib: a review of its use for symptomatic relief in the treatment of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. Drugs 71(18):2457–2489
8. Hopkins AL, Groom CR (2002) The druggable genome. Nat Rev Drug Discov 1(9):727–730
9. Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D et al (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nat Biotechnol 18(6):630–634
10. Geiser T (1990) Large-scale economic synthesis of antisense phosphorothioate analogues of DNA for preclinical investigations. Ann N Y Acad Sci 616:173–183
11. Marcus-Sekura CJ, Woerner AM, Shinozuka K, Zon G, Quinnan GV Jr (1987) Comparative inhibition of chloramphenicol acetyltransferase gene expression by antisense oligonucleotide analogues having alkyl phosphotriester, methylphosphonate and phosphorothioate linkages. Nucleic Acids Res 15(14):5749–5763
12. Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen JS et al (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc Natl Acad Sci U S A 84(21):7706–7710
13. Vitravene Study G (2002) Randomized dose-comparison studies of intravitreous fomivirsen for treatment of cytomegalovirus retinitis that has reactivated or is persistently active despite other therapies in patients with AIDS. Am J Ophthalmol 133(4):475–483
14. Vitravene Study G (2002) A randomized controlled clinical trial of intravitreous fomivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with AIDS. Am J Ophthalmol 133(4):467–474
15. Shen LX, Kandimalla ER, Agrawal S (1998) Impact of mixed-backbone oligonucleotides on target binding affinity and target cleaving specificity and selectivity by Escherichia coli RNase H. Bioorg Med Chem 6(10):1695–1705
16. McKay RA, Miraglia LJ, Cummins LL, Owens SR, Sasmor H, Dean NM (1999) Characterization of a potent and specific class of antisense oligonucleotide inhibitor of human protein kinase C-alpha expression. J Biol Chem 274(3):1715–1722
17. Bennett CF, Swayze EE (2010) RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol 50:259–293

18. Levin AA (1999) A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta 1489(1):69–84
19. Uhlmann E, Vollmer J (2003) Recent advances in the development of immunostimulatory oligonucleotides. Curr Opin Drug Discov Dev 6(2):204–217
20. Bodera P, Stankiewicz W, Kocik J (2012) Synthetic immunostimulatory oligonucleotides in experimental and clinical practice. Pharmacol Rep PR 64(5):1003–1010
21. Akdim F, Stroes ES, Sijbrands EJ, Tribble DL, Trip MD, Jukema JW et al (2010) Efficacy and safety of mipomersen, an antisense inhibitor of apolipoprotein B, in hypercholesterolemic subjects receiving stable statin therapy. J Am Coll Cardiol 55(15):1611–1618
22. Butler M, Stecker K, Bennett CF (1997) Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. Lab Invest J Tech Methods Pathol 77(4):379–388
23. Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2(4):279–289
24. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391(6669):806–811
25. Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101(1):25–33
26. Huang A, Chen Y, Wang X, Zhao S, Su N, White DW (2004) Functional silencing of hepatic microsomal glucose-6-phosphatase gene expression in vivo by adenovirus-mediated delivery of short hairpin RNA. FEBS Lett 558(1–3):69–73
27. Saxena S, Jonsson ZO, Dutta A (2003) Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J Biol Chem 278(45):44312–44319
28. Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev MMBR 67(4):657–685
29. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. Nat Biotechnol 22(3):326–330
30. Behlke MA (2008) Chemical modification of siRNAs for in vivo use. Oligonucleotides 18(4):305–319
31. Pasquinelli AE (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13(4):271–282
32. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5):843–854
33. Almeida MI, Reis RM, Calin GA (2011) MicroRNA history: discovery, recent applications, and next frontiers. Mutat Res 717(1–2):1–8
34. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E et al (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99(24):15524–15529
35. Bui TV, Mendell JT (2010) Myc: maestro of MicroRNAs. Genes Cancer 1(6):568–575
36. Feng Z, Zhang C, Wu R, Hu W (2011) Tumor suppressor p53 meets microRNAs. J Mol Cell Biol 3(1):44–50
37. Li Y, Kowdley KV (2012) MicroRNAs in common human diseases. Genomics Proteomics Bioinforma 10(5):246–253
38. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M et al (2005) Silencing of microRNAs in vivo with 'antagomirs'. Nature 438(7068):685–689
39. Lindow M, Kauppinen S (2012) Discovering the first microRNA-targeted drug. J Cell Biol 199(3):407–412
40. Kasinski AL, Kelnar K, Stahlhut C, Orellana E, Zhao J, Shimer E et al (2015) A combinatorial microRNA therapeutics approach to suppressing non-small cell lung cancer. Oncogene 34(27):3547–3555
41. Graham FL, van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52(2):456–467

42. Kantoff PW, Kohn DB, Mitsuya H, Armentano D, Sieberg M, Zwiebel JA et al (1986) Correction of adenosine deaminase deficiency in cultured human T and B cells by retrovirus-mediated gene transfer. Proc Natl Acad Sci U S A 83(17):6563–6567
43. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M et al (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science 270(5235):475–480
44. Felgner PL, Ringold GM (1989) Cationic liposome-mediated transfection. Nature 337(6205):387–388
45. Sibbald B (2001) Death but one unintended consequence of gene-therapy trial. CMAJ Can Med Assoc J = J Assoc Med Can 164(11):1612
46. Krieg AM (1999) Direct immunologic activities of CpG DNA and implications for gene therapy. J Gene Med 1(1):56–63
47. Pearson S, Jia H, Kandachi K (2004) China approves first gene therapy. Nat Biotechnol 22(1):3–4
48. Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F et al (2010) Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467(7313):318–322
49. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J et al (2014) Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med 371(21):1994–2004
50. Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C et al (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341(6148):1233151
51. Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T et al (2013) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341(6148):1233158
52. Gaj T, Gersbach CA, Barbas CF 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31(7):397–405
53. Perez EE, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O et al (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26(7):808–816
54. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G et al (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med 370(10):901–910
55. Li H, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS et al (2011) In vivo genome editing restores haemostasis in a mouse model of haemophilia. Nature 475(7355):217–221
56. Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L et al (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156(4):836–843
57. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J (2014) Clinical development success rates for investigational drugs. Nat Biotechnol 32(1):40–51
58. Jiang K (2013) Biotech comes to its 'antisenses' after hard-won drug approval. Nat Med 19(3):252
59. Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, Liebow A, Bettencourt BR, Sutherland JE et al (2014) Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet 383(9911):60–68
60. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309(5740):1577–1581
61. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327(5962):198–201
62. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K et al (2013) Treatment of HCV infection by targeting microRNA. N Engl J Med 368(18):1685–1694
63. Rokavec M, Li H, Jiang L, Hermeking H (2014) The p53/miR-34 axis in development and disease. J Mol Cell Biol 6(3):214–230

64. Cutting GR (2015) Cystic fibrosis genetics: from molecular understanding to clinical application. Nat Rev Genet 16(1):45–56
65. Sanders N, Rudolph C, Braeckmans K, De Smedt SC, Demeester J (2009) Extracellular barriers in respiratory gene therapy. Adv Drug Deliv Rev 61(2):115–127
66. Sinn PL, Arias AC, Brogden KA, McCray PB Jr (2008) Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. J Virol 82(21):10684–10692
67. Lechardeur D, Lukacs GL (2002) Intracellular barriers to non-viral gene transfer. Curr Gene Ther 2(2):183–194
68. Griesenbach U, Alton EW, Consortium UKCFGT (2009) Gene transfer to the lung: lessons learned from more than 2 decades of CF gene therapy. Adv Drug Deliv Rev 61(2):128–139
69. Hyde SC, Pringle IA, Abdullah S, Lawton AE, Davies LA, Varathalingam A et al (2008) CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat Biotechnol 26(5):549–551
70. Alton EW, Boyd AC, Cheng SH, Cunningham S, Davies JC, Gill DR et al (2013) A randomised, double-blind, placebo-controlled phase IIB clinical trial of repeated application of gene therapy in patients with cystic fibrosis. Thorax 68(11):1075–1077
71. Alton EW, Armstrong DK, Ashby D, Bayfield KJ, Bilton D, Bloomfield EV et al (2015) Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med 3:684–691
72. (2006) A billion dollar punt. Nat Biotechnol 24(12):1453
73. Monteleone G, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F et al (2015) Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. N Engl J Med 372(12):1104–1113
74. Gill S, June CH (2015) Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. Immunol Rev 263(1):68–89
75. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ et al (2014) Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 371(16):1507–1517
76. Evans CH, Robbins PD, Ghivizzani SC, Wasko MC, Tomaino MM, Kang R et al (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. Proc Natl Acad Sci U S A 102(24):8698–8703
77. Wehling P, Reinecke J, Baltzer AW, Granrath M, Schulitz KP, Schultz C et al (2009) Clinical responses to gene therapy in joints of two subjects with rheumatoid arthritis. Hum Gene Ther 20(2):97–101
78. Mease PJ, Wei N, Fudman EJ, Kivitz AJ, Schechtman J, Trapp RG et al (2010) Safety, tolerability, and clinical outcomes after intraarticular injection of a recombinant adeno-associated vector containing a tumor necrosis factor antagonist gene: results of a phase 1/2 study. J Rheumatol 37(4):692–703
79. Vegter S, Tolley K, Wilson Waterworth T, Jones H, Jones S, Jewell D (2013) Meta-analysis using individual patient data: efficacy and durability of topical alicaforsen for the treatment of active ulcerative colitis. Aliment Pharmacol Ther 38(3):284–293
80. Pauley KM, Cha S (2013) RNAi therapeutics in autoimmune disease. Pharmaceuticals 6(3):287–294
81. Bhavsar MD, Amiji MM (2008) Oral IL-10 gene delivery in a microsphere-based formulation for local transfection and therapeutic efficacy in inflammatory bowel disease. Gene Ther 15(17):1200–1209
82. Asadullah K, Sterry W, Volk HD (2003) Interleukin-10 therapy – review of a new approach. Pharmacol Rev 55(2):241–269
83. Laroui H, Geem D, Xiao B, Viennois E, Rakhya P, Denning T et al (2014) Targeting intestinal inflammation with CD98 siRNA/PEI-loaded nanoparticles. Mol Ther J Am Soc Gene Ther 22(1):69–80
84. Limmroth V, Barkhof F, Desem N, Diamond MP, Tachas G, Group ATLS (2014) CD49d antisense drug ATL1102 reduces disease activity in patients with relapsing-remitting MS. Neurology 83(20):1780–1788

Part III
Bispecific Antibodies

Bispecific Antibodies

Alain C. Tissot and Ulrich Brinkmann

1 Introduction

In this chapter we aim at describing a new class of biologics in development for rheumatoid arthritis (RA), bispecific antibodies (bsAbs). These recognize two different specificities, and in most cases targets, as implied in their name. These bring about a series of interesting questions related to target selection, potential mode of action, pharmacokinetics (PK), and whether their development differs from classical biologics. BsAb is a very dynamic field, and therefore it was not possible to review all preclinical developments in inflammatory diseases and RA in details here. As we feel a look at where bispecific antibodies have evolved from is instructive, we start this chapter with some of the main mode of actions and targets of biologics registered for RA, including combination of biologics which were tested in the clinic. We then address pathway and target selection and go over a series of points to consider where bsAbs may require special attention when compared to classical biologics for development. Related to mode of action and target is the selection of the right molecular format. We provide an overview on bsAb formats and then go more specifically over those used in molecules in development for RA and some inflammatory diseases. Finally, we provide an outlook on where this field might develop in the coming years.

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2 Rationale for Bispecific Biologics in Rheumatoid Arthritis

2.1 Mode of Action of Biologics in RA and Combination Therapies

Biologics have greatly improved the treatment and management of rheumatoid arthritis (RA), but there remains a large unmet need. In particular, a minority of patients reach ACR50 let alone ACR70 treatment criteria, and a number of them do not respond at all to therapy. In this respect, it is an attractive thought to combine the effect of multiple modes of action to achieve higher efficacy. Obviously one important aspect here is that the target product profile of the bispecific antibody should be differentiated in comparison to the mere combination therapy. For an indication like RA where chronic dosing for years is likely to remain necessary, availability of a single molecule is likely to remain attractive over co-formulation of two molecular entities for subcutaneous administration. There are also advantages when dealing with just a single molecular entity during clinical development and for manufacturing. Bispecific antibodies where the net effect is higher than the sum of the part, and could not be achieved with a combination of single molecular entities, are certainly a particular class of their own and extremely attractive. This has been illustrated in other areas such as oncology where, for example, bispecific antibodies bring T cells in contact with tumor cells [64, 66] and mediate their elimination, or is being explored in the effort of bringing large molecules to the brain in the area of neuroscience [42]. Common to these approaches is the interaction of the bispecific antibody with one or more cell-surface receptors. There lies certainly an unexplored potential beyond the more evident applications of the bispecific antibody technology targeting ligands in RA.

The modes of action of current biologics in RA cover a wide spectrum, ranging from B cell depletion [9], inhibition of T cell costimulation [69], and cytokine or cytokine receptor inhibition. The latter is the richest class of molecules, with inhibitors of TNFa, IL-1, and IL-6R on the market and IL-17A marketed for psoriasis and investigated for RA. Cytokines have pleiotropic effects on multiple cell types. This is akin to RA pathology, which is characterized by the involvement of T cells, B cells, synovial-like fibroblasts, macrophages, neutrophils, osteoclasts, osteoblasts, chondrocytes, mast cells, and endothelial cells. In addition, multiple pathways involved in pathology of the disease are impacting simultaneously these cells. This makes the case for combination therapies, to extend both the number of responders and the extent of response to therapy. And indeed combination therapies have been tested in clinical trials. The first attempts combined either an anti-TNFa, etanercept, with an anti-IL-1, anakinra [14], or the costimulation inhibitor abatacept on top of background biologics therapy [68]. We will come back to the outcome of these studies later in this chapter.

TNFa is certainly the most prominent cytokine in RA. It is worthwhile reviewing some of the effects of this cytokine, given its central role in the disease, evidenced by the efficacy of its inhibition for therapy. Hence, in rheumatoid synovial membrane

cultures, neutralization of TNFa inhibits secretion of IL-1b, IL-6, and IL-8, while treatment with IL-1 receptor antagonist anakinra did inhibit IL-6 and IL-8 but not TNFa. This illustrates that beyond pleiotropy, a certain hierarchy in cytokine effects exists [4, 38, 54]. At the same time, these cytokines reinforce each other's action by stimulating their respective secretion.

TNFa activates various leukocyte populations, endothelial cells, synovial-like fibroblasts, macrophages, chondrocytes, osteoclasts, and osteoblast. As a result, adhesion molecule, cytokine, and chemokine expressions are upregulated, resulting in leukocyte infiltration and inflammation. In addition, matrix protease secretion and activation of chondrocytes and osteoclasts drive cartilage destruction and bone erosion. Further, by suppressing Tregs, TNFa prevents anti-inflammatory mechanisms. Finally, it contributes to survival and invasiveness of synovial-like fibroblasts, a major cell type involved both in inflammation and formation of the pannus, and cartilage and bone destruction.

In clinical trials, the TNFa inhibitors infliximab, adalimumab, and etanercept have shown that TNFa inhibition results in a decrease in inflammation, both systemically (e.g., reduced acute phase markers, IL-6, IL-1RA, soluble TNFa receptors (TNFRs), serum adhesion molecules, and chemokines) and in the synovium (reduced TNFa expression, adhesion molecules, chemokines, cellular infiltration of, e.g., CD3+, CD68+, and granulocytes). In addition, a reduction in angiogenesis, observed as synovial vascularity or synovial VEGF, was evident. Finally, anti-TNFa therapy was effective in reducing bone and cartilage destruction and serum levels of MMP-1 and MMP-3 [44, 58].

RA patients have elevated levels of IL-1 correlating with disease activity [8], and its concentration in synovial fluid correlates with synovial pathology [27, 46]. Anakinra, which is a non-glycosylated form of the IL-1 receptor antagonist, has limited benefit on improving clinical signs of disease [39, 55]. However, it shows protective effects from bone erosion. Its impact may be more profound in diseases triggered by IL-1 such as autoinflammatory syndromes or gout [25].

Combination of an anti-TNFa and anakinra has shown significantly greater potency at inhibiting inflammation, bone resorption, and cartilage loss than infliximab in the human TNFa transgenic mouse model of arthritis [73]. In RA patients with active disease despite methotrexate treatment, however, there was no benefit of adding anakinra to etanercept, while the incidence of serious infections, neutropenia, and injection site reactions was increased [14]. These results highlight the complexity of translating animal data to the clinic. In addition, the combination of biologics drugs in the clinic is usually tested on a background of other, synthetic drugs such as methotrexate or corticosteroids, perhaps complicating translation further.

Interleukin-6 drives local endothelial cells and leukocyte activation, synovial proliferation, autoantibody production, and T cell activation and drives T cell differentiation to the Th17 instead of Treg phenotype in combination with transforming growth factor beta. It also mediates osteoclast differentiation, angiogenesis, and systemic effects that promote acute phase responses, anemia, cognitive dysfunction, and lipid-metabolism dysregulation. In addition, its levels are increased in the synovial fluid of RA patients and correlate with severity of synovitis and joint destruction

[38]. An antibody recognizing both membrane-bound and soluble IL-6 receptor, tocilizumab, has demonstrated efficacy and is approved for RA [65]. Interesting in the context of drug combination is its high activity as monotherapy in the absence of combination with synthetic disease-modifying antirheumatic drugs [65]. Also, an increase in Tregs and reduction in Th17 cells have been described in the peripheral blood of patients treated with tocilizumab [47].

IL-17 activates synovial fibroblasts, chondrocytes, and osteoclasts, mediating inflammation, bone erosion, and joint damage. It also drives monocyte activation and neutrophil differentiation, maturation, and activation. Synovial fibroblasts and monocyte activation induces cytokine and chemokine release, while synovial fibroblast activation in addition causes prostaglandin production and matrix metalloproteinase (MMP) synthesis. Antibodies neutralizing IL-17A are more advanced for psoriasis, where secukinumab has been approved than in RA, psoriatic arthritis, ankylosing spondylitis, or uveitis where its use is also being investigated [15, 20]. Ixekizumab, another antibody neutralizing IL-17A in development, showed reduced disease activity upon twelve weeks of treatment in RA [16].

T cells play an important role in the pathogenesis and pathology of RA. The costimulation inhibitor CTLA-4-Ig, abatacept, registered for RA, targets these pathways by inhibiting the interaction between CD80 and CD86 on antigen presenting cells with CD28 on T cells and thus inhibiting T cell activation. Reduction in levels of inflammatory mediators such as IL-6 [70] or synovial interferon gamma gene expression [3] is observed. Interestingly, abatacept has been studied as combination with background biologics therapy, but this led to an increase in the rate of serious adverse events [68].

Finally, one molecule registered for RA, rituximab, uses a cellular depletion mode of action. CD20-positive B cells are eliminated by the anti-CD20 monoclonal antibody, and subsequent efficacy has demonstrated a prominent role for B cells in the pathology of RA. Their depletion impacts autoantibody generation, antigen presentation, and cytokine release [9, 39].

2.2 Points to Consider for Selection of Pathways to Be Modulated

As evidenced above, target selection and choice of the binders are the most fundamental initial steps in designing a bispecific antibody. It is thereby important to have in mind the target product profile that needs to be fulfilled by the molecule. Because the net risk/benefit ratio of hitting two targets must be positive and clinically meaningful, both considerations of increased efficacy and an acceptable safety profile need to be factored in.

The two mechanisms to be combined should together therefore either have increased efficacy at acceptable safety or have comparable efficacy with a better safety profile. The latter may be seen however as challenging, as the safety of a

therapeutic intervention is often hard to predict unless a specific pathway has been identified as responsible for an adverse event.

As for increasing efficacy, it is important to evaluate the degree of interrelation of the two pathways to be modulated. One would certainly want to avoid combining intervention on two targets if all effects of inhibiting a given target A are recapitulated already by inhibiting an upstream or downstream target B. One way to circumvent this is to select mode of actions that are expected to be largely orthogonal. For example, inhibiting receptors present on different cell types impacting each the pathology could be one way to achieve this. These thoughts may be extended to pathological pathways, where, for example, orthogonality could be achieved by inhibiting one pathway only impacting bone and cartilage damage and another pathway impacting systemic inflammation, being understood that the latter would in many cases also at least indirectly affect bone and cartilage damage [61, 73]. Following a similar concept, a preclinical study tested the effect of inhibiting systemic inflammation via TNFa and angiogenesis via angiopoietin-2 using a bispecific inhibitor [28]. Such strategies bear the promise of limiting immunosuppression and the potential adverse events linked to it.

One other area where bispecific antibodies are attractive is when inhibition of two pathways is synergistic. This may occur when, for example, two cytokines enhance each other's function. The benefit is then that equivalent inhibition of a given phenotype can be obtained at lower degree of inhibition of one or the two pathways targeted. This again is favorable if inhibition of either pathway has an impact on safety. Furthermore, complete inhibition of the phenotype in vivo may require more than linear increases in doses when only one of the synergistic pathways is inhibited [12, 32, 73]. Finally, biological systems have a certain level of redundancy. Therefore, inhibition of one pathway may not be sufficient, or even lead to compensatory activation of another one, akin to an escape mechanism. In these cases combined inhibition may accordingly lead to higher efficacy.

3 Implications of the Mode of Action on Molecular Design and Target Selection

Having selected the pathways to be inhibited, a careful choice of the molecular targets has to follow. This is because the combined molecule may have different properties than each of the single components. Trying to inhibit two receptors on the same cell may become more complex in a combined molecule, for example, through mere spatial proximity that may or may not have advantages. If one of the receptor is internalizing and the other not, then the cellular fate of either of the receptors may be influenced by combining their inhibitors in a single molecule. This could have beneficial or detrimental effects on its PK properties, depending on whether receptor internalization and degradation are enhanced or diminished compared to single receptor inhibition. This may also affect potency and may lead to a new mode of

action [52]. Along the same lines, a cell depletion principle may bring additional complexities regarding PK, if combining it with, for example, a cytokine inhibition principle. The cells to be depleted may represent a large sink, and suddenly the dose necessary to inhibit the cytokine may turn out to be much larger. This may again change during therapy if the cells to be depleted and hence the sink are eliminated, leading at constant dose to a higher level of inhibition of the cytokine. But even for bsAbs targeting two soluble ligands, PK may play a role, if clearance of the molecule is accelerated upon ligand binding. Finally, some ligands may be both present as a soluble and cell bound form (e.g., TNFa [41, 50, 67]), and this may have to be taken into account for ligands with broad and high expression while evaluating impact on PK.

Bispecific antibodies targeting soluble ligands, in particular cytokines, have found more applications in RA and autoimmune diseases than bsAbs targeting cell-surface receptors. This may have to do with the multiplicity of cells involved in the pathology of these diseases and hence the difficulty to identify receptors whose distribution is consistent across multiple cell types. One question which needs to be addressed while selecting the two molecules to be combined is whether the stoichiometry of the targets is important. In other words, are the concentrations of the two cytokines different in the diseased tissue, and hence should affinity or even the number of binding sites for each target be adapted accordingly? In most cases the plasma concentration of bispecific molecules is well beyond the affinities of both binding components and the ligand concentration, so that a saturation situation is established. It is however important to consider that concentrations of therapeutic molecules vary between blood and tissue. In addition, some targets have a short half-life and rapid turnover, which is slowed down upon binding to an antibody. This may in turn have an impact on neutralization of the target, if complexes of the bsAb with it accumulate. In return, by law of mass action, there may be little reduction in free target concentration, as even at very high affinity a small part of the accumulated complexes will always dissociate. Recently, new technologies have been developed to overcome this, exploiting pH-dependent dissociation from ligands in endosomes (pH-dependent recycling) and engineering of binding to neonatal Fc receptor (sweeping) [21, 22]. Finally, further developments are using engineered calcium-dependent ligand binding [18] or Fc gamma receptor IIb-mediated uptake in combination with pH-dependent recycling [23].

Safety considerations also play a role during target selection. First of all, combining two immunosuppressive modes of action bears the risk of increased infectious adverse events. Further, beyond considerations on the pathway, when combining antibodies binding two receptors present on different cell types, the eventuality of cross-linking these cells has to be evaluated. Examples are available in the field of oncology, where T cell engagers cross-link a T cell with a tumor cell, leading to the activation of the T cell and elimination of the tumor cell [1, 40]. On the other hand, the presence of the second receptor on the same cell may mitigate this by favoring cis- over trans-interactions, akin to the situation where a classical inhibitory IgG targeting a receptor does not automatically lead to cross-cellular activation in spite of having two binding sites. Steric constraints may in addition prevent cell-cell cross-linking by the bsAb and may be influenced by epitope and

format selection. Finally, for cell-surface receptors as targets, with a mode of action envisaged being inhibition of these receptors in the absence of cell depletion, the use of bsAb formats devoid of Fcgamma receptor or complement receptor binding, collectively referred to as effector function, is advisable. Of note, there are multiple examples where this is achievable without compromising binding to the neonatal Fc receptor, thus avoiding any negative impact on PK [51].

Although these considerations may not apply to bispecific antibodies targeting ligands, other specific aspects need there also to be taken into account. Antibodies may change ligand bio distribution, and this may counteract its intended neutralization under some circumstances [11, 37, 49]. In particular, for toxic ligands, prolongation of exposure to the toxin may impact safety [34, 57].

Antibodies targeting ligands may also form immune complexes [48], which may alter clearance of both the antibody and its ligand, and this may also occur accordingly for bsAbs. Safety may also be affected if the immune complexes formed were to be deposited in tissues such as the kidney. In practice this does not seem to have affected the current bispecific antibodies in development, but may need to be considered. Immune complex formation will depend on the stoichiometry of the bsAb and its target, on the epitopes and geometries of the interaction.

4 Development Considerations

Being a single molecular entity, bsAb testing does not differ per se from the paradigm applied to classical monoclonal antibodies. The preclinical safety strategy and species selection are largely dictated by the same principles, where species cross-reactivity and availability of the target in a sensitive species play a major role. Likewise it is important to reach sufficient exposure levels in toxicological species in order to ensure safety multiples and to have a proper understanding of the PK of the molecule. For that matter, bioanalytical assays may have to be more sophisticated than assays usually developed for monoclonal antibodies owing to the presence of two different binding sites whose functionality may need to be assessed [59]. In addition, more complex assays assessing free and total ligand concentrations [62] may be useful in order to model doses for bsAbs recognizing ligands.

A variety of technologies have been described for the generation of bispecific antibodies, and these usually come with their own manufacturing setup, the description of which would by far go beyond the scope of this chapter. Important aspects to consider are however a potential increased complexity of potency assays used for the release of drug substance and drug product. This again will be highly dependent on the mode of action and specific targets recognized by the molecule. In particular, care has to be devoted so that the assay captures the functionality of both components of the bispecific antibody [13, 59]. Stability of the molecule is important in order to have the appropriate shelf life in line with the target product profile. Multiple bispecific antibody formats have already taken that hurdle, as described below [10, 29, 33, 51]. In addition, formulation is a specific topic deserving attention, in particular,

when the target product profile requires a subcutaneous route of administration. In this case, a high concentration formulation is required [33], the concentration of which is again determined by the dose envisaged in the clinic and other specific requirements of the molecule.

One potential issue for bsAb development that will certainly come to the mind of many readers is immunogenicity. Numerous marketed biologics [53] elicit a certain level of antidrug antibodies (ADAs), at manageable levels or incidence. In other words, generation of ADAs is not a black and white situation. Rather, as has been illustrated in so many cases, ADAs that may be detected in patients do not have to hinder development of the biologics [72]. Nonetheless, there are several ways how high ADA responses could negatively impact the development of a bsAb, and it is useful to keep this in mind. ADAs, if they are neutralizing, could prevent interaction of the drug with its targets at either of the binding site [17], or otherwise lead to a reduction in drug levels by accelerating clearance of the drug, preventing again efficacy. Finally, ADA-drug complexes could have safety implications, if they are deposited in tissues, or if ADAs decorate bsAbs that have attached to their targets on the surface of a cell.

A matter of interest for the design of bsAbs is whether any of its design features can have an impact on immunogenicity. The latter is by no means trivial to predict, and so the approach used for engineering bispecific antibodies has largely relied on the use of human or humanized components, careful selection of linkers [33, 117], and finally ensuring adequate product quality [24]. Of relevance here are certainly the levels of aggregates present in the final formulation, as well as the presence of contaminants [24, 72, 77]. The large body of experience gained with classical antibodies provides guidance here. Finally an important aspect in the development of bsAbs is the availability of a robust ADA testing approach [17, 31].

The B cell response underlying ADA formation may depend on the generation of T cells (Td) or be independent of their stimulation (Ti). The latter is characterized by limited isotypes and affinity and memory responses, if at all of short life span [24]. A Td response against a biologic requires the presence of immunogenic T cell epitopes, as these drive the generation of T helper cells specific for the biologic, which are needed to drive the generation of ADAs of the IgG isotype. In silico tools and in vitro assays have been established in order to predict T cell epitopes and immunogenicity. In order to elicit a T helper response, a potential epitope needs first to be processed and presented on class II MHC molecules. Assays have been developed to verify if epitopes predicted by in silico tools are indeed processed and presented [24, 76]. The next question is the immunogenicity of the epitope, for which in vitro assays have been also set up [24, 72, 76]. The validation of the predictability of the tools is a complex undertaking and mainly based on retrospective analyses, as obviously no direct comparison of biologics having been predicted as having high or low risk of immunogenicity has been performed in a clinical trial. Setting aside any aspects of assay setup, validation of the predictability of in vitro tools in assessing risk of immunogenicity requires a data set covering immunogenic and less immunogenic biologics, being understood that there are few biologics which elicit no ADAs. Important additional factors to be considered lie outside of

the drug itself and pertain to the patient population including its heterogeneity in HLA polymorphisms [24], potential immunosuppressive co-medications and the general disease state of the respective patient population that can be immunosuppressed (e.g., HIV) or immune enhanced (e.g. autoimmune diseases). As an additional factor, it has to be noted that the assays used to monitor ADAs in clinical studies vary between different companies in sensitivity so that incidences can only hardly be compared.

Although many bsAbs being developed for RA are still at an early stage, recent reports [75, 117] on the molecules having now reached phase II tell us that bsAbs developed so far have been able to take the immunogenicity hurdle and enter proof of concept studies in patients. This is also the case for bsAb developed in other indications [26]. A broader database will be needed to assess bsAb as a class of drugs, but as it appears the presence of an additional set of CDRs in the molecule does not amplify nor induce additional ADAs to levels precluding drug development.

5 Bispecific Antibody Formats: An Overview

Several recombinant bsAbs are presently in clinical development or marketed [78–80]. Although quite diverse in composition and format details [81–87], all bsAbs can be assigned to one of two general format classes which are differentiated by absence or presence of a constant region (Fc, Fig. 1).

BsAbs without Fc domains contain engineered variable domains or Fabs of different specificities, fused to each other via flexible linker peptides to generate a bispecific entity. An established format of this class is BiTEs which are composed of two single-chain Fvs (tandem scFvs) fused to each other via flexible linker peptides [88, 89]. This format has made it already to the market, blinatumomab targeting CD19 and CD3 [90], being approved for the therapy of ALL. Other recombinant bsAb formats in clinical development that lack Fc regions and functionalities are diabodies [89, 91–93]. These also harbor two Fvs with different specificities which however are composed of two different chains with engineered (short) linker peptides to force assembly to one functional bifunctional diabody molecule [89, 93]. Related entities are single-chain diabodies [90], TandAbs and DARTs [94]. TandAbs

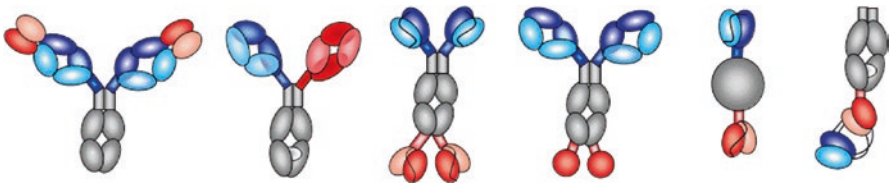

Fig. 1 Schematic overview of bispecific antibody (bsAb) formats that are currently in clinical development in inflammatory diseases. *Left* to *right*: DVD-IgG, Crossmab scFv2-Fc, FynoMab, scFv-HSA-scFv, Fc-Dart

are dimeric tetravalent entities that possess two binding sites for each antigen [93]. Fv- or Fab-based bsAbs can also be generated by the "dock-and-lock" method, which enforces desired heterodimerization of two binding entities via anchoring protein domains [95, 96]. BsAbs without Fc regions are usually quite small molecules. As the Fc region of IgGs is responsible (via FcRn recycling [97]) for the extended serum half-life of antibodies, bsAbs that lack Fc have generally a rather short serum half-life [98]. Serum half-life of bsAb derivatives without Fc regions can be modulated (increased) by addition of half-life extension moieties such as conjugated polyethylene glycol (PEG), addition of PEG-mimetic polypeptides, or by fusion to albumin or albumin-binding moieties [98–100]. DARTS are diabody-like molecules composed of two Fvs of different specificities which are linked to each other via peptide connectors as well as via interchain disulfides [94]. MGD010, a DART that modulates B cell activation, is currently in phase I and in clinical development for autoimmune diseases. It contains Fvs with binding functionalities for CD32B (Fcg receptor IIb) as well as CD79. These are combined in the interchain-disulfide assembled DART format (with added complementary charged coil stretches to facilitate correct molecule assembly). To confer benign PK properties (i.e., a long serum half-life), this DART unit is fused to an Fc region which carries mutations to abolish FcgammaR and C1q binding functionalities. The position of the Fc fusion is C-terminus to CH3 of one CH2-CH3 chain of the Fc heterodimer. Knobs into holes are introduced into a DART-fused and a complementary non-fused Fc to generate molecules with 1:1 DART-to-Fc stoichiometries. Inhibition of B cell activation is mediated by ligation of the inhibitory Fcg receptor to the CD79B component of the B cell receptor. Ex vivo B cell activation could be inhibited in samples from nonhuman primates treated with MGD010 (http://www.jimmunol.org/content/192/1_Supplement/200.9.short).

BsAbs with Fc domains contain in many instances all or parts of IgGs to which engineered domains were added to supply a second binding specificity. The first IgG-like bsAbs were generated by chemical conjugation of different antibodies or fusing dual hybridoma fusions (quadroma) [101]. The prototype format for recombinant IgG-containing bsAbs has also been generated already decades ago by Sherie L. Morrison and colleagues, and comprised additional scFv domains fused to the C-termini of the heavy chains of IgGs [102]. Further optimization of that "IgG-plus" concept leads to entities with improved stability, as well as to additional IgG-containing scAb formats [78–83]. The presence of the Fc region can be of advantage in bsAb production processes as technologies and experiences derived from standard processes for therapeutic antibodies can be applied (such as mammalian secretion systems and protein A affinity purification steps). The presence of an Fc region also confers benign PK parameters (long half-life) to bsAbs, due to larger size which prevents renal filtration and because of FcRn-mediated antibody recycling which prevents rapid degradation [103]. The Fc region in IgG-derived bsAbs may also enable Fc-mediated effector functionalities such as ADCC or CDC. This may be of advantage in certain therapeutic applications, such as in oncology where antibody-dependent cellular cytotoxicity (ADCC) and/or complement activation (CDC) adds to therapeutic efficacy [52, 104]. In cases where cyto-

toxic effector functions are not desired, these can be eliminated by genetic engineering eliminating ADCC and/or CDC while still maintaining FcRn-mediated PK properties.

The correct assembly of IgG-derived bsAbs can be achieved by expressing engineered heavy (or light) chains containing additional binding entities together with one matching complementary light (or heavy) chain. Applying this principle, the application of fused scFv domains generates "2 + 2" format molecules which resemble the Morrison format (Fig. 1). Fusion of separate VH and VL domains on top of regular V domains of IgGs generates also symmetric bispecific IgG-like molecules, termed DVD-Igs (DVD = dual variable domain [105].

In contrast to these symmetric IgG formats, the generation of IgG-derived bsAbs without a symmetric organisation of its binding sites (such as the 1 + 1 format, Fig. 1) requires two different heavy chains to become assembled as heterodimer. Simultaneously two light chains must become assembled to their cognate heavy chains. This poses the problem that H-chain assembly to each other and L-chain to H-chain assembly are by default nonspecific. Thus, co-expression of two different H and L chains generates only small amounts of the desired H-chain heterodimers with correct light-heavy chain combinations in their Fab arms. In fact, without further protein engineering, most IgG-like molecules will contain homodimer H chains with wrongly assembled L chains [106]. Two steps of antibody-engineering were necessary to overcome the chain association limitation. In a first step, desired heterodimerization of H chains can be achieved by knob-into-hole (or charge-exchange) technologies. This approach introduces different mutations into the complementary CH3 domains to generate asymmetric H chains which preferentially heterodimerize [107]. Variations of this approach include different mutations at different positions as well as charge-mediated attraction and repulsion effects or IgG-IgA-hybrid domains [108–112]. One method to achieve correct L-chain pairing to the forced H-chain heterodimers is to use one and the same L chain for both antigen-binding arms (common light-chain approach). An alternative approach is to selectively engineer H and L chains of one Fab arm to enforce the correct heterodimerization. For example, the CrossMab technology [51, 106] achieves correct L-chain pairing by exchanging the CH1 domain of one heavy chain with the CL domain of the corresponding light chain. Mutations in the CH1-CL and VH-VL interface can further support correct pairing of the light-to-heavy chain associations [112]. Finally, IgGs with dual specificities can also be generated by selecting VH and VL domains that recognize two different antigens. Such two-in-one antibodies are indistinguishable from normal IgGs [113]. In addition to "classical" bsAbs that are composed only of antibody-derived domains, bsAb derivatives can also be generated that contain antibody domains (or whole IgGs) and additionally some non-antibody-derived binding moieties. One example for that are bsAb-like molecules that harbor SH2-domain-derived ("Fynomer") binding modules attached to the C-termini of antibody domains [114]. The bsAb formats have evolved over the years and have incorporated learnings from previous molecules and exploited new technologies for generating these improvements, as detailed in Table 1.

Table 1 Hurdles in development of bispecific antibody formats

BsAb type	Technical approach	Example	Consequence
Heterohybridoma and conjugates	Nonhuman nonrecombinant IgG assembly	Catumaxomab and related heterohybridoma IgGs	Homogeneity and immunogenicity issues
First-generation recombinant	Nonhuman antibody-domain fusion proteins	"Morrison-type" murine scFv fusion proteins, first-generation diabodies, and scFv-scFv fusions	Frequent aggregation/instability (and immunogenicity) issues
Recombinant stability engineered humanized	Humanized and design for increased stability	Some TvIgGs containing stabilized humanized Fvs, some diabodies, and scFv-scFv fusion proteins	Remaining USP/DSP problems
Recombinant developability engineered humanized	Humanized and stability and developability optimized	CrossMabs, common LC bsAbs, DVDs, and others	Standard for most future bsAbs

6 Formats of Bispecific Antibodies That Are in Clinical Development for RA and Inflammatory Diseases

A vast variety of different bsAb formats with different target combinations have been generated and/or are currently in various stages of preclinical development in academia, biotech, as well as in the pharmaceutical industry. Some of these formats and target combinations have made it into clinical development. This section will focus on these and describe bsAb formats in more detail for molecules that are currently in clinical development stages for the treatment of inflammatory diseases and, in particular, RA. The formats that are described in this section are schematically shown in Fig. 1.

6.1 DVD-IgGs and Related TBTIs

DVD (dual variable domain)-IgGs are composed of IgGs with one binding specificity to which additional V domains have been fused "on top of" their regular V domains (Fig. 1). This bispecific antibody format is tetravalent with two binding entities for each antigen. The DVD format has been pioneered and first described by

C. Wu and T. Ghayur and their co-workers at Abbott and Abbvie [105]. DVD-IgGs harbor Fc regions to confer benign pharmacokinetic features. The orientation of their binding regions is optimized to enable ligand access and hence binding of both target antigens. One DVD-IgG currently undergoing clinical development in RA and psoriatic arthritis is ABT-122, a human IgG1/k which binds interleukin-17 with its "master IgG" and tumor necrosis factor with the "extra Fv" [115]. In vivo pharmacological activity was assessed in acute models, using a human TNFa/D-galactosamine lethality and a human IL-17-induced KC model. PK characteristics in rats were inconspicuous [19]. Efficacy in the mouse collagen-induced arthritis model was assessed using a surrogate antibody [6]. Another DVD-Ig is ABT981, in development for osteoarthritis and which binds interleukin-1α as well as interleukin-1β [116].

TBTIs are very similar to DVD-IgGs and contain additional V regions attached to the H- and L-chain N-termini of an IgG. This generates an N-terminal tandem configuration of V regions (hence the term TBTI for tetravalent bispecific tandem Ig). One TBTI currently undergoing clinical development in inflammatory diseases is SAR156597. This bsAb can simultaneously bind to and thereby interfere with the functionality of interleukin 4 and interleukin 13 [117].

6.2 Bi-nanobodies

These bsAbs contain two VH-like binding domains, each with one antigen specificity. The resulting molecule is bivalent bispecific, i.e., contains two binding arms. In contrast to DVD-IgGs, these molecules do not possess constant (Fc) regions. To nevertheless reach acceptable pharmacokinetic properties, loss of Fc is compensated by the addition of half-life extending modules. The Bi-nanobody that is currently undergoing clinical development in inflammatory diseases is ALX-0761. This molecule binds the interleukin-17 family members IL-17F and IL-17A [118].

6.3 CrossMabs

CrossMabs are IgG-shaped molecules with two binding arms, each recognizing a different antigen. They are composed of one asymmetric constant region which contains knob or hole mutations on each H chain, respectively. These mutations enforce the generation of correctly assembled heterodimeric H chains. Correct L-chain assembly of the two Fab arms (each binding a different antigen) is enabled by engineering one Fab arm in a manner that favors assembly of the correct heavy-light-chain pair and prevents wrong chain assembly. CrossMabs contain an Fc region that is competent to bind FcRn. Therefore, CrossMabs possess PK properties

of normal IgGs [51, 106]. For application in inflammatory diseases (and other applications that are not based on or supported by Fc mediated cytotoxic activities), Fc regions of CrossMabs can be mutated to incapacitate effector functionalities such as ADCC or CDC. One CrossMab that is in clinical development in wet age-related macular degeneration is RG7716. This molecule targets the receptor ligands VEGF-A as well as angiopoietin-2 (Ang2).

6.4 ScFv-IgGs

This bsAb format contains an Fc region to which scFvs with two specificities are fused. The Fc region enables benign pharmacokinetic behavior of the scFv-containing molecule, and the scFvs harbor the two specificities. One scFv-IgG that is currently in clinical development in inflammatory diseases is the IL-17/IL-23 binding bi-Mab. This molecule binds to and thereby inhibits (simultaneously) interleukin-17 as well as interleukin 23.

6.5 DART, Diabody-Based Format

DARTs are a diabody-based format. MGD010, a DART-targeting B cell activation, is currently in phase I and in clinical development for autoimmune diseases. It is a DART with a diabody-binding CD32B (Fcg receptor IIb) and CD79 and fused to an Fc portion to provide for a long half-life. Inhibition of B cell activation is mediated by ligation of the inhibitory Fcg receptor to the CD79B component of the B cell receptor. Ex vivo B cell activation could be inhibited in samples from nonhuman primates treated with MGD010 (http://www.jimmunol.org/content/192/1_Supplement/200.9.short).

6.6 FynoMabs

This “nonclassical” bsAb format is composed of an IgG to which (at the C-termini of CH3 domains) additional non-antibody-derived binding entities have been fused. These additional entities are SH3-domain-derived engineered domains (fynomers) that bind antigen in a similar manner as variable domains of antibodies. A FynoMab currently in clinical development in an inflammatory application is COVA322 [114]. This molecule binds to and thereby inhibits TNFalpha as well as interleukin-17a. In vivo pharmacological activity was shown in a human IL-17a and a human TNFa-induced KC model. PK characteristics in nonhuman primates have been analyzed using an assay detecting molecules able to bind both IL-17A and TNFa and were comparable to adalimumab and golimumab [114].

7 Outlook

Further developments in RA are bound to come from a better understanding of the biology of the disease and, in particular, of patient populations. Already new molecules in development for RA are modulating new therapeutic targets, and there could be more to come. On the other hand, increased specificity in modulating existing targets is another avenue which is being explored. For example, instead of using CTLA-4-Ig for inhibition of T cell costimulation, the generation of non-agonist monovalent anti-CD28 antibody fragments has been described. The aim there is to inhibit CD28 triggering, without interfering with binding of CD80 to PD-L1 on T cells, negative signaling into T cells of the CD80/CD86 interaction with CTLA-4 on T cells, and the suppressive function of Tregs [36, 63].

Protein engineering, beyond delivering the bispecific antibodies described above, provides further opportunities for achieving higher specificity. For example, targeting cytokines or antibodies to inflamed tissue and the joints has been explored in preclinical models of RA [35, 56, 63, 71]. In a further step, activation of an adalimumab prodrug directly at the inflamed vasculature using ICAM-1 to target the prodrug and linkers containing MMP-1 cleavage sites for activation has been described [43]. Of note, tissue targeting and biologics prodrugs are also explored in oncology [2, 7, 30, 45]. Strikingly, the "evolution" of antibody-based therapeutic approaches in inflammatory diseases appears to be similar to that of antibodies and antibody derivatives in cancer therapy. Here, the first generation of antibody-based therapeutics in cancer were "normal" antibodies directed at single targets on cancer cells (e.g., Herceptin, Erbitux, Rituxan) or antibodies at soluble targets modulating the environment of such cells (such as Avastin). The next generation of antibody-based cancer therapies included derivatives with modulated Fc regions, as well as bispecific antibodies and antibody-drug conjugates. Approved drugs that fall into the classes of next-generation antibodies include the bispecific blinatumomab, the Fc-engineered Gazyva, and the antibody-drug conjugates Kadcyla and Adcetris.

In analogy to the evolution of therapeutic antibodies toward more engineering in cancer indications, antibody-based therapies in inflammatory diseases have likewise evolved. Regular antibodies are already successfully applied (such as infliximab, rituximab, adalimumab, tocilizumab, secukinumab; see above). Engineered second-generation antibody-like molecules such as etanercept or abatacept have found early applications in inflammatory diseases. Additional engineering approaches include Fc modulation to increase antibody availability and efficacy [22] and Fc engineering to increase affinity for Fc gamma receptor IIb and thereby deactivate B cells [5]. Such Fc engineering concepts relate to Fc-engineered ADCC-enhanced Gazyva in cancer therapy (Table 2).

Furthermore, a variety of new bispecific antibody derivatives that modulate inflammation are currently in clinical development (see above and Table 2). If one follows the analogy to the evolution of cancer therapeutics, one next type of molecules that might emerge could be antibody-drug conjugates. In contrast to cancer therapeutics, however, ADCs in inflammatory indications might carry immune-modulating

Table 2 The "evolution" of antibody-based therapies in cancer has been paving the path for similar developments in inflammatory diseases

Antibody derivative	Cancer therapy	Inflammation
Regular IgG	Approved	Approved
Fc-fusion protein	Approved	Approved
Fc-engineered IgG	Approved	Clin. development
Bispecific antibody	Approved	Clin. development
Antibody-drug conjugate	Approved	None in clinical development

payloads instead of cytotoxic compounds. Antibody-mediated delivery of drugs might suppress inflammatory processes specifically at the site of disease, with the potential advantage of reducing systemic interference with immune functions. Interestingly, the application of conjugation technology to antibodies for RA seems to have started with conjugation of two proteins together in a bispecific antibody rather than in an ADC format [60]. Finally, as a further evolution paralleling what is happening in cancer immunotherapy, agonist antibodies may find broader application in the future, as is already being exemplified for a non-oncology indication [74].

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References

1. Bargou R, Leo E, Zugmaier G, Klinger M, Goebeler M, Knop S, Noppeney R, Viardot A, Hess G, Schuler M, Einsele H, Brandl C, Wolf A, Kirchinger P, Klappers P, Schmidt M, Riethmuller G, Reinhardt C, Baeuerle PA, Kufer P (2008) Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science 321:974–977
2. Bootz F, Neri D (2015) Immunocytokines: a novel class of products for the treatment of chronic inflammation and autoimmune conditions. Drug Discov Today
3. Buch MH, Boyle DL, Rosengren S, Saleem B, Reece RJ, Rhodes LA, Radjenovic A, English A, Tang H, Vratsanos G, O'Connor P, Firestein GS, Emery P (2009) Mode of action of abatacept in rheumatoid arthritis patients having failed tumour necrosis factor blockade: a histological, gene expression and dynamic magnetic resonance imaging pilot study. Ann Rheum Dis 68:1220–1227
4. Butler DM, Maini RN, Feldmann M, Brennan FM (1995) Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. Eur Cytokine Netw 6:225–230
5. Chu SY, Yeter K, Kotha R, Pong E, Miranda Y, Phung S, Chen H, Lee SH, Leung I, Bonzon C, Desjarlais JR, Stohl W, Szymkowski DE (2014) Suppression of rheumatoid arthritis B cells by XmAb5871, an anti-CD19 antibody that coengages B cell antigen receptor complex and Fcgamma receptor IIb inhibitory receptor. Arthritis Rheumatol 66:1153–1164
6. Cuff C, Bryant S, Hugunin M, Kamath RV, Voss J, Olson L, Hsieh CM (2014) Dual neutralization of TNF and IL-17 with A Dvd-Ig protein is efficacious in collagen induced arthritis. Ann Rheum Dis 73:363
7. Desnoyers LR, Vasiljeva O, Richardson JH, Yang A, Menendez EE, Liang TW, Wong C, Bessette PH, Kamath K, Moore SJ, Sagert JG, Hostetter DR, Han F, Gee J, Flandez J, Markham K, Nguyen M, Krimm M, Wong KR, Liu S, Daugherty PS, West JW, Lowman HB (2013)

Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index. Sci Transl Med 5:207ra144
8. Eastgate JA, Symons JA, Wood NC, Grinlinton FM, Di Giovine FS, Duff GW (1988) Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis. Lancet 2:706–709
9. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T (2004) Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 350:2572–2581
10. Fenn S, Schiller CB, Griese JJ, Duerr H, Imhof-Jung S, Gassner C, Moelleken J, Regula JT, Schaefer W, Thomas M, Klein C, Hopfner KP, Kettenberger H (2013) Crystal structure of an anti-Ang2 CrossFab demonstrates complete structural and functional integrity of the variable domain. PLoS ONE 8, e61953
11. Finkelman FD, Madden KB, Morris SC, Holmes JM, Boiani N, Katona IM, Maliszewski CR (1993) Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. J Immunol 151:1235–1244
12. Fischer JA, Hueber AJ, Wilson S, Galm M, Baum W, Kitson C, Auer J, Lorenz SH, Moelleken J, Bader M, Tissot AC, Tan SL, Seeber S, Schett G (2015) Combined inhibition of tumor necrosis factor alpha and interleukin-17 as a therapeutic opportunity in rheumatoid arthritis: development and characterization of a novel bispecific antibody. Arthritis Rheumatol 67:51–62
13. Gassner C, Lipsmeier F, Metzger P, Beck H, Schnueriger A, Regula JT, Moelleken J (2015) Development and validation of a novel SPR-based assay principle for bispecific molecules. J Pharm Biomed Anal 102:144–149
14. Genovese MC, Cohen S, Moreland L, Lium D, Robbins S, Newmark R, Bekker P (2004) Combination therapy with etanercept and anakinra in the treatment of patients with rheumatoid arthritis who have been treated unsuccessfully with methotrexate. Arthritis Rheum 50:1412–1419
15. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, Lee SH, Codding CE, Kellner H, Ikawa T, Hugot S, Ligozio G, Mpofu S (2014) One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. J Rheumatol 41:414–421
16. Genovese MC, Greenwald M, Cho CS, Berman A, Jin L, Cameron GS, Benichou O, Xie L, Braun D, Berclaz PY, Banerjee S (2014) A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. Arthritis Rheumatol 66:1693–1704
17. Gorovits B, Wakshull E, Pillutla R, Xu Y, Manning MS, Goyal J (2014) Recommendations for the characterization of immunogenicity response to multiple domain biotherapeutics. J Immunol Methods 408:1–12
18. Hironiwa N, Ishii S, Kadono S, Iwayanagi Y, Mimoto F, Habu K, Igawa T, Hattori K (2015) Calcium-dependent antigen binding as a novel modality for antibody recycling by endosomal antigen dissociation. mAbs 1–9
19. Hsieh CM, Cuff C, Tarcsa E (2014) Discovery and characterization of Abt-122, an anti-TNF/IL-17 Dvd-Ig™ molecule as a potential therapeutic candidate for rheumatoid arthritis. Ann Rheum Dis 73:495
20. Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, Antoni C, Draelos Z, Gold MH, Durez P, Tak PP, Gomez-Reino JJ, Foster CS, Kim RY, Samson CM, Falk NS, Chu DS, Callanan D, Nguyen QD, Rose K, Haider A, Di PF (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. Sci Transl Med 2:52ra72
21. Igawa T, Ishii S, Tachibana T, Maeda A, Higuchi Y, Shimaoka S, Moriyama C, Watanabe T, Takubo R, Doi Y, Wakabayashi T, Hayasaka A, Kadono S, Miyazaki T, Haraya K, Sekimori Y, Kojima T, Nabuchi Y, Aso Y, Kawabe Y, Hattori K (2010) Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. Nat Biotechnol 28:1203–1207

22. Igawa T, Maeda A, Haraya K, Tachibana T, Iwayanagi Y, Mimoto F, Higuchi Y, Ishii S, Tamba S, Hironiwa N, Nagano K, Wakabayashi T, Tsunoda H, Hattori K (2013) Engineered monoclonal antibody with novel antigen-sweeping activity in vivo. PLoS ONE 8, e63236
23. Iwayanagi Y, Igawa T, Maeda A, Haraya K, Wada NA, Shibahara N, Ohmine K, Nambu T, Nakamura G, Mimoto F, Katada H, Ito S, Tachibana T, Jishage K, Hattori K (2015) Inhibitory FcgammaRIIb-mediated soluble antigen clearance from plasma by a pH-dependent antigen-binding antibody and its enhancement by Fc engineering. J Immunol 195:3198–3205
24. Jawa V, Cousens LP, Awwad M, Wakshull E, Kropshofer H, De Groot AS (2013) T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation. Clin Immunol 149:534–555
25. Jesus AA, Goldbach-Mansky R (2014) IL-1 blockade in autoinflammatory syndromes. Annu Rev Med 65:223–244
26. Juric D, Dienstmann R, Cervantes A, Hidalgo M, Messersmith W, Blumenschein GR Jr, Tabernero J, Roda D, Calles A, Jimeno A, Wang X, Bohorquez SS, Leddy C, Littman C, Kapp AV, Shames DS, Penuel E, Amler LC, Pirzkall A, Baselga J (2015) Safety and pharmacokinetics/pharmacodynamics of the first-in-class dual action HER3/EGFR antibody MEHD7945A in locally advanced or metastatic epithelial tumors. Clin Cancer Res 21:2462–2470
27. Kahle P, Saal JG, Schaudt K, Zacher J, Fritz P, Pawelec G (1992) Determination of cytokines in synovial fluids: correlation with diagnosis and histomorphological characteristics of synovial tissue. Ann Rheum Dis 51:731–734
28. Kanakara J,P, Puffer BA, Yao XT, Kankanala S, Boyd E, Shah RR, Wang G, Patel D, Krishnamurthy R, Kaithamana S, Smith RG, Lafleur DW, Barbas CF III, Hilbert DM, Kiener PA, Roschke VV (2012) Simultaneous targeting of TNF and Ang2 with a novel bispecific antibody enhances efficacy in an in vivo model of arthritis. mAbs 4:600–613
29. Kienast Y, Klein C, Scheuer W, Raemsch R, Lorenzon E, Bernicke D, Herting F, Yu S, The HH, Martarello L, Gassner C, Stubenrauch KG, Munro K, Augustin HG, Thomas M (2013) Ang-2-VEGF-A CrossMab, a novel bispecific human IgG1 antibody blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and antimetastatic efficacy. Clin Cancer Res 19:6730–6740
30. Klein C, Waldhauer I, Nicolini V, Dunn C, Freimoser-Grundschober A, Herter S, Geven E, Boerman O, Nayak T (2013) Tumor-targeted, engineered IL-2 variant (IL-2v)-based immunocytokines for the immunotherapy of cancer. Cancer Res 73:486
31. Kloks C, Berger C, Cortez P, Dean Y, Heinrich J, Bjerring JL, Koppenburg V, Kostense S, Kramer D, Spindeldreher S, Kirby H (2015) A fit-for-purpose strategy for the risk-based immunogenicity testing of biotherapeutics: a European industry perspective. J Immunol Methods 417:1–9
32. Koenders MI, Marijnissen RJ, Devesa I, Lubberts E, Joosten LA, Roth J, Van Lent PL, Van De Loo FA, Van Den Berg WB (2011) Tumor necrosis factor-interleukin-17 interplay induces S100A8, interleukin-1beta, and matrix metalloproteinases, and drives irreversible cartilage destruction in murine arthritis: rationale for combination treatment during arthritis. Arthritis Rheum 63:2329–2339
33. Lacy SE, Wu C, Ambrosi DJ, Hsieh CM, Bose S, Miller R, Conlon DM, Tarcsa E, Chari R, Ghayur T, Kamath RV (2015) Generation and characterization of ABT-981, a dual variable domain immunoglobulin (DVD-Ig(TM)) molecule that specifically and potently neutralizes both IL-1alpha and IL-1beta. mAbs 7:605–619
34. Lobo ED, Soda DM, Balthasar JP (2003) Application of pharmacokinetic-pharmacodynamic modeling to predict the kinetic and dynamic effects of anti-methotrexate antibodies in mice. J Pharm Sci 92:1665–1676
35. Macor P, Durigutto P, De ML, Garrovo C, Biffi S, Cortini A, Fischetti F, Sblattero D, Pitzalis C, Marzari R, Tedesco F (2012) Treatment of experimental arthritis by targeting synovial endothelium with a neutralizing recombinant antibody to C5. Arthritis Rheum 64:2559–2567
36. Mary C, Coulon F, Poirier N, Dilek N, Martinet B, Blancho G, Vanhove B (2013) Antagonist properties of monoclonal antibodies targeting human CD28: role of valency and the heavy-chain constant domain. mAbs 5:47–55

37. May LT, Neta R, Moldawer LL, Kenney JS, Patel K, Sehgal PB (1993) Antibodies chaperone circulating IL-6. Paradoxical effects of anti-IL-6 “neutralizing” antibodies in vivo. J Immunol 151:3225–3236
38. Mcinnes IB, Schett G (2007) Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 7:429–442
39. Mcinnes IB, Schett G (2011) The pathogenesis of rheumatoid arthritis. N Engl J Med 365:2205–2219
40. Nagorsen D, Kufer P, Baeuerle PA, Bargou R (2012) Blinatumomab: a historical perspective. Pharmacol Ther 136:334–342
41. Nesbitt A, Fossati G, Bergin M, Stephens P, Stephens S, Foulkes R, Brown D, Robinson M, Bourne T (2007) Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. Inflamm Bowel Dis 13:1323–1332
42. Niewoehner J, Bohrmann B, Collin L, Urich E, Sade H, Maier P, Rueger P, Stracke JO, Lau W, Tissot AC, Loetscher H, Ghosh A, Freskgard PO (2014) Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle. Neuron 81:49–60
43. Onuoha SC, Ferrari M, Sblattero D, Pitzalis C (2015) Rational design of antirheumatic prodrugs specific for sites of inflammation. Arthritis Rheumatol 67:2661–2672
44. Paleolog E (2003) The therapeutic potential of TNF-alpha blockade in rheumatoid arthritis. Expert Opin Investig Drugs 12:1087–1095
45. Ribas A, Kirkwood JM, Atkins MB, Whiteside TL, Gooding W, Kovar A, Gillies SD, Kashala O, Morse MA (2009) Phase I/II open-label study of the biologic effects of the interleukin-2 immunocytokine EMD 273063 (hu14.18-IL2) in patients with metastatic malignant melanoma. J Transl Med 7:68
46. Rooney M, Symons JA, Duff GW (1990) Interleukin 1 beta in synovial fluid is related to local disease activity in rheumatoid arthritis. Rheumatol Int 10:217–219
47. Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, Ornetti P, Maillefert JF, Miossec P, Bonnotte B (2012) Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. Arthritis Rheum 64:2499–2503
48. Santora LC, Kaymakcalan Z, Sakorafas P, Krull IS, Grant K (2001) Characterization of noncovalent complexes of recombinant human monoclonal antibody and antigen using cation exchange, size exclusion chromatography, and BIAcore. Anal Biochem 299:119–129
49. Sato TA, Widmer MB, Finkelman FD, Madani H, Jacobs CA, Grabstein KH, Maliszewski CR (1993) Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo. J Immunol 150:2717–2723
50. Scallon BJ, Moore MA, Trinh H, Knight DM, Ghrayeb J (1995) Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. Cytokine 7:251–259
51. Schaefer W, Regula JT, Bahner M, Schanzer J, Croasdale R, Durr H, Gassner C, Georges G, Kettenberger H, Imhof-Jung S, Schwaiger M, Stubenrauch KG, Sustmann C, Thomas M, Scheuer W, Klein C (2011) Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. Proc Natl Acad Sci U S A 108:11187–11192
52. Schanzer JM, Wartha K, Croasdale R, Moser S, Kunkele KP, Ries C, Scheuer W, Duerr H, Pompiati S, Pollman J, Stracke J, Lau W, Ries S, Brinkmann U, Klein C, Umana P (2014) A novel glycoengineered bispecific antibody format for targeted inhibition of epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor type I (IGF-1R) demonstrating unique molecular properties. J Biol Chem 289:18693–18706
53. Schellekens H (2002) Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clin Ther 24:1720–1740
54. Schett G, Elewaut D, Mcinnes IB, Dayer JM, Neurath MF (2013) How cytokine networks fuel inflammation: toward a cytokine-based disease taxonomy. Nat Med 19:822–824
55. Schett G, Gravallese E (2012) Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. Nat Rev Rheumatol 8:656–664
56. Schwager K, Kaspar M, Bootz F, Marcolongo R, Paresce E, Neri D, Trachsel E (2009) Preclinical characterization of DEKAVIL (F8-IL10), a novel clinical-stage immunocytokine which inhibits the progression of collagen-induced arthritis. Arthritis Res Ther 11:R142

57. Shah DK, Balthasar JP (2014) PK/TD modeling for prediction of the effects of 8C2, an anti-topotecan mAb, on topotecan-induced toxicity in mice. Int J Pharm 465:228–238
58. Shealy DJ, Visvanathan S (2008) Anti-TNF antibodies: lessons from the past, roadmap for the future. Handb Exp Pharmacol 181:101–129
59. Silacci M, Lembke W, Woods R, Ttinger-Toller I, Baenziger-Tobler N, Batey S, Santimaria R, Von Der BU, Koenig-Friedrich S, Zha W, Schlereth B, Locher M, Bertschinger J, Grabulovski D (2015) Discovery and characterization of COVA322, a clinical-stage bispecific TNF/IL-17A inhibitor for the treatment of inflammatory diseases. mAbs 1–9
60. Stenderup K, Rosada C, Shanebeck K, Brady W, Van Brunt MP, King G, Marelli M, Slagle P, Xu H, Nairn NW, Johnson J, Wang AA, Li G, Thornton KC, Dam TN, Grabstein KH (2015) AZ17: a new bispecific drug targeting IL-6 and IL-23 with potential clinical use-improves psoriasis in a human xenograft transplantation model. Protein Eng Des Sel 28:467–480
61. Stolina M, Schett G, Dwyer D, Vonderfecht S, Middleton S, Duryea D, Pacheco E, Van G, Bolon B, Feige U, Zack D, Kostenuik P (2009) RANKL inhibition by osteoprotegerin prevents bone loss without affecting local or systemic inflammation parameters in two rat arthritis models: comparison with anti-TNFalpha or anti-IL-1 therapies. Arthritis Res Ther 11:R187
62. Stubenrauch K, Wessels U, Essig U, Vogel R, Waltenberger H, Hansbauer A, Koehler A, Heinrich J (2015) An immunodepletion procedure advances free angiopoietin-2 determination in human plasma samples during anti-cancer therapy with bispecific anti-Ang2/VEGF CrossMab. J Pharm Biomed Anal 102:459–467
63. Suchard SJ, Davis PM, Kansal S, Stetsko DK, Brosius R, Tamura J, Schneeweis L, Bryson J, Salcedo T, Wang H, Yang Z, Fleener CA, Ignatovich O, Plummer C, Grant S, Nadler SG (2013) A monovalent anti-human CD28 domain antibody antagonist: preclinical efficacy and safety. J Immunol 191:4599–4610
64. Suryadevara CM, Gedeon PC, Sanchez-Perez L, Verla T, Varez-Breckenridge C, Choi BD, Fecci PE, Sampson JH (2015) Are BiTEs the “missing link” in cancer therapy? Oncoimmunology 4, e1008339
65. Tanaka T, Narazaki M, Ogata A, Kishimoto T (2014) A new era for the treatment of inflammatory autoimmune diseases by interleukin-6 blockade strategy. Semin Immunol 26:88–96
66. Topp MS, Gokbuget N, Stein AS, Zugmaier G, O’Brien S, Bargou RC, Dombret H, Fielding AK, Heffner L, Larson RA, Neumann S, Foa R, Litzow M, Ribera JM, Rambaldi A, Schiller G, Bruggemann M, Horst HA, Holland C, Jia C, Maniar T, Huber B, Nagorsen D, Forman SJ, Kantarjian HM (2015) Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study. Lancet Oncol 16:57–66
67. Van Den Brande JM, Braat H, Van Den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, Van MC, Hommes DW, Peppelenbosch MP, Van Deventer SJ (2003) Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn’s disease. Gastroenterology 124:1774–1785
68. Weinblatt M, Combe B, Covucci A, Aranda R, Becker JC, Keystone E (2006) Safety of the selective costimulation modulator abatacept in rheumatoid arthritis patients receiving background biologic and nonbiologic disease-modifying antirheumatic drugs: a one-year randomized, placebo-controlled study. Arthritis Rheum 54:2807–2816
69. Weinblatt ME, Schiff M, Valente R, Van Der HD, Citera G, Zhao C, Maldonado M, Fleischmann R (2013) Head-to-head comparison of subcutaneous abatacept versus adalimumab for rheumatoid arthritis: findings of a phase IIIb, multinational, prospective, randomized study. Arthritis Rheum 65:28–38
70. Weisman MH, Durez P, Hallegua D, Aranda R, Becker JC, Nuamah I, Vratsanos G, Zhou Y, Moreland LW (2006) Reduction of inflammatory biomarker response by abatacept in treatment of rheumatoid arthritis. J Rheumatol 33:2162–2166
71. Wythe SE, Dicara D, Taher TE, Finucane CM, Jones R, Bombardieri M, Man YK, Nissim A, Mather SJ, Chernajovsky Y, Pitzalis C (2013) Targeted delivery of cytokine therapy to rheumatoid tissue by a synovial targeting peptide. Ann Rheum Dis 72:129–135

72. Yin L, Chen X, Vicini P, Rup B, Hickling TP (2015) Therapeutic outcomes, assessments, risk factors and mitigation efforts of immunogenicity of therapeutic protein products. Cell Immunol 295:118–126
73. Zwerina J, Hayer S, Tohidast-Akrad M, Bergmeister H, Redlich K, Feige U, Dunstan C, Kollias G, Steiner G, Smolen J, Schett G (2004) Single and combined inhibition of tumor necrosis factor, interleukin-1, and RANKL pathways in tumor necrosis factor-induced arthritis: effects on synovial inflammation, bone erosion, and cartilage destruction. Arthritis Rheum 50:277–290
74. Kolumam G et al (2015) EBioMedicine 2(7):730–743
75. Melanie Ruzek, Donna Conlon, Heikki Mansikka, Robert Padley and Carolyn Cuff. ABT-122, a Novel Dual Variable Domain (DVD)-IgTM, Targeting TNF and IL-17, Inhibits Peripheral Blood Mononuclear Cell Production of GM-CSF and Decreases Lymphocyte Expression of CXCR4 in Healthy Subjects ACR Meeting Abstract 1030
76. Kropshofer H, Singer T (2006) Overview of cell-based tools for pre-clinical assessment of immunogenicity of biotherapeutics. J Immunotoxicol 3(3):131–136
77. Mahler HC, Friess W, Grauschopf U, Kiese S (2009) Protein aggregation: pathways, induction factors and analysis. J Pharm Sci 98:2909–2934
78. Kontermann RE (2012) Dual targeting strategies with bispecific antibodies. mAbs 4:182–197
79. Roland E Kontermann, Ulrich Brinkmann (2015) Bispecific antibodies. Drug Discov Today 20:838–47
80. Weidle UH et al (2014) Tumor-antigen-binding bispecific antibodies for cancer treatment. Sem Oncol 41:653–660
81. Metz S et al (2011) Bispecific digoxigenin-binding antibodies for targeted payload delivery. Proc Natl Acad Sci U S A 108:8194–8199
82. Schanzer J et al (2011) Development of tetravalent, bispecific CCR5 antibodies with antiviral activity against CCR5 monoclonal antibody-resistant HIV-1 strains. Antimicrob Agents Chemother 55:2369–2378
83. Croasdale R et al (2012) Development of tetravalent IgG1 dual targeting IGF-1R-EGFR antibodies with potent tumor inhibition. Arch Biochem Biophys 526:206–218
84. Castoldi R et al (2012) Molecular characterization of novel trispecific ErbB-cMet-IGF1R antibodies and their antigen-binding properties. Protein Eng Des Sel 25:551–560
85. Trifabs Mayer K et al (2015) Trivalent IgG-shaped bispecific antibody derivatives: design, generation, characterization and application for targeted payload delivery. Int J Mol Sci 16(11):27497–27507
86. Kontermann RE (2010) Alternative antibody formats. Curr Opin Mol Ther 12:176–183
87. Metz S et al (2012) Bispecific antibody derivatives with restricted binding functionalities that are activated by proteolytic processing. Protein Eng Des Sel 25:571–580
88. Hayden MS et al (1994) Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumour activity from a COS cell transient expression system. Ther Immunol 1:3–15
89. Holliger P et al (1994) “Diabodies”: small bivalent and bispecific antibody fragments. Proc Natl Acad Sci U S A 90:6444–6448
90. Wolf E et al (2005) BiTEs: bispecific antibody constructs with unique anti-tumor activity. Drug Discov Today 10:1237–1244
91. Baeuerle PA, Reinhardt C (2009) Bispecific T-cell engaging antibodies for cancer therapy. Cancer Res 69:4941–4944
92. Baeuerle PA et al (2009) BiTE: teaching antibodies to engage T-cells for cancer therapy. Curr Opin Mol Ther 11:22–30
93. Kipriyanov SM et al (1999) Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. J Mol Biol 293:41–56
94. Johnson S et al (2010) Effector cell recruitment with novel Fv-based dual-affinity re-targeting protein leads to potent tumor cytolysis and in vivo B-cell depletion. J Mol Biol 399:436–449

95. Rossi EA et al (2006) Stably tethered multifunctional structures of defined composition made by the dock and lock method for use in cancer targeting. Proc Natl Acad Sci U S A 103:6841–6846
96. Rossi EA et al (2012) The dock-and-lock method combines recombinant engineering with site-specific covalent conjugation to generate multifunctional structures. Bioconjug Chem 23:309–323
97. Roopenian DC, Akilesh S (2007) FcRn: the neonatal Fc receptor comes of age. Nature reviews. Immunology 7(9):715–725
98. Kontermann RE (2011) Strategies for extended serum half-life of protein therapeutics. Curr Opin Biotechnol 22:868–876
99. Müller D et al (2007) Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. J Biol Chem 282:12650–12660
100. McDonagh CF et al (2012) Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. Mol Cancer Ther 11:582–593
101. Müller D, Kontermann RE (2007) Recombinant bispecific antibodies for cellular cancer immunotherapy. Curr Opin Mol Ther 9:319–326
102. Coloma MJ, Morrison SL (1997) Design and production of novel tetravalent bispecific antibodies. Nat Biotechnol 15:159–163
103. Kontermann RE (2009) Strategies to extend plasma half-lives of recombinant antibodies. BioDrugs 23:93–109
104. Ferrara C et al (2011) Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. Proc Natl Acad Sci U S A 108(31):12669–12674
105. Wu C et al (2007) Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin. Nat Biotechnol 25:1290–1297
106. Klein C et al (2012) Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies. mAbs 4:653–664
107. Ridgway JB et al (1996) 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 9:617–621
108. Davis JH et al (2010) SEEDbodies: fusion proteins based on strand-exchange engineered domain (SEED) CH3 heterodimers in an Fc analogue platform for asymmetric binders or immunofusions and bispecific antibodies. Protein Eng Des Sel 23:195–202
109. Merchant AM et al (1998) An efficient route to human bispecific IgG. Nat Biotechnol 16:677–681
110. Von Kreutenstein TS et al (2013) Improving biophysical properties of a bispecific antibody scaffold to aid developability: quality by molecular design. mAbs 5:646–654
111. Gunasekaran K et al (2010) Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent antibodies. J Biol Chem 285:19637–19646
112. Lewis SM et al (2014) Generation of bispecific IgG antibodies by structure-based design of an orthogonal Fab interface. Nat Biotechnol 32:191–198
113. Schaefer G et al (2011) A two-in-one antibody against HER3 and EGFR has superior inhibitory activity compared with monospecific antibodies. Cancer Cell 20:472–486
114. Silacci M, Lembke W, Woods R, Attinger-Toller I, Baenziger-Tobler N, Batey S, Santimaria R, von der Bey U, Koenig-Friedrich S, Zha W1, Schlereth B, Locher M, Bertschinger J, Grabulovski D (2015) Discovery and characterization of COVA322, a clinical-stage bispecific TNF/IL-17A inhibitor for the treatment of inflammatory diseases. MAbs 1–9. [Epub ahead of print]
115. Reichert JM, Dhimolea E (2012) The future of antibodies as cancer drugs. Drug Discov Today 17:954–963

116. Wu C et al (2009) Molecular construction and optimization of anti-human IL-1α/β dual variable domain immunoglobulin (DVD-Ig™) molecules. mAbs 1:339–347
117. Rao E et al (2012) Generation of a tetravalent bispecific antibody against IL4 and IL13 for the treatment of idiopathic pulmonary fibrosis. mAbs 4:14–16
118. Vanheusden K et al (2013) Pre-clinical proof-of concept of ALX-0761, a nanobody neutralising both IL-17A and IL-17F in a cynomolgus monkey collagen induced arthritis model. (2013) Poster n. 1287, presented at the Annual Meeting of the American College of Rheumatology (ACR); Oct 26–30; San Diego, CA
119. Lewis KE et al (2010) Engineering of stable bispecific antibodies targeting IL-17A and IL-23. Protein Eng Des Sel 23:115–127

Part IV
Cross-Functional Targets

Targeting CD13 with Asn-Gly-Arg (NGR) Peptide-Drug Conjugates

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1 Introduction

Endothelial cell activation and angiogenesis are common denominators of many pathological conditions, including rheumatoid arthritis, cancer, macular degeneration, atherosclerosis, cardiovascular diseases, diabetic ulcers, stroke, skin diseases, wound healing disorders, and others [1–4]. For this reason, many investigators have made efforts to identify new receptors that are selectively expressed by activated endothelial cells and to discover molecules capable of interacting with them, in an attempt to obtain new modulators of the vascular function or new ligands useful for delivering drugs and imaging agents to diseased tissues. Among the various receptors identified so far, aminopeptidase N (CD13), a multifunctional membrane-bound metalloproteinase, has attracted the interest of many investigators, owing to the fact that this enzyme is upregulated in angiogenic blood vessels and is barely expressed (or not at all) in normal blood vessels [5–9]. Furthermore, recent findings have shown that endothelial CD13 is upregulated in various inflammatory conditions [9].

A growing body of evidence suggests that the endothelial form of CD13 expressed by angiogenic vessels is recognized by peptides containing Asn-Gly-Arg (NGR), a motif originally discovered by selecting peptide-phage libraries in tumor-bearing mice [10]. Because of these properties, NGR peptides have been exploited

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by many investigators as vehicles for ligand-directed targeted delivery of a variety of therapeutic and imaging agents to angiogenic vessels.

In this chapter, we discuss the structural and functional properties of NGR peptides, including the most recently developed peptides, and their potential applications as drug delivery system in inflammatory diseases with an angiogenesis components. In addition, we discuss the manufacturing problems related to the strong propensity of NGR to undergo asparagine deamidation (with consequent formation isoDGR, an integrin-binding motif [11]), as well as the pharmacological and toxicological implications of this structural change in peptide-drug conjugates.

2 The Aminopeptidase N (CD13) Receptor of NGR

2.1 CD13 Structure

CD13 (EC 3.4.11.2) is a type II ectoenzyme of ~150–240 kDa involved in the degradation of neutral or basic N-terminal residues of bioactive peptides. This protein is a member of the M1 family of zinc metallopeptidases and consists of an enzymatic extracellular domain, a transmembrane region, and a short cytoplasmic domain that has been implicated in signal transduction [12]. Differential *O*-glycosylation of this enzyme results in various isoforms that are differentially recognized by antibodies [13]. The crystal structures of porcine aminopeptidase N and its complex with a peptide substrate have been recently described [14]. The ectodomain has a seahorse-like shape, with four distinct domains (head, side, body, and tail) that form dimers through the interactions between the head domains. The zinc-dependent catalytic site resides in a cavity with wide openings allowing easy access to peptide substrates. This cavity can potentially open up further to bind the exposed N-terminus of proteins [14]. Recently, the crystal structures of the dimeric ectodomain of human aminopeptidase N in the presence of angiotensin IV and two peptidomimetic inhibitors (amastatin and bestatin) have been reported [15]. These studies suggest that a rapid interconversion between open and closed forms of the ectodomains could be critically involved in the mechanism of peptide processing and signal transduction.

2.2 CD13 Expression in Normal and Pathological Conditions

CD13 is expressed by most cells of myeloid origin including granulocytes, monocytes, macrophages, and their hematopoietic precursors [16–18]. It is also abundantly expressed in the brush border of epithelial cells from small intestine and renal proximal tubules, in bile duct canaliculi, in prostatic epithelial cells, in mast cells, in activated endothelial cells, and, in some cases, in pericytes, smooth muscle cells, and fibroblasts [17–20]. In most of these cells, CD13 immunoreactivity localizes to the cell membrane. However, cytoplasmic staining and detection of soluble CD13 in human plasma have also been reported [6, 16–20].

Various studies have shown that CD13 expression is increased at disease sites in patients with collagen vascular diseases (CVD, a group of inflammatory disorders which affect the skin, joints, serous membranes, and a variety of organs such as the heart, kidney, and lung), including rheumatoid arthritis, polymyositis/dermatomyositis, systemic sclerosis, and systemic lupus erythematosus [21]. Higher levels of serum aminopeptidase activity have been detected in these patients. Furthermore, increased aminopeptidase activity has been detected in pleural effusions from patients with systemic lupus erythematosus, or in the synovial fluids from rheumatoid arthritis patients [21]. Increased expression of CD13 has been also observed in synovial fibroblasts from rheumatoid arthritis patients and in alveolar macrophages from CVD patients with interstitial lung diseases [21, 22].

CD13 is little or barely expressed by normal vessels, whereas it is upregulated in inflammation-associated vessels [9]. For example, in a study performed on inflamed skin and tonsils, anti-CD13 antibodies stained vessels in about half of the tissues analyzed [9]. In these tissues, the anti-CD13 antibodies stained stroma and vessels along with some inflammatory cells [9]. Other studies performed in a murine model of myocardial infarction based on permanent coronary occlusion have shown that CD13 is expressed in some vessels of the infarcted area/border zone, along with macrophages and dendritic cells [23].

Several studies have shown upregulation of CD13 in the vasculature of many solid tumors. Depending on tumor type, staining of cancer cells, stroma, and/or vasculature has been observed [6, 9, 24]. Interestingly, immunohistochemical analysis of the same tumor tissue specimens with three anti-CD13 monoclonal antibodies (WM15, 3D8, and BF10) showed different staining patterns, pointing to the existence of different immunoreactive forms of CD13 [9]. For example, in many tumors, WM15 stained almost all intra-tumor and peri-tumor capillaries and only partially large vessels, whereas the other antibodies reacted with arteries and venules and to a lesser extent with capillaries. In about half of the neoplastic tissues analyzed, these antibodies could also stain the stroma. Remarkably, the three antibodies failed to stain endothelial cells of normal colon, whereas they reacted with endothelial cells of colon adenocarcinoma vessels and with activated human umbilical vein endothelial cells [9]. These data indicate that CD13 is a heterogeneous antigen and that various immunoreactive forms are expressed in a differential manner in different tissues. Notably, CD13 is upregulated not only in angiogenic vessels of neoplastic tissues [5, 6, 8] but also in angiogenic vessels associated with other pathological conditions, such myocardial infarction [8, 25].

2.3 Role of CD13 in Angiogenesis/Inflammation and NGR Recognition

A growing body of evidence suggests that CD13 upregulation is not just a marker or an epiphenomenon of angiogenesis and inflammation, but that it can also have important pathophysiological roles. Indeed, CD13 plays a variety of functions in the regulation of hormones and cytokines, in viral infection, in antigen presentation, in cell differentiation and proliferation, in apoptosis, in endocytosis, in cell adhesion

and migration, and in tissue invasion [17, 18, 26–32]. Furthermore, substantial experimental evidence suggests that this enzyme is implicated in the pathogenesis of cancer, leukemia, myocardial infarction, rheumatoid arthritis, and other inflammatory diseases. For this reason, the interest in this enzyme as a therapeutic target has been progressively increasing. The detailed description of these biological effects and of the various enzyme inhibitors so far developed is out of the scope of the present discussion, and we refer the interested reader to excellent reviews on this topic [18, 24]. The following discussion mainly focuses on the vascular functions of CD13, which could be relevant for the biological properties of NGR peptides.

At this regard, a large number of in vitro and in vivo experiments have shown that CD13 may have an important role in the angiogenesis process [5, 18, 27, 33–37]. In endothelial cells, CD13 is activated by angiogenic signals and functions as a regulator of cell invasion and filopodia formation, which are critical for angiogenesis [27, 38]. The involvement of CD13 in angiogenesis is also supported by the observation that inhibition of this enzyme with anti-CD13 antibodies or with bestatin impairs angiogenesis, whereas hypoxia and angiogenic factors induce CD13 expression in endothelial cells [18, 27]. Furthermore, CD13-null mice show reduced angiogenic responses to growth factors and are significantly deficient in promoting retinal neovascularization under hypoxic conditions [39]. CD13-null mice also display reduced tumor growth after implantation with melanoma and Lewis lung carcinoma cells [39]. The role of CD13 in angiogenesis is not limited to the "vascular" form of this enzyme. Indeed, direct involvement of a $CD11b^{+}CD13^{+}$ myeloid subset of bone marrow-derived cells in tumor angiogenesis, tumor growth, and metastasis has been recently shown [40]. Thus, a CD13 form expressed by vascular cells and a form expressed by bone marrow-derived myeloid cells seem to exert key roles in angiogenesis. Remarkably, peptides containing the CNGRC motif (disulfide-bridged) recognize $CD13^{+}$ cells of angiogenic vessels, but not $CD13^{+}$ bone marrow-derived myeloid cells [40], suggesting that the two forms are different and that CNGRC is selective for the "vascular" form.

The capability of CNGRC to selectively recognize a CD13 form associated with the angiogenic vasculature, and not other CD13-positive tissues, is also supported by the results of immunohistochemical and biodistribution studies of compounds containing CNGRC, showing that this peptide can bind CD13-positive tumor blood vessels, but not other CD13-rich tissues [6, 41]. Quantitative magnetic resonance imaging studies in tumor mouse models with NGR-labeled paramagnetic quantum dots confirmed the capability of CNGRC to recognize angiogenic blood vessels in tumor tissues [42]. Remarkably, magnetic resonance imaging studies with a CNGRC-labeled paramagnetic quantum dots allowed also selective, noninvasive detection of infarcted heart [25]. Other works have shown that a CNGRC peptide tagged with a fluorochrome can co-localize with CD13 and the endothelial marker CD31 in a murine model of cardiac angiogenesis [8], whereas a CNGRC phage can home to CD13-positive blood vessels of angiogenic retina [7]. These findings suggest that NGR can recognize angiogenic vessels of neoplastic as well as nonneoplastic tissues. Thus, NGR peptides can potentially target not only tumor vessels but also other physiologic or pathologic angiogenic vessels in different tissues.

Interestingly, it has been shown that CD13 expressed in endothelial cells and monocytes mediates homotypic cell adhesion in a manner that is independent on the enzymatic activity [31]. In particular, CD13 clustering on monocyte with anti-CD13 antibodies results in cell activation and in increased adhesion to the endothelium, a mechanism that involves the formation of complexes containing both monocyte and endothelial CD13. Furthermore, CD13-blocking antibodies reduced peritoneal leukocyte infiltration in a murine model of peritonitis, suggesting that CD13 has a crucial role in leukocyte trans-endothelial migration at inflammatory sites [31]. These results are remarkable as they imply a role for CD13 in inflammatory cell trafficking through the endothelial barrier, thereby pointing to a crucial role of this enzyme in inflammation. Accordingly, it has been shown that CD13 is essential for the proper trafficking of inflammatory cells in infarcted heart tissues following permanent coronary artery occlusion in mice [23]. Notably, loss of CD13 results in adverse remodeling of the left ventricular wall [23].

Whether NGR peptides can recognize or not CD13 expressed by endothelial cells and/or other cells in the various inflammatory tissues is difficult to predict. Indeed, the notion that CNGRC can selectively recognize a CD13 form associated with the angiogenic vasculature in tumors cannot necessarily be extrapolated to all pathological conditions associated with CD13 upregulation, considering that various CD13-positive tissues are not, or poorly, recognized by this peptide. Studies aimed at clarifying this important issue are, therefore, of great experimental and pharmacological interest.

2.4 Structural Basis of CD13/NGR Interactions

The structural basis of the selectivity of NGR peptides for the CD13 expressed in the angiogenic vasculature is still unclear. Analysis of the crystal structure of His-tagged porcine CD13 ectodomain complexed with CNGRCG showed that the NGR tripeptide sequence can interact with the enzymatic active site, but resists to degradation owing to a distorted scissile peptide bond. Notably, this peptide can inhibit the activity of CD13 with a Ki value of 38.7 μM [43]. Although this is an important proof of the capability of NGR to recognize CD13, these findings cannot explain the good affinity and selectivity of NGR for CD13 expressed in the neovasculature. This is another important issue that still remains to be clarified. At this regards, it is important to keep in mind that natural CD13, unlike the recombinant ectodomain used in these studies, is a type II membrane glycoprotein with an N-terminal membrane anchor. This leads to an archlike structure on the cell surface that may undergo large conformational changes, owing to the fact that each monomer can assume open or closed conformations [15]. An interesting possibility is that conformational changes in different tissues, possibly owing to the presence of tissue-specific cofactors or signaling molecules, may result in differential NGR-binding affinity. Further, work is necessary to assess this hypothesis.

3 Use of NGR Peptides as Drug Delivery Systems

Peptides containing the NGR sequence have been used by several investigators for delivering a variety of compounds to tumor blood vessels, including chemotherapeutic drugs, liposomes, antiangiogenic compounds, DNA complexes, viral particles, and imaging compounds (*see* Table 1 and references thereof). NGR peptides have been also fused to cytokines, such as TNFα, IFNγ, and IFNα-2a, in an attempt to improve their antitumor therapeutic index. Remarkably, some NGR-drug conjugates are been tested in patients. For example, a fusion protein consisting of the extracellular domain of tissue factor (truncated tissue factor, tTF) and the peptide GNGRAHA (tTF-NGR) has been tested in cancer patients [44]. This product, but not untargeted tTF, induced thrombosis of blood vessels and tumor growth retardation or regression in murine models of solid tumors and decreased tumor perfusion in patients [44–46]. Another NGR-drug conjugate that is being tested in patients is NGR-TNF, a CNGRCG-tumor necrosis factor (TNF)-alpha fusion product originally developed by our group [47]. The results of preclinical studies performed with these drugs suggest that NGR-mediated vascular targeting is a valuable strategy for delivering bioactive amounts of cytokines to tumor endothelial cells without causing the activation of counter-regulatory mechanisms and toxic reactions [47, 48]. In particular, these studies have shown that targeted delivery of minute amounts of TNF to tumor vessels is sufficient to alter the endothelial barrier function and favor the penetration of various chemotherapeutic drugs in tumors as well as the infiltration of lymphocytes in neoplastic tissues [48–50]. Consequently, it has been shown that low-dose NGR-TNF exerts synergistic effects with chemotherapy and immunotherapy. Noteworthy, the antitumor activity of NGR-TNF in animal models is largely inhibited by an anti-CD13 antibody, which supports the major role of CD13 as a targeting receptor of this drug [41]. Because of these properties, NGR-TNF is currently tested in various phase II and III clinical studies in patients with solid tumors, alone and in combination with chemotherapy or immunotherapy, with evidence of activity (www.molmed.com). The biological and pharmacological properties of this product and the results of phase I and II clinical studies have been recently reviewed [47]. These studies showed that NGR-TNF is well tolerated. Chills and fever were the most frequently observed toxicities, and no patients developed anti-NGR-TNF antibodies during treatment. Dynamic contrast-enhanced magnetic resonance imaging showed a vascular response to NGR-TNF. Single-agent phase II studies with low-dose NGR-TNF (0.8 μg/m^2, 1 h infusion, every 3 weeks or weekly), conducted in malignant pleural mesothelioma (MPM), hepatocellular carcinoma, and colorectal cancer, showed radiological anti-vascular effects and significant disease control. In particular, a phase II study on MPM patients showed disease control in about half of previously treated patients, which was maintained in the triweekly cohort for 4.4 months and for 9.1 months in the weekly cohort [51]. These results are remarkable considering that, currently, there are no standard options for patients with MPM who are failing a frontline pemetrexed-based regimen and also considering the easily manageable toxicity profile of

Table 1 Examples of NGR peptides that have been used by various investigators for delivering drugs or imaging compounds to neovessels

Peptide[a]	Type of conj[b]	Use[c]	Drug or imaging compound coupled to peptide	Targeted tissue/cell line[d]	Preclinical/clinical study	Ref
Cyclic (S-S bond)						
CNGRC	C	T/I	Doxorubicin, doxorubicin-loaded poly(lactic-co-glycolic acid) nanoparticles, fluorescent probes	MDA-MD-435, HT-1080	Mouse	[10, 55, 56]
CNGRC	C		5-Fluoro-2′-deoxyuridine prodrugs	HT-1080	In vitro assay	[57]
$CNGRC_{NH2}$	C		Carboplatinum	HUVEC, HepG2	In vitro assay	[58]
CNGRCG	FP	T	Tumor necrosis factor-alpha	WEHI 164, B16F1, RMA,TS/A, various solid tumors[e]	Mouse/human (phase I, II, III)	[41, 47, 52–54, 59–68]
	FP		Apoprotein-bleomycin conjugate	BEL-7402	In vitro assay	[69]
	C		DNA-loaded poly(lactic acid)-poly(ethylene glycol) nanoparticles	HUVEC	In vitro assay	[70]
CNGRCGK	C		Cy 5.5	HT-1080	Mouse	[71]
CNGRCGG	FP	T	Proapoptotic peptide	MDA-MB-435	Mouse	[72]
CNGRCGGG	FP	T	Plasminogen kringle 5	LLC, colo 205,	Mouse	[73]
			Actin fragment	HepG2	In vitro assay	[74]
CNGRCGGY	C	I	^{131}I	HT-1080	Mouse	[75]
YGGCNGRC	C	I	^{99m}Tc	HepG2	Mouse	[76]
acCNGRCGGK	C	I	^{68}Ga, Qdot, SPIOs, ^{111}Ind	Infarcted myocardium, LS174T	Rat/mouse	[8, 42, 77–79]

(continued)

Table 1 (continued)

Peptide[a]	Type of conj[b]	Use[c]	Drug or imaging compound coupled to peptide	Targeted tissue/cell line[d]	Preclinical/clinical study	Ref
acCNGRCGGG	C		Oregon green 488 and gadolinium	–		[80]
GACNGRCVSG	FP		Moloney murine leukemia virus envelope escort proteins	HUVEC, KSY1	In vitro assay	[109]
CNGRCVSGCAGRC	FP	I	VEGI	HT-1080	Mouse	[81]
CNGRCVSGCAGRC	FP		Adenovirus capsid protein for delivering angiostatin K1-5 cDNA	LLC	Mouse	[82]
GNCNGRCVSGCAGRC	FP	T	Tumstatin-5, interferon alpha 2a	S180, SPC-A-1, A549, LLC	Mouse	[105–107]
acCNGRCG-hTNF$_{1-10}$KY	C		Qdot	HUVEC	In vitro assay	[83]
CNGRCG	FP	T	Lydiamycin	MCF-7	Mouse	[84]
CDCNGRCFC	FP		Adenoviral fiber protein	RD	In vitro assay	[85]
CRNGRGPDC (iNGR)		T	Nanoparticles	4T1	Mouse	[86]
SGCNGRC	FP	T	Interferon gamma	RMA, WEHI-164	Mouse	[87]
GGCNGRC	C	T	Docetaxel-carboxymethyl chitosan, polymeric micelles containing doxorubicin	B16, HT-1080	Mouse	[88, 89]
GGGCNGRC	C	I	^{64}Cu, Cy5.5	HT-1080	Mouse	[90, 91]
Cyclic (peptide bond)						
KNGRE$_{NH2}$	C	I	Liposomal doxorubicin, oregon green 488, ^{68}Ga	HT-1080, NeDe	In vitro assay, rat	[92, 93]
NGRYK	C	I	^{99m}Tc	OVCAR-3	Mouse	[94]
Linear						
NGR	FP	T	Endostatin	LM3	Mouse	[95]
NGR$_{NH2}$	C		Platinum (IV)	BCE, HMVEC	In vitro assay	[96]
GNGRGG	FP	T	Tumor necrosis factor-alpha	B16F1	Mouse	[97]

GNGRGC	C	I	Liposomal iohexol	H520	Mouse	[98]
GNGRGGYC	C	T	Dual-ligand liposomal doxorubicin modified with penetrating peptide	RCC	Mouse	[99]
ac(GNGRG)2KGK	C	I	^{99m}Tc	HepG2	Mouse	[100]
GNGRGG-hTNF$_{1-11}$YC	C	T	Liposomal doxorubicin, liposome containing doxorubicin and siRNA	SH-SY5Y, PC3, HCT-116, HT-1080	Mouse	[101–103]
GNGRAHA	FP	T	Truncated tissue factor	M21, A549, HT1080	Mouse/human (phase I)	[44, 104]
NGRAHA	FP		Parvovirus AAV type 2	KS1767, RD	In vitro assay	[108]
NGRGGS	FP	T	Monodisperse spherical micelles	FaDu	Mouse	[110]
Other modifications						
NGR(NO_2) $COOCH_3$	C	T	5-Fluorouracil	–	Mouse	[111]

[a]The residue used for conjugation is underlined; *ac* acetylated residue, *NH_2* amidated residue

[b]*C* chemical conjugate, *FP* fusion protein

[c]*T* therapy, *I* imaging

[d]Examples of cell lines and/or tumor models used: *MDA-MD-435* human breast carcinoma, *HT-1080* human fibrosarcoma, *HUVEC* human umbilical vein endothelial cells, *HepG2* human hepatocellular carcinoma, *WEHI 164* mouse fibrosarcoma, *B16F1* mouse melanoma, RMA mouse lymphoma, *TS/A* mouse mammary adenocarcinoma, *BEL-7402* human liver carcinoma cells, *LLC* mouse Lewis lung carcinoma, *Colo 205* human colorectal adenocarcinoma, *LS174T* human colorectal adenocarcinoma, *MCF-7* human breast cancer, *RD* human embryonal rhabdomyosarcoma cells, *4T1* mouse mammary carcinoma, *NeDe* mouse mesenchymal mesoblastic nephroma tumor, *OVCAR-3* human ovarian carcinoma, LM3 murine mammary adenocarcinoma, *BCE* bovine capillary endothelial cells, *HMVEC* human microvascular endothelial cells, *H520* human squamous cell carcinoma, RCC human renal cell carcinoma, SH-SY5Y human neuroblastoma, *HCT-116* human colon cancer, PC3 human prostate cancer, *M21* human melanoma, *A549* human lung adenocarcinoma, *S180* mouse sarcoma, *SPC-A-1* human lung adenocarcinoma, *KS1767* and *KSY1* Kaposi's human sarcoma cells, *FaDu* human hypopharyngeal carcinoma

[e]Pleural mesothelioma (randomized phase III), non-small cell lung cancer, ovarian cancer, soft tissue sarcomas (randomized phase II), colorectal cancer, liver cancer, small cell lung cancer, pleural mesothelioma, ovarian cancer (phase II)

NGR-TNF. Based on these results, a randomized double-blind phase III study of human NGR-TNF plus best investigator's choice (called "BIC") versus placebo plus BIC in previously treated patients with advanced malignant pleural mesothelioma has been undertaken (http://www.clinicaltrialsfeeds.org/clinical-trials/show/NCT01098266). Finally, phase I and II studies of NGR-hTNF in combination with chemotherapy (e.g., doxorubicin or cisplatin) in patients with refractory solid tumors showed that also this drug combination has interesting clinical activity and safe toxicity profile [47, 52–54].

Thus, a variety of different compounds have been coupled to NGR peptides by different investigators, with good results. It is noteworthy that these products rely on the use of various peptides having NGR embedded in different molecular scaffolds, such as disulfide-bridged CNGRC, acetylated-CNGRC, CVLNGRMEC, CNGRCGK, head-to-tail cyclized cKNGRE, or linear GGCNGRC, GNGRG, NGRAHA, KNGRE, NGR, and several others (Table 1). These peptides have been chemically coupled to drugs and particles, or fused to the N-terminal or C-terminal sequences of proteins, or even incorporated in internal loops of proteins by genetic engineering technology. The good results obtained with many of these products highlight the utility and versatility of NGR as a targeting motif for drug development.

The concept that NGR is a versatile motif is also underscored by the recent development of a peptide in which targeting motif, scaffold, and effector domain overlap. This peptide, called "internalizing" NGR (iNGR), consists of disulfide-bridged CRNGRGPDC, a sequence that comprises NGR embedded into a cryptic C-end rule (CendR) motif [86]. The CendR motif is an amino acid sequence that, upon cleavage and generation of an R/KXXR/K-OH C-terminal sequence, can interact with neuropilin-1 and activate a tissue penetration pathway that delivers the peptide and the attached payload into solid tumors [112]. Thus, once CD13 binding has brought iNGR to the tumor vasculature, the peptide is proteolytically cleaved to expose the cryptic CendR motif (RNGR). This causes the gain of affinity for neuropilin-1 and, after binding, the activation of a tissue penetration pathway. Experimental data obtained in animal models have shown that indeed iNGR homes to tumor vessels and penetrates into tumor tissues more effectively than the standard CNGRC peptide [86]. Remarkably, iNGR induced greater tumor penetration of coupled nanoparticles, as well as of coadministered compounds, such as doxorubicin. Consequently, in murine tumor models, doxorubicin was significantly more efficacious when given in combination with iNGR than when given alone [86].

4 Potential Use of NGR Peptides in Inflammation

The experimental evidence showing that NGR can target neovessels in neoplastic and in nonneoplastic tissues, e.g., in myocardial infarction and retinal angiogenesis, and that vessels in certain inflammatory lesions have increased expression of CD13 suggests that NGR peptides might be exploited as drug delivery systems not only in

cancer but also in other pathological conditions. One interesting possibility is that NGR peptides could be exploited for delivering drugs to inflammatory sites, particularly in diseases with an angiogenesis component, such as rheumatoid arthritis and myocardial infarction. In rheumatoid arthritis, a chronic systemic inflammatory disorder that primarily affects joints, endothelial cells are active participants in the inflammatory process (by regulation of leukocyte extravasation, cytokine production, angiogenesis, protease and extracellular matrix synthesis, vessel permeability, and antigen presentation), thereby representing an important target for therapies based on peptide-mediated delivery of anti-inflammatory cytokines [113]. However, while consistent experimental evidence shows that NGR peptides can target infarcted heart tissues, it remains to be demonstrated that in patients with rheumatoid arthritis, CD13 is expressed in sufficient amounts by endothelial cells to enable efficient targeting and that NGR peptides can recognize this CD13 form. The same applies to other inflammatory disorders. Indeed, the experimental evidence that CNGRC can selectively recognize a CD13 form associated with the angiogenic vasculature in tumor-bearing mice and not (or much less) the CD13 forms abundantly expressed by other tissues (e.g., in the kidney, intestine, and liver) cannot necessarily be extrapolated to all pathological conditions associated with CD13 upregulation in the vasculature. On the other hand, we cannot exclude that NGR might recognize CD13 expressed by cells other than endothelial cells in inflamed tissues. Only properly designed experiments aimed at evaluating the in vitro and in vivo binding of NGR peptides to inflamed tissues may help to address these questions. Elucidating the mechanism underlying the selective interaction of NGR peptides with CD13 expressed by neovessels may also help to answer these questions and to predict which tissues might be targeted by NGR. An interesting possibility is that different structural/conformational forms of CD13 exist in diseased and normal tissues that might be differentially targeted by NGR peptides. Elucidating this point may also help to design new targeting peptides and peptide-drug conjugates as well as to speculate on their potential interfering effects on the physiological functions of CD13 in angiogenesis and inflammation.

5 Role of the NGR Molecular Scaffold on the Biochemical, Biological, and Immunological Properties of Peptide-Drug Conjugates

Although the results of most of the studies discussed above show the utility and versatility of the NGR motif as an efficient system for drug delivery to neovessels, it is important to stress the concept that the use of different molecular scaffolds of NGR might lead to the generation of CD13 ligands with different biochemical, biological, immunological, and toxicological properties. For example, it has been shown that the affinity of cyclic CNGRCG peptide for CD13-positive endothelial cells is greater than that of linear GNGRGG [97], suggesting that different flanking residues and/or peptide cyclization/linearization might affect NGR affinity and

selectivity for CD13. According to this view, a CNGRCG peptide could bind recombinant porcine CD13 and inhibit its in vitro enzymatic activity more efficiently than GNGRG [43].

Changes in the molecular scaffold might also change the immunogenicity of NGR. Remarkably, immunogenicity studies, carried out in mice, rabbits, and humans, have shown that the CNGRC motif is poorly, or not at all, immunogenic [47, 114]. It is also remarkable the fact that we failed to elicit antibodies in mice and rabbits even after repeated administrations of high doses of CNGRC peptides coupled to immunogenic carrier proteins. The poor immunogenicity of NGR is an important property for a peptide ligand that has to be repeatedly injected in patients. An explanation for the low immunogenicity of CNGRC comes from the results of molecular dynamics simulation experiments, which predict that the most populated structures of this peptide are highly superimposable to the structure of an NGR loop of human fibronectin [115]. These data suggest that this peptide might be viewed as a self-antigen by the immune system. However, the low immunogenicity of CNGRC peptide cannot be extrapolated to other peptides having NGR embedded in a different molecular scaffold. Adequate studies on CD13-binding affinity and immunogenicity are necessary to compare the value of different NGR peptides. Another important point that should always be taken into account is the potential impact of the molecular scaffold on the stability of NGR. Indeed, a growing body of evidence indicates that the asparagine (Asn) residue of NGR has a strong propensity to undergo deamidation reactions, with potentially important pharmacological and toxicological implications that will be discussed in the following paragraphs.

6 The NGR-to-isoDGR Transition and Receptor Switch

It is well known that the Asn residue of proteins and peptides can spontaneously undergo posttranslational modifications to form isoaspartyl residues (isoAsp or isoD) [11]. The formation of this nonstandard β-amino acid can occur in vivo, e.g., in extracellular matrix proteins during tissue aging, as well as in vitro during protein or peptide preparation and storage, including NGR peptides [11]. Asn deamidation at NGR sites occurs by a nucleophilic attack of the backbone NH center at the carbonyl group of Asn side chain, which leads to formation of a succinimide ring (Fig. 1). Hydrolysis can then occur at both carbonyl groups of the cyclic imide, leading to the formation of a mixture of Asp and isoAsp residues, typically with a ratio of approximately 1:3, thereby changing the NGR sequence to DGR or isoDGR.

In general, the Asn deamidation reaction in proteins or peptides can take hours, days, or even years, depending on the molecular microenvironment and other factors. For example, the presence of a Gly residue following Asn (as in NGR) can generally accelerate this reaction [11, 116–118]. Remarkably, the CNGRC peptide and a fragment of fibronectin containing a GNGRG loop can undergo deamidation reactions in a very rapid manner (half-life, 4–5 h at 37 °C in cell culture medium) [115, 119]. This is probably one of the fastest Asn deamidation reactions so far described. Considering the importance of the molecular microenvironment on the

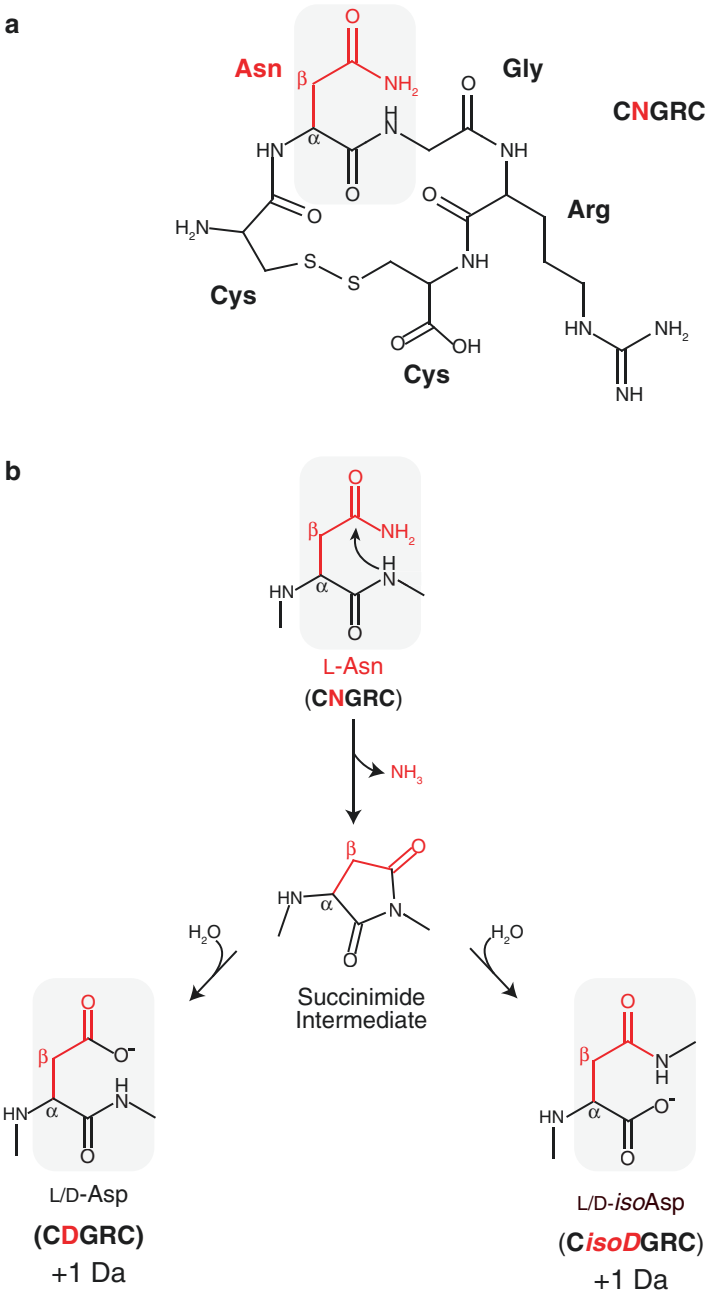

Fig. 1 Schematic representation of the cyclic CNGRC peptide (**a**) and its deamidation reaction (**b**). Nucleophilic attack of the backbone NH center on the Asn side chain (*red*) leads to formation of a succinimide intermediate, which after hydrolysis may lead to formation of negatively charged CDGRC (with an alpha-peptide bond) and C*iso*DGRC (with a beta-peptide bond)

kinetics of Asn deamidation, it is likely that the different peptides so far exploited by different investigators as payload delivery systems are characterized by different degradation kinetics. This view is supported by the results of peptide degradation studies performed with cyclic CNGRC and linear GNGRG peptides, showing markedly different qualitative and quantitative degradation patterns [119].

Although Asn deamidation in proteins typically causes a loss of function, substantial experimental data suggest that isoAsp formation at the NGR sites in peptides and in certain proteins, such as fibronectin and ceruloplasmin, might have a gain of function [115, 120]. Indeed, isoDGR can mimic RGD, an integrin recognition motif, and recognize the RGD-binding site of integrins, such as αvβ3, αvβ5, αvβ6, αvβ8, and α5β1, but not other RGD-independent integrins [115, 119, 121, 122]. For example, the CisoDGRC peptide can recognize the αvβ3 integrin with a Kd value of ~9 nM and the other integrins with lower affinity [119]. NMR structure analysis of cyclic CisoDGRC, CRGDC, CDGRC, and CNGRC peptides and αvβ3-integrin docking experiments showed that CisoDGRC, but not CDGRC and CNGRC, fits into the RGD-binding pocket and favorably interacts with this integrin [11, 115, 121, 122]. Therefore, isoDGR, unlike DGR and NGR, is a natural fit for the RGD-binding pocket of αvβ3 integrin. This implies that the transformation of NGR into isoDGR results in a receptor switch, from CD13 to integrins, with important biological implications.

A crucial point that deserves to be highlighted is that isoDGR peptides can recognize members of the RGD-dependent integrin family in a differential manner, depending on the molecular scaffold in which isoDGR is inserted [119, 123]. For example, cyclic CisoDGRC can bind αvβ3 integrin with an affinity 10–100-fold higher than that for other members of the RGD-dependent integrin family. Replacement of the Cys by two Gly residues (as in GisoDGRG) leads to a marked loss of affinity for all integrins and a change of specificity [119]. It appears, therefore, that the molecular scaffold of NGR can affect various biochemical and biological properties not only of NGR itself but also of its degradation products, including receptor affinity and selectivity, immunogenicity, and stability. Considering the CD13-to-integrin receptor switch after NGR-to-isoDGR transition, the deamidation reactions in peptide-drug conjugates (which may potentially occur during their preparation, storage, or even in vivo after administration to patients) might have important biological, pharmacological, and toxicological implications. Of note, a method to avoid, or markedly reduce, Asn deamidation during storage is to store peptide and peptide-drug conjugates in water or in HEPES buffer at pH 7.4 [119].

7 Biological and Pharmacological Implications of the NGR-to-isoDGR Transition in NGR-Drug Conjugates

Integrins are involved in many physiological and pathological processes, such as inflammation, thrombosis, osteoporosis, angiogenesis, and cancer. Notably, a growing body of evidence suggests that αvβ3 is overexpressed in the tumor vascular

endothelium and has an important role in angiogenesis and tumor growth [124–129]. Considering the notion that RGD peptides with a variable degree of affinity and selectivity for this integrin have been used for delivering a variety of drugs and nanoparticles to tumor vessels [128, 130–132], the NGR-to-isoDGR transition in long-circulating NGR-drug conjugates might represent an advantage, as this mechanism might enable a dual-receptor (CD13 and αvβ3) targeting mechanism. However, as discussed above, the NGR motif embedded in different molecular scaffolds may recognize, after deamidation, also other integrins with unpredictable effects. Thus, the NGR-to-isoDGR transition is a crucial issue that should always be addressed when new peptide-drug conjugates are being developed.

Another important concern related to this issue regards the mechanism of action of NGR-drug conjugates. While some studies have ruled out the risk that the biological properties of NGR-drug conjugates are actually mediated, at least in part, by isoDGR/integrin interactions, most studies reported in the literature have not adequately addressed this crucial point. Mass spectrometry is often used to assess NGR peptide identity after synthesis, taking advantage of the fact that the NGR-to-isoDGR transition is accompanied by a gain of 1 Da. However, this analytical control, although necessary, is not sufficient to rule out the possibility that isoDGR is formed during in vitro or in vivo assays. Notably, in some studies reported in the literature, mass spectrometry analysis of NGR conjugates revealed a mass 1 Da greater than expected, which may raise doubts about product identity. Many studies have also used CD13-positive and CD13-negative cells, e.g., HT1080 and MCF7, to assess NGR/CD13 interactions with new peptide-drug conjugates. However, in most studies, the potential involvement of integrins expressed by these cells was not investigated. We think that more controls and more tools (e.g., neutralizing antibodies, receptor-null mice, receptor-silencing reagents, etc.) should be used to unequivocally demonstrate the role of NGR/CD13 interactions and to rule out the potential contribute of isoDGR/integrin interactions. In other words, detailed information on the stability of new peptide-drug conjugates or peptide fusion proteins, in vitro and in vivo, and on their receptor-binding properties are necessary to unequivocally elucidate their mechanism of action.

8 Concluding Remarks

The large number of tumor homing peptides containing the NGR motif so far developed and their successful exploitation for targeted delivery of many compounds to tumors highlight the utility and versatility of NGR as a tumor neovasculature-targeting motif, a strategy that takes advantage from the fact that the CD13 receptor of NGR is upregulated in many tumor tissues. The finding that CD13 is upregulated also in various inflammatory conditions opens the possibility that NGR peptides might be exploited as drug delivery systems also in inflammatory diseases with an angiogenesis component, such as myocardial infarction and rheumatoid arthritis, provided that the capability of NGR to recognize CD13 in all these conditions is

demonstrated. In any case, a key point that must be carefully considered when planning the development of new NGR-drug conjugates is the choice of the molecular scaffolds of NGR, as this may have dramatic effects on receptor-binding affinity and selectivity, on peptide immunogenicity, and, above all, on peptide stability. Indeed, the strong propensity of NGR to undergo deamidation reactions and the consequent transition to isoDGR remain a major issue, considering that this spontaneous reaction may occur during drug preparation, storage, analysis, or even in vivo after administration to animals or patients, with potentially important mechanistic, pharmacologic, and toxicologic implications.

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Conflict of Interest A. Corti and F. Curnis are the inventors of various patents on the use of NGR peptides for drug delivery. M. Fiocchi declares no conflict of interests.

References

1. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286
2. Italiano JE Jr, Richardson JL, Patel-Hett S et al (2008) Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 111:1227–1233
3. Ribatti D (2009) Endogenous inhibitors of angiogenesis: a historical review. Leuk Res 33:638–644
4. Crippa L, Bianco M, Colombo B et al (2013) A new chromogranin A-dependent angiogenic switch activated by thrombin. Blood 121:392–402
5. Pasqualini R, Koivunen E, Kain R et al (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60:722–727
6. Curnis F, Arrigoni G, Sacchi A et al (2002) Differential binding of drugs containing the NGR motif to CD13 isoforms in tumor vessels, epithelia, and myeloid cells. Cancer Res 62:867–874
7. Lahdenranta J, Sidman RL, Pasqualini R et al (2007) Treatment of hypoxia-induced retinopathy with targeted proapoptotic peptidomimetic in a mouse model of disease. FASEB J 21:3272–3278
8. Buehler A, van Zandvoort MA, Stelt BJ et al (2006) cNGR: a novel homing sequence for CD13/APN targeted molecular imaging of murine cardiac angiogenesis in vivo. Arterioscler Thromb Vasc Biol 26:2681–2687
9. Di Matteo P, Arrigoni GL, Alberici L et al (2011) Enhanced expression of CD13 in vessels of inflammatory and neoplastic tissues. J Histochem Cytochem 59:47–59
10. Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380
11. Corti A, Curnis F (2011) Isoaspartate-dependent molecular switches for integrin-ligand recognition. J Cell Sci 124:515–522
12. Santos AN, Langner J, Herrmann M et al (2000) Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. Cell Immunol 201:22–32

13. O'Connell PJ, Gerkis V, d'Apice AJ (1991) Variable O-glycosylation of CD13 (aminopeptidase N). J Biol Chem 266:4593–4597
14. Chen L, Lin YL, Peng G et al (2012) Structural basis for multifunctional roles of mammalian aminopeptidase N. Proc Natl Acad Sci U S A 109:17966–17971
15. Wong AH, Zhou D, Rini JM (2012) The X-ray crystal structure of human aminopeptidase N reveals a novel dimer and the basis for peptide processing. J Biol Chem 287:36804–36813
16. Chen H, Kinzer CA, Paul WE (1996) p161, a murine membrane protein expressed on mast cells and some macrophages, is mouse CD13/aminopeptidase N. J Immunol 157:2593–2600
17. Riemann D, Kehlen A, Langner J (1999) CD13 – not just a marker in leukemia typing. Immunol Today 20:83–88
18. Mina-Osorio P (2008) The moonlighting enzyme CD13: old and new functions to target. Trends Mol Med 14:361–371
19. Taylor A (1993) Aminopeptidases: structure and function. FASEB J 7:290–298
20. Dixon J, Kaklamanis L, Turley H et al (1994) Expression of aminopeptidase-n (CD 13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin. J Clin Pathol 47:43–47
21. Dan H, Tani K, Hase K et al (2003) CD13/aminopeptidase N in collagen vascular diseases. Rheumatol Int 23:271–276
22. Shimizu T, Tani K, Hase K et al (2002) CD13/aminopeptidase N-induced lymphocyte involvement in inflamed joints of patients with rheumatoid arthritis. Arthritis Rheum 46:2330–2338
23. Pereira FE, Cronin C, Ghosh M et al (2013) CD13 is essential for inflammatory trafficking and infarct healing following permanent coronary artery occlusion in mice. Cardiovasc Res 100:74–83
24. Wickstrom M, Larsson R, Nygren P et al (2011) Aminopeptidase N (CD13) as a target for cancer chemotherapy. Cancer Sci 102:501–508
25. Oostendorp M, Douma K, Wagenaar A et al (2010) Molecular magnetic resonance imaging of myocardial angiogenesis after acute myocardial infarction. Circulation 121:775–783
26. Luan Y, Xu W (2007) The structure and main functions of aminopeptidase N. Curr Med Chem 14:639–647
27. Bhagwat SV, Lahdenranta J, Giordano R et al (2001) CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. Blood 97:652–659
28. Razak K, Newland AC (1992) The significance of aminopeptidases and haematopoietic cell differentiation. Blood Rev 6:243–250
29. Razak K, Newland AC (1992) Induction of CD13 expression on fresh myeloid leukaemia: correlation of CD13 expression with aminopeptidase-N activity. Leuk Res 16:625–630
30. Wulfaenger J, Niedling S, Riemann D et al (2008) Aminopeptidase N (APN)/CD13-dependent CXCR4 downregulation is associated with diminished cell migration, proliferation and invasion. Mol Membr Biol 25:72–82
31. Mina-Osorio P, Winnicka B, O'Conor C et al (2008) CD13 is a novel mediator of monocytic/endothelial cell adhesion. J Leukoc Biol 84:448–459
32. Mina-Osorio P, Ortega E (2005) Aminopeptidase N (CD13) functionally interacts with FcgammaRs in human monocytes. J Leukoc Biol 77:1008–1017
33. Bauvois B (2004) Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis? Oncogene 23:317–329
34. Bauvois B, Dauzonne D (2006) Aminopeptidase-N/CD13 (EC 3.4.11.2) inhibitors: chemistry, biological evaluations, and therapeutic prospects. Med Res Rev 26:88–130
35. Fukasawa K, Fujii H, Saitoh Y et al (2006) Aminopeptidase N (APN/CD13) is selectively expressed in vascular endothelial cells and plays multiple roles in angiogenesis. Cancer Lett 243:135–143
36. Mahoney KM, Petrovic N, Schacke W et al (2007) CD13/APN transcription is regulated by the proto-oncogene c-Maf via an atypical response element. Gene 403:178–187

37. Yang E, Shim JS, Woo HJ et al (2007) Aminopeptidase N/CD13 induces angiogenesis through interaction with a pro-angiogenic protein, galectin-3. Biochem Biophys Res Commun 363:336–341
38. Petrovic N, Schacke W, Gahagan JR et al (2007) CD13/APN regulates endothelial invasion and filopodia formation. Blood 110:142–150
39. Rangel R, Sun Y, Guzman-Rojas L et al (2007) Impaired angiogenesis in aminopeptidase N-null mice. Proc Natl Acad Sci U S A 104:4588–4593
40. Dondossola E, Corti A, Sidman RL et al (2014) Bone marrow-derived CD13 cells sustain tumor progression: a potential non-malignant target for anticancer therapy. Oncoimmunology 3:e27716
41. Curnis F, Sacchi A, Borgna L et al (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol 18:1185–1190
42. Oostendorp M, Douma K, Hackeng TM et al (2008) Quantitative molecular magnetic resonance imaging of tumor angiogenesis using cNGR-labeled paramagnetic quantum dots. Cancer Res 68:7676–7683
43. Liu C, Yang Y, Chen L et al (2014) A unified mechanism for aminopeptidase N-based tumor cell motility and tumor-homing therapy. J Biol Chem 289:34520–34529
44. Bieker R, Kessler T, Schwoppe C et al (2009) Infarction of tumor vessels by NGR-peptide-directed targeting of tissue factor: experimental results and first-in-man experience. Blood 113:5019–5027
45. Dreischaluck J, Schwoppe C, Spieker T et al (2010) Vascular infarction by subcutaneous application of tissue factor targeted to tumor vessels with NGR-peptides: activity and toxicity profile. Int J Oncol 37:1389–1397
46. Schwoppe C, Kessler T, Persigehl T et al (2010) Tissue-factor fusion proteins induce occlusion of tumor vessels. Thromb Res 125(Suppl 2):S143–S150
47. Corti A, Curnis F, Rossoni G et al (2013) Peptide-mediated targeting of cytokines to tumor vasculature: the NGR-hTNF example. BioDrugs 27:591–603
48. Curnis F, Sacchi A, Corti A (2002) Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. J Clin Investig 110:475–482
49. Sacchi A, Gasparri A, Gallo-Stampino C et al (2006) Synergistic antitumor activity of cisplatin, paclitaxel, and gemcitabine with tumor vasculature-targeted tumor necrosis factor-alpha. Clin Cancer Res 12:175–182
50. Calcinotto A, Grioni M, Jachetti E et al (2012) Targeting TNF-alpha to neoangiogenic vessels enhances lymphocyte infiltration in tumors and increases the therapeutic potential of immunotherapy. J Immunol 188:2687–2694
51. Gregorc V, Zucali PA, Santoro A et al (2010) Phase II study of asparagine-glycine-arginine-human tumor necrosis factor alpha, a selective vascular targeting agent, in previously treated patients with malignant pleural mesothelioma. J Clin Oncol: Off J Am Soc Clin Oncol 28:2604–2611
52. Zucali PA, Simonelli M, De Vincenzo F et al (2013) Phase I and pharmacodynamic study of high- dose NGR-hTNF in patients with refractory solid tumours. Br J Cancer 108:58–63
53. Lorusso D, Scambia G, Amadio G et al (2012) Phase II study of NGR-hTNF in combination with doxorubicin in relapsed ovarian cancer patients. Br J Cancer 107:37–42
54. Gregorc V, De Braud FG, De Pas TM et al (2011) Phase I study of NGR-hTNF, a selective vascular targeting agent, in combination with cisplatin in refractory solid tumors. Clin Cancer Res 17:1964–1972
55. Gupta M, Chashoo G, Sharma PR et al (2014) Dual targeted polymeric nanoparticles based on tumor endothelium and tumor cells for enhanced antitumor drug delivery. Mol Pharm 11:697–715
56. Zhang Z, Harada H, Tanabe K et al (2005) Aminopeptidase N/CD13 targeting fluorescent probes: synthesis and application to tumor cell imaging. Peptides 26:2182–2187
57. Zhang Z, Hatta H, Tanabe K et al (2005) A new class of 5-fluoro-2′-deoxyuridine prodrugs conjugated with a tumor-homing cyclic peptide CNGRC by ester linkers: synthesis, reactivity, and tumor-cell-selective cytotoxicity. Pharm Res 22:381–389

58. Ndinguri MW, Solipuram R, Gambrell RP et al (2009) Peptide targeting of platinum anticancer drugs. Bioconjug Chem 20:1869–1878
59. Mammoliti S, Andretta V, Bennicelli E et al (2011) Two doses of NGR-hTNF in combination with capecitabine plus oxaliplatin in colorectal cancer patients failing standard therapies. Ann Oncol 22:973–978
60. Santoro A, Citterio G, Rimassa L et al. (2009) Phase II trial of NGR-hTNF, a selective vascular targeting agent (VTA), administered at low dose in pre-treated patients with hepatocellular carcinoma (HCC). ASCO 2009 Annual Meeting, Abstract 247
61. Santoro A, Rimassa L, Sobrero AF et al (2010) Phase II study of NGR-hTNF, a selective vascular targeting agent, in patients with metastatic colorectal cancer after failure of standard therapy. Eur J Cancer 46:2746–2752
62. Gregorc V, Ceresoli G, Zucali PA et al (2009) Phase II study of NGR-hTNF, a selective vascular targeting agent (VTA), in previously treated patients with malignant pleural mesothelioma MPM). J Clin Oncol 27:15s, suppl; abstr 7582
63. Rimassa L, Sobrero A, Santoro A et al (2009) Phase II study of NGR-hTNF, a selective vascular targeting agent (VTA), administered as single agent in patients (pts) with colorectal cancer (CRC) refractory to standard regimens. J Clin Oncol 27:15s, suppl; abstr 4088
64. Gregorc V, Santoro A, Bennicelli E et al (2009) Phase Ib study of NGR-hTNF, a selective vascular targeting agent, administered at low doses in combination with doxorubicin to patients with advanced solid tumours. Br J Cancer 101:219–224
65. Citterio G, De Braud F, Gregorc V et al (2009) Phase Ib study of NGR-hTNF, a selective vascular targeting agent (VTA), in combination with cisplatin in patients with refractory solid tumors. J Clin Oncol 27:15s, suppl; abstr 3570
66. Desar IM, van Herpen CM, van Asten JJ et al (2011) Factors affecting the unexpected failure of DCE-MRI to determine the optimal biological dose of the vascular targeting agent NGR-hTNF in solid cancer patients. Eur J Radiol 80:655–661
67. Gregorc V, Citterio G, Vitali G et al (2010) Defining the optimal biological dose of NGR-hTNF, a selective vascular targeting agent, in advanced solid tumours. Eur J Cancer 46:198–206
68. Santoro A, Pressiani T, Citterio G et al (2010) Activity and safety of NGR-hTNF, a selective vascular-targeting agent, in previously treated patients with advanced hepatocellular carcinoma. Br J Cancer 103:837–844
69. Li B, Zheng YB, Li DD et al (2014) Preparation and evaluation of a CD13/APN-targeting and hydrolase-resistant conjugate that comprises pingyangmycin and NGR motif-integrated apoprotein. J Pharm Sci 103:1204–1213
70. Liu C, Yu W, Chen Z et al (2011) Enhanced gene transfection efficiency in CD13-positive vascular endothelial cells with targeted poly(lactic acid)-poly(ethylene glycol) nanoparticles through caveolae-mediated endocytosis. J Control Release 151:162–175
71. von Wallbrunn A, Waldeck J, Holtke C et al (2008) In vivo optical imaging of CD13/APN-expression in tumor xenografts. J Biomed Opt 13:011007
72. Ellerby HM, Arap W, Ellerby LM et al (1999) Anti-cancer activity of targeted pro-apoptotic peptides. Nat Med 5:1032–1038
73. Jiang W, Jin G, Ma D et al (2012) Modification of cyclic NGR tumor neovasculature-homing motif sequence to human plasminogen kringle 5 improves inhibition of tumor growth. PLoS One 7:e37132
74. Lei H, Cao P, Miao G et al (2010) Expression and functional characterization of tumor-targeted fusion protein composed of NGR peptide and 15-kDa actin fragment. Appl Biochem Biotechnol 162:988–995
75. Hu Z, Yang W, Ma X et al (2013) Cerenkov luminescence tomography of aminopeptidase N (APN/CD13) expression in mice bearing HT1080 tumors. Mol Imaging 12:173–181
76. Ma W, Wang Z, Yang W et al (2014) Biodistribution and SPECT imaging study of (99m)Tc labeling NGR peptide in nude mice bearing human HepG2 hepatoma. Biomed Res Int 2014:618096
77. Tillmanns J, Schneider M, Fraccarollo D et al (2015) PET imaging of cardiac wound healing using a novel [68Ga]-labeled NGR probe in rat myocardial infarction. Mol Imaging Biol 17:76–86

78. Wolters M, Oostendorp M, Coolen BF et al (2012) Efficacy of positive contrast imaging techniques for molecular MRI of tumor angiogenesis. Contrast Media Mol Imaging 7:130–139
79. Hendrikx G, De Saint-Hubert M, Dijkgraaf I et al (2015) Molecular imaging of angiogenesis after myocardial infarction by (111)In-DTPA-cNGR and (99m)Tc-sestamibi dual-isotope myocardial SPECT. EJNMMI Res 5:2
80. Dirksen A, Langereis S, de Waal BF et al (2004) Design and synthesis of a bimodal target-specific contrast agent for angiogenesis. Org Lett 6:4857–4860
81. Ma W, Li G, Wang J et al (2014) In vivo NIRF imaging-guided delivery of a novel NGR-VEGI fusion protein for targeting tumor vasculature. Amino Acids 46:2721–2732
82. Jullienne B, Vigant F, Muth E et al (2009) Efficient delivery of angiostatin K1-5 into tumors following insertion of an NGR peptide into adenovirus capsid. Gene Ther 16:1405–1415
83. Curnis F, Sacchi A, Gasparri A et al (2008) Isoaspartate-glycine-arginine: a new tumor vasculature- targeting motif. Cancer Res 68:7073–7082
84. Sheng W, Shang Y, Li L et al (2014) An EGFR/CD13 bispecific fusion protein and its enediyne- energized analog show potent antitumor activity. Anticancer Drugs 25:82–91
85. Majhen D, Gabrilovac J, Eloit M et al (2006) Disulfide bond formation in NGR fiber-modified adenovirus is essential for retargeting to aminopeptidase N. Biochem Biophys Res Commun 348:278–287
86. Alberici L, Roth L, Sugahara KN et al (2013) De novo design of a tumor-penetrating peptide. Cancer Res 73:804–812
87. Curnis F, Gasparri A, Sacchi A et al (2005) Targeted delivery of IFNgamma to tumor vessels uncouples antitumor from counterregulatory mechanisms. Cancer Res 65:2906–2913
88. Liu F, Li M, Liu C et al (2014) Tumor-specific delivery and therapy by double-targeted DTX-CMCS-PEG-NGR conjugates. Pharm Res 31:475–488
89. Wang X, Wang Y, Chen X et al (2009) NGR-modified micelles enhance their interaction with CD13-overexpressing tumor and endothelial cells. J Control Release: Off J Control Release Soc 139:56–62
90. Chen K, Ma W, Li G et al (2013) Synthesis and evaluation of 64Cu-labeled monomeric and dimeric NGR peptides for MicroPET imaging of CD13 receptor expression. Mol Pharm 10:417–427
91. Li G, Xing Y, Wang J et al (2014) Near-infrared fluorescence imaging of CD13 receptor expression using a novel Cy5.5-labeled dimeric NGR peptide. Amino Acids 46:1547–1556
92. Negussie AH, Miller JL, Reddy G et al (2010) Synthesis and in vitro evaluation of cyclic NGR peptide targeted thermally sensitive liposome. J Control Release 143:265–273
93. Mate G, Kertesz I, Enyedi KN et al (2015) In vivo imaging of Aminopeptidase N (CD13) receptors in experimental renal tumors using the novel radiotracer (68)Ga-NOTA-c(NGR). Eur J Pharm Sci 69:61–71
94. Faintuch BL, Oliveira EA, Targino RC et al (2014) Radiolabeled NGR phage display peptide sequence for tumor targeting. Appl Radiat Isot 86:41–45
95. Yokoyama Y, Ramakrishnan S (2005) Addition of an aminopeptidase N-binding sequence to human endostatin improves inhibition of ovarian carcinoma growth. Cancer 104:321–331
96. Mukhopadhyay S, Barnes CM, Haskel A et al (2007) Conjugated platinum(IV)-peptide complexes for targeting angiogenic tumor vasculature. Bioconjug Chem 19:39–49
97. Colombo G, Curnis F, De Mori GM et al (2002) Structure-activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif. J Biol Chem 277:47891–47897
98. Dunne M, Zheng J, Rosenblat J et al (2011) APN/CD13-targeting as a strategy to alter the tumor accumulation of liposomes. J Control Release 154:298–305
99. Takara K, Hatakeyama H, Kibria G et al (2012) Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy. J Control Release 162:225–232
100. Ma W, Kang F, Wang Z et al (2013) (99m)Tc-labeled monomeric and dimeric NGR peptides for SPECT imaging of CD13 receptor in tumor-bearing mice. Amino Acids 44:1337–1345

101. Pastorino F, Brignole C, Marimpietri D et al (2003) Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy. Cancer Res 63:7400–7409
102. Garde SV, Forte AJ, Ge M et al (2007) Binding and internalization of NGR-peptide-targeted liposomal doxorubicin (TVT-DOX) in CD13-expressing cells and its antitumor effects. Anticancer Drugs 18:1189–1200
103. Chen Y, Wu JJ, Huang L (2010) Nanoparticles targeted with NGR motif deliver c-myc siRNA and doxorubicin for anticancer therapy. Mol Ther 18:828–834
104. Persigehl T, Ring J, Bremer C et al (2014) Non-invasive monitoring of tumor-vessel infarction by retargeted truncated tissue factor tTF-NGR using multi-modal imaging. Angiogenesis 17:235–246
105. Meng J, Ma N, Yan Z et al (2006) NGR enhanced the anti-angiogenic activity of tum-5. J Biochem 140:299–304
106. Meng J, Yan Z, Wu J et al (2007) High-yield expression, purification and characterization of tumor- targeted IFN-alpha2a. Cytotherapy 9:60–68
107. Zhang B, Gao B, Dong S et al (2011) Anti-tumor efficacy and pre-clinical immunogenicity of IFNalpha2a-NGR. Regul Toxicol Pharm 60:73–78
108. Grifman M, Trepel M, Speece P et al (2001) Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. Mol Ther 3:964–975
109. Liu L, Anderson WF, Beart RW et al (2000) Incorporation of tumor vasculature targeting motifs into moloney murine leukemia virus env escort proteins enhances retrovirus binding and transduction of human endothelial cells. J Virol 74:5320–5328
110. Simnick AJ, Amiram M, Liu W et al (2011) In vivo tumor targeting by a NGR-decorated micelle of a recombinant diblock copolypeptide. J Control Release 155:144–151
111. Luan Y, Jing F, Zhang J et al (2012) Design, synthesis, and activity evaluation of a new 5-fluorouracil prodrug containing an Asn-Gly-Arg(NO2)COOCH3 tripeptide. Protein Pept Lett 19:1122–1131
112. Teesalu T, Sugahara KN, Ruoslahti E (2013) Tumor-penetrating peptides. Front Oncol 3:216
113. Wythe SE, DiCara D, Taher TE et al (2013) Targeted delivery of cytokine therapy to rheumatoid tissue by a synovial targeting peptide. Ann Rheum Dis 72:129–135
114. Di Matteo P, Curnis F, Longhi R et al (2006) Immunogenic and structural properties of the Asn-Gly- Arg (NGR) tumor neovasculature-homing motif. Mol Immunol 43:1509–1518
115. Curnis F, Longhi R, Crippa L et al (2006) Spontaneous formation of L-isoaspartate and gain of function in fibronectin. J Biol Chem 281:36466–36476
116. Robinson AB (1979) Molecular clocks, molecular profiles, and optimum diets: three approaches to the problem of aging. Mech Ageing Dev 9:225–236
117. Robinson NE (2002) Protein deamidation. Proc Natl Acad Sci U S A 99:5283–5288
118. Robinson NE, Robinson AB (2001) Molecular clocks. Proc Natl Acad Sci U S A 98:944–949
119. Curnis F, Cattaneo A, Longhi R et al (2010) Critical role of flanking residues in NGR-to-isoDGR transition and CD13/integrin receptor switching. J Biol Chem 285:9114–9123
120. Barbariga M, Curnis F, Spitaleri A et al (2014) Oxidation-induced structural changes of ceruloplasmin foster NGR motif deamidation that promotes integrin binding and signaling. J Biol Chem 289:3736–3748
121. Spitaleri A, Ghitti M, Mari S et al (2011) Use of metadynamics in the design of isoDGR-based alphavbeta3 antagonists to fine-tune the conformational ensemble. Angew Chem 50:1832–1836
122. Spitaleri A, Mari S, Curnis F et al (2008) Structural basis for the interaction of isoDGR with the RGD-binding site of alphavbeta3 integrin. J Biol Chem 283:19757–19768
123. Curnis F, Sacchi A, Longhi R et al (2013) IsoDGR-tagged albumin: a new alphavbeta3 selective carrier for nanodrug delivery to tumors. Small 9:673–678
124. Brooks PC, Stromblad S, Klemke R et al (1995) Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. J Clin Invest 96:1815–1822

125. Eliceiri BP, Cheresh DA (2000) Role of alpha v integrins during angiogenesis. Cancer J 6(Suppl 3):S245–S249
126. Friedlander M, Brooks PC, Shaffer RW et al (1995) Definition of two angiogenic pathways by distinct alpha v integrins. Science 270:1500–1502
127. Friedlander M, Theesfeld CL, Sugita M et al (1996) Involvement of integrins alpha v beta 3 and alpha v beta 5 in ocular neovascular diseases. Proc Natl Acad Sci U S A 93:9764–9769
128. Neubauer S, Rechenmacher F, Beer AJ et al (2013) Selective imaging of the angiogenic relevant integrins alpha5beta1 and alphavbeta3. Angew Chem Int Ed Engl 52:11656–11659
129. Kumar CC (2003) Integrin alpha v beta 3 as a therapeutic target for blocking tumor-induced angiogenesis. Curr Drug Targets 4:123–131
130. Desgrosellier JS, Cheresh DA (2010) Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 10:9–22
131. Ruoslahti E, Bhatia SN, Sailor MJ (2010) Targeting of drugs and nanoparticles to tumors. J Cell Biol 188:759–768
132. Liu Z, Wang F, Chen X (2008) Integrin alpha(v)beta(3)-targeted cancer therapy. Drug Dev Res 69:329–339

Energy Homeostasis of Immune Cells: Translating Cell Bioenergetics into Clinical Application in Rheumatoid Arthritis

Mauricio Rosas-Ballina

1 Introduction

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory condition primarily affecting body joints but also other organs. The estimated prevalence of rheumatoid arthritis in the United States is 1.5 million adults [1], with an estimated annual economic burden of 39.2 billion (in 2005 USD) [2]. Despite important advances in the understanding of the pathophysiology and treatment of chronic inflammatory disease [3], rheumatoid arthritis is still considered as disease of unknown molecular pathogenesis with no available cure [4–6].

The early events in the pathogenesis in rheumatoid arthritis are not completely understood. During the prearticular phase of rheumatoid arthritis, a process that can begin many years prior to clinical manifestation of disease, a loss of tolerance to citrullinated self-antigens occurs [7]. This event is associated with risk alleles and exposure to environmental factors such as smoking, silica exposure, and certain infectious diseases [8]. Later on, in the transitional phase of rheumatoid arthritis, an unknown stimulus triggers a local and chronic inflammatory response against synovial joints. Infectious, neurologic, microvascular, and biomechanical factors have been implicated in this process, which is followed by the clinical phase of rheumatoid arthritis. This is characterized by synovitis or leukocyte infiltration into the synovial compartment, cartilage degradation, and erosive bone damage. This inflammatory response affecting joints can also be accompanied by other systemic, inflammation-driven derangements like vascular disease and metabolic syndrome, which complete the syndrome of rheumatoid arthritis [5].

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The autoimmune nature of rheumatoid arthritis implies an underlying adaptive immune response against self-antigens mediated by T and B cells. In particular, cytokines produced by Th1 cells lead to monocyte/macrophage and fibroblast activation, which in turn drives production of TNF-α, IL-6, and IL-1β [9]. These cytokines can further activate chondrocytes that release matrix metalloproteinases (MMP) that contribute to the degradation and damage of the matrix component of joints [10]. Additionally, the inflammatory environment can induce macrophage differentiation into osteoclasts that mediate bone resorption and damage [11]. This is further enhanced by Th17 cell-derived IL-17A that, in synergy with TNF-α, activates fibroblasts and chondrocytes [12]. B cells can also contribute to disease pathogenesis and progression through cytokine release, presentation of autoantigen, and production of autoreactive antibodies that add to the inflammatory insult through immune complex deposition in cartilage [5, 13]. Effector mechanisms of activated macrophages, neutrophils, and mast cells include production of pro-inflammatory cytokines, reactive oxygen and nitrogen species, prostaglandins, proteases, and histamine, among others [14–16].

It is evident that RA has a complex pathophysiology in which many cell subsets, cytokines, receptors, signaling pathways, and effector molecules are involved. Despite this complexity, many targets have been identified, and they are the object of current biological therapeutic approaches (reviewed in [4]). Examples of these are therapies that target or neutralize B and T cells (anti-CD20 antibody and CTLA4-Ig fusion protein, respectively), TNF-α, IL-1β, IL-6, IL-17, and Janus kinases 1 and 3. However, treatment with biologic agents achieves remission only in a minority of patients [17]. Thus, targeting a single molecule does not necessarily lead to a cure, likely because of redundant cytokine networks that can perpetuate inflammatory responses. Similarly, compensatory mechanisms can trigger alternative inflammatory pathways, therefore maintaining the pathologic process [3]. Apart from the inherent molecular complexity of the disease, other reasons for unsuccessful therapies against RA have been identified [18]. One of the most pressing challenges is the lack of information determining which therapy to use in which patient at which disease stage. Therefore, development of novel biomarkers to stratify patients and to monitor disease progression is particularly needed in the clinical ambit of RA.

This chapter provides a brief overview of the current understanding in the field of immune cell bioenergetics with emphasis on T lymphocytes and macrophages. This relatively new conceptual approach to inflammation can be applied to chronic inflammatory disease in general and to rheumatoid arthritis in particular. However, the promising novel therapeutic concepts stemming from this body of knowledge consist thus far of basic and preclinical findings. The last part of the chapter touches upon how aspects of immune cell bioenergetics could be used by translational scientists to develop biomarkers for diagnosis and disease progression assessment. It also provides an approach for target identification and suggests possible drug delivery options.

1.1 Why Bioenergetics?

Regardless of size and organization complexity, all living systems are constrained at their most fundamental structural and functional level by fixed biochemical boundaries. Homeostasis, the stable internal status of living systems, can thus be studied

at this elementary biochemical level by determining how metabolic substrates are distributed within systems to extract energy and to form mass. This is the object study of bioenergetics. If signaling pathways, each with its own informational input and output, dictate function, and if signaling pathways evolved on top of already existing metabolic networks, it follows that function is inherently linked to the biochemical state of the cell. Bioenergetics applied to the immune system considers the fundamental biochemical processes of immune cells as the underlying platform over which signaling pathways and effector molecules (i.e., cytokines) operate. In this context, cytokines and other mediators are messengers of the metabolic state of the cell, and their coordinated effects on other cells constitute what we know as an immune response (Fig. 1). Immune responses in coordination with other systems (e.g., nervous and endocrine systems [19, 20]), themselves dependent on fundamental biochemical constraints, serve to maintain a stable state of optimal production, distribution, and consumption of resources among cells, organs, and systems, a state that we call health [21]. Thus, cell bioenergetics can provide a framework to understand inflammation at a fundamental biochemical level, which has the potential to be translated successfully into clinical application against diseases with an inflammatory component such as RA.

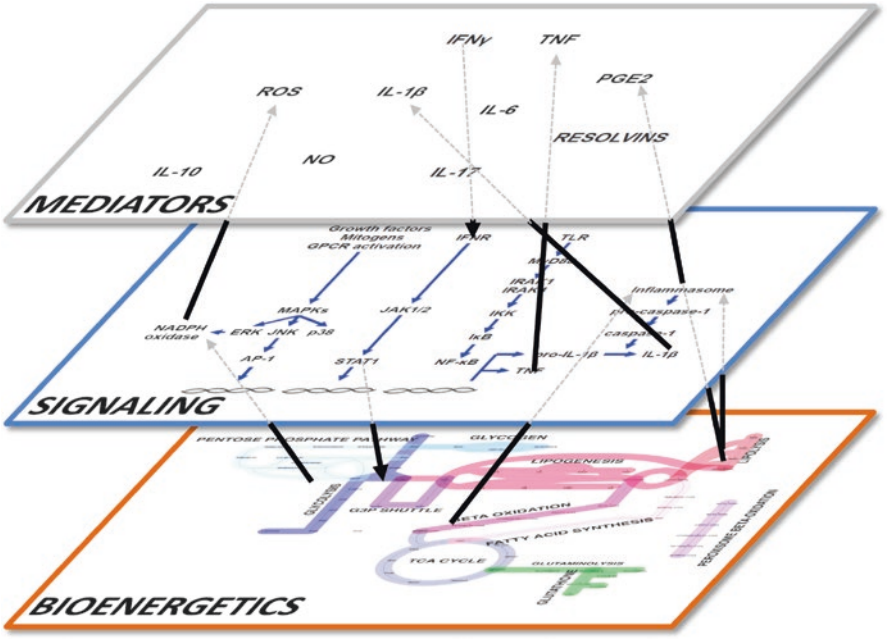

Fig. 1 Bioenergetics as the fundamental physicochemical constraint upon which signaling pathways and immune responses are built. The figure describes the bidirectional interaction between diverse functional layers of immune cells. From an evolutionary perspective, cells developed signaling modules built upon a layer of metabolic pathways that sustain the essential physicochemical requirements of life. In this context, cytokines and other immune mediators convey information about the energetic state of the cell and their coordinated effects constitute an immune response. Homeostasis (or disease) thus arises from the optimal (or suboptimal) production, distribution, and consumption of resources among cells of the body

1.1.1 Bioenergetics Modulates Immune Cell Function

The following section provides a brief overview of macrophage and T lymphocyte bioenergetics as these leukocytes are central to the pathogenesis of RA and the focus of most studies on immune cell bioenergetics (Fig. 2). Other publications have comprehensively reviewed the role of bioenergetics on macrophage and T cell function, while reports start to emerge on the role of bioenergetics in fibroblast function in RA and in B cell function in other contexts [22–26].

Macrophages

A recurrent observation made in many immune cell types studied thus far is that cells are undergoing proliferation shift to a glycolytic metabolism. This metabolic reprogramming serves the purpose of rapidly producing ATP. At the same time, it provides reductive potential in the form of NADPH required for anabolic processes

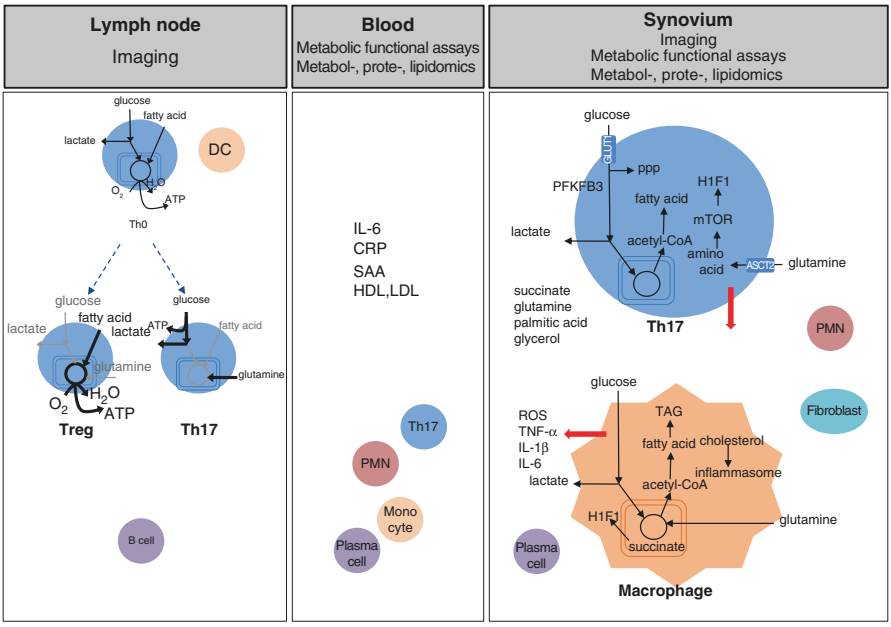

Fig. 2 Overview of the metabolic pattern of immune cells central to RA pathogenesis. The figure displays the metabolic pattern associated to T cells and macrophages under various sterile and non-sterile inflammatory conditions. Other cells like B cells, neutrophils, and fibroblasts are important players in the pathogenesis of RA as well, and the role of bioenergetics in their function is already being investigated. The image indicates what metabolic patterns are expected from these cells in different body compartments (lymph node, blood, and synovium), and suggests which experimental approaches could be used in order to gain insight into their actual metabolic state. Please refer to text for details about these approaches

(i.e., fatty acid and nucleotide synthesis) through the pentose phosphate pathway. In contrast, quiescent and long-lasting cells oxidize glucose and other substrates in the mitochondrion. While cells performing glycolysis present a high reductive energetic charge and a pro-inflammatory phenotype, mitochondrial oxidation is associated with a phenotype characterized by anti-inflammation, tissue repair, and humoral immunity.

Macrophage function ranges from pathogen clearance to resolution of inflammation and tissue repair [27]. This wide repertoire of activities requires different metabolic needs that macrophages satisfy by rerouting resources through different metabolic pathways [28]. Bioenergetics studies on macrophages have mainly addressed the metabolic requirements during activation and how these metabolic requirements are satisfied. Stimulation of macrophages with pro-inflammatory agents such as lipopolysaccharide (LPS) and interferon gamma (IFNγ) leads to polarization into the pro-inflammatory M1 macrophage phenotype. Among the metabolic features of M1 macrophages are upregulation of key proteins involved in glucose uptake such as glucose transporter 1 and 3 [29] and lactate release [30]. Together with upregulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 [31], a key enzyme that maintains glucose flow through glycolysis, these changes lead to increase glucose uptake and lactate release. Functionally, glucose utilization by activated macrophages supports an inflammatory profile. For example, incubation with 2-DG, a glucose analogue that does not undergo glycolysis, inhibits inflammasome activation and IL-1β production [32]. Recently, a deletion in chromosome 12 has been shown to protect against rheumatoid arthritis. Intriguingly, GLUT3, the transporter with highest affinity for glucose [33], is among the three genes encoded in this 129 Kb deletion [34], suggesting that glucose uptake is involved in pathophysiology of rheumatoid arthritis. M1 activation is also favored by LPS-induced downregulation of the pentose phosphate pathway enzyme sedoheptulose kinase (CARKL), and knockdown of CARKL leads to increased production of TNF-α, IL-12, IL-6, IL-1β, and reactive oxygen species (ROS) upon stimulation with LPS [35].

In addition to increased glycolytic activity, the mitochondrial oxidative metabolism of M1 macrophages is inhibited. In macrophages and dendritic cells, this inhibition is dependent on iNOS-derived nitric oxide [36, 37] that inhibits mitochondrial respiratory complexes I and IV through S-nitrosylation [38]. As a consequence, ATP is mainly produced through incomplete oxidation of glucose into lactate yielding 2 moles of ATP per mole of glucose, as opposed to 32 moles of ATP when glucose is completely oxidized in the mitochondrion. Despite this difference in efficiency, the elevated glycolytic rate leads to higher ATP levels, which are essential in preventing cell death [36, 37]. This metabolic program, first described by Otto Warburg in tumor cells, is known as aerobic glycolysis as it consists of glucose fermentation as a source of ATP in the presence of oxygen.

Another important molecular effector in the metabolic program of M1 macrophages is the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1) composed of HIF-1α and HIF-1β. Under normoxic conditions, HIF-1α is expressed constitutively and is hydroxylated by HIF-1α prolyl hydroxylases (PHD), leading to HIF-1α degradation through the ubiquitin protease pathway mediated by

pVHL. Under hypoxia, PHD is inhibited, and HIF-1α is stabilized and dimerizes with HIF-1β upregulating various metabolic enzymes allowing for sustained glycolysis [39, 40]. Additionally, active HIF-1 blocks the entry of pyruvate into the TCA cycle by inducing the expression of pyruvate dehydrogenase kinase 1 [41] that phosphorylates pyruvate dehydrogenase impeding the conversion of pyruvate to acetyl-CoA. PHD is also inhibited by succinate, and conditions leading to succinate accumulation, like activation of macrophages with LPS, also induce HIF-1α stabilization and transcription of IL-1β, a gene controlled by HIF-1α [32]. In a model of heart ischemia/reperfusion, succinate accumulation led to increased mitochondrial reactive oxygen species (ROS) levels through reverse mitochondrial transport at mitochondrial complex I causing tissue injury [42]. Consequently, blocking accumulation of succinate decreased IL-1β production and provided protection against ischemia reperfusion [32, 42]. Importantly, HIF-1α is expressed in synovial membrane of patients with rheumatoid arthritis, but not in healthy controls [43]. In this study comprising 26 subjects, the staining pattern of HIF-1α correlated with that of CD68 in sequential tissue sections, suggesting that HIF-1α is expressed solely in synovial macrophages. Studies with larger patient populations including patients at different disease stages utilizing current tissue imaging techniques allowing for multiple staining (i.e., imaging mass cytometry [44]) should further clarify the role of HIF-1α in rheumatoid arthritis. Furthermore, a combination of 20 metabolites obtained from synovial fluid was able to discriminate between RA patients and patients with other forms of arthritis [45]. Remarkably, succinate levels increased 73 times in synovial fluid of patients with RA and were the metabolite with the highest fold change among these 20 metabolites. Together, these findings suggest a succinate-driven HIF-1α stabilization in synovial inflammation and emphasize the role of bioenergetics in the local inflammatory process in patients with RA.

Lipid accumulation is a common finding in macrophages during in infectious and sterile inflammation [46]. Activated macrophages accumulate lipid droplets, single-membrane organelles mainly containing cholesterol esters, and triacylglycerol (TAG) [47]. Lipids, particularly TAG, can accumulate from de novo fatty acid synthesis in phagocytes activated with various TLR agonists in vitro [48, 49]. Lipid accumulation is also typical of foam cells in atherosclerosis, which take up cholesterol from circulating lipoproteins. Cholesterol in turn drives inner cell processes that lead to endoplasmic reticulum stress, increased ROS production and inflammasome activation [50]. At systemic level, the lipid derangement characteristics of RA, namely, increased total and HDL cholesterol and high TAG [51], are part of the acute phase response common to systemic inflammation, indicating the existence of a lipid metabolic program at the organ level that underlies atherosclerosis, obesity, and autoimmune disease [52]. In fact, RA patients have increased risk to develop coronary artery disease [53, 54], presumably through the atherogenic effects on the liver and adipose tissue exerted by pro-inflammatory cytokines produced in the synovium [55].

The inflammatory response in the synovium is central to the pathogenesis of RA. This response is driven in part by macrophages through a variety of effector mediators reminiscent of M1 activation. These include ROS, nitric oxide, IL-1β,

IL-6, IL-23, TNF-α, and prostaglandins, among others [5]. As described above, bioenergetics pathways modulate the generation of some of these effectors. Modulation of these pathways therefore represents a therapeutic strategy to treat synovial inflammation and can be beneficial for patients with RA. This approach could be advantageous over the specific targeting of single downstream effectors, i.e., anti-cytokine antibodies, since targeting one metabolic enzyme or its product can lead to modulation of various effector molecules like in the case of inhibition of succinate dehydrogenase with malonate or knockdown of CARKL, as previously mentioned. Similarly, metformin, which has a long track safety record, decreased mitochondrial ROS levels, inhibited pro-IL-1β and increased IL-10 production in LPS-activated macrophages [56], and reduced TNF-α and IL-1β levels in a mouse model collagen-induced arthritis [57]. Thus, a possibility exists for safely modulating various effector molecules via a drug that targets bioenergetics pathways.

T Lymphocytes

Research on bioenergetics has identified two main metabolic programs associated with T cell function, which could possibly be modulated in order to modify disease progression or severity in rheumatoid arthritis. On the one hand, resting lymphocytes preferentially perform a catabolic metabolic pattern characterized by generation of ATP through oxidative phosphorylation. For example, mitochondrial oxidation of fatty acids is upregulated in T regulatory (Treg) cells and provides ATP required for the long-term survival of memory CD8 T cells [58, 59]. On the other hand, increased glycolysis and glutaminolysis together with low substrate oxidation by mitochondria and de novo fatty acid synthesis are characteristics of T cell activation. This anabolic program effectively supplies energy and biomass required for T cell proliferation and enhanced immune function and is dependent in part on the transcription factor Myc and HIF-1 that control transcription of enzymes participating in glutaminolysis and glycolysis [60, 61]. Indeed, Th1 and Th17 effector cells from GLUT1-deficient mice show defects in proliferation, differentiation, and survival in vitro and in vivo, while Treg cells are functional and retain the capacity to suppress T effector cells in models of graft versus host disease and colitis [62]. Furthermore, in a mouse model of colitis, lack of GLUT1 on effector T cells reduces their expansion and prevents their contribution to the development of disease. Moreover, inhibition of glycolysis with 2-deoxyglucose suppresses T cell differentiation into Th17 effector cells and promotes generation of Treg cells. Finally, HIF-1α deficiency impairs glycolysis, hinders Th17 cell differentiation, and enhances Treg cell polarization [61].

mTOR is another important modulator of metabolism. mTOR is a serine/threonine kinase activated by a variety of cues such as oxidative stress, amino acid levels, and other nutrients together with PI3K/Akt and cytokines [63]. mTOR forms two different kinase complexes known as mTORC1 and mTORC2 which are necessary for T cell differentiation into Th1, Th2, and Th17 effectors [64, 65]. In order to undergo clonal expansion and differentiate into Th1 and Th17 effector cells, CD4 T

cells require Slc7a5, a transporter of large neutral amino acids, and cells deficient of Slc7a5 have impaired mTORC1 activation. Remarkably, CD4 T cells deficient in Slc7a5 retain the capacity to differentiate into Treg cells. Glutamine uptake is mediated through the ASC amino acid transporter 2 (ASCT2). Upon T cell activation, glutamine uptake increases and glutamine oxidation through glutaminolysis provides substrate for nucleotides, polyamines [60], and amino acids, the last of which can, in turn, activate mTOR [66]. Deficiency of ASCT2 impairs Th1 and Th17 differentiation by inhibiting mTORC1 activation. ASCT2 deficiency attenuates severity in an experimental model of allergic encephalomyelitis by hindering Th17 differentiation, but lack of ASCT2 does not impede differentiation into Th2 and Treg cells [67]. These results underscore the importance of amino acid metabolism in establishing a pro-inflammatory T cell phenotype through mTOR [68]. In experimental models of rheumatoid arthritis, inhibition of mTOR with rapamycin, or everolimus, improved paw swelling [69, 70] and reduced articular bone erosion and cartilage loss [69]. In a randomized, placebo-controlled, double-blinded study, treatment of RA patients with everolimus plus methotrexate improved patient's and physician's global assessment of disease activity in comparison to methotrexate plus placebo and showed mild and reversible adverse events [71]. Although it is not known whether mTOR activation in T cells was targeted in these studies, inhibition of T cell activation with sirolimus has proven beneficial in patients and in experimental models of systemic lupus erythematosus [72, 73].

T cells are present in the inflamed synovium and play an important role in rheumatoid arthritis. Specifically, Th1 and Th17 cells perpetuate the inflammatory milieu in the synovium, which is exacerbated by a decrease in Treg cell function [74]. Thus far, targeting of total T cells, for example, with antibodies against CD4 or CD5, has had limited success [75] that could be a consequence of targeting Treg cells as well. A potential strategy for selectively targeting defined T cell subsets could be based on the different bioenergetics requirements between Th1/Th17 and Treg cells. It is conceivable that inflammation could be hampered by targeting metabolic pathways or metabolite uptake mechanisms related to glycolysis and glutaminolysis in Th1 and Th17. Even if these interventions would target Th1 or Th17 cells, they would not affect Treg cell function as Tregs require a different bioenergetics program. Conversely, enhancement of Treg cell development, for example, by enhancing beta-oxidation with AMPK activation [59] would in principle not alter Th1 and Th17 cell function, potentially reducing adverse effects.

1.1.2 Translation into Clinical Application

Despite the growing scientific interest that immune cell bioenergetics is generating, and the promise of its delivering novel therapeutic approaches, the path to making this knowledge clinically useful has not been systematically explored. I contend that knowledge derived from immune cell bioenergetics can help overcome present limitations of drug development in RA. These limitations include an incomplete knowledge of disease mechanism, lack of new targets and reliable biomarkers, and

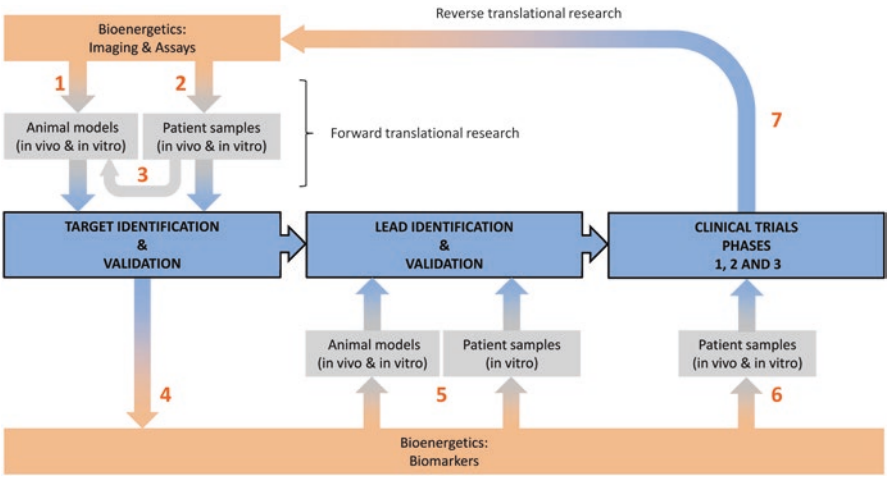

Fig. 3 Application of bioenergetics concepts at different stages of the drug development process. The image shows stages of the drug development process, from target validation through phase 1 (first in human) and 2 (proof of concept) to phase 3 studies. (1) Assays and imaging techniques based on bioenergetics applied to animal models can be used to advance our understanding of RA pathophysiology and to identify drug targets (forward translational research). (2) Drug targets can also be identified by using bioenergetics assays on human samples and imaging techniques directly on patients. (3) Information obtained with human samples and patients can be used to create or refine available animal models. (4) Deeper understanding of the role of bioenergetics on RA pathogenesis can lead then to development of new biomarkers. (5) These could be used during lead identification and validation (6) and during clinical development as biomarkers to establish safety and mechanism of action of new drug entities and to stratify patients based on disease stage or response to treatment. (7) Finally, knowledge thus generated can be used to reassess disease pathophysiology and to develop new assays and experimental models (reverse translational research)

inadequate means for patient stratification [76]. Following the tenets of translational medicine [77, 78], the remaining part of this chapter proposes ways to bridge the gap between advances in immune cell bioenergetics at the basic research level and its clinical application in rheumatoid arthritis (Fig. 3).

1.2 Biomarker Development

Development of drug candidates is often interrupted when efficiency cannot be proven in phase II or even in large-scale confirmatory phase III studies [79]. This high attrition rate is better explained by the homogeneous in vivo and in vitro experimental conditions in which drug candidates are generally developed compared to the heterogeneous patient populations in which they are actually tested. This is particularly the case in RA because of the large phenotypic variation among patients likely derived from the heterogeneous molecular pathogenesis of RA [80]. This heterogeneity is not only a hindrance for establishing efficacy of new molecular

entities in clinical trials but is also a source of variability in patients' response to available treatments. Early staging of rheumatoid arthritis through the use of biomarkers is thus advantageous as different therapeutic agents have different benefit depending on the disease stages in which they are prescribed [18]. Moreover, there is a need to develop biomarkers to identify the so-called early window of opportunity during which intervention might have a significant effect on disease progression [81]. For these reasons, identification of biomarkers can likely accelerate drug development, improve efficacy, and reduce treatment costs.

Biomarkers are objectively measured variables that function as indicators of normal or pathological biological processes, or as indicators of response to therapeutic intervention [82]. Biomarkers can serve many important roles in different stages of drug development [83]. For instance, biomarkers are used as readout of target engagement by a drug, thus helping selection of drug candidates and establishing proof of mechanism (response biomarkers). By defining a disease stage, biomarkers are also used to stratify patients (stratification biomarkers), to monitor disease progression, and to assess and predict response to therapy or drug toxicity.

The development of biomarkers for RA is cumbersome because the pathogenesis of RA is still unclear, and as consequence biomarkers in RA are lacking [5]. Biomarker development is further complicated by the need of physiological variables whose measurement is not only readily accessible and clinically feasible, but that reflect biological processes in anatomical sites that are disease relevant. For example, CRP and erythrocyte sedimentation report on systemic inflammatory status of a patient but do not provide information about local inflammation in joints. Moreover, biomarkers should preferably be part of the disease mechanism. This confers to a biomarker disease specificity and the capacity to report reliably on whether an intervention is effectively acting upon the actual disease process. Thus, an ideal biomarker in RA should be easy to measure, should reflect disease activity, and should inform about the local disease process even when no systemic involvement occurs.

The current initiative on precision medicine seeks to provide treatments targeted to specific needs of individual patients in order to improve clinical outcome and reduce adverse effects [84, 85]. Datasets obtained through omics studies (proteomics, metabolomics, lipidomics) are particularly suited to make patient variability tractable. Ideally, these datasets are obtained from cohorts of patients with known treatment history and defined disease stage according to current stratification criteria. This ensures that results inferred from these unbiased top-down analyses can be accurately assigned to disease phenotypes. Molecular entities so identified could then be used as biomarkers or even as surrogate end points if adequately validated to show strong and significant correlation with true clinical end points [82].

Large-scale metabolite profiling has been used in RA with the goal of developing biomarkers. This approach is based on the premise that circulating and tissue metabolites reflect immune cell activity and can thus inform about the inflammatory process [86, 87]. Urine metabolites have been shown to predict response to anti-TNF-α therapies in RA and psoriatic arthritis (PsA) [88]; and plasma [89] or synovial fluid [45] metabolite profiling can differentiate RA from other articular inflammatory

conditions, or predict disease severity [90]. In addition to citrulline and lactate, metabolites found in synovial fluid that are associated to the TCA cycle such as succinate, and glutamine, and to lipid metabolism like cholesterol, palmitic acid, and glycerol have been identified as biomarkers for RA [45, 90, 91]. Some of these metabolites are by-products of the metabolic pattern common to inflammatory activation of macrophages and effector T cells, as explained in the previous section, suggesting that immune cell metabolism and therefore the inflammatory process can be probed through the metabolite signature of synovial fluid and plasma.

Several different imaging techniques currently in use in discovery research are suitable for visualization of bioenergetics processes. For example, multimodal nonlinear optical microscopy has been used to observe lipid droplet formation and free fatty acid trafficking in macrophages and adipocytes in visceral adipose tissue [92]. As mentioned above, lipid droplet formation is a hallmark of sterile and infectious inflammation [93]. Therefore detection of lipid accumulation could be used as an indicator of phagocyte activation. Interestingly, while lipid droplet accumulation has been observed in leukocytes in synovial fluid of RA patients [94], neither the cell type that accumulates them nor their function is known. Other techniques currently in use that allow study of lipid droplets in situ are 1H NMR and Raman spectroscopy [95]. These techniques could help investigate a role for lipid accumulation in disease progression in experimental disease models. Moreover, identification of cell subsets accumulating lipid could serve as a basis for studying a potential link between the atherogenic serum lipid profile of patients with RA and the increased pro-inflammatory phenotype of synovial phagocytes [51, 96, 97].

In comparison to plasma or tissue biomarkers, imaging provides direct information about the inflamed tissue, and depending on the imaging technique, it can obviate the need of collecting samples. There are however challenges for clinical translation in biomarker imaging. These are invasiveness of the imaging technique, quantitation, and labeling of cells [98]. Newer techniques like near-infrared fluorescence combined with indocyanine green, a dye already approved for intravenous administration, have allowed noninvasive imaging of the lymphatic system and lymph node staging of cancer [99]. Oxidative environments are characteristic of inflammatory processes and are driven by the metabolism of activated immune cells, like in synovial fluid [100, 101]. Thus, it can be envisioned the coupling of near-infrared fluorescence with optical probes activated in oxidative environments [102]. Imaging the inflammatory process in this way could help identify critical events in immune cell bioenergetics and in the pathophysiology of RA. If clinically validated, these events could in turn help define disease stage, assess therapeutic response, and possibly guide the type and timing of therapeutic intervention.

Another imaging technique with potential application in RA is based on the distinct spectral properties of NADH and FAD, both of which can be analyzed by fluorescence lifetime microscopy (FLIM) [103]. Since glycolysis relies only on NADH as electron donor while mitochondrial oxidation utilizes both NADH and FAD, obtaining the NADH/FAD or redox ratio can provide information about the metabolic state of the cell [104]. For example, a high NADH/FAD ratio has been observed in cancer cells [105–107], which are typically glycolytic. Similarly, due to decreased

mitochondrial oxidative function and enhanced glycolysis, it is expected that classically activated innate immune cells present a higher NADH/FAD ratio. Thus, FLIM could provide a straightforward indication of the metabolic state of immune cells obtained in clinical samples or in experimental disease models. Interestingly, FLIM has been adapted to flexible fiber-optic devices to characterize elastin-, collagen-, lipid-, and macrophage-rich areas of atherosclerotic plaques [108]. Such minimally invasive procedures could also find a clinical application in RA.

Imaging of the uptake of fluorine-18 fluorodeoxyglucose (18F-FDG), a radiolabeled glucose analogue, has previously been applied in RA to assess physiological changes in the synovium [109]. This technique is based on the principle that 18F-FDG uptake parallels glucose uptake and thus is used to monitor the metabolic activity of tissue. Provided enough spatial resolution, this imaging technique could be applied to study glucose uptake in immune cells. Indeed, increased 18F-FDG uptake has been ascribed to proliferating pannus and to inflammatory activity in an experimental model of RA [110]. Further, measurement of 18F-FDG uptake, which measures macrophage activity in atherosclerotic plaque [111], has shown subclinical vascular inflammation in patients with RA [112, 113]. More recent studies have aimed to use 18F-FDG uptake coupled to PET/CT imaging to establish optimal timing of therapy [114]. 18F-FDG uptake correlates with clinical response to anti-TNF-α therapy and disease activity [115]. Finally, noninvasive in vivo metabolic imaging is now feasible with intravenous injection of 13C-labeled substrates detected by hyperpolarized magnetic resonance spectroscopic imaging [116, 117]. This method allows for detection of several labeled metabolites downstream from the labeled precursor through which a more complete characterization of cell metabolism is obtained. The safety and feasibility of this technique were recently demonstrated in a first-in-man study in patients with prostate cancer [118] and could be useful to monitor disease progression and response to treatment in RA patients.

Given the strong mounting evidence implicating metabolic pathways in modulating immune cell function, biomarkers developed on the basis of immune cell bioenergetics are likely to be related to the underlying disease mechanism of RA. It is also foreseeable that bioenergetics changes occurring as a consequence of targeting metabolic pathways within pro-inflammatory immune cells will be detectable and measureable through any of the abovementioned biomarker strategies or others yet to be developed under the premise of cell bioenergetics.

1.3 Target Identification and Drug Delivery

As the number of in vivo and in vitro studies on immune cell bioenergetics continues to expand, a growing list of potential drug targets for various inflammatory conditions has emerged. Some of these potential targets satisfy many of the characteristics of an ideal drug target [119]. These features include: proven function in pathophysiology; the target is disease modifying; availability of assays to probe binding and function; and availability of biomarkers to confirm that a drug has hit

its target. A number of recent studies have already provided evidence for potential new targets in chronic inflammation and RA. For example, CD4 T cells from lupus-prone mice have increased glycolysis and mitochondrial oxidative metabolism, and their inhibition with 2-deoxy-D-glucose (2DG) and metformin, respectively, reduced IFNγ levels upon activation in vitro and biomarkers of disease in vivo [120]. Similarly, metformin decreased the severity of collagen-induced arthritis in mice, decreased serum levels of TNF-α and IL-1β, and reduced the number of Th17 cells and increased Treg cell numbers in spleen [57, 121].

Animal disease models do not necessarily recapitulate disease in humans. Therefore in order to fully capitalize on the link between cellular energetics and immune cell activity, and bring this body of knowledge to the clinical front in RA, it will be necessary to determine to what extent current and future findings related to immune cell bioenergetics pathways observed in experimental models reflect those observed in RA patients. Standard RA animal models like collagen-induced arthritis and collagen antibody-induced arthritis have been used successfully to develop therapies [122], and it is likely that these models can serve as a starting point.

An alternative to identifying targets using experimental models is to actually perform target identification in humans. For instance, it has been found that naïve CD4 T cells from patients with RA downregulate 6-phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) and upregulate glucose-6-phosphate dehydrogenase (G6PD) that redirect glycolytic flux toward the pentose phosphate pathway. These T cells synthesize significantly less ATP from glucose than T cells from age-matched controls. Instead, they accumulate NADPH produced through the pentose phosphate pathway and have diminished ROS levels. This reductive potential hinders activation of ataxia telangiectasia mutated (ATM), which in turn biases CD4 T cell differentiation toward Th1 and Th17 cells [123, 124]. The findings of these studies suggest that addressing deficient ATP levels and redox homeostasis could be a therapeutic strategy against RA. Moreover, the possibility exists that the as of yet unknown mechanism of differential PFKFB3 and G6PD expression precedes the clinical manifestation of RA. If this were the case, screening for such mechanism could represent a strategy to identify preclinical RA [125]. This could then be useful to identify and treat those individuals with an early autoimmune response before additional risk factors trigger overt clinical arthritis [5].

Bioenergetics-related targets (and biomarkers) in RA could also be identified in prospective cohort studies with homogeneous groups of patients stratified by disease stage or type of treatment received. In vitro assays could be run in whole blood or isolated blood leukocytes under basal conditions and after activation with LPS or anti-CD3 to determine basic metabolic parameters such as oxygen consumption rate, acidification rate (a readout for glycolysis), mitochondrial membrane potential, reactive oxygen species levels, and neutral lipid content. Availability of fluorescent dyes that measure these parameters can be multiplexed with multicolor flow cytometry to interrogate different leukocyte subsets. This information could be later integrated with other known biomarkers and data from proteomics, metabolomics, and lipidomics studies of serum or tissue samples. Results obtained in this way could be used as a basis for more detailed mechanistic studies performed in human cells and/

or experimental disease models to reveal targets (and biomarkers) relevant to disease initiation or progression. Because cell metabolism depends on the substrate(s) available in the medium in which cells are maintained, it is important that in vitro assays are performed under pertinent culture conditions. Thus, a culture medium whose composition correlates quantitatively and qualitatively with the particular disease state should be considered. Results from metabolomic studies in RA can already provide some of this information. This approach could sieve out useful, relevant information from cell culture artifacts.

Redundant and compensatory molecular networks regulate inflammation, which suggests that targeting one cytokine only might not necessarily lead to significant health improvement, and thus engaging more than one target might be required [3, 126]. Therapy combination of established disease-modifying antirheumatic drugs seems to be as effective as biological targeting a single molecule [127, 128]. At the same time, therapy combination could also translate into a lower benefit and risk balance due to potential safety issues. Considering that bioenergetics pathways underlie function, targeting metabolic pathways in immune cells could effectively modulate several downstream cytokines or other mediators in the same way that removing a hub can disrupt parts of or a whole network. On the other side, many, if not all, cell types in the body rely to a different extent on the same metabolic pathways that support pathological phenotypes in immune cells during chronic inflammation. Therefore, attempts to modulate the bioenergetic state of immune cells to ameliorate inflammation will probably have to be cell specific so that undesirable side effects can be avoided.

In order to overcome this limitation, drugs modulating metabolic pathways could be packaged within carriers containing either antibodies, peptides, or polymers to specifically target cell subsets (reviewed in [129]). Liposomes or nanoparticles containing drugs against key metabolic enzymes (e.g., dichloroacetate against PDH or the glucose inhibitor 2-DG) could be functionalized with an antibody against αVβ3 that is selectively expressed in synovial macrophages [130]. This strategy has been applied in an experimental model of arthritis by encapsulating methotrexate in nanoparticles conjugated to the peptide arginine-glycine-aspartic acid (RGD), which targets αVβ3. These nanoparticles contain gold deposits that release heat and methotrexate with near-infrared excitation. In this way, drug can be delivered in a temporal and spatial specific fashion when near-infrared excitation is applied to inflamed joints [131]. It is conceivable that nanoparticles can also be coupled to antibodies to target specific cell types and that siRNAs can be used as cargo as well [132]. Similarly, drugs targeting bioenergetics pathways could be conjugated to antibodies or peptides for specific cell delivery [133]. Finally, another strategy for specific delivery could be to use a bispecific antibody to target metabolite transporters on the one side (e.g., Slc7a5 or ASCT2 for glutamine or GLUT1/3 for glucose) and a specific cell marker on the other with the goal of decreasing metabolite uptake in specific cell subsets [134]. These approaches could of course be used to treat joint inflammation, but the exciting possibility exists to use these approaches in order to release therapeutic agents at the correct anatomical site (e.g., lymph node, lung, joint) and at the correct disease stage in order to limit disease progression.

Because of their likely novel mechanism of action and selectivity for immune cells, the initial dose of drugs targeting bioenergetics pathways will have to be carefully evaluated before testing them in phase 1, first-in-man studies [135]. Preclinical safety data, which includes safety pharmacology and toxicology studies, will have to include in vitro studies with relevant human cell types or tissues. Additionally, immunodeficient mice engrafted with a functional human immune system, or humanized mouse strains [136], could be used to obtain safety (and efficacy) data of drug candidates targeting bioenergetics pathways. This can be useful not only because of the known differences between the human and the mouse immune systems but also because human target cells would then be investigated in a whole-body metabolic environment on which they depend to obtain substrates in order to function. This is particularly relevant considering the dyslipidemia that accompanies RA and the role of lipid metabolism on T cell and macrophage function. In fact, a humanized mouse model of RA already exists [137], heralding a useful new approach for translational research in RA. After conversion of the no adverse event level (NOAEL) in the more sensitive animal species tested to the human equivalent dose, judicious assessment of preclinical safety data will help choose an appropriate safety factor to calculate the initial dose. Finally, once the initial dose is determined, a cautious and conservative clinical trial design with adequate intervals between dosing of subjects will have to be implemented.

2 Concluding Remarks

Underlying bioenergetic boundaries based on physicochemical principles define the constraints through which living systems have evolved and continue to evolve. These boundaries determine the biochemical space in which homeostasis operates to regulate physiological variables such as temperature, acidity, or cytokine production. Not surprisingly, diverse medical fields like rheumatology, cardiology, endocrinology, and infectious diseases start to converge on cell bioenergetics to understand disease pathogenesis and find cures against RA, cardiovascular disease, metabolic syndrome, and sepsis, respectively. All these medical conditions, which represent a pressing public health issue, share a common denominator: inflammation.

Research on immune cell metabolism and its relationship to immune function began decades ago. Back then, research on this topic did not gain enough momentum so that the knowledge it generated was not translated into clinical application. Today, renewed interest on immune cell bioenergetics comes at a time when advances in technology allow for simpler, comprehensive, and affordable ways to measure metabolic function of immune cells. Translational, multidisciplinary research should be able to bring this body of knowledge into the drug development process and eventually into therapeutic reality against chronic inflammation and RA.

Conflict of Interest None to declare.

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References

1. Sacks JJ, Luo YH, Helmick CG (2010) Prevalence of specific types of arthritis and other rheumatic conditions in the ambulatory health care system in the United States, 2001–2005. Arthritis Care Res (Hoboken) 62:460–464
2. Birnbaum H, Pike C, Kaufman R, Marynchenko M, Kidolezi Y, Cifaldi M (2010) Societal cost of rheumatoid arthritis patients in the US. Curr Med Res Opin 26:77–90
3. Tabas I, Glass CK (2013) Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science (New York, NY) 339:166–172
4. Burmester GR, Feist E, Dorner T (2014) Emerging cell and cytokine targets in rheumatoid arthritis. Nat Rev Rheumatol 10:77–88
5. McInnes IB, Schett G (2011) The pathogenesis of rheumatoid arthritis. N Engl J Med 365:2205–2219
6. Smolen JS, Aletaha D (2015) Rheumatoid arthritis therapy reappraisal: strategies, opportunities and challenges. Nat Rev Rheumatol 11:276–289
7. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H et al (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 48:2741–2749
8. Catrina AI, Ytterberg AJ, Reynisdottir G, Malmstrom V, Klareskog L (2014) Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nat Rev Rheumatol 10:645–653
9. Smolen JS, Steiner G (2003) Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2:473–488
10. Otero M, Goldring MB (2007) Cells of the synovium in rheumatoid arthritis. Chondrocytes. Arthritis Res Ther 9:220
11. Fujikawa Y, Sabokbar A, Neale S, Athanasou NA (1996) Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis. Ann Rheum Dis 55:816–822
12. Chabaud M, Fossiez F, Taupin JL, Miossec P (1998) Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 161:409–414
13. Ishikawa H, Smiley JD, Ziff M (1975) Electron microscopic demonstration of immunoglobulin deposition in rheumatoid cartilage. Arthritis Rheum 18:563–576
14. Cascao R, Rosario HS, Souto-Carneiro MM, Fonseca JE (2010) Neutrophils in rheumatoid arthritis: more than simple final effectors. Autoimmun Rev 9:531–535
15. Schuerwegh AJ, Ioan-Facsinay A, Dorjee AL, Roos J, Bajema IM, van der Voort EI et al (2010) Evidence for a functional role of IgE anticitrullinated protein antibodies in rheumatoid arthritis. Proc Natl Acad Sci U S A 107:2586–2591
16. Kinne RW, Brauer R, Stuhlmuller B, Palombo-Kinne E, Burmester GR (2000) Macrophages in rheumatoid arthritis. Arthritis Res 2:189–202
17. Listing J, Strangfeld A, Rau R, Kekow J, Gromnica-Ihle E, Klopsch T et al (2006) Clinical and functional remission: even though biologics are superior to conventional DMARDs overall success rates remain low – results from RABBIT, the German biologics register. Arthritis Res Ther 8:R66
18. van Vollenhoven RF (2011) Unresolved issues in biologic therapy for rheumatoid arthritis. Nat Rev Rheumatol 7:205–215
19. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ (2012) Rethinking inflammation: neural circuits in the regulation of immunity. Immunol Rev 248:188–204
20. Straub RH (2014) Rheumatoid arthritis – a neuroendocrine immune disorder: glucocorticoid resistance, relative glucocorticoid deficiency, low-dose glucocorticoid therapy, and insulin resistance. Arthritis Res Ther 16(Suppl 2):I1
21. Buchman TG (2002) The community of the self. Nature 420:246–251
22. Lemons JM, Feng XJ, Bennett BD, Legesse-Miller A, Johnson EL, Raitman I et al (2010) Quiescent fibroblasts exhibit high metabolic activity. PLoS Biol 8:e1000514

23. Dufort FJ, Gumina MR, Ta NL, Tao Y, Heyse SA, Scott DA et al (2014) Glucose-dependent de novo lipogenesis in B lymphocytes: a requirement for atp-citrate lyase in lipopolysaccharide-induced differentiation. J Biol Chem 289:7011–7024
24. Jang KJ, Mano H, Aoki K, Hayashi T, Muto A, Nambu Y et al (2015) Mitochondrial function provides instructive signals for activation-induced B-cell fates. Nat Commun 6:6750
25. Biniecka M, Canavan M, McGarry T, Gao W, McCormick J, Cregan S et al (2016) Dysregulated bioenergetics: a key regulator of joint inflammation. Ann Rheum Dis Published Online First: [24 March 2016] doi:10.1136/annrheumdis-2015-208476
26. Garcia-Carbonell R, Divakaruni AS, Lodi A, Vicente-Suarez I, Saha A, Cheroutre H et al (2016) Critical role of fibroblast-like synoviocytes glycolytic metabolism in rheumatoid arthritis. Arthritis Rheumatol 68:1614
27. Martinez FO, Helming L, Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27:451–483
28. Odegaard JI, Chawla A (2011) Alternative macrophage activation and metabolism. Annu Rev Pathol 6:275–297
29. Fu Y, Maianu L, Melbert BR, Garvey WT (2004) Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3, and 5 in the immune response and foam cell formation. Blood Cells Mol Dis 32:182–190
30. Tan Z, Xie N, Banerjee S, Cui H, Fu M, Thannickal VJ et al (2015) The monocarboxylate transporter 4 is required for glycolytic reprogramming and inflammatory response in macrophages. J Biol Chem 290:46–55
31. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P et al (2010) Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol 185:605–614
32. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G et al (2013) Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature 496:238
33. Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ (2008) The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 295:E242–E253
34. Veal CD, Reekie KE, Lorentzen JC, Gregersen PK, Padyukov L, Brookes AJ (2014) A 129-kb deletion on chromosome 12 confers substantial protection against rheumatoid arthritis, implicating the gene SLC2A3. Hum Mutat 35:248–256
35. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P et al (2012) The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. Cell Metab 15:813–826
36. Garedew A, Moncada S (2008) Mitochondrial dysfunction and HIF1alpha stabilization in inflammation. J Cell Sci 121:3468–3475
37. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE et al (2012) Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood 120:1422–1431
38. Clementi E, Brown GC, Feelisch M, Moncada S (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc Natl Acad Sci U S A 95:7631–7636
39. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH et al (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 12:149–162
40. Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ (2009) An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. Nucleic Acids Res 37:4587–4602
41. Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3:177–185
42. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL et al (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 515:431–435

43. Hollander AP, Corke KP, Freemont AJ, Lewis CE (2001) Expression of hypoxia-inducible factor 1alpha by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. Arthritis Rheum 44:1540–1544
44. Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B et al (2014) Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nat Methods 11:417–422
45. Kim S, Hwang J, Xuan J, Jung YH, Cha HS, Kim KH (2014) Global metabolite profiling of synovial fluid for the specific diagnosis of rheumatoid arthritis from other inflammatory arthritis. PLoS One 9:e97501
46. Melo RC, D'Avila H, Wan HC, Bozza PT, Dvorak AM, Weller PF (2011) Lipid bodies in inflammatory cells: structure, function, and current imaging techniques. J Histochem Cytochem 59:540–556
47. Martin S, Parton RG (2006) Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 7:373–378
48. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY et al (2014) TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat Immunol 15:323–332
49. Funk JL, Feingold KR, Moser AH, Grunfeld C (1993) Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation. Atherosclerosis 98:67–82
50. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG et al (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464:1357–1361
51. Kerekes G, Nurmohamed MT, Gonzalez-Gay MA, Seres I, Paragh G, Kardos Z et al (2014) Rheumatoid arthritis and metabolic syndrome. Nat Rev Rheumatol 10:691–696
52. Tall AR, Yvan-Charvet L (2015) Cholesterol, inflammation and innate immunity. Nat Rev Immunol 15:104–116
53. Hahn BH, Grossman J, Ansell BJ, Skaggs BJ, McMahon M (2008) Altered lipoprotein metabolism in chronic inflammatory states: proinflammatory high-density lipoprotein and accelerated atherosclerosis in systemic lupus erythematosus and rheumatoid arthritis. Arthritis Res Ther 10:213
54. Sattar N, McInnes IB (2005) Vascular comorbidity in rheumatoid arthritis: potential mechanisms and solutions. Curr Opin Rheumatol 17:286–292
55. Sattar N, McCarey DW, Capell H, McInnes IB (2003) Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. Circulation 108:2957–2963
56. Kelly B, Tannahill GM, Murphy MP, O'Neill LA (2015) Metformin inhibits the production of reactive oxygen species from NADH: ubiquinone oxidoreductase to limit induction of interleukin-1beta (IL-1beta) and boosts interleukin-10 (IL-10) in lipopolysaccharide (LPS)-activated macrophages. J Biol Chem 290:20348–20359
57. Kang KY, Kim YK, Yi H, Kim J, Jung HR, Kim IJ et al (2013) Metformin downregulates Th17 cells differentiation and attenuates murine autoimmune arthritis. Int Immunopharmacol 16:85–92
58. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS et al (2009) Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature 460:103–107
59. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF et al (2011) Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. J Immunol 186:3299–3303
60. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D et al (2011) The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity 35:871–882
61. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR et al (2011) HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J Exp Med 208:1367–1376

62. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D et al (2014) The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. Cell Metab 20:61–72
63. Powell JD, Pollizzi KN, Heikamp EB, Horton MR (2012) Regulation of immune responses by mTOR. Annu Rev Immunol 30:39–68
64. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B et al (2009) The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30:832–844
65. Powell JD, Delgoffe GM (2010) The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity 33:301–311
66. Bar-Peled L, Sabatini DM (2014) Regulation of mTORC1 by amino acids. Trends Cell Biol 24:400–406
67. Nakaya M, Xiao Y, Zhou X, Chang JH, Chang M, Cheng X et al (2014) Inflammatory T cell responses rely on amino acid transporter ASCT2 facilitation of glutamine uptake and mTORC1 kinase activation. Immunity 40:692–705
68. Sinclair LV, Rolf J, Emslie E, Shi YB, Taylor PM, Cantrell DA (2013) Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. Nat Immunol 14:500–508
69. Cejka D, Hayer S, Niederreiter B, Sieghart W, Fuereder T, Zwerina J et al (2010) Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. Arthritis Rheum 62:2294–2302
70. Carlson RP, Baeder WL, Caccese RG, Warner LM, Sehgal SN (1993) Effects of orally administered rapamycin in animal models of arthritis and other autoimmune diseases. Ann N Y Acad Sci 685:86–113
71. Bruyn GA, Tate G, Caeiro F, Maldonado-Cocco J, Westhovens R, Tannenbaum H et al (2008) Everolimus in patients with rheumatoid arthritis receiving concomitant methotrexate: a 3-month, double-blind, randomised, placebo-controlled, parallel-group, proof-of-concept study. Ann Rheum Dis 67:1090–1095
72. Warner LM, Adams LM, Sehgal SN (1994) Rapamycin prolongs survival and arrests pathophysiologic changes in murine systemic lupus erythematosus. Arthritis Rheum 37:289–297
73. Fernandez D, Bonilla E, Mirza N, Niland B, Perl A (2006) Rapamycin reduces disease activity and normalizes T cell activation-induced calcium fluxing in patients with systemic lupus erythematosus. Arthritis Rheum 54:2983–2988
74. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR (2008) Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci U S A 105:19396–19401
75. Panayi GS (2006) Even though T-cell-directed trials have been of limited success, is there reason for optimism? Nat Clin Pract Rheumatol 2:58–59
76. Miossec P, Verweij CL, Klareskog L, Pitzalis C, Barton A, Lekkerkerker F et al (2011) Biomarkers and personalised medicine in rheumatoid arthritis: a proposal for interactions between academia, industry and regulatory bodies. Ann Rheum Dis 70:1713–1718
77. Wehling M (2010) Principles of translational science in medicine: from bench to bedside. Cambridge University Press, Cambridge/New York, xxii, 382 p., 24 p. of plates p
78. Butler D (2008) Translational research: crossing the valley of death. Nature 453:840–842
79. Arrowsmith J (2012) A decade of change. Nat Rev Drug Discov 11:17–18
80. Tak PP (2005) Analyzing synovial tissue samples. What can we learn about early rheumatoid arthritis, the heterogeneity of the disease, and the effects of treatment? J Rheumatol Suppl 72:25–26
81. Raza K, Filer A (2015) The therapeutic window of opportunity in rheumatoid arthritis: does it ever close? Ann Rheum Dis 74:793–794
82. Biomarkers Definitions Working G (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69:89–95

83. Robinson WH, Lindstrom TM, Cheung RK, Sokolove J (2013) Mechanistic biomarkers for clinical decision making in rheumatic diseases. Nat Rev Rheumatol 9:267–276
84. Collins FS, Varmus H (2015) A new initiative on precision medicine. N Engl J Med 372:793–795
85. Jameson JL, Longo DL (2015) Precision medicine – personalized, problematic, and promising. N Engl J Med 372:2229–2234
86. Semerano L, Romeo PH, Boissier MC (2015) Metabolomics for rheumatic diseases: has the time come? Ann Rheum Dis 74:1325–1326
87. Fitzpatrick M, Young SP (2013) Metabolomics – a novel window into inflammatory disease. Swiss Med Wkly 143:w13743
88. Kapoor SR, Filer A, Fitzpatrick MA, Fisher BA, Taylor PC, Buckley CD et al (2013) Metabolic profiling predicts response to anti-tumor necrosis factor alpha therapy in patients with rheumatoid arthritis. Arthritis Rheum 65:1448–1456
89. Madsen RK, Lundstedt T, Gabrielsson J, Sennbro CJ, Alenius GM, Moritz T et al (2011) Diagnostic properties of metabolic perturbations in rheumatoid arthritis. Arthritis Res Ther 13:R19
90. Kang J, Zhu L, Lu J, Zhang X (2015) Application of metabolomics in autoimmune diseases: insight into biomarkers and pathology. J Neuroimmunol 279:25–32
91. Volchenkov R, Dung Cao M, Elgstoen KB, Goll GL, Eikvar K, Bjorneboe O et al (2016) Metabolic profiling of synovial tissue shows altered glucose and choline metabolism in rheumatoid arthritis samples, Scandinavian Journal of Rheumatology, doi:10.3109/03009742.2016.1164242
92. Urasaki Y, Johlfs MG, Fiscus RR, Le TT (2012) Imaging immune and metabolic cells of visceral adipose tissues with multimodal nonlinear optical microscopy. PLoS One 7:e38418
93. Bozza PT, Viola JP (2010) Lipid droplets in inflammation and cancer. Prostaglandins Leukot Essent Fatty Acids 82:243–250
94. Coimbra A, Lopes-Vaz A (1971) The presence of lipid droplets and the absence of stable sudanophilia in osmium-fixed human leukocytes. J Histochem Cytochem 19:551–557
95. Hakumaki JM, Kauppinen RA (2000) 1H NMR visible lipids in the life and death of cells. Trends Biochem Sci 25:357–362
96. Asquith DL, Ballantine LE, Nijjar JS, Makdasy MK, Patel S, Wright PB et al (2013) The liver X receptor pathway is highly upregulated in rheumatoid arthritis synovial macrophages and potentiates TLR-driven cytokine release. Ann Rheum Dis 72:2024–2031
97. Wu BJ, Ong KL, Shrestha S, Chen K, Tabet F, Barter PJ et al (2014) Inhibition of arthritis in the Lewis rat by apolipoprotein A-I and reconstituted high-density lipoproteins. Arterioscler Thromb Vasc Biol 34:543–551
98. Benson RA, McInnes IB, Brewer JM, Garside P (2015) Cellular imaging in rheumatic diseases. Nat Rev Rheumatol 11:357–367
99. Sevick-Muraca EM (2012) Translation of near-infrared fluorescence imaging technologies: emerging clinical applications. Annu Rev Med 63:217–231
100. Griffiths HR (2005) ROS as signalling molecules in T cells – evidence for abnormal redox signalling in the autoimmune disease, rheumatoid arthritis. Redox Rep 10:273–280
101. Datta S, Kundu S, Ghosh P, De S, Ghosh A, Chatterjee M (2014) Correlation of oxidant status with oxidative tissue damage in patients with rheumatoid arthritis. Clin Rheumatol 33:1557–1564
102. Lee D, Khaja S, Velasquez-Castano JC, Dasari M, Sun C, Petros J et al (2007) In vivo imaging of hydrogen peroxide with chemiluminescent nanoparticles. Nat Mater 6:765–769
103. Rueden CT, Conklin MW, Provenzano PP, Keely PJ, Eliceiri KW (2009) Nonlinear optical microscopy and computational analysis of intrinsic signatures in breast cancer. Conf Proc IEEE Eng Med Biol Soc 2009:4077–4080
104. Quinn KP, Sridharan GV, Hayden RS, Kaplan DL, Lee K, Georgakoudi I (2013) Quantitative metabolic imaging using endogenous fluorescence to detect stem cell differentiation. Sci Rep 3:3432
105. Ostrander JH, McMahon CM, Lem S, Millon SR, Brown JQ, Seewaldt VL et al (2010) Optical redox ratio differentiates breast cancer cell lines based on estrogen receptor status. Cancer Res 70:4759–4766

106. Palmer S, Litvinova K, Rafailov EU, Nabi G (2015) Detection of urinary bladder cancer cells using redox ratio and double excitation wavelengths autofluorescence. Biomed Opt Express 6:977–986
107. Walsh AJ, Cook RS, Manning HC, Hicks DJ, Lafontant A, Arteaga CL et al (2013) Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. Cancer Res 73:6164–6174
108. Phipps J, Sun Y, Saroufeem R, Hatami N, Fishbein MC, Marcu L (2011) Fluorescence lifetime imaging for the characterization of the biochemical composition of atherosclerotic plaques. J Biomed Opt 16:096018
109. Polisson RP, Schoenberg OI, Fischman A, Rubin R, Simon LS, Rosenthal D et al (1995) Use of magnetic resonance imaging and positron emission tomography in the assessment of synovial volume and glucose metabolism in patients with rheumatoid arthritis. Arthritis Rheum 38:819–825
110. Matsui T, Nakata N, Nagai S, Nakatani A, Takahashi M, Momose T et al (2009) Inflammatory cytokines and hypoxia contribute to 18F-FDG uptake by cells involved in pannus formation in rheumatoid arthritis. J Nucl Med 50:920–926
111. Rudd JH, Warburton EA, Fryer TD, Jones HA, Clark JC, Antoun N et al (2002) Imaging atherosclerotic plaque inflammation with [18F]-fluorodeoxyglucose positron emission tomography. Circulation 105:2708–2711
112. Rose S, Sheth NH, Baker JF, Ogdie A, Raper A, Saboury B et al (2013) A comparison of vascular inflammation in psoriasis, rheumatoid arthritis, and healthy subjects by FDG-PET/CT: a pilot study. Am J Cardiovasc Dis 3:273–278
113. Maki-Petaja KM, Elkhawad M, Cheriyan J, Joshi FR, Ostor AJ, Hall FC et al (2012) Anti-tumor necrosis factor-alpha therapy reduces aortic inflammation and stiffness in patients with rheumatoid arthritis. Circulation 126:2473–2480
114. Wang SC, Xie Q, Lv WF (2014) Positron emission tomography/computed tomography imaging and rheumatoid arthritis. Int J Rheum Dis 17:248–255
115. Okamura K, Yonemoto Y, Arisaka Y, Takeuchi K, Kobayashi T, Oriuchi N et al (2012) The assessment of biologic treatment in patients with rheumatoid arthritis using FDG-PET/CT. Rheumatology (Oxford) 51:1484–1491
116. Gutte H, Hansen AE, Henriksen ST, Johannesen HH, Ardenkjaer-Larsen J, Vignaud A et al (2015) Simultaneous hyperpolarized (13)C-pyruvate MRI and (18)F-FDG-PET in cancer (hyperPET): feasibility of a new imaging concept using a clinical PET/MRI scanner. Am J Nucl Med Mol Imaging 5:38–45
117. Dafni H, Larson PE, Hu S, Yoshihara HA, Ward CS, Venkatesh HS et al (2010) Hyperpolarized 13C spectroscopic imaging informs on hypoxia-inducible factor-1 and myc activity downstream of platelet-derived growth factor receptor. Cancer Res 70:7400–7410
118. Nelson SJ, Kurhanewicz J, Vigneron DB, Larson PE, Harzstark AL, Ferrone M et al (2013) Metabolic imaging of patients with prostate cancer using hyperpolarized [1-(1)(3)C]pyruvate. Sci Transl Med 5:198ra08
119. Gashaw I, Ellinghaus P, Sommer A, Asadullah K (2012) What makes a good drug target? Drug Discov Today 17(Suppl):S24–S30
120. Yin Y, Choi SC, Xu Z, Perry DJ, Seay H, Croker BP et al (2015) Normalization of CD4+ T cell metabolism reverses lupus. Sci Transl Med 7:274ra18
121. Son HJ, Lee J, Lee SY, Kim EK, Park MJ, Kim KW et al (2014) Metformin attenuates experimental autoimmune arthritis through reciprocal regulation of Th17/Treg balance and osteoclastogenesis. Mediators Inflamm 2014:973986
122. Kollias G, Papadaki P, Apparailly F, Vervoordeldonk MJ, Holmdahl R, Baumans V et al (2011) Animal models for arthritis: innovative tools for prevention and treatment. Ann Rheum Dis 70:1357–1362
123. Yang Z, Fujii H, Mohan SV, Goronzy JJ, Weyand CM (2013) Phosphofructokinase deficiency impairs ATP generation, autophagy, and redox balance in rheumatoid arthritis T cells. J Exp Med 210:2119–2134
124. Yang Z, Shen Y, Oishi H, Matteson EL, Tian L, Goronzy JJ et al (2016) Restoring oxidant signaling suppresses proarthritogenic T cell effector functions in rheumatoid arthritis. Sci Transl Med 8:331ra38

125. Gerlag DM, Raza K, van Baarsen LG, Brouwer E, Buckley CD, Burmester GR et al (2012) EULAR recommendations for terminology and research in individuals at risk of rheumatoid arthritis: report from the Study Group for Risk Factors for Rheumatoid Arthritis. Ann Rheum Dis 71:638–641
126. Nathan C, Ding A (2010) Nonresolving inflammation. Cell 140:871–882
127. Miossec P (2015) Drug treatments for rheumatoid arthritis: looking backwards to move forwards. BMJ 350:h1192
128. Scott DL, Ibrahim F, Farewell V, O'Keeffe AG, Walker D, Kelly C et al (2015) Tumour necrosis factor inhibitors versus combination intensive therapy with conventional disease modifying anti-rheumatic drugs in established rheumatoid arthritis: TACIT non-inferiority randomised controlled trial. BMJ 350:h1046
129. Ferrari M, Onuoha SC, Pitzalis C (2015) Trojan horses and guided missiles: targeted therapies in the war on arthritis. Nat Rev Rheumatol 11:328–337
130. Wilder RL (2002) Integrin alpha V beta 3 as a target for treatment of rheumatoid arthritis and related rheumatic diseases. Ann Rheum Dis 61(Suppl 2):ii96–ii99
131. Lee SM, Kim HJ, Ha YJ, Park YN, Lee SK, Park YB et al (2013) Targeted chemo-photothermal treatments of rheumatoid arthritis using gold half-shell multifunctional nanoparticles. ACS Nano 7:50–57
132. Scheinman RI, Trivedi R, Vermillion S, Kompella UB (2011) Functionalized STAT1 siRNA nanoparticles regress rheumatoid arthritis in a mouse model. Nanomedicine (Lond) 6:1669–1682
133. Firer MA, Gellerman G (2012) Targeted drug delivery for cancer therapy: the other side of antibodies. J Hematol Oncol 5:70
134. Byrne H, Conroy PJ, Whisstock JC, O'Kennedy RJ (2013) A tale of two specificities: bispecific antibodies for therapeutic and diagnostic applications. Trends Biotechnol 31:621–632
135. Expert Group on Phase I clinical trials (Chairman: Professor Gordon W. Duff) (2006)
136. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL (2012) Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol 12:786–798
137. Misharin AV, Haines GK 3rd, Rose S, Gierut AK, Hotchkiss RS, Perlman H (2012) Development of a new humanized mouse model to study acute inflammatory arthritis. J Transl Med 10:190

The Intestinal Microbiome, the Immune System and Spondyloarthropathy

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1 Introduction

The microbial communities that live in and on our bodies play complex roles in maintaining our health and in causing disease. The recent application of high-throughput DNA sequencing to examine these communities, both in terms of species present and in their activities, has proven to be a very powerful tool for examining the influences of microbes on human health. Whilst we are only just beginning to understand the role of our microbial 'second' genome, what is clear is that certain shifts and alterations in our microbiome are associated with, and may ultimately cause or cure, disease. The interaction between human host and microbes is multifaceted, however, and such interactions must therefore be examined in overall context of disease, diet, medications as well as underlying host genetics.

Bacteria inhabit all parts of the human body including our skin, our mouths and the intestinal and reproductive tracts [1]. It has long been suspected that microbiome interactions with the host can influence or directly cause disease, and recent research has shown the considerable extent of such microbial influence. For example, alterations in microbial communities and their function have been linked to a variety conditions: from inflammatory bowel disease (IBD) and heart disease to colon cancer and obesity [2–5]. In addition, the manner in which we enter this world appears to shape our microbial population. Thus, whether we are born by vaginal delivery or caesarean section may alter our propensity to develop chronic diseases such as obesity, allergies

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and asthma [6–9]. However, for reasons outlined below, the microbes that live in our intestinal tract have drawn the most interest and research to date [1, 3, 10, 11].

It has been estimated that roughly 30 % of all the microbes living in and on our body inhabit in our intestinal tract. Given that the gut is the primary site for interaction between these microorganisms and the immune system [12, 13], elucidating the role of the intestinal microbiome in immune-mediated diseases is important for understanding disease pathogenesis but also as an avenue for potential therapies. Of particular interest is the role the intestinal microbiome plays in metabolism, pathogen resistance as well as the body's immune response and role in driving immune-mediated diseases.

Understanding how the intestinal microbiome is assembled and maintained is becoming increasingly relevant. To date, only a handful of studies have examined the composition, diversity and function of the human gut microbiome and its impact on our overall health. Recent studies in humans and mice have shown that changes in intestinal microbes can lead to changes in metabolism and obesity [3, 14, 15]. Changes in the composition and metabolic capacity of the intestinal microbiome can also influence the development of autoimmune disease. For example, the production of immunomodulatory products, such as short chain fatty acids, is altered and this affects the immune system and inflammation [16, 17]. This illustrates that our smallest inhabitants can have large impacts on our health.

2 The Intestinal Microbiome Is a Dynamic and Responsive System

The gastrointestinal tract is heavily populated with microbes and is the primary site for interaction between these microorganisms and the immune system [12, 13]. Microbes and humans have evolved over time to live in symbiosis with each other. Many of these diverse communities carry out specific tasks that benefit the host, as well as the microbial communities. A delicate balancing act exists between host and the intestinal microbiome. It is well documented that changes in the microbial composition, particularly by a pathogen, elicit an innate immune response [18]. However, it is still unknown if dysbiosis in the intestinal microflora has the capacity to illicit the same response as an infection as the gut attempts to defend itself and restore homeostasis [19]. Homeostasis of the normal flora in the gut microbiome is essential, and both the bacteria that inhabit the gut and the human immune system have developed strategies to regulate and vigorously protect this delicate balance.

The resident intestinal microbes have been shown to sculpt host immune systems from an early age [20, 21]. It follows that microbiome maintenance and the preservation/return of homeostasis are being increasingly accepted as vital for intestinal and overall health [22] and that dysregulation of intestinal microbial homeostasis may play a role in autoimmunity pathogenesis. Bacteria inhabiting the intestine have several techniques to maintain homeostasis including competitive exclusion, biosurfactant production and modulation of tight junctions [23].

The human body has a number of physiological processes that respond to a disruption in gut homeostasis or to a bacterial insult. If a pathogen is detected by antigen-presenting cells (APC), such as a dendritic cell, naïve T cells are then activated and recruited to the area. IL-17 secreting Th17 cells are sequestered to induce epithelial cells to recruit neutrophils to neutralise the disruption, and IL-22 is secreted to promote epithelial repair and induce the secretion of antibacterial proteins [24]. The epithelial cells lining the intestine can also respond to a disturbance or insult and attempt to restore homeostasis by secreting a range of soluble factors such as mucins and antimicrobial peptides (AMPs) that aim to prevent the invasion of microbes into the intestinal crypt [25–28]. The physiological processes required for maintaining intestinal homeostasis and the microbiome are reviewed below.

3 The Physical Barrier

The human gastrointestinal tract is lined with a single layer of intestinal epithelial cells (IECs) that form a physical barrier between the intestinal lumen and the next tissue layer, the lamina propria. The *interaction* between IECs, intestinal microbes and the local immune cells is integral to intestinal homeostasis. However, *these interactions* have also been shown to contribute to disease pathogenesis [29, 30]. IECs secrete soluble factors such as mucins and AMPs that are critical to intestinal homeostasis and microbial community balance [26–28]. The mucus which lines the gastrointestinal tract provides both a physical barrier protecting the underlying tissue and a key immunological role in educating antigen-presenting cells such as dendritic cells (DC, discussed below) to develop tolerance towards food and commensal antigens [31]. Depletion of the mucus layer, the thickness of which is influenced by bacteria, cytokines, genetic factors and antibiotics and other medicinals, can lead to an inflammatory bowel disease (IBD)-like phenotype and endoplasmic reticulum stress, potentially driving production of the cytokine IL-23 [32]. IL-23 excess alone is sufficient to induce the inflammatory disease spondylarthritis in mice [33], and genetic evidence indicates that IL-23 plays a key role in the development of spondylarthritis in humans (further evidence for the role of is reviewed in the section “The Microbiome in Immune-Mediated Disease”).

The cell-to-cell junctions between IECs constantly open and close in response to a number of stimuli including diet, neural signals, mast cell productions as well as a variety of cellular pathways, all of which can be exploited by microbial as well as viral pathogens [34–37]. In IBD and celiac disease, epithelial tight junctions are known to be dysregulated, which causes increased permeability between IECs and results in a ‘leaky gut’ [38, 39]. Whilst the causes and ramifications of a ‘leaky gut’ in the context of disease are still under investigation, it has been suggested that an increase in intestinal permeability may lead to bacteria, either pathogens or normal resident bacteria, entering from the lumen and triggering an inflammatory immune response [38, 40]. Intestinal permeability in AS patients has always been considered a side effect due to the long-term use of non-steroidal anti-inflammatory drugs

(NSAIDs) [41]. However, studies examining first-degree relatives of AS patients showed they also have increased gut permeability, suggesting that there may be an underlying genetic process operating in the gut [42, 43].

4 Innate Immunity

The intestinal lamina propria is densely populated by dendritic cells (DC), which sample and survey the environment, forming a widespread microbe-sensing network. DC are able to recognise a broad repertoire of bacteria, sensing with receptors such as toll-like receptors (TLRs) and monitoring the bacteria on the mucosal surface [44]. Intestinal DC coordinate the production of intestinal-specific IgA in order to restrict bacterial interaction with the intestinal epithelial cell surface [45]. Activated DC are also able to secrete a number of key cytokines and chemokines involved in inflammation and migration such as IL-23 and IL-6 [46].

Macrophages are gastrointestinal sentinels patrolling in high numbers and frequently coming in contact with 'stray' bacteria (commensal and otherwise) that have breached the epithelial cell barrier. Circulating macrophages phagocytose and kill such bacteria using mechanisms that include production of antimicrobial proteins and reactive oxygen species [47]. However, intestinal macrophages have several unique characteristics including the expression of the anti-inflammatory cytokine IL-10, both constitutively and after bacterial stimulation [48, 49]. The loss-of-function mutations in *IL10R*, leading to early-onset IBD, highlight the importance of this pathway [50] and demonstrate that common variants in *IL10* are associated with IBD [51, 52].

5 Adaptive Immunity

5.1 IL-23-Responsive Cells

IL-23 is a crucial cytokine in the development of cells that secrete IL-17 and IL-22. IL-23 signals through a receptor consisting of the specific IL-23 receptor (IL-23R) subunit and IL-12Rβ1, also shared with IL-12R [53]. Loss-of-function polymorphisms in *IL23R,* as well as other genes in the pathway, are associated with protection from AS [54], psoriasis [55], IBD [56] and Behcet's disease [57] amongst other conditions. IL-23-, IL-17- and IL-22-producing cells have been shown to be enriched in the intestinal mucosa [33], with IL-17 and IL-22 known to be key regulators of homeostasis and intestinal 'health'. IL-17 and IL-22 work in concert to maintain intestinal homoeostasis by maintaining epithelial barrier tight junctions [58] and inducing antimicrobial proteins such as β-defensins and REG proteins [59]. In the gut, innate-like immune cells act as sentinels, responding very rapidly to alterations to the microbial composition of the gut, within 4–8 h, with rapid secretion of IL-17 [60–62]. The IL-22-IL-22R pathway contributes to the

regulation of inflammation and tissue repair. IL-22 is expressed by innate and adaptive cells and seems to act almost exclusively on non-haematopoietic cells, with basal IL-22R expression in the skin, pancreas, intestine, liver, lung and kidney [63]. IL-22 can act synergistically with IL-17A, IL-17F or tumour necrosis factor (TNF) to promote the expression of many of the genes that encode molecules involved in host defence in the skin, airway or intestine. This demonstrates the functional importance of IL-22 in promoting barrier immunity and mucosal integrity. This rapid reaction to pathogens also includes secreting factors that recruit large numbers of neutrophils through increasing the activity of IL-1, IL-6 and tumour necrosis factor (TNF). These factors promote tissue infiltration, which, in turn, is critical for rapid and effective control of bacterial and fungal pathogens [64]. Key immune cells in this process include γδ T cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and lymphoid tissue inducer (LTi)-like cells.

5.2 γδ T Cells

Large numbers of γδ T cells reside in epithelial surfaces such as the gut and skin, where they can account for almost half of T cells. γδ T cells are interesting in that they posses aspects of the innate and adaptive immune system, making them ideal to respond to alterations in the intestines. They express an antigen-specific T cell receptor (TCR), as well as many properties of cells of the innate immune system including expression of major innate immunity receptors such as TLRs [65] and dectin-1 [66], which recognises microbial peptides. γδ T cells are potent inflammatory cytokine producers including IFN-γ, TNF-α and IL-17 [62, 67]. γδ T cells are known to be pathogenic in the collagen-induced arthritis model [68] and mouse models of colitis [69]. It has also been reported that IL-17-secreting γδ T cells are enriched and possibly pathogenic in patients with AS [70]. Taken together, this supports a role for γδ T cells in early responses to alterations in the intestinal microbiome.

5.3 Natural Killer T Cells

Natural killer T (NKT) cells are rapid responders to antigenic stimuli and are capable of producing a range of immunoregulatory cytokines after recognising glycolipid structures presented to them by the nonclassical antigen-presenting molecule CD1d [71–74]. Within the intestinal tract, NKT cells convey protection in Th1-mediated models of inflammatory bowel disease but pathogenic in Th2 models [75, 76]. Stimulation of NKT cells with microbial products has been shown to shape the NKT cell phenotype as well as their functional maturation [77]. A protective role for NKT cells have has been described in models of arthritis [78] and spondyloarthropathy [79, 80]; their functional maturation in the gut provides evidence for a role for mucosal T cell priming in inflammatory joint disease.

5.4 *Mucosal-Associated Invariant T Cells*

Mucosal-associated invariant T (MAIT) cells are innate-like T cells found in the intestinal tract, liver and blood and are known to secrete inflammatory cytokines in response to antigenic stimulation, such as IL-17 and IFN-γ [81–83]. MAIT cells have an invariant T cell receptor (Vα7.2 in humans) that recognises antigen presented by the nonclassical MHC-like molecule, MR1 [84]. MAIT cells display a memory phenotype in the blood [83], expressing the transcription factor ZBTB16 [85], which facilitates the rapid secretion of cytokines in response to antigenic stimuli. Furthermore, they express high levels of IL-23R [86]. MAIT cells respond to a broad range of microbial stimuli, including bacteria and yeasts [81, 82]. Whilst the exact role of MAIT cells in mucosal barrier homeostasis is unknown, the acquisition of phenotypic markers of memory coupled with the demonstrated expansion of MAIT cells in utero demonstrates the interaction of the immune system with developing commensal microflora [87, 88].

5.5 *Lymphoid Tissue Inducer-Like Cells*

Lymphoid tissue inducer (LTi)-like cells are that reside in multiple sites of the human body including the intestinal lamina propria, lymph nodes and spleen. LTi-like cells express several features of IL-17-secreting cells including the expression of IL-23R, RORγt, AHR and CCR6 [60, 89]. Intestinal LTi cells are a heterogeneous population of innate lymphoid cells (ILC) with or without CD4 expression [90]. A common characteristic of ILCs is the expression RORγt, a transcription factor important for the development of these cells. ILCs have been linked to gut inflammation through colitis models where IL-23-responsive ILCs secrete IL-17, IL-22 and IFN-γ and promote intestinal inflammation [91]. RORγt-expressing ILCs are abundant in the intestinal lamina propria and produce IL-17 and/or IL-22 in order to preserve mucosal integrity against extracellular pathogens. These NKp46+ ILCs have been found to be critical for host defence against pathogens such as *Citrobacter rodentium* infection through secretion of IL-22 [89, 92].

6 Interaction Between Intestinal Microbes and the Immune System

There is strong evidence from murine studies to indicate that interaction between the gut microbiome and the host determines the overall level of activation of immune cells producing cytokines. Segmented filamentous bacteria (SFB) are commensal bacteria that induce IL-17 secretion. Mice that lacked SFB had low levels of intestinal IL-17 and were more susceptible to infection with pathogenic *Citrobacter* spp.

[20, 93]. When SFB were reintroduced to these mice, the number of gut-resident IL-17-producing cells increased and provided resistance to infection. This was through the induction of specific epithelia cell-specific genes, as well as host inflammatory response genes, which were found to be upregulated by the bacteria, which lead to an inflammatory environment and a reduction of tolerance [20, 94]. Lachnospiraceae, Ruminococcaceae and Prevotellaceae are families of bacteria that have been observed in IBD gut microbiomes and are strongly associated with colitis and Crohn's disease (CD) [95–97], with Prevotellaceae especially known to elicit a strong inflammatory response in the gut [97]. The demonstration that bacteria can directly influence the host response highlights the effect commensal bacteria have on the immune response and their role in inflammation. Conversely, members of the gut microbiota, such as *Clostridium*, have been found to play a protective role by inducing T regulatory cells (Tregs) in the colonic mucosa. Tregs are an important counterbalance in the immune system and promote homeostasis. It was found that a specific mix of cluster IV and XIVa of *Clostridium* was sufficient to promote Treg accumulation in the colon [98].

7 Defining the 'Normal' Microbiome

Given the dynamic and responsive nature of the intestinal microbiome [99, 100], understanding how the intestinal microbiome is assembled and maintained is becoming increasingly relevant not only for general health but also potentially for the treatment of complex chronic diseases. In recent years, only a handful of studies have examined the composition, diversity and function of the human gut microbiome. The most recent was the National Institutes of Health lead Human Microbiome Project [10, 101, 102].

The Human Microbiome Project was the first study to identify and catalogue microbiomes throughout the whole body, rather than focusing on specific body sites. The body sites of interest were the mouth, skin, stool and vaginal tract. Samples were collected on multiple occasions with the aim of investigating within-subject variation, between-subject variation as well as microbiome variation over time. A total of 4,788 specimens were sequenced with females sampled from 18 different habitats and males from 15 habitats [103]. Diversity and abundance of each habitat's signature microbes were found to vary greatly amongst the healthy subjects, with strong niche specialisation found both within and between individuals, demonstrating the dynamic nature of the microbiome [99, 103–105]. Metagenomic metabolic pathways were found to be stable amongst individuals, despite the variation seen within community structure, suggesting the presence of a 'core microbiome' or a 'core metabolic microbiome' in an adaptive landscape. When clinical metadata was available, ethnic background proved to be one of the strongest predictors of both pathways and microbes. This suggests that, unsurprisingly, environmental factors such as diet, location/region and host genetics play a role in sculpting the microbiome. This is relevant to defining a 'normal'

microbiome, which is likely to be context specific, and may be better defined metabolically than phylogenetically.

8 Interaction Between Host Genetics and the Intestinal Microbiome

The main tool used to investigate the microbiome is the sequencing of the universal bacterial marker gene encoding the 16S ribosomal RNA (rRNA) [106]. The 16S rRNA gene is found in all bacteria and Archaea, and sequence divergence between microbial and eukaryotic ribosomal RNA genes allows bacterial profiling even when samples are contaminated with host DNA [106]. 16S rRNA sequences are used to differentiate between organisms across all major phyla of bacteria and to classify strains down to species level [106]. 16S taxonomy has drastically changed our understanding of the microbes that live in and on our body, revealing what microbes are present in a community and how the communities compare. It does not, however, directly characterise the metabolic capacity of the community [107]. To further examine the interaction between host and microbiome, dissecting the interaction and reaction between host and microbiome transcription is key [108, 109].

Metagenomic analyses of the human intestine have previously identified that genes and pathways involved in the transport and metabolism of simple carbohydrate substrates are enriched in the intestine microbiome [3, 107, 110] and that alterations in these pathways have been linked with obesity, suggesting their importance for the appropriate functioning of this microbial ecosystem. However, elucidation of the specific activity and metabolic role of individual microbial members within the intestinal microbiome is still limited. Many of the microbial transcriptomic studies have examined stool [3, 107, 110], as the use of intestinal biopsies is still technically very challenging due to the shear amount of human material in samples. Examining host transcriptomics in the gut in combination with microbial transcriptions will further our understanding into how host genetics influences intestinal microbial community composition and function [111] and how this contributes to disease. These studies further define the range of structural and functional configurations that are present in normal microbial communities in a healthy population [10, 103].

9 The Microbiome in Immune-Mediated Disease

9.1 Ankylosing Spondylitis

The involvement of the intestinal microbiome has been suspected as playing a role in the pathogenesis of AS, although a definite link has yet to be established [112, 113]. To date, only a few studies have directly examined this hypothesis by community profiling the microbiome in either tissue or stool samples of patients with

AS. One early study using denaturing gradient gel electrophoresis to profile the microbiome using faecal samples found no differences between AS cases and healthy controls [112]. However, microbial community profiling using next-generation 16S rRNA sequencing of terminal ileal (TI) biopsies from AS cases and healthy controls demonstrates an association between intestinal dysbiosis AS [114]. It was found the intestinal microbial communities of AS patients differed significantly ($P<0.001$) from those of healthy controls, driven by higher abundance of five families of bacteria Lachnospiraceae ($P=0.001$), Ruminococcaceae ($P=0.012$), Rikenellaceae ($P=0.004$), Porphyromonadaceae ($P=0.001$) and Bacteroidaceae ($P=0.001$) and decreases in abundance of two families Veillonellaceae ($P=0.01$) and Prevotellaceae ($P=0.004$) [114]. Interestingly, an overall increase in microbial diversity was observed in AS patients, in contrast to the findings in IBD discussed below, without an overall change in microbial load. This indicates that the microbial dysbiosis seen in the AS intestinal microbiome is not due to an overgrowth or dominance of specific bacteria. Further investigations showed that correlations between these aforementioned families of bacteria were found to sculpt the AS microbial signature. These families of bacteria are also found to be present in all of the AS samples studied.

A number of antibody studies have proposed an increased carriage of *Klebsiella* species in AS patients; however, this has not been universally supported [113]. In the sequencing study of AS TI samples by Costello et al. [114], there was no association observed between *Klebsiella* or any other members of the Enterobacteriaceae family of bacteria, to which *Klebsiella* belongs [114]. This casts further doubt on the role of *Klebsiella* in AS pathogenesis.

Interestingly, increases in *Prevotellaceae* and decreases in *Rikenellaceae* have also recently been reported in the intestinal microbiome in the HLA-B27 transgenic rat model of SpA [115]. Increased levels of *B. vulgatus* have linked to colitis in the *HLA-B27*/β_2 microglobulin transgenic rats. Studies have shown that when germ-free *HLA-B27*/β_2 microglobulin transgenic rats were colonised with an intestinal bacterial cocktail containing *B. vulgatus*, the rats developed more severe colitis [116]. Additionally in BALB/c ZAP-70^{W163C}-mutant (SKG) mice, the interaction between immunogenetic background and host microbiome has been demonstrated to influence SpA and Crohn's-like disease outcomes. Both animal models suggest that underlying host genetics may play a role in sculpting the gut microbiome and disease progression and/or severity [117].

9.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the only inflammatory arthritis for which modern metagenomic studies have been reported. Community profiling studies of RA patients' intestinal microbiome revealed differences in the composition of microbiome in RA patients when compared to healthy controls. This included an observed lower abundance of *Bifidobacterium* and *Bacteroides* bacteria observed in the RA

cases [118, 119]. However, these studies were undertaken using faecal samples and not intestinal biopsies, possibly influencing the populations and the proportions observed [4, 120]. It is not just the intestinal microbiome that has an implicated in RA pathogenesis. There is microbial profiling data that suggests that the oral microbiome, specifically a periodontal infection with the common pathogen *Porphyromonas gingivalis*, may be important in RA pathogenesis. *P. gingivalis*, which has also been identified in synovial fluids of RA patients [121], has the ability to citrullinate host peptides (in common with smoking), which subsequently generates autoantigens that drive autoimmunity in RA [122].

9.3 Inflammatory Bowel Disease

Several lines of evidence indicate that the gut microbiome plays an important role in IBD, including the association of genes involved in mucosal immunity with IBD (such as *CARD15*, *CARD9*, *IL23R* and *ATG16L1*), the therapeutic effect of antibiotics on the condition and the beneficial effect of faecal stream diversion in CD. Previous studies of human IBD have been undertaken using standard culture techniques [123] or molecular analysis [124, 125]. These studies noted alterations in intestinal microbiota when compared to non-IBD patients, a finding recently confirmed using 16S rRNA sequencing of intestinal biopsies [126]. A recent study examined the intestinal microbiome in treatment naïve new-onset CD [4]. 16S community profiling intestinal and faecal samples showed that the signature comprised of an increased abundance in bacteria including Enterobacteriaceae, Pasteurellaceae, Veillonellaceae and Fusobacteriaceae and decreased in abundance of Erysipelotrichales, Bacteroidales and Clostridiales and correlate strongly with disease status [4].

As well as specific bacteria triggering disease, it has also been speculated that antimicrobial reactivity can develop during intestinal inflammation. There have been a number of antibodies described over the years that have shown to have clinical significance in IBD. These include anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-I2 antibodies (which are associated with anti-*Pseudomonas* activity), perinuclear antineutrophil cytoplasmic antibodies (pANCA), anti-*Escherichia coli* outer membrane porin C (anti-OmpC) antibodies, and anti-flagellin antibodies (anti-CBir1) [127, 128]. Given the overlap between IBD and AS, the prevalence of several of the above antimicrobial antibodies was examined in the serum of 80 AS patients and healthy controls [129]. There was no difference detected in the number of antibody positive results between AS patients and healthy controls; however, elevated levels of anti-I2 and ASCA antibodies were detected in AS patients. A recent study has shown anti-CBir1 antibodies are elevated in patients with AS with IBD compared to patients who only have AS [130]. Moreover, patients with AS had increased levels of anti-CBir1 antibodies when compared to healthy controls. This data suggests that AS patients may have increased exposure of the immune system to commensal bacteria, causing the antibody production. Increased levels of

anti-CBir1 antibody in AS patients, compared to healthy controls, could be accounted for, at least in part, by microscopic gut inflammation even in the absence of clinical gastrointestinal symptoms [131].

10 Psoriasis and Psoriatic Arthritis

Other SpA-related conditions where the microbiome is thought to play a significant role are psoriatic arthritis (PsA) and psoriasis (Ps). Historically, it has been suggested that throat infections, particularly streptococcal infection, trigger psoriasis in a genetically susceptible individual [132]. Recent microbiome profiling studies suggest significant differences between the cutaneous microbiota of psoriasis cases and controls. Patients who had psoriasis were found to have decreased levels of *Staphylococci* and *Propionibacteria* in affected skin sites [133]. However, it is not just the skin microbiota that have been found to differ between cases and controls. 16S rRNA community profiling studies of the intestinal microbiota in patients with both PsA and Ps showed the intestinal communities to be less diverse with a relative decrease in abundance of *Coprococcus* species, when compared to healthy controls. PsA patients were further characterised by a significant reduction in *Akkermansia*, *Ruminococcus* and *Pseudobutyrivibrio*, which is not too dissimilar to microbiome profiles previously described in IBD [134].

11 Disentangling Cause and Consequence

Studies in both humans and mice suggest that common aspects of a modern Western lifestyle, including antibiotic use [135, 136], high-fat and fibre-poor diets [14–16], can persistently alter commensal microbial communities. In turn, these microbial disturbances may increase susceptibility to pathogens [137], obesity [3, 14, 138] and autoimmune disease [139–141]. This makes dissecting cause and effect challenging, given normal variation over time and the significant environmental factors that can shape the microbiome.

The successful use of the gut microbiome as a biomarker and/or therapeutic target requires a more detailed understanding of factors that shape the community composition (such as age, diet, geographical location, gender) as well as underlying host genetics. Investigations to date have largely focused on the bacterial composition of the microbiome, and so relatively little is known about the viruses and fungi that also inhabit the gut. Despite the considerable limitations in sequencing and functional annotation of the eukaryotic viruses present in the gastrointestinal tract, recent deep-sequencing efforts have revealed the existence of a complex enteric virome [142, 143]. Much further research along these lines is required to improve our knowledge of the intestinal microbiome as well as microbiome host interactions critical for overall health.

12 Treating and Detecting Disease Through the Microbiome

The intestinal microbiome is a dynamic responsive ecosystem [99, 104, 105]. Remarkably, although the microbiome shifts and responds with daily living [99], the overall composition remains reasonably stable for months and possibly even years [104, 144]. By better understanding the boundaries of 'normal', this helps us to identify a dysbiotic microbiome and, more importantly, the bacteria that help maintain it and which cause disease. However, the majority of our understanding of intestinal microbiome composition and stability to date is based on stool studies from single points in time, not mucosal biopsies or longitudinal studies [3, 15, 103, 104, 144–146]. Although stool is an easily accessible sample, it does not inform us about what is happening in intestinal mucosal environment and the sites of inflammation.

There have been a several studies demonstrating that the microbiome profiles differ greatly between stool and mucosal biopsy, possibly skewing our understanding [4, 115, 120]. This distinction becomes integral given that intestinal microbiome modulation is increasingly looked at as a target for novel therapies such as faecal microbiota transplants (FMT). These novel therapies are being increasingly used as a final line of treatment for patients suffering with chronic and recurrent *Clostridium difficile* infections (CDI) where conventional front line treatments have failed. Once in a fringe and highly experiment treatment, the number and frequency of FMT are on the rise [147]. In response, biobanks for stool have been created at Massachusetts General Hospital in Boston and Emory University Hospital in Atlanta, where 'healthy' stool is screened and stored for medical use in approved cases [148]. Non-randomised studies of CDI patients treated with either antibiotics only or FMT showed a remarkable success rate of about 90 % [149–151]. To date the majority of testimonials regarding FMT outcomes are positive although there are still many unknowns. Outside of *C. difficile* treatment, there are no published clinical trials for many conditions that FMT is being proposed such as AS, IBD, irritable bowel syndrome, Crohn's disease and even multiple sclerosis. Screening of the donor faeces is currently not standardised and can be rudimentary. Humans carry different combinations of bacteria, viruses and parasites at any given moment in time [103]. Whilst the particular microbial composition that has evolved in one person may not be harmful, transplanting it into another person with a different genetic make-up may also transplant potentially pathogenic microbes or combinations of microbes causing unexpected outcomes. One example of this is a reported case where a patient with CDI underwent a successful FMT; however, post transplant, the patient developed new-onset obesity [152]. In this particular case, the faecal material for transplant was provided by an otherwise healthy but overweight donor; and these transplanted microbes may have led to an unintentional and rapid weight gain [152]. This effect has also been document in mouse studies where microbiomes from obese mice, when transplanted into a lean host, can cause obesity [14]. This highlights the need for long-term safety data for FMT recipients [153]. Given the increasing evidence that the gut microbiome

plays a role in metabolic diseases (such as obesity) and immune-mediated diseases at the least, it needs to be established whether FMT may inadvertently induce other diseases.

The importance of underlying host genetics in community composition [108, 109] raises some important questions when considering new procedures aimed at modifying microbiomes. If underlying host genetics influences the structure and composition of the intestinal microbiome, would microbiome modulation through diet changes or FMT in complex genetic diseases be successful long term? In diseases such as AS and IBD, the expectation is that since the microbiome influences the immune system, transplanting with a healthy intestinal flora would lead to a less inflammatory intestinal environment reducing gut disease. This would hopefully improve gastrointestinal symptoms and maybe even disease. The caveat here is that many genes associated with AS and IBD are involved in microbial sensing and processing, as well as mucosal immunology. The concern is that underlying host genetics may eventually override the transplant.

The pursuit to understand the pathogenesis of spondylarthritis and thereby enabling accurate, early diagnosis and effective treatment requires better understanding of how underlying host genetics influences the immune system and the intestinal microbiome. What is clear is the need to move away from generalised treatment approaches and more towards individually tailored solutions. Potentially, 16S microbial profiling of samples, tissue or stool, either alone or in combination with genetic tests, may lead to early identification of individuals at high risk of developing spondylarthritis. Further, such technology may lead to subsequent monitoring and preventative intervention regimes that can be individually tailored to the patient.

References

1. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT (2012) Structure, function and diversity of the healthy human microbiome. Nature 486:207–214
2. Burns M, Lynch J, Starr T, Knights D, Blekhman R (2015) Virulence genes are a signature of the microbiome in the colorectal tumor microenvironment. Genome Med 7(1):55
3. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE et al (2009) A core gut microbiome in obese and lean twins. Nature 457(7228):480–484
4. Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van† Treuren W, Ren B et al (2014) The treatment-naive microbiome in new-onset Crohn s disease. Cell Host Microbe 15(3):382–392
5. Knights D, Silverberg M, Weersma R, Gevers D, Dijkstra G, Huang H et al (2014) Complex host genetics influence the microbiome in inflammatory bowel disease. Genome Med 6(12):107
6. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N et al (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci 107(26):11971–11975
7. Huh SY, Rifas-Shiman SL, Zera CA, Edwards JWR, Oken E, Weiss ST, et al (2012) Delivery by caesarean section and risk of obesity in preschool age children: a prospective cohort study. Arch Dis Child 97(7):610–616

8. Bager P (2011) Birth by caesarean section and wheezing, asthma, allergy, and intestinal disease. Clin Exp Allergy 41(2):147–148
9. Arrieta M-C, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S et al (2015) Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med 7(307):307ra152–307ra152
10. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende D et al (2011) Enterotypes of the human gut microbiome. Nature 473:174–180
11. Ursell LK, Haiser HJ, Van Treuren W, Garg N, Reddivari L, Vanamala J et al (2014) The intestinal metabolome: an intersection between microbiota and host. Gastroenterology 146(6):1470–1476
12. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9(5):313–323
13. Clemente J, Ursell L, Parfrey L, Knight R (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148:1258–1270
14. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3:213–223
15. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M et al (2012) Human gut microbiome viewed across age and geography. Nature 486(7402):222–227
16. Maslowski KM, Mackay CR (2011) Diet, gut microbiota and immune responses. Nat Immunol 12(1):5–9
17. Kranich J, Maslowski KM, Mackay CR (2011) Commensal flora and the regulation of inflammatory and autoimmune responses. Semin Immunol 23(2):139–145
18. Perdomo OJ, Cavaillon JM, Huerre M, Ohayon H, Gounon P, Sansonetti PJ (1994) Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. J Exp Med 180(4):1307–1319
19. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV (2008) Paneth Cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci 105(52):20858–20863
20. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U et al (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139(3):485–498
21. Duan J, Chung H, Troy E, Kasper DL (2010) Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing [gamma]/[delta] T cells. Cell Host Microbe 7(2):140–150
22. Cho I, Blaser M (2012) The human microbiome: at the interface of health and disease. Nat Rev Genet 13:10
23. Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ (2011) Microbiota restoration: natural and supplemented recovery of human microbial communities. Nat Rev Micro 9(1):27–38
24. Laurence A, O'Shea JJ, Watford WT (2008) Interleukin-22: a sheep in wolf's clothing. Nat Med 14(3):247–249
25. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E et al (2010) Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 11(1):76–82
26. Maloy KJ, Powrie F (2011) Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474(7351):298–306
27. Koslowski MJ, Beisner J, Stange EF, Wehkamp J (2010) Innate antimicrobial host defense in small intestinal Crohn's disease. Int J Med Microbiol 300(1):34–40
28. Wehkamp J, Koslowski M, Wang G, Stange EF (2008) Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. Mucosal Immunol 1(1s):S67–S74
29. Artis D (2008) Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 8(6):411–420

30. Hooper LV, Macpherson AJ (2010) Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol 10(3):159–169
31. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K et al (2013) Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science 342(6157):447–453
32. Heazlewood C, Cook M, Eri R, Price G, Tauro S, Taupin D et al (2008) Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 5:e54
33. Sherlock J, Joyce-Shaikh B, Turner S, Chao C, Sathe M, Grein J et al (2012) IL-23 induces spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells. Nat Med 18:1069–1076
34. Fasano A, Nataro JP (2004) Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins. Adv Drug Deliv Rev 56(6):795–807
35. Collins SM. IV (2001) Modulation of intestinal inflammation by stress: basic mechanisms and clinical relevance. 2001-03-01 00:00:00. G315-G8 p
36. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM et al (2003) Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol 171(11):6164–6172
37. Nusrat A, Turner JR, Madara JL. IV (2000) Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. 2000-11-01 00:00:00. G851-G7 p
38. Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I (1992) Intestinal permeability in patients with Crohn's disease and their first degree relatives. Gut 33(3):320–323
39. Lubrano E, Ciacci C, Ames P, Mazzacca G, Oriente P, Scarpa R (1996) The arthritis of coeliac disease: prevalence and pattern in 200 adult patients. Rheumatology 35:1314–1318
40. Podolsky DK (2002) Inflammatory bowel disease. N Eng J Med 347(6):417–429
41. Mielants H, Veys EM, Goemaere S, Goethals K, Cuvelier C, De Vos M (1991) Gut inflammation in the spondyloarthropathies: clinical, radiologic, biologic and genetic features in relation to the type of histology. A prospective study. J Rheumatol 18(10):1542–1551
42. Vaile J, Meddings J, Yacyshyn B, Russell A, Maksymowych W (1999) Bowel permeability and CD45RO expression on circulating CD20+ B cells in patients with ankylosing spondylitis and their relatives. J Rheumatol 26:128–135
43. Martinez-Gonzalez O, Cantero-Hinojosa J, Paule-Sastre P, Gomez-Magan JC, Salvatierra-Rios D (1994) Intestinal permeability in patients with ankylosing spondyllitis and their healthy relatives. Rheumatology 33(7):644–647
44. Chabot S, Wagner JS, Farrant S, Neutra MR (2006) TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. J Immunol 176(7):4275–4283
45. Suzuki K, Ha S, Tsuji M, Fagarasan S (2007) Intestinal IgA synthesis: a primitive form of adaptive immunity that regulates microbial communities in the gut. Semin Immunol 19:127–135
46. Wesa A, Galy A (2002) Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. BMC Immunol 3:14
47. Cruz CM, Rinna A, Forman HJ, Ventura ALM, Persechini PM, Ojcius DM (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 282(5):2871–2879
48. Hirotani T, Lee PY, Kuwata H, Yamamoto M, Matsumoto M, Kawase I et al (2005) The nuclear IkB protein IkBNS selectively inhibits lipopolysaccharide-induced IL-6 production in macrophages of the colonic lamina propria. J Immunol 174(6):3650–3657
49. Denning T, Wang Y-C, Patel S, Williams I, Pulendran B (2007) Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol 8:1086–1094
50. Glocker E, Kotlarz D, Boztug K, Gertz E, Schaffer A, Noyan F et al (2009) Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 361:2033–2045

51. Consortium TUIGCtWTCC (2009) Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. Nat Genet 41(12):1330–1334
52. Franke A, Balschun T, Sina C, Ellinghaus D, Hasler R, Mayr G et al (2010) Genome-wide association study for ulcerative colitis identifies risk loci at 7q22 and 22q13 (IL17REL). Nat Genet 42(4):292–294
53. Oppmann B, Lesley R, Blom B, Timans J, Xu Y, Hunte B et al (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13:715–725
54. Burton P, Clayton D, Cardon L, Craddock N, Deloukas P, Duncanson A et al (2007) Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. Nat Genet 39:1329–1337
55. Cargill M, Schrodi S, Chang M, Garcia V, Brandon R, Callis K et al (2007) A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. Am J Hum Gen 80:273–290
56. Duerr R, Taylor K, Brant S, Rioux J, Silverberg M, Daly M et al (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 314:1461–1463
57. Xavier JM, Shahram F, Davatchi F, Rosa A, Crespo J, Abdollahi BS et al (2012) Association study of IL10 and IL23R–IL12RB2 in Iranian patients with Behçet's disease. Arthritis Rheum 64(8):2761–2772
58. Ogawa A, Andoh A, Araki Y, Bamba T, Fujiyama Y (2004) Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. Clin Immunol 110:55–62
59. Zheng S, Wang J, Horwitz D (2008) Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. J Immunol 180:7112–7116
60. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II et al (2009) Lymphoid tissue inducer–like cells are an innate source of IL-17 and IL-22. J Exp Med 206(1):35–41
61. Kobori A, Yagi Y, Imaeda H, Ban H, Bamba S, Tsujikawa T et al (2010) Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. J Gastroenterol 45(10):999–1007
62. Lockhart E, Green AM, Flynn JL (2006) IL-17 production is dominated by γδ T cells rather than CD4 T cells during mycobacterium tuberculosis infection. J Immunol 177(7):4662–4669
63. Sonnenberg GF, Fouser LA, Artis D (2011) Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol 12(5):383–390
64. Cua DJ, Tato CM (2010) Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol 10(7):479–489
65. Mokuno Y, Matsuguchi T, Takano M, Nishimura H, Washizu J, Ogawa T et al (2000) Expression of toll-like receptor 2 on gammadelta T cells bearing invariant Vgamma6/Vdelta1 induced by Escherichia coli infection in mice. J Immunol 165:931–940
66. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M (2009) Interleukin-17-producing [gamma][delta] T cells selectively expand in response to pathogen products and environmental signals. Immunity 31(2):321–330
67. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y (2007) Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. J Immunol 178:4466–4472
68. Roark C, French J, Taylor M, Bendele A, Born W, O'Brien R (2007) Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. J Immunol 179:5576–5583
69. Nanno M, Kanari Y, Naito T, Inoue N, Hisamatsu T, Chinen H et al (2008) Exacerbating role of gammadelta T cells in chronic colitis of T-cell receptor alpha mutant mice. Gastroenterology 134:481–490

70. Kenna T, Davidson S, Duan R, Bradbury L, McFarlane J, Smith M et al (2012) Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive gamma/delta T cells in patients with active ankylosing spondylitis. Arthritis Rheum 64:1420–1429
71. Bendelac A, Savage P, Teyton L (2007) The biology of NKT cells. Ann Rev Immunol 25:297–336
72. Rachitskaya A, Hansen A, Horai R, Li Z, Villasmil R, Luger D et al (2008) Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6- independent fashion. J Immunol 180:5167–5171
73. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T et al (2003) Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat Med 9:582–588
74. Baxter A, Kinder S, Hammond K, Scollay R, Godfrey D (1997) Association between alphabetaTCR+CD4-CD8- T-cell deficiency and IDDM in NOD/Lt mice. Diabetes 46:572–582
75. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W (2002) Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity 17(5):629–638
76. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M et al (2005) Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of Vα14 natural killer T cells in mice. Inflamm Bowel Dis 11(1):35–41
77. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B et al (2012) Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. Gastroenterology 143:418–428
78. Coppieters K, Van Beneden K, Jacques P, Dewint P, Vervloet A, Vander Cruyssen B et al (2007) A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner. J Immunol 179:2300–2309
79. Jacques P, Venken K, Van Beneden K, Hammad H, Seeuws S, Drennan M et al (2010) Invariant natural killer T cells are natural regulators of murine spondylarthritis. Arthritis Rheum 62:988–999
80. Singh A, Misra R, Aggarwal A (2011) Th-17 associated cytokines in patients with reactive arthritis/undifferentiated spondyloarthropathy. Clin Rheumatol 30(6):771–776
81. Gold M, Cerri S, Smyk-Pearson S, Cansler M, Vogt T, Delepine J et al (2010) Human mucosal associated invariant T cells detect bacterially infected cells. PLoS Biol 8:e1000407
82. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M et al (2010) Antimicrobial activity of mucosal associated invariant T cells. Nat Immunol 11:701–708
83. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D et al (2011) Human MAIT cells are xenobiotic resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood 117:1250–1259
84. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F et al (2003) Selection of evolutionarily conserved mucosalassociated invariant T cells by MR1. Nature 422:164–169
85. Savage A, Constantinides M, Han J, Picard D, Martin E, Li B et al (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 29:391–403
86. Billerbeck E, Kang Y, Walker L, Lockstone H, Grafmueller S, Fleming V et al (2010) Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. Proc Natl Acad Sci U S A 107:3006–3011
87. Le Bourhis L, Guerri L, Dusseaux M, Martin E, Soudais C, Lantz O (2011) Mucosal associated invariant T cells: unconventional development and function. Trends Immunol 32:212–218
88. Leeansyah E, Loh L, Nixon DF, Sandberg JK (2014) Acquisition of innate-like microbial reactivity in mucosal tissues during human fetal MAIT-cell development. Nat Commun 5:3143

89. Qiu J, Heller Jennifer J, Guo X, Chen Z-ming E, Fish K, Fu Y-X et al (2012) The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity 36(1):92–104
90. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, Kleinschek M et al (2011) ROR[gamma]t+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nat Immunol 12(4):320–326
91. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ et al (2010) Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 464(7293):1371–1375
92. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz J et al (2009) A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 457:722–725
93. Wu H-J, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y et al (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 32(6):815–827
94. Wang S, Charbonnier L-M, Noval Rivas M, Georgiev P, Li N, Gerber G et al (2015) MyD88 adaptor-dependent microbial sensing by regulatory T cells promotes mucosal tolerance and enforces commensalism. Immunity 43(2):289–303
95. Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci 104(34):13780–13785
96. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D et al (2012) Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. PLoS One 7(6):e39242
97. Elinav E, Strowig T, Kau Andrew L, Henao-Mejia J, Thaiss Christoph A, Booth Carmen J et al (2011) NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 145(5):745–757
98. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y et al (2011) Induction of colonic regulatory T cells by indigenous clostridium species. Science 331:337–341
99. David L, Materna A, Friedman J, Campos-Baptista M, Blackburn M, Perrotta A et al (2014) Host lifestyle affects human microbiota on daily timescales. Genome Biol 15(7):R89
100. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE et al (2014) Diet rapidly and reproducibly alters the human gut microbiome. Nature 505(7484):559–563
101. Group TNHW, Peterson J, Garges S, Giovanni M, McInnes P, Wang L et al (2009) The NIH human microbiome project. Genome Res 19(12):2317–2323
102. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464(7285):59–65
103. Consortium HMP (2012) Structure, function and diversity of the healthy human microbiome. Nature 486(7402):207–214
104. Caporaso JG, Lauber C, Costello E, Berg-Lyons D, Gonzalez A, Stombaugh J et al (2011) Moving pictures of the human microbiome. Genome Biol 12(5):R50
105. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R (2009) Bacterial community variation in human body habitats across space and time. Science 326(5960):1694–1697
106. Woese CR (1987) Bacterial evolution. Microbiol Rev 51(2):221–271
107. Booijink CCGM, Boekhorst J, Zoetendal EG, Smidt H, Kleerebezem M, de Vos WM (2010) Metatranscriptome analysis of the human fecal microbiota reveals subject-specific expression profiles, with genes encoding proteins involved in carbohydrate metabolism being dominantly expressed. Appl Environ Microbiol 76(16):5533–5540
108. Spor A, Koren O, Ley R (2011) Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Micro 9(4):279–290
109. Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J et al (2010) Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci 107(44):18933–18938

110. Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CCGM, Troost FJ et al (2012) The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J 6(7):1415–1426
111. Leimena M, Ramiro-Garcia J, Davids M, van den Bogert B, Smidt H, Smid E et al (2013) A comprehensive metatranscriptome analysis pipeline and its validation using human small intestine microbiota datasets. BMC Genomics 14(1):530
112. Stebbings S, Munro K, Simon MA, Tannock G, Highton J, Harmsen H et al (2002) Comparison of the faecal microflora of patients with ankylosing spondylitis and controls using molecular methods of analysis. Rheumatology 41(12):1395–1401
113. Stone MA, Payne U, Schentag C, Rahman P, Pacheco-Tena C, Inman RD (2004) Comparative immune responses to candidate arthritogenic bacteria do not confirm a dominant role for Klebsiella pneumonia in the pathogenesis of familial ankylosing spondylitis. Rheumatology 43(2):148–155
114. Costello M-E, Ciccia F, Willner D, Warrington N, Robinson PC, Gardiner B et al (2015) Brief report: intestinal dysbiosis in ankylosing spondylitis. Arthritis Rheum 67(3):686–691
115. Lin P, Bach M, Asquith M, Lee AY, Akileswaran L, Stauffer P et al (2014) HLA-B27 and human β2-microglobulin affect the gut microbiota of transgenic rats. PLoS One 9(8): e105684
116. Rath H, Herfarth H, Ikeda J, Grenther W, Hamm T, Balish E et al (1996) Normal luminal bacteria, especially bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J Clin Invest 98:945–953
117. Rehaume LM, Mondot S, Aguirre de Cárcer D, Velasco J, Benham H, Hasnain SZ et al (2014) ZAP-70 genotype disrupts the relationship between microbiota and host, leading to spondyloarthritis and ileitis in SKG mice. Arthritis Rheum 66(10):2780–2792
118. Eerola E, Mottonen T, Hannonen P, Luukkainen R, Kantola I, Vuori K et al (1994) Intestinal flora in early rheumatoid arthritis. Rheumatology 33:1030–1038
119. Vaahtovuo J, Munukka E, Korkeamaki M, Luukainen R, Toivanen P (2008) Fecal microbiota in early rheumatoid arthritis. J Rheumatol 35(8):1500–1505
120. Eckburg P, Bik E, Bernstein C, Purdom E, Dethlefsen L, Sargent M et al (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638
121. Martinez-Martinez RE, Abud-Mendoza C, Patiño-Marin N, Rizo-Rodríguez JC, Little JW, Loyola-Rodríguez JP (2009) Detection of periodontal bacterial DNA in serum and synovial fluid in refractory rheumatoid arthritis patients. J Clin Periodontol 36(12):1004–1010
122. Wegner N, Wait R, Sroka A, Eick S, Nguyen K-A, Lundberg K et al (2010) Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. Arthritis Rheum 62(9):2662–2672
123. Gaiffer MH, Holdsworth CD, Duerden BI (1991) The assessment of faecal flora in patients with inflammatory bowel disease by a simplified bacteriological technique. J Med Microbiol 35(4):238–243
124. Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P et al (2003) Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut 52(2):237–242
125. Ott SJ, Musfeldt M, Ullmann U, Hampe J, Schreiber S (2004) Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora. J Clin Microbiol 42(6):2566–2572
126. Walker A, Sanderson J, Churcher C, Parkes G, Hudspith B, Rayment N et al (2011) High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiol 11:7
127. Quinton JF (1998) Anti-Saccharomyces cerevisiae mannan antibodies combined with anti-neutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. Gut 42:788–791
128. Targan SR (2005) Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. Gastroenterology 128:2020–2028

129. Mundwiler M, Mei L, Landers C, Reveille J, Targan S, Weisman M (2009) Inflammatory bowel disease serologies in ankylosing spondylitis patients: a pilot study. Arthritis Res Ther 11(6):R177
130. Wallis D (2013) Elevated serum anti-flagellin antibodies implicate subclinical bowel inflammation in ankylosing spondylitis: an observational study. Arthritis Res Ther 15:R166
131. van Praet L (2013) Microscopic gut inflammation in axial spondyloarthritis: a multiparametric predictive model. Ann Rheum Dis 72:414–417
132. Krueger JG (2002) The immunologic basis for the treatment of psoriasis with new biologic agents. J Am Acad Dermatol 46(1):1–26
133. Fahlen A, Engstrand L, Baker B, Powles A, Fry L (2012) Comparison of bacterial microbiota in skin biopsies from normal and psoriatic skin. Arch Dermatol Res 304:15–22
134. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM et al (2015) Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. Arthritis Rheum 67(1):128–139
135. Dethlefsen L, Relman DA (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci 108(Supplement 1):4554–4561
136. Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L (2010) Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS One 5(3):e9836
137. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A et al (2012) Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to clostridium difficile-induced colitis. Infect Immun 80(1):62–73
138. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL et al (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 341(6150):1241214
139. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A et al (2012) Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il102/2 mice. Nature 487(7405):104–108
140. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C et al. (2013) Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Mathis D, editor. 2013-11-05 15:22:27
141. Scher JU, Abramson SB (2011) The microbiome and rheumatoid arthritis. Nat Rev Rheumatol 7(10):569–578
142. Norman JM, Handley SA, Virgin HW (2014) Kingdom-agnostic metagenomics and the importance of complete characterization of enteric microbial communities. Gastroenterology 146(6):1459–1469
143. Virgin HW (2014) The virome in mammalian physiology and disease. Cell 157(1):142–150
144. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL et al (2013) The long-term stability of the human gut microbiota. Science (New York, NY) 341(6141):1237439
145. Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. Nature 444:1022–1023
146. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312(5778):1355–1359
147. van Nood E, Speelman P, Nieuwdorp M, Keller J (2014) Fecal microbiota transplantation: facts and controversies. Curr Opin Gastroenterol 30(1):34–39. doi:10.1097/MOG.0000000000000024
148. Smith M, Kassam Z, Edelstein C, Burgess J, Alm E (2014) OpenBiome remains open to serve the medical community. Nat Biotech 32(9):867
149. Kassam Z, Lee CH, Yuan Y, Hunt RH (2013) Fecal microbiota transplantation for clostridium difficile infection: systematic review and meta-analysis. Am J Gastroenterol 108(4):500–508
150. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM et al (2013) Duodenal infusion of donor feces for recurrent clostridium difficile. N Eng J Med 368(5):407–415

151. Youngster I, Sauk J, Pindar C, Wilson RG, Kaplan JL, Smith MB et al (2014) Fecal microbiota transplant for relapsing clostridium difficile infection using a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. Clin Infect Dis 58(11):1515–1522
152. Alang N, Kelly CR (2015) Weight gain after fecal microbiota transplantation. Open Forum Infect Dis 2(1):ofv004
153. Petrof E, Gloor G, Vanner S, Weese S, Carter D, Daigneault M et al (2013) Stool substitute transplant therapy for the eradication of clostridium difficile infection: 'RePOOPulating' the gut. Microbiome 1(1):1–12

Stem Cell Therapy in the Treatment of Rheumatic Diseases and Application in the Treatment of Systemic Lupus Erythematosus

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Abbreviations

ACR	American College of Rheumatology
Allo-HSCT	Allogeneic hematopoietic stem cell therapy
ANCA	Antineutrophil cytoplasmic antibody
ARD	Autoimmune rheumatic diseases
ASC	Adult stem cells
ASSIST	American Scleroderma Stem Cell Versus Immune Suppression Trial
ASTIRA	Autologous Stem Cell Transplantation International Rheumatoid Arthritis
Auto-HSCT	Autologous hematopoietic stem cell therapy
AZA	Azathioprine
BAFF	B-cell-activating factor

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BD	Behcet's disease
BLyS	Soluble B lymphocyte stimulator
CsA	Cyclosporine A
CYC	Cyclophosphamide
DM	Dermatomyositis
DMARD	Disease-modifying antirheumatic drugs
EBMT/EULAR	European Bone Marrow Transplant/European League Against Rheumatism
EMA	European Medicines Agency
ESC	Embryonic stem cells
Flu	Fludarabine
GVHD	Graft-versus-host disease
HCQ	Hydroxychloroquine
HSC	Hematopoietic stem cells
HSCT	HSC transplant
ILD	Interstitial lung disease
iPSC	Induced pluripotent stem cells
IRAKs	IL-1R-associated kinases
ISSCR	International Society for Stem Cell Research
JIA	Juvenile idiopathic arthritis
JSSc	Juvenile systemic sclerosis
LEF	Leflunomide
MMF	Mycophenolate mofetil
MS	Multiple sclerosis
MSC	Mesenchymal stem cells
MSCT	MSC transplant
MTX	Methotrexate
MyD88	Myeloid differentiation factor 88
PM	Polymyositis
PSV	Primary systemic vasculitis
QOL	Quality of life
RA	Rheumatoid arthritis
rbATG	Rabbit antithymocyte globulin
RTX	Rituximab
SCOT	"Scleroderma: Cyclophosphamide or Transplantation" Trial
SCT	Stem cell transplant
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
SS	Sjögren's syndrome
SSc	Systemic sclerosis
TAP2	Transporter associated with antigen processing 2
TBI	Total body irradiation
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cells
TRM	Transplant-related mortality

1 Biology of Stem Cells and History of Stem Cell Therapy

Stem cell therapy is one of the most fascinating areas in modern medicine. Stem cells are different from other cells in that (a) they are undifferentiated, (b) they can divide for long periods, and (c) they are capable of becoming specialized cell types. These unique characteristics have generated significant excitement in the scientific community to examine the biology underlying their distinct characteristics and more importantly, their application for cell-based therapy.

Three primary categories of stem cells exist: embryonic stem cells (ESC), adult stem cells (ASC), and induced pluripotent stem cells (iPSC) (Table 1). ESC are derived from the blastocysts during embryo development. ESC are pluripotent because they have the potential to self-renew and also to differentiate into any cell type. In the laboratory, ESC lines can remain undifferentiated under specific conditions. Undifferentiated ESC can directly undergo differentiation into specific functional cell types. It is envisioned that differentiated ESC can be used to cure diseases. Examples of clinical applications of ESC include diabetes, heart diseases, traumatic spinal cord injury, muscular dystrophy, and hearing and vision loss. iPSC are adult cells that have been genetically reprogrammed to dedifferentiate into behaving like ESC. Mouse iPSC were first reported in 2006 [1], and soon after, the first human iPSC were successfully generated in 2007 [2].

Research on ASC can be traced back to the 1950s when two kinds of stem cells were discovered in the bone marrow. The first one being hematopoietic stem cells (HSC) and the other being bone marrow stromal cells, which are also known as mesenchymal stem cells (MSC). Since then, ASC have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. HSC can differentiate into all blood cell lineages such as red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages [3]. MSC are multipotent and can give rise to a variety of cell types such as bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and stromal cells.

The potential applications of stem cells in clinical medicine are enormous. The unique property that allows stem cells to differentiate into specific cell types offers the possibility of a renewable source of replacement cells and tissues in cell-based therapy. Indeed, over 40 years ago, HSC transfer was initially conducted in the form of bone marrow transplantation with successful allogeneic transplantations performed for an infant with X-linked lymphopenic immune deficiency [4]. Stem cell therapy generated great enthusiasm in the 1980s as a targeted and permanent treatment for many previously incurable autoimmune disorders. In 1986, Jacobs et al. reported that allogeneic HSC transplant in a patient with drug-induced aplastic anemia and severe rheumatoid arthritis not only reversed the hematological abnormality but also simultaneously resulted in a 2-year period of relief from joint pain [5]. HSC therapy also resulted in significant clinical improvements in other autoimmune diseases [6–10]. In 1996, the First International Symposium on HSC Therapy in autoimmune rheumatic diseases (ARD) was convened in Basel, which led to the

Table 1 Comparison of three categories of stem cells and their clinical applications

	Adult stem cells	Embryonic stem cells	Induced pluripotent stem cells
Source	Various tissues and includes bone marrow, umbilical cord, and blood stem cells	Blastocysts from fertilized eggs	Viral or nonviral reprogramming of somatic cells
Potency	Multipotent	Pluripotent	Pluripotent
Laboratory features	1. Finite – may not live long in culture. Difficult to obtain in large numbers	1. Immortal – endless division in culture without losing function	1. Immortal – endless division in culture without losing function
	2. Less flexible – more difficult to reprogram to another tissue type	2. Plasticity – can be easily manipulated	2. Most difficult among these three to obtain or create
Immunogenic/rejection	Low risk (but with possible second autoimmune disease development)	High risk	Low risk
Ethical issues	No serious ethical issues involved	Destruction of developing life	No serious ethical issues involved
Clinical research/application	HSC therapy	Diabetes, heart diseases, traumatic spinal cord injury, muscular dystrophy, hearing loss, and vision loss	Relative new to science
	Systemic sclerosis		
	Rheumatoid arthritis		
	Systemic lupus		
	Erythematosus		
	Sjogren's syndrome		
	Juvenile idiopathic		
	Arthritis		
	MSC therapy		
	Multiple sclerosis		
	Osteoarthritis		
	Sjogren's syndrome		

development of the first consensus guidelines for HSCT in autoimmunity recommending standardized protocols and established the European Bone Marrow Transplant/European League Against Rheumatism (EBMT/EULAR) registry [11]. Since then, over 1,500 HSC transplants for ARD, including systemic sclerosis (SSc), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), and juvenile idiopathic arthritis (JIA), have been registered [12]. Despite its promise of potential long-term benefit, acute toxicity such as infection

and bleeding during the aplastic period, complications due to opportunistic infections during the T-cell reconstitution phase, and the possibility of developing a second autoimmune disease should be carefully considered [13, 14].

Multipotent MSC have recently gained significant attention in the treatment of ARD. MSC was initially isolated from guinea pig bone marrow as spindle-shaped cells with progenitor properties that adhered to plastic and formed fibroblast colonies [15]. MSC are not truly pluripotent, and most MSC described to date are actually multipotent progenitors obtained from a wide range of tissues such as bone marrow, umbilical cord, placenta, cord blood, adipose tissue, synovium, and teeth. Human MSC are now phenotypically characterized as $CD105^+$, $CD73^+$ and $CD90^+$, $CD45^-$, $CD34^-$, $CD14^-$ or $CD11b^-$, $CD79a^-$ or $CD19^-$, and negative for HLA class II molecules. Human MSC must also be plastic-adherent cells and have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts [16]. However, these criteria are not unique because CD105, CD73, or CD90 is also expressed on other cell populations, while other cell markers are also expressed in MSC [17]. Moreover, there are various sources of MSC with differentiation potentials that are different from the criteria described above [18]. Essentially, MSC represent a heterogeneous progenitor cell population with immunomodulatory properties, which are able to suppress T- and B-cell proliferation, inhibit the differentiation of monocytes into immature dendritic cells, and affect the functions of NK cells [19, 20]. MSC express low levels of cell surface HLA class I molecules and are negative for HLA class II molecules. Meanwhile, MSC do not express co-stimulatory molecules CD80, CD86, or CD40. Hence, MSC can easily escape immune surveillance [21]. Biologically, the regenerative, immune privileged, immunomodulatory, and tissue-protective properties of MSC suggest that these cells are effective therapeutic reagents in human diseases [20–24].

Preclinical studies have demonstrated the therapeutic efficacy of MSC in various rheumatic autoimmune disorders in animal models including multiple sclerosis (MS) [25–27], osteoarthritis [28–31], rheumatoid arthritis (RA) [32–36], and Sjogren's syndrome (SS) [37, 38]. The hallmark of the clinical application of MSC therapy was phase I study in which 23 patients experienced full remission after treatment of various hematological malignancies in 1995. Moreover, there were no adverse events after intravenous infusion of ex vivo expended bone marrow-derived MSC [39]. The first published report describing the application of MSC in therapeutic intervention was on breast cancer patients receiving high-dose chemotherapy. This study showed that MSC therapy was safe and had the potential to enhance HSC engraftment [40]. Thereafter, animal studies, in vitro, and clinical studies on MSC have increased rapidly.

2 Clinical Studies on SCT for Common Rheumatic Diseases

SCT has been successfully applied in patients with ARD. Here we will review a few clinical trials in SSc, RA, inflammatory myopathies, primary systemic vasculitis (PSV), SS, and pediatric ARDs, such as JIA.

2.1 Systemic Scleroderma (SSc)

SSc is a rare chronic systemic autoimmune disease with a prevalence rate of around 5 per 100,000 and an incidence of 1 in 100,000. Based on epidemiological data, approximately 3.4 million individuals are affected globally. Despite advances in early diagnosis and appropriate therapy, the prognosis of SSc patients remains poor, and the disease is associated with a high mortality [41]. The amenable treatment for SSc is immunosuppressive therapy. For example, the standard of regimens for interstitial lung disease (ILD) in SSc is CYC. Nevertheless, two randomized trials and meta-analyses showed no improvement in prognosis of SSc with CYC treatment [42].

In 1994, Ratanatharathorn et al. [43] reported the first successful HSC treatment in SSc patients with untreatable pulmonary hypertension which led to the gradual acceptance of HSC therapy as an optional treatment regimen for severe SSc. Complete or partial remission was observed in small case series and non-randomized clinical trials of HSCT treatment for SSc patients, although there were high rates of transplant-related mortality (TRM) [44]. Data from a single-center retrospective study of SSc patients who received auto-HSCT showed significant skin and lung disease amelioration in 78.3 % of patients at 6 months, and 91 % of patients achieved an overall good response. However, cardiac events result in 6 % TRM [45]. Another subsequent analysis of 57 SSc patients receiving HSCT from the EBMT/EULAR registry showed that sustained improvement in skin score and visceral organ functions was observed in two-thirds of the patients for up to 3 years after HSCT but with a TRM of 8.7 % [46]. TRM was reduced by pretransplant evaluation, early intervention, and the use of amenable conditioning regimen.

More recently, one phase II and two phase III randomized control trials were conducted to evaluate the efficacy, safety, and long-term side effects of auto-HSCT: the American Scleroderma Stem Cell Versus Immune Suppression Trial (ASSIST) [47], Autologous Stem Cell Transplantation International Scleroderma (ASTIS) [48, 49], and the "Scleroderma: Cyclophosphamide or Transplantation" trial (SCOT) [50].

ASSIST is a published open-label, randomized, controlled trial. The result of this phase II clinical trial demonstrated that unmanipulated auto-HSCT steadily ameliorated skin flexibility and pulmonary function defects in patients with SSc. This was found in patients treated with CYC whose disease progressed before being switched to HSCT. After 2 years of follow-up, patients receiving HSC therapy had durable remission in pulmonary function, reduction in interstitial lung lesions visualized on high-resolution CT imaging, and improved quality of life (QOL). More importantly, no TRM was reported [47]. Based on the success of phase II clinical trials [47], a phase III study is in progress to compare the safety and efficacy of the ASSIST trial pretransplant protocols of CYC and G rabbit antithymocyte globulin (rbATG) with the addition of rituximab [50]. In the multicenter phase III ASTIS trial [51], there is an increase in overall and survival benefit in patients administered with CYC

200 mg/kg and rbATG with CD34+ auto-HSCT compared to those with monthly pulse CYC treatment of HSCT that had a 10 % TRM. More stringent patient selection and safer conditioning regimens may reduce the TRM of ASTIS [52].

A controlled phase III SCOT trial was conducted to compare intensive immunotherapy and HSCT to monthly pulse CYC [50]. For future intertrial comparison, the SCOT trial shared identical end points and control regimen with the ASTIS trial, but the SCOT trial protocol employed transplant conditioning with total body irradiation (TBI), which differed from both the ASTIS and ASSIST trials that contained ATG as part of their immunoablative protocols [53].

A number of case reports [54, 55] indicated clear, positive therapeutic effects, without immediate toxicity nor severe infection in SSc patients receiving allo-HSCT and MSCT. Further studies using larger samples in randomized controlled trials are required to validate the efficacy and safety of allo-HSCT and MSCT.

2.2 Rheumatoid Arthritis (RA)

RA is a chronic, debilitating, systemic ARD affecting 1 % of the population [56]. Despite aggressive disease-modifying antirheumatic drugs (DMARD) approaches and efficient biologic agents in RA [56], a considerable proportion of RA patients still suffer from a severe, destructive, refractory disease [52, 57]. Besides biological agents, lymphoablative regimens combined with SCT have been employed as a therapeutic modality for refractory RA. The rationale for this approach is based on the concept of lymphoablation by high-dose chemotherapy, with a subsequent revival of naive T cells derived from reinfused hematopoietic progenitor cells [58].

In 1997, a disabled patient with refractory RA received auto-HSCT and became almost free of joint symptoms in half a year [59]. Since then, phase I/II clinical trials were set up to evaluate the feasibility, safety, and efficacy of auto-HSCT in patients with RA. From the 2001 EBMT/EULAR data, 43 patients from 11 centers underwent auto-HSCT. Among 39 patients evaluated, significant improvement in clinical response was observed in half of the patients, but the disease recurrence rate was around two-thirds within 2 years. One patient died as a consequence of sepsis [60]. In the 2004 data analysis of EBMT/EULAR, 73 refractory RA patients from 15 centers were given auto-HSCT and assessed for treatment response using the American College of Rheumatology (ACR) criteria. Two-thirds achieved an ACR50 improvement response. However, most patients restarted DMARD within 6 months due to persistent or relapsing disease activity. Interestingly, most patients were relatively sensitive to DMARD, which had proven refractory prior to HSCT [61].

In a CYC dose escalation followed by unmanipulated auto-HSCT study, the cohort receiving subablative dosage (100 mg/kg) developed disease recurrence within 3–4 months, while the cohort at the higher dosage (200 mg/kg) had durable remission for 17–19 months [62]. The most common protocol for auto-HSCT

treatment in RA includes CD34 selection and a lymphoablative, rather than myeloablative, conditioning regimen. Data from these heterogeneous studies indicate the feasibility and safety of auto-HSCT in RA. No severe adverse event or TRM was noted [61, 63].

Preclinical data and anecdotal evidence showed that allo-HSCT might be more effective than auto-HSCT [64]. In 1977, four patients with RA underwent allo-HSCT for gold-induced marrow aplasia. Three patients died from transplant-related toxicity. The one surviving patient had complete remission at 2 years follow-up [65]. Three other patients with RA receiving allo-HSCT reached long-term amelioration of their disease [66]. However, the TRM from nonmyeloablative allo-HSCT was 10–20 %. The risks of TRM and graft-versus-host disease (GVHD) may discourage physicians from recommending allo-HSCT to RA patients unless all other standard treatments have proven to be noneffective [64].

It is difficult to differentiate the poor prognosis and HSCT-responsive RA patients from refractory ones. Therefore, HSCT for RA patients should only be considered with extra caution. Prospective, randomized controlled long-term follow-up trials are urgently needed to evaluate the risk–benefit ratio. Unfortunately, the EBMT Autologous Stem Cell Transplantation International Rheumatoid Arthritis (ASTIRA) phase III trials were suspended because of failure to recruit sufficient patients [67].

To date, only limited clinical trials of MSCT in RA have been registered [68]. A single-center cohort study demonstrated that 136 patients with intractable RA receiving DMARD plus umbilical cord MSC had a rapid and effective remission. Moreover, repeated treatments achieved better clinical response and more clearly improved the QOL of intractable RA, without serious side effects [69]. It should be noted that the utilization of biologic agents has significantly altered the natural history of RA [70]. Therefore, SCT regimens only have a finite therapeutic potential in a portion of patients with RA, specifically those who fail to respond to currently available therapies [44, 61].

2.3 Sjögren's Syndrome (SS)

SS is the most common chronic, slowly progressive ARD, which typically affects the exocrine glands leading to xerostomia, keratoconjunctivitis sicca, and systemic features. Prevalence of SS varying from 0.1 to 4.8 % has been estimated using different criteria for classification among different study populations, and patients with SS have a 20- to 40-fold increased risk of developing lymphoma [71]. Currently, clinical management of SS remains challenging because of a lack of effective therapeutic agents.

Only a limited number of case reports of HSCT in SS are available. Three SS patients with refractory systemic vasculitis or lymphoma receiving auto-HSCT developed amelioration of the vasculitis and lymphoma but not the SS [72]. Two other patients with severe and refractory SS were able to tolerate high-dose

immunosuppressive drugs and auto-HSCT and had temporary alleviation of disease [73].

Recently, clinical data from 24 refractory SS patients receiving MSCT demonstrated the feasibility, safety, and efficacy of MSCT. In this study, most SS patients reach durable increased salivary flow rate, considerable improvements in disease activity, and organ function after MSCT [38].

2.4 Primary Systemic Vasculitis (PSV)

PSV, as well as its related conditions, Behcet's disease (BD) and relapsing polychondritis, belongs to a heterogeneous group of autoimmune diseases with severe organ damage and an often fatal course [74]. With the development of early diagnosis and optimal standard therapy, the outcome of PSV has been dramatically transformed into a controllable disease. However, one quarter of patients with PSV are resistant [75] to current treatment, and half of them suffer from disease recurrence despite at least 2 years of therapy. There is therefore an obvious unmet need in the treatment of PSV [76].

SCT in PSV is limited. Retrospective analysis of 15 patients with various PSV and related diseases from EBMT showed that 14 of them first received auto-HSCT, while one additional patient received allo-HSCT. Remission rate was beyond 90 %, but one-third had a recurrence of the disease [77]. In a single-center study, four patients with refractory SV with neurological system involvement received nonmyeloablative auto-HSCT. Among them, three patients recovered completely. One patient with BD did not respond to HSCT. No TRM or adverse reactions were noted [72].

The first report of a patient with antineutrophil cytoplasmic antibody (ANCA)-associated renal vasculitis treated beyond conventional therapy with MSCT demonstrated an abrupt and striking recovery from disease, which was clearly confirmed with a second infusion [75]. However, another small clinical study showed that MSCT could not reverse BD's retinal vasculitis process, which might be due to the late and advanced stage of disease [78].

2.5 Polymyositis (PM) and Dermatomyositis (DM)

Inflammatory myopathies are a heterogeneous group of rare conditions including PM and DM characterized by muscle weakness and inflammation [79]. The approximate incidence in the United States is five to ten cases per million. Unfortunately, PM/DM can induce marked disability and mortality unless properly recognized and timely and aggressive therapy is given [80]. In severe refractory cases of PM/DM, auto-HSCT may be a salvage strategy.

In one case report, a severe refractory PM patient with anti-Jo-1 antibody received auto-HSCT after T-cell-depleted myeloablative conditioning with CYC. The patient's strength and respiratory function significantly improved. Chest CT imaging showed remarkable reduction of interstitial shadows [81]. Five other patients with rapidly progressive and refractory ILD due to PM/DM were also successfully treated with auto-HSCT. After treatment, the patients' dyspnea disappeared, and arterial blood gas analysis and pulmonary function testing significantly improved. CT imaging showed a remarkable reduction of interstitial infiltrates [82].

An open-label pilot study of ten patients with intractable PM/DM who underwent allo-MSCT was conducted. Most patients achieved clinical remission along with improved laboratory parameters and tapering of medications. However, none of the patients could completely withdraw therapy after following up for about 1 year. It should also be noted that two patients died following MSCT from disease relapse after infection [83]. To date, the sample size with PVS and PM/DM receiving SCT is still too minute to draw any definitive conclusion.

2.6 SCT in Pediatric ARD

In children, ARD such as JIA, juvenile systemic sclerosis (JSSc), juvenile SLE, and others are a major cause of morbidity, which is due to both the disease itself and conventional treatment strategies and especially holds true for the subset of patients with severe or refractory disease [84]. Although recent advances in the understanding of the pathogenesis of these diseases have led to significant progress in treat-to-target approach, some ARD patients continue to be refractory to standard treatment. The rate of death in pediatric ARD is about 2–4 % [85]. In recent decades, SCT has been successfully employed in severe and refractory pediatric ARD as a novel salvage strategy.

On the other hand, one needs to seriously consider that conditioning regimens at pretransplantation are associated with a high rate of growth retardation, infertility, and late tumors in children with ARD [86]. With advances in SCT techniques, the rate of morbidity and mortality associated with transplantation procedures has been decreasing. Not only are the pretreatment strategies safer and less intense, but antibiotics and antifungal drugs are also more effective. To date, the preferred conditioning regimen used in JIA is a nonmyeloablative regimen of CYC, rbATG, and fludarabine (Flu) [87].

Although it is widely believed that allo-HSCT is a more effective and potentially curative regimen compared to auto-HSCT, there is no statistical difference in long-term survival. Unfortunately, the risk of allo-HSCT-related adverse event is still high, and the risks from GVHD and TRM were generally not acceptable for the pediatric ARD [87]. So far, there are no clinical trials on the use of MSCT in pediatric ARD.

2.7 Juvenile Idiopathic Arthritis (JIA)

JIA is the most common ARD in children [88]. Despite the application of novel treatment regimens, the prognosis of JIA is still poor, especially in children with systemic and polyarticular onset. The mortality of JIA is approximately 0.2 % [85]. Since 1997, HSCT has been successfully applied in intractable JIA. The first four patients with severe refractory JIA receiving auto-HSCT all had complete remission and went off drug therapy [89]. Retrospective analysis of a multicenter cohort of 34 patients with JIA after auto-HSCT demonstrated that 53 % reached drug-free full remission, 18 % had partial recovery, and 21 % did not respond to the procedure, with a TRM of 9 %. All partial and complete recurrence of disease happened in the first 18 months post-HSCT [90]. In a multicenter, prospective, phase II clinical trial, 22 children with refractory progressive JIA underwent T-cell-depleted auto-HSCT with a regimen of myeloablative and immunoablative including CYC, ATG, and TBI. After a median follow-up of 80 months, eight patients reached durable full remission, seven responded partially, and five experienced a relapse of disease. However, there was a 9 % TRM [91].

HSCT has significantly improved QOL for refractory JIA. However, as expected, already damaged joints did not improve nor worsen. If managed before the DMARD treatment and before any severe permanent joint destruction, HSCT is likely to reverse what would otherwise have become a permanent defect. Therefore, it is important to screen carefully those patients who are apt to benefit from HSCT [87].

2.8 Juvenile Systemic Sclerosis (JSSc)

Compared with the adult form, JSSc appears to have a better outcome. However, children with diffuse skin thickening and lung involvement had a 5-year mortality of 10 % [92]. Up to this point in time, those with JSSc treated with HSCT have been included in two groups of clinical studies. The data from the EBMT database showed that five JSSc received auto-HSCT, and three of them achieved clinical remission at 23 months follow-up [93], while one had a recurrence of the disease [46]. Another five JSSc patients were included in a clinical study on 26 patients with SSc. After auto-HSCT, three children achieved full remission after more than 5 years of follow-up [93].

2.9 Juvenile Dermatomyositis (JDM) and Juvenile Vasculitis (JV)

Recently, two children with severe progressive refractory DM were administered auto-HSCT. Both had a dramatic improvement in QOL, and sustained recovery was

noted [94]. Notably a 9-year-old girl with severe resistant granulomatosis and polyangiitis became disease-free after allo-HSCT following reduced-intensity conditioning [95].

3 Stem Cell Transplantation in the Treatment of Systemic Lupus Erythematosus

3.1 The Natural History and Epidemiology of SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease of unknown etiology and clinical heterogeneity. Patients with SLE are presented with diverse clinical symptoms: skin lesions, arthritis, renal disorder, neurologic disorder, and hematologic changes. Major biomarkers include antinuclear antibodies, anti-dsDNA antibody and anti-Sm antibody. Immune-mediated injury in multiple organs leads to high mortality and morbidity [96–104]. Besides the immune imbalance, evidence from familial studies together with high concordance among monozygotic twins suggests the contribution of genetics in SLE [105–108]. To date, there are many single genes, such as coagulation factor II gene (F2), TAP2 (transporter associated with antigen processing 2) gene, VKORC1gene, and autosomal gene, which are implicated in the pathogenesis of SLE [109–112]. Various environmental agents and toxicants, such as cigarette smoke, alcohol, plastic and electronic products, cosmetic agents, occupationally and non-occupationally related chemicals, ultraviolet light, infections, sex hormones, and certain medications and vaccines, are found to be associated with SLE onset or flares [113–115].

The incidence and prevalence of SLE varies considerably worldwide, ranging from 15 to 100 per 100,000 individuals among different racial groups [116]. SLE appears to be more prevalent in certain ethnic groups, such as the African-Americans, African-Caribbeans, and Asians, but it is also reported that there is a trend toward higher incidence and prevalence of SLE in Europe and Australia compared to the United States [117]. The reported prevalence of SLE in Asian countries varies from 20 to 59 per 100,000 [118, 119].

3.2 The Deficiency of Traditional Treatment in SLE

Traditional therapies for the treatment of SLE, notably corticosteroids and immunosuppressive drugs, have led to a significant improvement in survival over the last two to three decades and decreased the progression to end-stage multi-organ failure. The most widely and classically used immunosuppressors include cyclophosphamide (CYC), mycophenolate mofetil (MMF), leflunomide (LEF), methotrexate (MTX), cyclosporine A (CsA), azathioprine (AZA), and hydroxychloroquine (HCQ). Each of these agents, however, can carry high toxicities and many side

effects. They include osteoporosis and dyslipidemia induced by corticosteroids, myelotoxicity and gonadal injury induced by CYC, gastrointestinal discomfort and liver dysfunction by MTX or LEF, and hypertension and nephrotoxicity by CsA. The main concern for the side effects of corticosteroids and immunosuppressive therapies is infection [120]. CYC, particularly in combination with high-dose steroids, is reported to have the strongest effect in suppressing immune responses against microorganisms. The most common infections in patients with SLE treated with these traditional drugs include virus herpes zoster, mycobacterium tuberculosis, cytomegalovirus, Epstein–Barr virus, and fungal infections [120–124]. These infections may worsen the disease and aggravate the economic burden of patients. Furthermore, steroids and immunosuppressive drugs are not universally effective, with partial or no response in many cases.

3.3 The Efficacy and Deficiencies of the New Treatments (Including Small Molecules and Biological Agents)

Over the past decade, due to a better understanding of SLE immunopathogenesis, many new drugs have been developed to target specific immune cells, co-stimulatory modulation, or cytokines thought to be central to the disease pathogenesis, with the aim of achieving better control of the disease with fewer side effects.

B cells have long been considered central to the pathogenesis of SLE and have been regarded as an important target for biologic drugs. Several B-cell-targeted drugs have been developed. Rituximab (RTX), a monoclonal antibody targeting the B-cell-specific receptor CD20 (anti-CD20), has been reported to be an effective treatment for patients with active SLE who failed to respond to standard therapy. A pooled analysis of the efficacy of RTX from European cohorts diagnosed with biopsy-proven lupus nephropathy showed that administration of RTX resulted in high response rates and significant improvement in 24-h proteinuria, serum albumin, and protein/creatinine ratio [125]. Despite these promising data, two other large randomized controlled studies designed to assess the efficacy of RTX in nonrenal lupus [126] and lupus nephritis [127] did not achieve their respective primary endpoints. In the ACR and EULAR guidelines for the management of patients with refractory lupus nephritis (class III/IV) who have not responded to CYC nor MMF, RTX is still recommended [128]. Belimumab, another B-cell-targeted therapy, is a human immunoglobulin (Ig)-G1λ monoclonal antibody that binds soluble B lymphocyte stimulator (BLyS) and inhibits its biologic activities. The efficacy of belimumab is demonstrated in two large randomized control trials with more than 800 patients in each study [129, 130]. Pooled data showed a beneficial effect in 50.6 % of belimumab-treated patients versus 46.2 % in the placebo arm. However, the benefits obtained with belimumab are modest and only attained in patients with mild disease who are already receiving standard therapy [130]. Epratuzumab is a monoclonal antibody that targets

CD22, a B-cell-specific surface antigen involved in B-cell signaling. In a phase IIb trial to assess the efficacy and safety of epratuzumab, the overall treatment effect was not statistically significant [131]. Multicenter phase III studies with epratuzumab in patients with SLE are currently ongoing.

Tumor necrosis factor alpha (TNFα) is an interesting and controversial cytokine in the field of SLE due to its apparent dual role [132]. While TNFα blockade has been successful as a mainstay treatment for RA [133], the assessment of this therapy in SLE patients has not been straightforward. A recent study demonstrated the safety and efficacy of anti-TNFα therapy in SLE [134]. It suggests that any consideration of anti-TNFα for the treatment of SLE patients must be for a short duration only and not recommended for patients with antiphospholipid syndrome [135].

Despite potential benefits of biological inhibitors in the treatment of SLE, concerns exist regarding the occurrence of infections in patients treated with these agents [136, 137]. Both patients and primary physicians need to be aware of the possibility that serious infection may develop. If such a problem is diagnosed, the biologic inhibitor should be discontinued until adequate treatment has been completed [138].

Recently, many small molecule inhibitors have been designed to treat SLE based on multiple targets in Toll-like receptor (TLR) signaling pathways, including TLRs, myeloid differentiation factor 88 (MyD88), and IL-1R-associated kinases (IRAKs). These new chemical drugs, which can be taken orally, include CpG-52364 and IMO-9200 (targeting TLR7/8/9), SM934 (targeting TLR9), E-6446 and AT-791 (targeting TLR7/9 and IL-6), and ST-2825 (interfering with the recruitment of IRAK4 and IRAK1 via MyD88). They penetrate the cell membrane, effectively targeting endosomal TLRs and downstream signaling proteins [139]. Almost all these drugs are in preclinical animal studies or in phase I clinical studies and still require further exploration [140–142]. There are other recombinant small molecule inhibitors like abatacept, which blocks T-cell co-stimulatory ligands (CD80 and CD86) on B cells. Unfortunately, abatacept did not meet primary and secondary endpoints in a phase II clinical trial of SLE patients [143].

3.4 The Mechanisms of SCT in the Treatment of SLE

The rationale for auto-HSCT is its broad effect on the repopulated immune system, complex regulatory potentials, and long-term beneficial effect via down-regulating immune reactivity. The $CD4^+$ and $CD19^+$ cells were significantly reduced [144], and the expression of CD69 declined or normalized. Th2 cell cytokines like IL-4 decreased, while Th1 cell cytokines like interferon γ (IFN-γ) increased after auto-HSCT [145]. The peripheral T-cell receptor repertoire was normalized [146]. Thymic-derived $Foxp3^+$ regulatory T cells (Treg) regenerated [147], or a newly differentiated population of $LAP^{high}CD103^{high}CD8^{TGF\text{-}\beta}$ Treg generated after autologous HSCT [148]. Likewise, responders exhibited

normalization of the previously disturbed B-cell homeostasis with numeric recovery of the naive B-cell compartment [147]. These data reveal that both depletion of the autoreactive immunologic memory and a profound resetting of the adaptive immune system are required to reestablish self-tolerance by auto-HSCT in SLE.

Unlike auto-HSCT, allo-HSCT appears to offer curative potential in that the autoaggressive "old" immune system is replaced by a "new" one [149]. Auto-HSCT aims at restoring tolerance to self but does not affect genetic risk factors for the development of lupus, and therefore relapses are not unexpected, whereas allo-HSCT transfers a completely new immune system to the recipient with a chance for a cure.

MSC have become a major interest in their potential for immune-modulating, anti-inflammatory, and tissue-protective properties. The therapeutic effect of allogeneic MSCT was primarily dependent on its systemic immunoregulatory effects on various immune regulatory cells. Allogeneic MSC dose-dependently inhibited T-cell proliferation [150] and inhibited Akt/GSK3β signaling pathway mediated G1/S transition of lupus T cells [151]. The frequency of $CD4^+$ T cells decreased, and inflammatory cytokines were regulated by allogeneic MSCT in both animal models and humans [150, 152, 153]. MSC can regulate T-cell function via two pathways. First, MSC directly inhibit the functions of antigen-specific T cells. Second, MSC inhibit T-cell functions indirectly by stimulating the expansion of Treg [153–156]. In addition to T cells, MSC also suppress B-cell proliferation and plasma cell differentiation [157]. Serum and local levels of B-cell-activating factor (BAFF) and IL-10 significantly declined after MSC transfusion [158], potentially explaining the reduction in specific autoantibody production. Modulation of lymphocyte function may also be mediated by other regulatory factors secreted by MSC, including TGF-β, indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor (HGF), prostaglandin E2 (PEG2), nitric oxide (NO), IL-10, heme oxygenase 1 (HO-1), and HLA-G [159]. MSC may therefore exert some of their clinical effects by interfering with the production or function of such factors.

3.5 History of SCT in the Treatment of SLE

In 1996, an international collaboration began to explore the concept of immune ablation in patients suffering from severe autoimmune disease and not responding to conventional therapy [11]. It was hoped that following reconstitution of the immune system, a "resetting" of the autoimmune process would occur. In 1997, the first auto-HSCT for SLE was performed by Marmont et al. in Genoa, Italy [160]. Although many protocols were employed, they basically ranged from less aggressive (e.g., 200 mg/kg CYC plus antithymocyte globulin (ATG)) to more intensive (e.g., total body irradiation (TBI) plus CYC/ATG and CD34 selection). However, the initial choice of autologous HSC, with low toxicity, resulted in a high rate of relapse.

Theoretically, allo-HSCT offers the replacement of an autoreactive immune system and provides curative potential for patients with severe and drug-resistant ARD. Some SLE patients also received allo-HSCT previously. MSC were first used in humans for hematopoietic stem cell graft enhancement over 15 years ago [161]. Following many positive animal models of inflammation, organ transplant, autoimmunity, critical ischemia, radiation damage, and tissue scarring, MSC entered clinical trials for inflammatory disorders first in GVHD and then later in MS, Crohn's disease, SLE, and SSc [24].

3.6 The Current Status of SCT in the Treatment of SLE

In the past two decades, more than 2,000 patients received HSCT, and about 500 patients received MSCT worldwide. For autologous HSCT, phase I/II prospective and retrospective studies have supported autologous HSCT as a potential treatment option for severely affected lupus patients, as profound and prolonged clinical responses were noted [162]. SLE disease activity index (SLEDAI) score, 24-h proteinuria, serum creatinine, serum complements, and autoimmune antibodies, including antinuclear antibody and anti-dsDNA antibody, decreased, and there was a sustained withdrawal of immunosuppressive medication for most patients [145, 162]. The 5-year follow-up data from the CIBMTR database, with 50 patients enrolled, showed that the overall survival was 84 %, the probability of disease-free survival was 50 %, and treatment-related mortality was 4 % [163]. Recently a retrospective survey reviewed the efficacy and safety of autologous HSCT in 28 SLE patients from eight centers reported to the European Group for Blood and Marrow Transplantation (EBMT) registry between 2001 and 2008. The 5-year overall survival was 81 ± 8 %, and disease-free survival was 29 ± 9 %, with non-relapse mortality of 15 ± 7 % [164], suggesting a satisfactory clinical efficacy of autologous HSCT for lupus patients.

In lupus-like animal models, allogeneic HSCT both reversed disease symptoms and prevented disease development. In 2007, Vanikar et al. reported a single-center retrospective study of allo-HSCT in 27 drug-resistant SLE patients along with follow-up for 4.9 years. The average disease-free interval was 7.35 months (range, 2.1–12.7 months), and serum anti-double-strand DNA antibody titers declined [165]. The EBMT data showed two SLE patients who underwent allo-HSCT. However, one patient died of infection at 2.9 months, and the other patient had progression of disease when followed up for 3 years.

As a new stem cell therapy option, in 2007, allogeneic MSCT was first administered in severe and drug-resistant lupus patients. Data from phase I clinical studies showed that disease activity was satisfactorily controlled, and proteinuria and serum autoimmune antibodies declined after allogeneic MSCT [154, 166]. The transfusion of umbilical cord-derived MSC also resulted in clinical benefits in patients with severe lupus, who were otherwise poorly responsive to conventional therapy [153]. A further phase II study, with up to 4 years of follow-up,

demonstrated a good clinical safety profile, with an overall rate of survival of 94 %, and about 50 % patients achieving and remaining clinical remission at 4 years visit, although relapses of disease occurred in 23 % [167]. Based on these studies, there appears to be no difference in clinical efficacy between allogeneic bone marrow and umbilical cord-derived MSCT. MSC infusion induced remission in multi-organ dysfunctions including lupus nephritis [168], diffuse alveolar hemorrhage [169], and refractory cytopenia [170]. Recently a multicenter clinical study showed that 32.5 % patients achieved major clinical response and another 27.5 % patients achieved partial clinical response during 12 months follow-up. Again, a proportion of patients (17.5 %) experienced disease relapse within 6 months of a prior clinical response and required repeated MSCT [171]. However, combining MSCT and HSCT may achieve higher efficacy for SLE patients. Autologous MSCT was also applied in two lupus patients but received no clinical efficacy [172]. Recently, combined transplantation of autologous HSCT and allogeneic MSCT was used in a Chinese female lupus patient and achieved disease remission for 36 months [173], suggesting a novel and effective therapy option for refractory SLE.

4 Lessons Learned from Stem Cell Transplantation in Systemic Lupus Erythematosus

In the past two decades, SCT has represented an important breakthrough for patients suffering from severe and refractory SLE. Because it is an invasive procedure, SCT inevitability comes with risks, including treatment-related morbidity and mortality. However, with careful patient selection and adoption of conditioning regimens, TRM can be reduced. Indeed, lessons learned now from utilizing SCT in SLE will contribute to better outcomes in future clinical studies (Tables 2 and 3).

4.1 The Potential Benefits and Limitations of SCT in SLE

Currently there are more than ten clinical trials listed on clinicaltrials.gov designed to evaluate SCT as a cure for SLE (Table 2). Stem cells under consideration include MSC and HSC from bone marrow and umbilical cord. The basic premise for HSCT is to reconstruct the immune system by replacing abnormal lymphocytes in patients with SLE, whereas the goal behind using MSC is to modulate the patient's existing microenvironment in the immune system, for example, by suppressing autoreactivity or upregulating the number of Treg [174]. In addition to MSC and HSC, iPSC provide an alternative source for stem cells. IPSC enable us to model normal and diseased cellular growth as well as the development of SLE. Along with extensive assessment of patient-specific disease pathogenesis, this approach may provide a personalized therapeutic choice for SLE patients in the future.

Table 2 Clinical trials of SCT in SLE

Study title	Purpose	Stem cells used	Status	Sponsor/reference
Pilot study of total body irradiation in combination with cyclophosphamide, antithymocyte globulin, and autologous CD34-selected peripheral blood stem cell transplantation in children with refractory autoimmune disorders	To determine the safety and long-term complications of total body irradiation in combination with cyclophosphamide, antithymocyte globulin, and autologous CD34-selected peripheral blood stem cell transplantation in children with refractory autoimmune disorders	Autologous (or syngeneic) CD34-selected peripheral blood stem cell	Started November 2000, completed in July 2010	Fred Hutchinson Cancer Research Center NCT00010335
Immune ablation and hematopoietic stem cell support in patients with SLE: a phase II study	To examine the immunosuppressive therapy to the point of complete immune ablation and HSC recovery	Autologous HSC	Started September 2002, completed in November 2007	Richard Burt, MD Northwestern University NCT00271934
Lymphocyte depletion and stem cell transplantation to treat severe SLE	To study a new approach to treating patients with severe SLE, which involves collecting stem cells from the patient, completely shutting down the patient's immune system (rituximab, fludarabine, and cyclophosphamide), and then giving back the patient's stem cells	G-CSF (growth colony stimulating factor) was used to boost production of autologous HSC	Started January 2004, completed in October 2013	National Cancer Institute (NCI) NCT00076752
Allogeneic blood stem cell transplantation for patients with life-threatening SLE	To examine the outcomes after replacing the abnormal immune cells of patients with SLE that cause the disease with normal immune cells that are generated from the transplant blood stem cells from the healthy donor	HSC	Started June 2004, no recent verification on the recruitment status	National Heart, Lung, and Blood Institute City of Hope National Medical Center NCT00325741

Cyclophosphamide and rATG/ rituximab in patients with SLE	To examine whether treatment using chemotherapy followed by stem cell infusion will result in improvement of lupus disease. After intense chemotherapy destroys the cells in the immune system that may be causing this disease; stem cell infusion will start to produce a normal immune system that will no longer attack body	HSC	Started August 2005, recruiting	Richard Burt, MD Northwestern University NCT00278538
Mesenchymal stem cells transplantation for refractory SLE	To explore the outcomes after eliminating abnormal cells in immune system and restoring the body with a new population of progenitor cells in patients with SLE	Allogeneic MSC	Started March 2007, no recent verification on the recruitment status	Nanjing Medical University National Natural Science Foundation of China NCT00698191
Mesenchymal stem cell transplantation reverses multi-organ dysfunction in systemic lupus erythematosus mice and humans	To evaluate the efficacy and safety of using allogeneic MSCT in patients with treatment-refractory SLE during a 12–18 months follow-up period	Allogeneic MSC	Completed in 2008	California Institute for Regenerative Medicine (RN1-00572) NIDCR/ NIH R01DE017449 and R21 DE017632 National Natural Science Foundation of China (30772014)
				Chinese Education Ministry (20050315001), Jiangsu Province 135 Talent Foundation (RC2007002) [154]
Umbilical cord-derived MSC transplantation for active and refractory SLE	To explore safety and efficacy of allogeneic umbilical cord-derived MSC to treat patients with active and refractory SLE who have been resistant to multiple standard treatments	Human umbilical cord-derived MSC	Started January 2012, no recent verification on the recruitment status	The affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School NCT01741857 [171]

Table 3 Lessons learned from current SCT in SLE

SCT has become a viable treatment for SLE in the past two decades that employs the use of HSC, MSC, and iPSC
Limitations of SCT exist including disease relapse, side effects due to conditioning therapy, treatment-related mortalities, and GVHD
Those who choose to undergo SCT should seek out legitimate studies and institutions with proper counseling and follow-up by their medical providers
To ensure the development of novel approaches, standardized treatment protocols, and safety criteria for SCT, international research centers should be established through the support of government or private agencies

Despite the positive outlook for SCT, limitations do exist. The biggest challenge for auto-HSCT is the high rate of disease relapse as well as serious side effects arising from conditioning therapy [175]. Jayne et al. reported that although 66 % patients achieved clinical remission by 6 months, 32 % of patients subsequently relapsed, and transplant-related mortality (TRM) was 12 % at 1 year [176]. The US single-arm data showed a 4 % (2/50) TRM [163]. The 7-year retrospective data from EBMT showed that the relapse incidence (RI) was 56 ± 11 %, and non-relapse mortality was 15 ± 7 % [164]. The mechanism for disease flare after auto-HSCT is not clearly understood. Niu et al. showed that the function of HSC is altered by both genetic and inflammatory factors in lupus mice [177]. Moreover, bone marrow $CD34^+$ cells expressed a higher percentage of surface markers for CD95, CD123, and CD166 compared to healthy controls [178], thereby implying that abnormal autologous HSC in lupus patients may lead to higher rates of relapse after HSCT. Although allo-HSCT can completely restore the immune system, its clinical application has unfortunately demonstrated a high rate of treatment-related mortality and a high risk for GVHD, which limits its widespread use.

SLE patients with a hypersensitive state or a severe allergic history are not suitable for SCT. Caution should be exercised with SCT, where intensive immunotherapy may increase the risk of life-threatening cardiac complications, bleeding events, and severe infections. Therefore, the following exclusion criteria for SCT in SLE should be carefully considered:

1. Organ dysfunction: Patients with advanced organ failure (heart, lung, and kidney) or active gastrointestinal bleeding should be excluded from SCT.
2. Uncontrolled infection: Any patient with an uncontrolled acute or chronic infection, including HIV, human T-lymphotropic virus type 1 and 2, hepatitis B, and hepatitis C, should be excluded.
3. Pregnancy: Pregnancy should always be excluded within 7 days of administering immunosuppressant or SCT.

These guidelines and recommendations will promote careful patient selection and clinical outcome, which are crucial for the most appropriate clinical niche of SCT in SLE.

In MSC therapy, most clinical protocols have depended on in vitro culturing of MSC to expand the cell population from the donor to get the required number of

cells for therapeutic applications. This ex vivo manipulation process has to be carefully monitored to maintain the desired therapeutic property in vitro (e.g., immunomodulation). Based on reported phase I/II clinical studies, the safety and efficacy data acquired from allogeneic MSCT in severe and drug-resistant SLE patients are encouraging and thus provide a foundation for double-blinded, randomized placebo-controlled trials. However, numerous questions still need to be addressed. First, what is the most appropriate MSC source for use in clinical applications? Second, what should the dose of infused MSC be? Third, is a preconditioning regimen necessary before MSCT? Fourth, what is the optimal time to administer MSCT? When lupus has progressed or at disease onset? Furthermore, Should it be applied to only drug-resistant cases? More double-blinded and controlled clinical studies are needed to confirm proper treatment protocols.

4.2 Medical Personnel Influence: Communicating with Patients and Public Education

All the aforementioned SCT appear to offer curative potential, but as mentioned previously, limitations and possible risks to the patients' lives exist. This therapeutic strategy is in its early stages of clinical studies, and much more data and experience need to be acquired. Patients with SLE refractory to conventional therapy may opt to participate in clinical trials in hopes of a cure to their chronic symptoms. However, they should be diligent in seeking out legitimate studies undertaken by reputable academic institutions. Their medical providers should also assist in this process as well as advise their patients on the goals and end points of those studies. The rapid increase in centers carrying out SCT throughout various countries will require supervisory and ethics committees to monitor the production of stems cells, protocol safety, and adverse events. To ensure patients are not vulnerable to possible unproven therapies, these guidelines have to be strictly reinforced because any deviations could lead to inconclusive results. Relevant clinical experiences, both success and failures, should be communicated openly in professional conferences. Groups such as the International Society for Stem Cell Research (ISSCR) and European Medicines Agency (EMA) provide guidelines regarding these matters.

Confusing medical terms, physical or mental stresses, and financial obligations may overwhelm patients who eventually do undergo SCT therapy. Specific guidelines and instructions should be made available to the physicians, patients, and caregivers to help all parties understand what to expect throughout the SCT journey. Qualified healthcare professionals and counselors should be trained and be available to prepare the patients and assist them with questions on SCT throughout and post-SCT follow-up. Various ill side effects and discomfort may occur from the time the patient first receives a chemotherapeutic regimen, which is performed to prepare the body for SCT (also known as "conditioning"), to the actual SCT. At any of these phases, good communication between the medical team and the patient will ensure effective, high-quality care.

4.3 Active Participation and Support from the Society and Government for SLE

With its incidence nearly tripled in the last 40 years of the twentieth century and its estimated incidence rates at 1–25 per 100,000 in North America, South America, Europe, and Asia [117, 179], active support from the society and government agencies will expedite understanding of the etiology and pathogenesis of SLE, leading to the development of therapeutic interventions and improving quality of life for patients. To establish an international SCT center for SLE or ARD will not only provide a platform for researchers and clinicians to collaborate and exchange data and experience but also standardize and regulate SCT protocols in order to ensure patient safety. For example, the current quality of stem cells with regard to the source (donor's age or disease severity), heterogeneity, potency, and cell phenotype (cell surface markers) used for either animal studies or clinical trials is varied among labs. Further coordinated international studies from both the scientific and clinical community will help to develop novel approaches and standardize treatment protocols and safety criteria for the use of SCT in patients with SLE and other rheumatologic diseases.

5 Summary

The evidence base for the benefit of stem cell therapies for SLE has increased progressively over the last 5 years, with an initial interest in high-dose immunosuppression supported by HSCT followed by growing work in MSCT. There is therapeutic benefit from both HSCT and MSCT approaches, although the safety and tolerability profiles vary considerably. Current uncontrolled studies show improvement in SLE patients that had only been followed for short lengths of time. Larger randomized, controlled trials with long-term follow-up are warranted in order to establish safety criteria for the use of SCT. These multicenter studies should be designed to minimize discrepancies resulting from the use of different protocols and to compare clinical safety and efficacy between steroids combined with MSC or HSC treatment and steroids combined with traditional immunosuppressive drug therapy, such as CYC or MMF. To be sure, further elucidation of the molecular mechanisms between stem cells and the host immune system will also be necessary to understand the pathogenesis of SLE and perhaps other novel therapeutic applications.

References

1. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
2. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K et al (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324

3. Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132:631–644
4. Gatti RA, Meuwissen HJ, Allen HD, Hong R, Good RA (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet 2:1366–1369
5. Jacobs P, Vincent MD, Martell RW (1986) Prolonged remission of severe refractory rheumatoid arthritis following allogeneic bone marrow transplantation for drug-induced aplastic anaemia. Bone Marrow Transplant 1:237–239
6. Gratwohl A, Passweg J, Bocelli-Tyndall C, Fassas A, van Laar JM, Farge D et al (2005) Autologous hematopoietic stem cell transplantation for autoimmune diseases. Bone Marrow Transplant 35:869–879
7. Tamm M, Gratwohl A, Tichelli A, Perruchoud AP, Tyndall A (1996) Autologous haemopoietic stem cell transplantation in a patient with severe pulmonary hypertension complicating connective tissue disease. Ann Rheum Dis 55:779–780
8. Tyndall A (2012) Application of autologous stem cell transplantation in various adult and pediatric rheumatic diseases. Pediatr Res 71:433–438
9. Tyndall A, Black C, Finke J, Winkler J, Mertlesmann R, Peter HH et al (1997) Treatment of systemic sclerosis with autologous haemopoietic stem cell transplantation. Lancet 349:254
10. Muraro PA, Douek DC, Packer A, Chung K, Guenaga FJ, Cassiani-Ingoni R et al (2005) Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp Med 201:805–816
11. Tyndall A, Gratwohl A (1997) Blood and marrow stem cell transplants in autoimmune disease. A consensus report written on behalf of the European League Against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT). Br J Rheumatol 36:390–392
12. Liu B, Shu S, Kenny TP, Chang C, Leung PS (2014) Stem cell therapy in autoimmune rheumatic diseases: a comprehensive review. Clin Rev Allergy Immunol 47:244–257
13. Bartoloni E, Shoenfeld Y, Gerli R (2011) Inflammatory and autoimmune mechanisms in the induction of atherosclerotic damage in systemic rheumatic diseases: two faces of the same coin. Arthritis Care Res 63:178–183
14. Brinkman DM, de Kleer IM, ten Cate R, van Rossum MA, Bekkering WP, Fasth A et al (2007) Autologous stem cell transplantation in children with severe progressive systemic or polyarticular juvenile idiopathic arthritis: long-term follow-up of a prospective clinical trial. Arthritis Rheum 56:2410–2421
15. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA et al (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2:83–92
16. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317
17. Gronthos S, Zannettino AC, Graves SE, Ohta S, Hay SJ, Simmons PJ (1999) Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J Bone Miner Res: Off J Am Soc Bone Miner Res 14:47–56
18. Sarugaser R, Hanoun L, Keating A, Stanford WL, Davies JE (2009) Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. PLoS One 4:e6498
19. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S et al (2002) Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 30:42–48
20. Ben-Ami E, Berrih-Aknin S, Miller A (2011) Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases. Autoimmun Rev 10:410–415
21. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P et al (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99:3838–3843

22. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M et al (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363:1439–1441
23. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 57:11–20
24. Tyndall A, Uccelli A (2009) Multipotent mesenchymal stromal cells for autoimmune diseases: teaching new dogs old tricks. Bone Marrow Transplant 43:821–828
25. Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E et al (2009) Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells 27:2624–2635
26. Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, Scolding NJ (2008) Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. Neurosci Lett 448:71–73
27. Rafei M, Campeau PM, Aguilar-Mahecha A, Buchanan M, Williams P, Birman E et al (2009) Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. J Immunol 182:5994–6002
28. Ruiz M, Cosenza S, Maumus M, Jorgensen C, Noel D (2016) Therapeutic application of mesenchymal stem cells in osteoarthritis. Expert Opin Biol Ther 16:33–42
29. Diekman BO, Wu CL, Louer CR, Furman BD, Huebner JL, Kraus VB et al (2013) Intra-articular delivery of purified mesenchymal stem cells from C57BL/6 or MRL/MpJ superhealer mice prevents posttraumatic arthritis. Cell Transplant 22:1395–1408
30. Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464–3474
31. Wyles CC, Houdek MT, Behfar A, Sierra RJ (2015) Mesenchymal stem cell therapy for osteoarthritis: current perspectives. Stem Cells Cloning: Adv Appl 8:117–124
32. Chen M, Su W, Lin X, Guo Z, Wang J, Zhang Q et al (2013) Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. Arthritis Rheum 65:1181–1193
33. El-Denshary ES, Rashed LA, Elhussiny M (2014) Mesenchymal stromal cells versus betamethasone can dampen disease activity in the collagen arthritis mouse model. Clin Exp Med 14:285–295
34. Greish S, Abogresha N, Abdel-Hady Z, Zakaria E, Ghaly M, Hefny M (2012) Human umbilical cord mesenchymal stem cells as treatment of adjuvant rheumatoid arthritis in a rat model. World J Stem Cells 4:101–109
35. Liu LN, Wang G, Hendricks K, Lee K, Bohnlein E, Junker U et al (2013) Comparison of drug and cell-based delivery: engineered adult mesenchymal stem cells expressing soluble tumor necrosis factor receptor II prevent arthritis in mouse and rat animal models. Stem Cells Transl Med 2:362–375
36. Liu Y, Mu R, Wang S, Long L, Liu X, Li R et al (2010) Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 12:R210
37. Khalili S, Liu Y, Kornete M, Roescher N, Kodama S, Peterson A et al (2012) Mesenchymal stromal cells improve salivary function and reduce lymphocytic infiltrates in mice with Sjogren's-like disease. PLoS One 7:e38615
38. Xu J, Wang D, Liu D, Fan Z, Zhang H, Liu O et al (2012) Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjogren syndrome. Blood 120:3142–3151
39. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI (1995) Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone Marrow Transplant 16:557–564
40. Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI et al (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-

expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol Off J Am Soc Clin Oncol 18:307–316
41. Mayes MD, Lacey JV Jr, Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ et al (2003) Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. Arthritis Rheum 48:2246–2255
42. Nannini C, West CP, Erwin PJ, Matteson EL (2008) Effects of cyclophosphamide on pulmonary function in patients with scleroderma and interstitial lung disease: a systematic review and meta-analysis of randomized controlled trials and observational prospective cohort studies. Arthritis Res Ther 10:R124
43. Ratanatharathorn V, Uberti J, Karanes C, Abella E, Lum LG, Momin F et al (1994) Prospective comparative trial of autologous versus allogeneic bone marrow transplantation in patients with non-Hodgkin's lymphoma. Blood 84:1050–1055
44. Farge D, Labopin M, Tyndall A, Fassas A, Mancardi GL, Van Laar J et al (2010) Autologous hematopoietic stem cell transplantation for autoimmune diseases: an observational study on 12 years' experience from the European Group for Blood and Marrow Transplantation Working Party on Autoimmune Diseases. Haematologica 95:284–292
45. Henes JC, Schmalzing M, Vogel W, Riemekasten G, Fend F, Kanz L et al (2012) Optimization of autologous stem cell transplantation for systemic sclerosis – a single-center long term experience in 26 patients with severe organ manifestations. J Rheumatol 39:269–275
46. Farge D, Passweg J, van Laar JM, Marjanovic Z, Besenthal C, Finke J et al (2004) Autologous stem cell transplantation in the treatment of systemic sclerosis: report from the EBMT/EULAR registry. Ann Rheum Dis 63:974–981
47. Burt RK, Shah SJ, Dill K, Grant T, Gheorghiade M, Schroeder J et al (2011) Autologous non-myeloablative haemopoietic stem-cell transplantation compared with pulse cyclophosphamide once per month for systemic sclerosis (ASSIST): an open-label, randomised phase 2 trial. Lancet 378:498–506
48. Scleroderma clinical trials: Astis Study. Available at http://www.sclero.org/medical/treatments/clinical-trials/current/astis/a-to-z.html. Accessed May 2014
49. van Laar JM, Farge D, Sont JK, Naraghi K, Marjanovic Z, Larghero J et al (2014) Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. JAMA 311:2490–2498
50. ClinicalTrials.gov:. Scleroderma: Cyclophosphamide or Transplantation (SCOT). Available at http://www.clinicaltrials.gov/ct2/show/NCT00114530?term=SCOT+trial&rank=6. Accessed Aprile 2014
51. Naraghi K, van Laar JM (2013) Update on stem cell transplantation for systemic sclerosis: recent trial results. Curr Rheumatol Rep 15:326
52. Atzeni F, Benucci M, Salli S, Bongiovanni S, Boccassini L, Sarzi-Puttini P (2013) Different effects of biological drugs in rheumatoid arthritis. Autoimmun Rev 12:575–579
53. Craciunescu OI, Steffey BA, Kelsey CR, Larrier NA, Paarz-Largay CJ, Prosnitz RG et al (2011) Renal shielding and dosimetry for patients with severe systemic sclerosis receiving immunoablation with total body irradiation in the scleroderma: cyclophosphamide or transplantation trial. Int J Radiat Oncol Biol Phys 79:1248–1255
54. Nash RA, McSweeney PA, Nelson JL, Wener M, Georges GE, Langston AA et al (2006) Allogeneic marrow transplantation in patients with severe systemic sclerosis: resolution of dermal fibrosis. Arthritis Rheum 54:1982–1986
55. Scuderi N, Ceccarelli S, Onesti MG, Fioramonti P, Guidi C, Romano F et al (2013) Human adipose-derived stromal cells for cell-based therapies in the treatment of systemic sclerosis. Cell Transplant 22:779–795
56. Moura RA, Graca L, Fonseca JE (2012) To B or not to B the conductor of rheumatoid arthritis orchestra. Clin Rev Allergy Immunol 43:281–291
57. Atzeni F, Sarzi-Puttini P, Mutti A, Bugatti S, Cavagna L, Caporali R (2013) Long-term safety of abatacept in patients with rheumatoid arthritis. Autoimmun Rev 12:1115–1117
58. Burt RK, Testori A, Craig R, Cohen B, Suffit R, Barr W (2008) Hematopoietic stem cell transplantation for autoimmune diseases: what have we learned? J Autoimmun 30:116–120

59. Gratwohl A, Tyndall A (1997) Hematopoietic stem cell transplantations in treatment of autoimmune diseases. Z Rheumatol 56:173–177
60. Tyndall A, Blood EEIDBEGf, Marow T, European League Against R (2001) Autologous hematopoietic stem cell transplantation for severe autoimmune disease with special reference to rheumatoid arthritis. J Rheumatol Suppl 64:5–7
61. Snowden JA, Passweg J, Moore JJ, Milliken S, Cannell P, Van Laar J et al (2004) Autologous hemopoietic stem cell transplantation in severe rheumatoid arthritis: a report from the EBMT and ABMTR. J Rheumatol 31:482–488
62. Snowden JA, Biggs JC, Milliken ST, Fuller A, Brooks PM (1999) A phase I/II dose escalation study of intensified cyclophosphamide and autologous blood stem cell rescue in severe, active rheumatoid arthritis. Arthritis Rheum 42:2286–2292
63. Moore J, Brooks P, Milliken S, Biggs J, Ma D, Handel M et al (2002) A pilot randomized trial comparing CD34-selected versus unmanipulated hemopoietic stem cell transplantation for severe, refractory rheumatoid arthritis. Arthritis Rheum 46:2301–2309
64. McSweeney P (2001) Nonmyeloablative allogeneic hematopoietic cell transplants: any role for rheumatoid arthritis? J Rheumatol Suppl 64:49–54
65. Baldwin JL, Storb R, Thomas ED, Mannik M (1977) Bone marrow transplantation in patients with gold-induced marrow aplasia. Arthritis Rheum 20:1043–1048
66. Snowden JA, Kearney P, Kearney A, Cooley HM, Grigg A, Jacobs P et al (1998) Long-term outcome of autoimmune disease following allogeneic bone marrow transplantation. Arthritis Rheum 41:453–459
67. Rosa SB, Voltarelli JC, Chies JA, Pranke P (2007) The use of stem cells for the treatment of autoimmune diseases. Braz J Med Biol Res 40:1579–1597
68. Liang J, Li X, Zhang H, Wang D, Feng X, Wang H et al (2012) Allogeneic mesenchymal stem cells transplantation in patients with refractory RA. Clin Rheumatol 31:157–161
69. Wang L, Wang L, Cong X, Liu G, Zhou J, Bai B et al (2013) Human umbilical cord mesenchymal stem cell therapy for patients with active rheumatoid arthritis: safety and efficacy. Stem Cells Dev 22:3192–3202
70. Chighizola CB, Favalli EG, Meroni PL (2014) Novel mechanisms of action of the biologicals in rheumatic diseases. Clin Rev Allergy Immunol 47:6–16
71. Mavragani CP, Moutsopoulos HM (2010) The geoepidemiology of Sjogren's syndrome. Autoimmun Rev 9:A305–A310
72. Statkute L, Oyama Y, Barr WG, Sufit R, Ho S, Verda L et al (2008) Autologous nonmyeloablative haematopoietic stem cell transplantation for refractory systemic vasculitis. Ann Rheum Dis 67:991–997
73. Zhang ZY, Li RB, Jiang Y, Leng XM, Mei YF, Zhao YP et al (2007) High-dose immunosuppression and autologous peripheral blood stem cell transplantation for immunological reconstitution in two patients with primary Sjogren's syndrome. Zhonghua Nei Ke Za Zhi 46:926–929
74. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F et al (2013) 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. Arthritis Rheum 65:1–11
75. Gregorini M, Maccario R, Avanzini MA, Corradetti V, Moretta A, Libetta C et al (2013) Antineutrophil cytoplasmic antibody-associated renal vasculitis treated with autologous mesenchymal stromal cells: evaluation of the contribution of immune-mediated mechanisms. Mayo Clin Proc 88:1174–1179
76. Hiemstra TF, Jayne D (2009) Newer therapies for vasculitis. Best Pract Res Clin Rheumatol 23:379–389
77. Daikeler T, Kotter I, Bocelli Tyndall C, Apperley J, Attarbaschi A, Guardiola P et al (2007) Haematopoietic stem cell transplantation for vasculitis including Behcet's disease and polychondritis: a retrospective analysis of patients recorded in the European Bone Marrow Transplantation and European League Against Rheumatism databases and a review of the literature. Ann Rheum Dis 66:202–207

78. Davatchi F, Nikbin B, Shams H, Sadeghi Abdollahi B, Mohyeddin M, Shahram F (2013) Mesenchymal stem cell therapy unable to rescue the vision from advanced Behcet's disease retinal vasculitis: report of three patients. Int J Rheum Dis 16:139–147
79. Tansley S, Gunawardena H (2014) The evolving spectrum of polymyositis and dermatomyositis-moving towards Clinicoserological syndromes: a critical review. Clin Rev Allergy Immunol 47:264–273
80. Dimachkie MM, Barohn RJ (2012) Idiopathic inflammatory myopathies. Semin Neurol 32:227–236
81. Baron F, Ribbens C, Kaye O, Fillet G, Malaise M, Beguin Y (2000) Effective treatment of Jo-1-associated polymyositis with T-cell-depleted autologous peripheral blood stem cell transplantation. Br J Haematol 110:339–342
82. Bingham S, Griffiths B, McGonagle D, Snowden JA, Morgan G, Emery P (2001) Autologous stem cell transplantation for rapidly progressive Jo-1-positive polymyositis with long-term follow-up. Br J Haematol 113:840–841
83. Wang D, Zhang H, Cao M, Tang Y, Liang J, Feng X et al (2011) Efficacy of allogeneic mesenchymal stem cell transplantation in patients with drug-resistant polymyositis and dermatomyositis. Ann Rheum Dis 70:1285–1288
84. Ravelli A, Martini A (2007) Juvenile idiopathic arthritis. Lancet 369:767–778
85. Petty RE (1999) Prognosis in children with rheumatic diseases: justification for consideration of new therapies. Rheumatology (Oxford) 38:739–742
86. Burt RK, Marmont A, Oyama Y, Slavin S, Arnold R, Hiepe F et al (2006) Randomized controlled trials of autologous hematopoietic stem cell transplantation for autoimmune diseases: the evolution from myeloablative to lymphoablative transplant regimens. Arthritis Rheum 54:3750–3760
87. Milanetti F, Abinun M, Voltarelli JC, Burt RK (2010) Autologous hematopoietic stem cell transplantation for childhood autoimmune disease. Pediatr Clin North Am 57:239–271
88. Martini A (2012) Systemic juvenile idiopathic arthritis. Autoimmun Rev 12:56–59
89. Wulffraat N, van Royen A, Bierings M, Vossen J, Kuis W (1999) Autologous haemopoietic stem-cell transplantation in four patients with refractory juvenile chronic arthritis. Lancet 353:550–553
90. De Kleer IM, Brinkman DM, Ferster A, Abinun M, Quartier P, Van Der Net J et al (2004) Autologous stem cell transplantation for refractory juvenile idiopathic arthritis: analysis of clinical effects, mortality, and transplant related morbidity. Ann Rheum Dis 63:1318–1326
91. Wulffraat NM, van Rooijen EM, Tewarie R, Brinkman D, Prakken B, Kuis W (2008) Current perspectives of autologous stem cell transplantation for severe Juvenile Idiopathic Arthritis. Autoimmunity 41:632–638
92. Martini G, Foeldvari I, Russo R, Cuttica R, Eberhard A, Ravelli A et al (2006) Systemic sclerosis in childhood: clinical and immunologic features of 153 patients in an international database. Arthritis Rheum 54:3971–3978
93. Vonk MC, Marjanovic Z, van den Hoogen FH, Zohar S, Schattenberg AV, Fibbe WE et al (2008) Long-term follow-up results after autologous haematopoietic stem cell transplantation for severe systemic sclerosis. Ann Rheum Dis 67:98–104
94. Holzer U, van Royen-Kerkhof A, van der Torre P, Kuemmerle-Deschner J, Well C, Handgretinger R et al (2010) Successful autologous stem cell transplantation in two patients with juvenile dermatomyositis. Scand J Rheumatol 39:88–92
95. Lawitschka A, Peters C, Seidel MG, Havranek A, Heitger A, Fazekas T et al (2011) Long-term remission in pediatric Wegener granulomatosis following allo-SCT after reduced-intensity conditioning. Bone Marrow Transplant 46:462–463
96. Chang NH, Li TT, Kim JJ, Landolt-Marticorena C, Fortin PR, Gladman DD et al (2015) Interferon-alpha induces altered transitional B cell signaling and function in Systemic Lupus Erythematosus. J Autoimmun 58:100–110
97. Clement M, Charles N, Escoubet B, Guedj K, Chauveheid MP, Caligiuri G et al (2015) CD4+CXCR3+ T cells and plasmacytoid dendritic cells drive accelerated atherosclerosis associated with systemic lupus erythematosus. J Autoimmun 63:59–67

98. Figgett WA, Deliyanti D, Fairfax KA, Quah PS, Wilkinson-Berka JL, Mackay F (2015) Deleting the BAFF receptor TACI protects against systemic lupus erythematosus without extensive reduction of B cell numbers. J Autoimmun 61:9–16
99. Shaabani N, Honke N, Dolff S, Gorg B, Khairnar V, Merches K et al (2015) IFN-gamma licenses CD11b(+) cells to induce progression of systemic lupus erythematosus. J Autoimmun 62:11–21
100. Yu C, Gershwin ME, Chang C (2014) Diagnostic criteria for systemic lupus erythematosus: a critical review. J Autoimmun 48-49:10–13
101. Chi S, Yu Y, Shi J, Zhang Y, Yang J, Yang L et al (2015) Antibodies against C1q are a valuable serological marker for identification of systemic lupus erythematosus patients with active lupus nephritis. Dis Markers 2015:450351
102. Cozzani E, Drosera M, Gasparini G, Parodi A (2014) Serology of lupus erythematosus: correlation between immunopathological features and clinical aspects. Autoimmune Dis 2014:321359
103. Kis-Toth K, Tsokos GC (2014) Engagement of SLAMF2/CD48 prolongs the time frame of effective T cell activation by supporting mature dendritic cell survival. J Immunol 192:4436–4442
104. Koga T, Hedrich CM, Mizui M, Yoshida N, Otomo K, Lieberman LA et al (2014) CaMK4-dependent activation of AKT/mTOR and CREM-alpha underlies autoimmunity-associated Th17 imbalance. J Clin Invest 124:2234–2245
105. Sestak AL, Shaver TS, Moser KL, Neas BR, Harley JB (1999) Familial aggregation of lupus and autoimmunity in an unusual multiplex pedigree. J Rheumatol 26:1495–1499
106. Reichlin M, Harley JB, Lockshin MD (1992) Serologic studies of monozygotic twins with systemic lupus erythematosus. Arthritis Rheum 35:457–464
107. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P et al (1992) A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum 35:311–318
108. Ghodke-Puranik Y, Niewold TB (2015) Immunogenetics of systemic lupus erythematosus: a comprehensive review. J Autoimmun 64:125–136
109. Demirci FY, Dressen AS, Kammerer CM, Barmada MM, Kao AH, Ramsey-Goldman R et al (2011) Functional polymorphisms of the coagulation factor II gene (F2) and susceptibility to systemic lupus erythematosus. J Rheumatol 38:652–657
110. Ocal L, Russell K, Beynon H, Cruickshank K, Lanchbury JS, Walport M et al (1996) Genetic analysis of TAP2 in systemic lupus erythematosus patients from two ethnic groups. Br J Rheumatol 35:529–533
111. Kaiser R, Taylor KE, Deng Y, Zhao J, Li Y, Nititham J et al (2013) Brief report: single-nucleotide polymorphisms in VKORC1 are risk factors for systemic lupus erythematosus in Asians. Arthritis Rheum 65:211–215
112. Hughes T, Adler A, Merrill JT, Kelly JA, Kaufman KM, Williams A et al (2012) Analysis of autosomal genes reveals gene-sex interactions and higher total genetic risk in men with systemic lupus erythematosus. Ann Rheum Dis 71:694–699
113. Lu-Fritts PY, Kottyan LC, James JA, Xie C, Buckholz JM, Pinney SM et al (2014) Association of systemic lupus erythematosus with uranium exposure in a community living near a uranium-processing plant: a nested case-control study. Arthritis Rheumatol 66:3105–3112
114. Mak A, Tay SH (2014) Environmental factors, toxicants and systemic lupus erythematosus. Int J Mol Sci 15:16043–16056
115. Parks CG, De Roos AJ (2014) Pesticides, chemical and industrial exposures in relation to systemic lupus erythematosus. Lupus 23:527–536
116. Ward MM (2004) Prevalence of physician-diagnosed systemic lupus erythematosus in the United States: results from the third national health and nutrition examination survey. J Womens Health 13:713–718
117. Danchenko N, Satia JA, Anthony MS (2006) Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus 15:308–318

118. Shim JS, Sung YK, Joo YB, Lee HS, Bae SC (2014) Prevalence and incidence of systemic lupus erythematosus in South Korea. Rheumatol Int 34:909–917
119. Mok CC (2007) Management of systemic lupus erythematosus in Chinese patients. Expert Rev Clin Immunol 3:925–935
120. Kang I, Park SH (2003) Infectious complications in SLE after immunosuppressive therapies. Curr Opin Rheumatol 15:528–534
121. Ghosh K, Patwardhan M, Pradhan V (2009) Mycobacterium tuberculosis infection precipitates SLE in patients from endemic areas. Rheumatol Int 29:1047–1050
122. Mohamed DF, Habeeb RA, Hosny SM, Ebrahim SE (2014) Incidence and risk of infection in egyptian patients with systemic lupus erythematosus. Clin Med Insights Arthritis Musculoskelet Dis 7:41–48
123. Gross AJ, Hochberg D, Rand WM, Thorley-Lawson DA (2005) EBV and systemic lupus erythematosus: a new perspective. J Immunol 174:6599–6607
124. Deng H, You Y, Liu P, Zhao H, Zhou Y, Xie Y et al (2013) Clinical study of invasive fungal infection secondary to systemic lupus erythematosus. Zhong Nan Da Xue Xue Bao Yi Xue ban = J Cent South Univ Med Sci 38:182–185
125. Diaz-Lagares C, Croca S, Sangle S, Vital EM, Catapano F, Martinez-Berriotxoa A et al (2012) Efficacy of rituximab in 164 patients with biopsy-proven lupus nephritis: pooled data from European cohorts. Autoimmun Rev 11:357–364
126. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC et al (2010) Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum 62:222–233
127. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J et al (2012) Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 64:1215–1226
128. Paran D, Naparstek Y (2015) Is B cell-targeted therapy effective in systemic lupus erythematosus? Israel Med Assoc J: IMAJ 17:98–103
129. Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D et al (2011) A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. Arthritis Rheum 63:3918–3930
130. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE et al (2011) Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. Lancet 377:721–731
131. Wallace DJ, Kalunian K, Petri MA, Strand V, Houssiau FA, Pike M et al (2014) Efficacy and safety of epratuzumab in patients with moderate/severe active systemic lupus erythematosus: results from EMBLEM, a phase IIb, randomised, double-blind, placebo-controlled, multicentre study. Ann Rheum Dis 73:183–190
132. Aringer M, Smolen JS (2012) Therapeutic blockade of TNF in patients with SLE-promising or crazy? Autoimmun Rev 11:321–325
133. Taylor PC, Feldmann M (2009) Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. Nat Rev Rheumatol 5:578–582
134. Uppal SS, Hayat SJ, Raghupathy R (2009) Efficacy and safety of infliximab in active SLE: a pilot study. Lupus 18:690–697
135. Gottschalk TA, Tsantikos E, Hibbs ML (2015) Pathogenic inflammation and its therapeutic targeting in systemic lupus erythematosus. Front Immunol 6:550
136. Takayanagi N (2015) Biological agents and respiratory infections: causative mechanisms and practice management. Respir Invest 53:185–200
137. Jacobs BA, Opdam FL, Rodenhuis S, Baars JW (2015) Recurrent upper respiratory tract infections during and after rituximab therapy. Ned Tijdschr Geneeskd 159:A8546
138. Bresnihan B, Cunnane G (2003) Infection complications associated with the use of biologic agents. Rheum Dis Clin North Am 29:185–202

139. Wu YW, Tang W, Zuo JP (2015) Toll-like receptors: potential targets for lupus treatment. Acta Pharmacol Sin 36:1395–1407
140. Parkinson T (2008) The future of toll-like receptor therapeutics. Curr Opin Mol Ther 10:21–31
141. Hou LF, He SJ, Li X, Wan CP, Yang Y, Zhang XH et al (2012) SM934 treated lupus-prone NZB x NZW F1 mice by enhancing macrophage interleukin-10 production and suppressing pathogenic T cell development. PLoS One 7:e32424
142. Lamphier M, Zheng W, Latz E, Spyvee M, Hansen H, Rose J et al (2014) Novel small molecule inhibitors of TLR7 and TLR9: mechanism of action and efficacy in vivo. Mol Pharmacol 85:429–440
143. Merrill JT, Burgos-Vargas R, Westhovens R, Chalmers A, D'Cruz D, Wallace DJ et al (2010) The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 62:3077–3087
144. Su G, Luan Z, Wu F, Wang X, Tang X, Wu N et al (2013) Long-term follow-up of autologous stem cell transplantation for severe paediatric systemic lupus erythematosus. Clin Rheumatol 32:1727–1734
145. Traynor AE, Schroeder J, Rosa RM, Cheng D, Stefka J, Mujais S et al (2000) Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. Lancet 356:701–707
146. Wada H, Terasako K, Kamiya Y, Sato M, Kimura SI, Okuda S et al (2011) Immune recovery after autologous PBSC transplantation without in vitro graft manipulation for refractory systemic lupus erythematosus. Bone Marrow Transplant 46:1450–1454
147. Alexander T, Thiel A, Rosen O, Massenkeil G, Sattler A, Kohler S et al (2009) Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system. Blood 113:214–223
148. Zhang L, Bertucci AM, Ramsey-Goldman R, Burt RK, Datta SK (2009) Regulatory T cell (Treg) subsets return in patients with refractory lupus following stem cell transplantation, and TGF-beta-producing CD8+ Treg cells are associated with immunological remission of lupus. J Immunol 183:6346–6358
149. Tyndall A, Saccardi R (2005) Haematopoietic stem cell transplantation in the treatment of severe autoimmune disease: results from phase I/II studies, prospective randomized trials and future directions. Clin Exp Immunol 141:1–9
150. Zhou K, Zhang H, Jin O, Feng X, Yao G, Hou Y et al (2008) Transplantation of human bone marrow mesenchymal stem cell ameliorates the autoimmune pathogenesis in MRL/lpr mice. Cell Mol Immunol 5:417–424
151. Ji S, Guo Q, Han Y, Tan G, Luo Y, Zeng F (2012) Mesenchymal stem cell transplantation inhibits abnormal activation of Akt/GSK3beta signaling pathway in T cells from systemic lupus erythematosus mice. Cell Physiol Biochem: Int J Exp Cell Physiol Biochem Pharmacol 29:705–712
152. Chang JW, Hung SP, Wu HH, Wu WM, Yang AH, Tsai HL et al (2011) Therapeutic effects of umbilical cord blood-derived mesenchymal stem cell transplantation in experimental lupus nephritis. Cell Transplant 20:245–257
153. Sun L, Wang D, Liang J, Zhang H, Feng X, Wang H et al (2010) Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis Rheum 62:2467–2475
154. Sun L, Akiyama K, Zhang H, Yamaza T, Hou Y, Zhao S et al (2009) Mesenchymal stem cell transplantation reverses multiorgan dysfunction in systemic lupus erythematosus mice and humans. Stem Cells 27:1421–1432
155. Gu Z, Akiyama K, Ma X, Zhang H, Feng X, Yao G et al (2010) Transplantation of umbilical cord mesenchymal stem cells alleviates lupus nephritis in MRL/lpr mice. Lupus 19:1502–1514

156. Choi EW, Shin IS, Park SY, Park JH, Kim JS, Yoon EJ et al (2012) Reversal of serologic, immunologic, and histologic dysfunction in mice with systemic lupus erythematosus by long-term serial adipose tissue-derived mesenchymal stem cell transplantation. Arthritis Rheum 64:243–253
157. Feng X, Che N, Liu Y, Chen H, Wang D, Li X et al (2014) Restored immunosuppressive effect of mesenchymal stem cells on B cells after olfactory 1/early B cell factor-associated zinc-finger protein down-regulation in patients with systemic lupus erythematosus. Arthritis Rheumatol 66:3413–3423
158. Ma X, Che N, Gu Z, Huang J, Wang D, Liang J et al (2013) Allogenic mesenchymal stem cell transplantation ameliorates nephritis in lupus mice via inhibition of B-cell activation. Cell Transplant 22:2279–2290
159. Schena F, Gambini C, Gregorio A, Mosconi M, Reverberi D, Gattorno M et al (2010) Interferon-gamma-dependent inhibition of B cell activation by bone marrow-derived mesenchymal stem cells in a murine model of systemic lupus erythematosus. Arthritis Rheum 62:2776–2786
160. Marmont AM, van Lint MT, Gualandi F, Bacigalupo A (1997) Autologous marrow stem cell transplantation for severe systemic lupus erythematosus of long duration. Lupus 6:545–548
161. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK et al (2005) Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant: J Am Soc Blood Marrow Transplant 11:389–398
162. Traynor AE, Barr WG, Rosa RM, Rodriguez J, Oyama Y, Baker S et al (2002) Hematopoietic stem cell transplantation for severe and refractory lupus. Analysis after five years and fifteen patients. Arthritis Rheum 46:2917–2923
163. Burt RK, Traynor A, Statkute L, Barr WG, Rosa R, Schroeder J et al (2006) Nonmyeloablative hematopoietic stem cell transplantation for systemic lupus erythematosus. JAMA 295:527–535
164. Alchi B, Jayne D, Labopin M, Demin A, Sergeevicheva V, Alexander T et al (2013) Autologous haematopoietic stem cell transplantation for systemic lupus erythematosus: data from the European Group for Blood and Marrow Transplantation registry. Lupus 22:245–253
165. Vanikar AV, Modi PR, Patel RD, Kanodia KV, Shah VR, Trivedi VB et al (2007) Hematopoietic stem cell transplantation in autoimmune diseases: the Ahmedabad experience. Transplant Proc 39:703–708
166. Liang J, Zhang H, Hua B, Wang H, Lu L, Shi S et al (2010) Allogenic mesenchymal stem cells transplantation in refractory systemic lupus erythematosus: a pilot clinical study. Ann Rheum Dis 69:1423–1429
167. Wang D, Zhang H, Liang J, Li X, Feng X, Wang H et al (2013) Allogeneic mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus: 4 years of experience. Cell Transplant 22:2267–2277
168. Gu F, Wang D, Zhang H, Feng X, Gilkeson GS, Shi S et al (2014) Allogeneic mesenchymal stem cell transplantation for lupus nephritis patients refractory to conventional therapy. Clin Rheumatol 33:1611–1619
169. Shi D, Wang D, Li X, Zhang H, Che N, Lu Z et al (2012) Allogeneic transplantation of umbilical cord-derived mesenchymal stem cells for diffuse alveolar hemorrhage in systemic lupus erythematosus. Clin Rheumatol 31:841–846
170. Li X, Wang D, Liang J, Zhang H, Sun L (2013) Mesenchymal SCT ameliorates refractory cytopenia in patients with systemic lupus erythematosus. Bone Marrow Transplant 48:544–550
171. Wang D, Li J, Zhang Y, Zhang M, Chen J, Li X et al (2014) Umbilical cord mesenchymal stem cell transplantation in active and refractory systemic lupus erythematosus: a multicenter clinical study. Arthritis Res Ther 16:R79

172. Carrion F, Nova E, Ruiz C, Diaz F, Inostroza C, Rojo D et al (2010) Autologous mesenchymal stem cell treatment increased T regulatory cells with no effect on disease activity in two systemic lupus erythematosus patients. Lupus 19:317–322
173. Wang Q, Qian S, Li J, Che N, Gu L, Wang Q et al (2015) Combined transplantation of autologous hematopoietic stem cells and allogenic mesenchymal stem cells increases T regulatory cells in systemic lupus erythematosus with refractory lupus nephritis and leukopenia. Lupus 24:1221–1226
174. Wang D, Huang S, Yuan X, Liang J, Xu R, Yao G et al (2015) The regulation of the Treg/Th17 balance by mesenchymal stem cells in human systemic lupus erythematosus. Cell Mol Immunol
175. Zeher M, Papp G, Szodoray P (2011) Autologous haemopoietic stem cell transplantation for autoimmune diseases. Expert Opin Biol Ther 11:1193–1201
176. Jayne D, Passweg J, Marmont A, Farge D, Zhao X, Arnold R et al (2004) Autologous stem cell transplantation for systemic lupus erythematosus. Lupus 13:168–176
177. Niu H, Fang G, Tang Y, Xie L, Yang H, Morel L et al (2013) The function of hematopoietic stem cells is altered by both genetic and inflammatory factors in lupus mice. Blood 121:1986–1994
178. Sun LY, Zhou KX, Feng XB, Zhang HY, Ding XQ, Jin O et al (2007) Abnormal surface markers expression on bone marrow CD34+ cells and correlation with disease activity in patients with systemic lupus erythematosus. Clin Rheumatol 26:2073–2079
179. Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS (2010) Understanding the epidemiology and progression of systemic lupus erythematosus. Semin Arthritis Rheum 39:257–268

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