Resistance to Targeted Anti-Cancer Therapeutics 9
Series Editor: Benjamin Bonavida

Emmanuel Donnadieu *Editor*

Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy

Springer

Resistance to Targeted Anti-Cancer Therapeutics

Series Editor
Benjamin Bonavida

More information about this series at http://www.springer.com/series/11727

Emmanuel Donnadieu
Editor

Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy

Springer

Editor
Emmanuel Donnadieu
Institut Cochin
INSERM U1016
Université Paris Descartes
Paris, France

ISSN 2196-5501 ISSN 2196-551X (electronic)
Resistance to Targeted Anti-Cancer Therapeutics
ISBN 978-3-319-42221-3 ISBN 978-3-319-42223-7 (eBook)
DOI 10.1007/978-3-319-42223-7

Library of Congress Control Number: 2016949129

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Preface

After years of disappointments, cancer immunotherapy has finally gained considerable attention due to the development and use of approaches that target T cells, key players in the battle against cancer. In particular, immune checkpoint-targeting antibodies and adoptive T-cell therapies are starting to transform the treatment of advanced cancers. However, despite recent successes, many patients with cancer fail to respond to these treatments. A major challenge now is to identify underlying mechanisms responsible for resistance to cancer immunotherapy in order to overcome them and propose more efficient strategies.

For an effective direct destruction of cancer cells, CD8 T cells must fulfill several functions. First, they should be able to migrate efficiently into and within tumors in order to make contact with malignant cells. Second, they should be able to respond adequately to tumor antigens by releasing cytotoxic granules. In cancer patients, accumulating evidence suggests that responsiveness to tumor antigens is altered and current immunotherapies mainly aim at boosting T-cell activities. But relieving the brake of T-cell suppression will not be effective if lymphocytes are unable to migrate and interact with tumor cells.

The objective of this book titled *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy* is to focus on this important aspect which has been overlooked for years. The volume starts with a chapter by Stein, Moalli, and Ackerknecht who provide an overview of the main features of T-cell trafficking in basal conditions and during an efficient immune response. T lymphocytes are among the cells in the body that migrate the fastest, and it is now established that these cellular displacements are crucial for T cells to mount a protective immune response. Our understanding of how T cells move in a variety of organs including malignant tumors mainly comes from the two-photon microscopy technique. Loyher, Combadière, and Boissonnas present this powerful approach and discuss the dynamic behavior of T cells within an intact tumor environment.

This volume is also focused on the different obstacles the environment of advanced tumors creates to limit T cells from migrating and making contact with their malignant targets. A specific attention is devoted to defective entry of T cells into tumors and its underlying mechanisms. Fabian and Storkus discuss the unique

characteristics of the tumor-associated vasculature acting as a barrier for T cells. Even when T cells succeed in crossing the tumor vessels, lymphocytes are rarely in contact with tumor cells being instead greatly enriched in the surrounding stroma, composed of non-cancer cells. Over the last few years, different stromal cells and stromal components have been identified as limiting T cells from contacting malignant cells. Pommier and Fearon focus on carcinoma-associated fibroblasts and the negative impact of these stromal cells on T lymphocytes. Apart from detrimental elements, other structures in the tumor stroma that display strong similarities with lymphoid organs favor the trafficking and surveillance activity of T cells. Dubois, Kaplon, Couillault, and colleagues review the current knowledge about these tertiary lymphoid structures and their importance in T-cell antitumor activities.

Finally, a special attention is paid to current and future therapeutic interventions to improve migration of T cells into tumors and thus to enhance the effectiveness of cancer immunotherapy. Several chapters in this book discuss the ideas of modulating a number of components in the lymphocyte migration machinery. Cantor addresses the modulation of integrin functions to improve homing of T cells into tumors. Garetto, Sardi, Morone, and Kallikourdis focus on chemokines and the enforced expression of their receptors on T cells. Finally, Chen, Dotti, and Savoldo explain how the genetic manipulations of chimeric antigen receptor-modified T cells with carefully chosen genes can overcome poor migration and persistence of T lymphocytes into tumors.

The field of cancer immunotherapy has undergone a truly explosive growth in recent years. The development of therapies that target T cells raises hopes for these approaches to become a significant treatment for a variety of cancers. However, in order for these strategies to be fully successful, we need to understand how T cells traffic into and within tumors, how T cells are blocked in these processes, and how these obstacles can be overcome. I believe that this book will help readers to do this.

I would like to thank the authors, who are among the top leaders in their areas of research, for their exceptional contributions. This volume represents one in a book series entitled Resistance to Targeted Anti-Cancer Therapeutics of which Professor Benjamin Bonavida of the University of California, Los Angeles, serves as the series editor (published by Springer Publishing Company). I wish to thank Professor Bonavida for his friendly help and encouragement. Also, I acknowledge the valuable cooperation and coordination with Fiona Sarne, Joy Evangeline Bramble, and Ravishankar Kamalakannan, publishing editors, Cancer Research, Springer Science+Business Media.

Paris, France Emmanuel Donnadieu

Contents

Chapter 1
Basic Rules of T Cell Migration

Jens V. Stein, Federica Moalli, and Markus Ackerknecht

Abstract The positive correlation of lymphocyte infiltration into solid tumors with the patient survival, as well as recent successes of checkpoint inhibitors enhancing antitumor responses, have kindled a huge interest in cancer immunotherapy. In fact, adoptive cell therapy (ACT) of tumor-recognizing T cells has led to complete recession of tumors in a subset of melanoma patients. Yet, the molecular mechanisms that guide T cells to infiltrate tumor tissue for the detection and destruction of cancer cells are only incompletely understood. Here, we will give an overview on the basic rules of T cell migration, focusing on extra- and intracellular guidance cues gained primarily from intravital two photon imaging, and relate this with the efficient unfolding of adaptive immune responses. From this, we outline the challenges that T cells face entering and maneuvering inside non-lymphoid tissues including tumor sites.

Keywords T cell trafficking • Integrins • Small GTPases • Intravital imaging • Extracellular matrix • Guanine exchange factors

J.V. Stein (✉) • F. Moalli
Theodor Kocher Institute, University of Bern, Bern 3012, Switzerland
e-mail: jstein@tki.unibe.ch; federica.moalli@tki.unibe.ch

M. Ackerknecht
Theodor Kocher Institute, University of Bern, Bern 3012, Switzerland

Friedrich Miescher Institute for Biomedical Research,
Maulbeerstrasse 66, Basel 4058, Switzerland
e-mail: markus.ackerknecht@email.de

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_1

Abbreviations

ACT	Adoptive cell therapy
APC	Antigen-presenting cell
ATX	Autotaxin
DC	Dendritic cell
ECM	Extracellular matrix
FRC	Fibroblastic reticular cell
GALT	Gut-associated lymphoid tissue
GEF	Guanine exchange factor
GPCR	G-protein coupled receptor
HEV	High endothelial venule
LN	Lymph node
LPA	Lysophosphatidic acid
SLO	Secondary lymphoid organ
TIL	Tumor-infiltrating lymphocyte

1.1 Introduction

The adaptive immune system fulfills the critical task to protect against infectious microbes, including viruses and bacteria, while maintaining tolerance against self. The initiation of primary immune responses takes place in lymphoid tissues, such as skin-draining peripheral lymph nodes (LNs). There, the cellular arm of the adaptive immune system consisting of $CD4^+$ and $CD8^+$ T lymphocytes has evolved to quickly recognize pathogen-derived peptides displayed on major histocompatibility complexes (pMHC) by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. The encounter of DCs or macrophages bearing cognate pMHC induces a "stop" signal for migrating T cells, i.e., these cells rapidly decrease their scanning behavior and engage in long-term interactions with DCs, which imprint a differentiation program leading to effector cell generation. Once activated, most $CD8^+$ and $CD4^+$ T cells leave lymphoid tissue after clonal expansion to accumulate at the site of infection, where they fight pathogens by a variety of mechanisms including cytotoxic activity and cytokine secretion. A second central function of the adaptive immune system is to kill or keep in check cancerogeneous cells, which present aberrant pMHC on their surface owing to their altered and increased metabolic activity (Fig. 1.1). In fact, the concept to exploit the immune system to fight cancers—cancer immunotherapy—is supported by the recent observation that the presence of $CD8^+$ T cells correlates positively with the control of triple-negative breast cancers when compared in more than 12,000 patients [1]. Similarly, the presence of Th1-skewed $CD4^+$ T cells and other immune cells in colon carcinoma patients is a better predictor of the patient's survival than commonly used histopathological methods for staging [2]. Accordingly, a recent publication has found

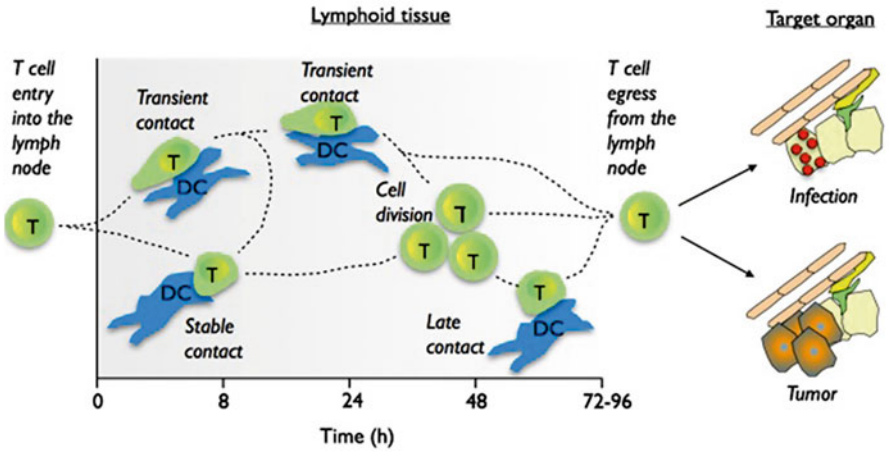

Fig. 1.1 Scheme of adaptive immune responses against pathogen-induced infections and transformed tumor cells. Naïve T cells enter draining lymphoid tissues, e.g., lymph nodes, where they encounter DCs presenting cognate pMHC molecules before becoming activated and migrating to their respective target organs

that Th1 cytokines induce senescence in tumor cells [3]. Taken together, these findings underscore the importance of successful entry of $CD4^+$ and $CD8^+$ T cells into tumors, as well as the importance of further investigations into molecular mechanisms allowing for robust T cell responses against tumor cells. However, adoptive cell therapy (ACT), consisting of re-introducing autologous in vitro-activated T cells into cancer patients, showed only mixed successes in various trials [4]. In most instances, the reasons for this failure remain unknown but may include defective re-activation in lymphoid tissue post transfer or impaired accumulation in tumor target tissue. The shaping of tumor antigenicity by immunoediting or other tumor-suppressive mechanisms, including suppression by tumor-associated macrophages or stroma cells, may also lead to defective killing of cancer cells by immune cells [5, 6]. Yet, in recent years, there has been a renewed interest in cancer immunotherapy, in large part owing to the successful clinical trials targeting immunosuppressive signaling pathways, in particular inhibitors of CTLA-4 and PD-1—PD-L1 signaling [7]. As example, blocking PD-1 is currently the only treatment proven to prolong the life expectancy of prostate cancer patients. The somewhat unexpected success of such treatments has prompted Science magazine to promote cancer immunotherapy as breakthrough of the year 2013 [8]. How do these therapies work? Studies from chronic viral infections have shown that both CTLA-4 and PD-1 are expressed on "exhausted" $CD8^+$ T cells and function as negative regulators of TCR signaling by engaging intracellular phosphatases, rather than kinases. Furthermore, CTLA-4 has been shown to counteract the "stop" signal delivered by pMHC-bearing DCs, which is required for full activation [9]. Similarly, PD-1 (but not CTLA-4) blockade increased $CD4^+$ T cell arrest in a skin inflammation model, suggesting that T cell arrest on target cells positively correlates with effector function

[10]. A prerequisite for T cell-mediated tumor cell destruction is its ability to enter tumor tissue and interact directly with tumor cells, since T lymphocytes have evolved to scan the surfaces of other cells for pMHC and act therefore on a strictly local level [11]. Here, we will present an overview on the adaptive immune response from a T cell's perspective, with a focus on basic extracellular and intracellular signaling circuits guiding their activation and migration.

1.2 T Cell Recirculation Through Secondary Lymphoid Organs

The primary activation of naïve T cells takes place in secondary lymphoid organs (SLOs), including peripheral lymph nodes (LNs), spleen and gut-associated lymphoid tissue (GALT). These tissues sample tissue lymph, blood and gut, respectively, to provide a niche for the onset of adaptive immune responses by serving as antigen libraries, where lymphocytes screen within a relatively small volume for cognate pMHC presented on DCs. Since naïve T cells scan pMHC on DCs through direct cellular contact, they have evolved to be highly motile both between organs as well as within organ parenchyma [12]. As consequence, individual T cells show high motility between single SLOs, while the average number of lymphocytes in a given SLO remains stable over time. During steady state, naïve T cells selectively recirculate through LNs, spleen and GALT from blood, where they spend a few hours before leaving through afferent lymphatics, which drain back into the blood circulation (in case of spleen, naïve T cells leave directly through venous sinusoids). In average, each T cell completes the round trip from the blood to SLOs back to blood one-three times per day [13].

Naïve lymphocytes entry from the blood into the LN parenchyma is a multistep process. High endothelial venules (HEVs) in LNs express peripheral node addressin (PNAd). Binding of L-selectin (CD62L) on T cells leads to selectin-mediated tethering and rolling of lymphocytes along the endothelium. The chemokines CCL21 (expressed by HEVs) and CCL19 (expressed by stromal cells and transported to HEVs) bind the chemokine receptor 7 (CCR7) on T cells, leading to G-protein mediated conformational activation of αLβ2-integrin (leukocyte function-associated antigen type 1, LFA-1) on T cells [14, 15]. This activation induces a conformational change of integrins that enables rapid binding of LFA-1 to the intracellular adhesion molecule (ICAM)-1 and -2, leading to shear-resistant T cell arrest. Finally, adherent lymphocytes become polarized and crawl along the luminal surface of the endothelium before transmigrating into the surrounding parenchyme. During this process, T cells need to negotiate a path through the basement membrane of HEVs [16, 17]. The establishment of twophoton microscopy (2PM) of lymphoid tissue has allowed to directly assess the motility parameters of scanning T cells (Fig. 1.2).

After entering LN parenchyma, T cells move along a loosely spaced stromal cell scaffold formed by fibroblastic reticular cells (FRCs) of the T cell zone, with speeds of 12–15 μm/min [16]. Since T cells follow the FRC network but do not appear to move along a chemotactic gradient, this migration pattern is referred to random-guided

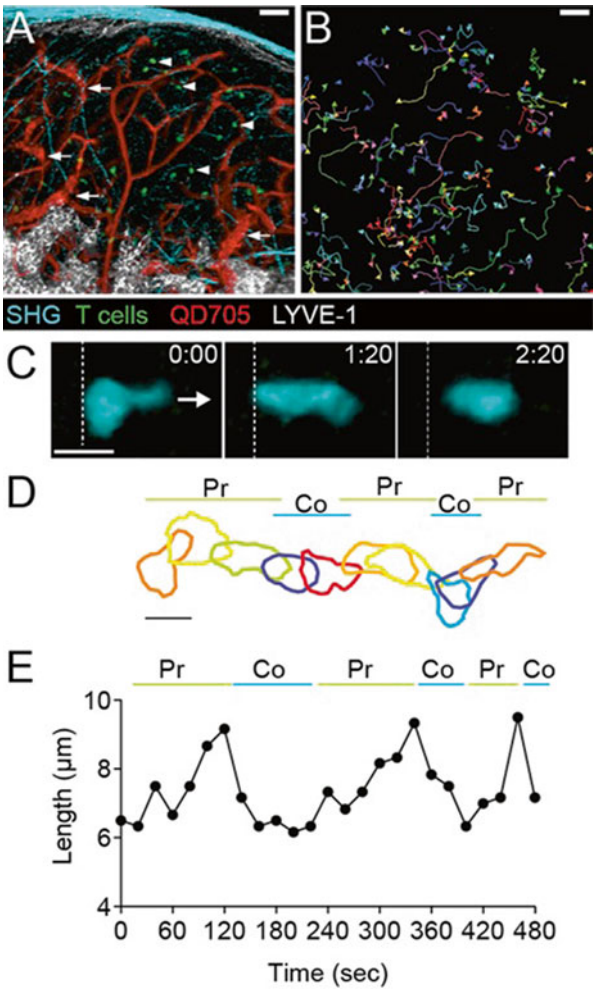

Fig. 1.2 Protrusion-contraction sequences define lymphocyte motility in lymphoid tissue. (**a**) 2PM projection of a mouse lymph node after adoptive transfer of T cells (*green*; examples labeled with *arrowheads*). The blood vasculature is labeled *red*, and *arrows* depict HEV (defined by their "cobblestone" shape). SHG, second harmonic generation, which labels collagen fibers. (**b**) T cell tracks over 20 min from image sequence in (**a**), showing random motility. Scale bar in (**a**) and (**b**), 40 μm. (**c**) 2PM image sequence of a migrating T cell in lymphoid tissue. The *arrow* indicates migration direction, while the *dashed line* delineates the back of the cell at t = 0:00. Time in min and sec, scale 5 μm. (**d**) Outline of T cell in consecutive frames showing protrusion (Pr) and contraction (Co). Scale, 5 μm. (**e**) Frame-to-frame analysis of cell length showing oscillations induced by protrusions and contractions

walk. DCs attach closely to the FRC network to facilitate interactions by scanning T cells [18]. FRCs produce CCL19 and CCL21, and both DCs and FRCs express the LFA-1 ligands ICAM-1 and ICAM-2 [17, 19, 20]. CCR7 ligands and LFA-1—ICAM-1 interactions contribute to basal migration, although even in either absence,

T cells retain a considerable average speed of ~10–12 μm/min [21–23]. Similarly, T cells lacking the $G\alpha_{i2}$ subunit acting downstream of CCR7 move only marginally slower than WT T cells in LN parenchyma [24]. Thus, additional pathways are likely to contribute to T cell motility in vivo, perhaps through cell-intrinsic basal F-actin treadmilling in combination with a confined 3D environment. In this context, lysophosphatidic acid (LPA) produced by the exoenzyme autotaxin (ATX) on stromal cells, including HEV and FRCs, has been shown to contribute to transmigration and basal lymphocyte motility in lymphoid tissue [25–29]. LPA binds to T cell-expressed LPA2, a member of the GPCR family, and induces Rho activation, which cooperates with CCL21 to induce to contractility—dependent lymphocyte migration (see below). Pharmacological blocking of ATX or LPA receptors or lack of LPA2 reduces T cell speeds by approximately 30 % in vivo, and addition of LPA increases T cell polarization and speeds in vitro [27–29]. These observations are in line with recent descriptions of increased cell motility generated by augmented contractility of the trailing edge in confined environments [30, 31].

Lymphocytes spend an average dwell time of 8–12 h in resting LNs, and leave via medullary and cortical lymphoid sinuses in an sphingosine-1 phosphate receptor-1 (S1P1)-dependent manner [32]. S1P1, which is closely related to LPA receptors, functions as a rheostat of tissue dwell time; S1P1 on T cells becomes rapidly internalized after binding S1P in blood and lymph but becomes gradually increased in S1P-low tissue. Thus, T cells become increasingly responsive to S1P gradients close to efferent lymphatic vessels, favoring egress. Interestingly, this mechanism is also involved in the retention of T cells in inflamed LNs. T cell activation induces the upregulation of CD69, which complexes with S1P1 in *cis*, leading to its internalization. Furthermore, TCR signaling induces transient transcriptional repression of S1P1 [33]. Such a mechanism ensures that activated T cells do not leave LNs before full activation has been completed. Regulation of S1P signaling on lymphocytes is therefore partially responsible for the so-called "LN shutdown", which leads to a transient stop of lymphocyte egress [33].

1.3 Molecular Basis of Protrusion: Contraction Cycles that Confer T Cell Motility

Careful quantification of polarized leukocyte shapes during migration has typically uncovered consecutive protrusion–contraction cycles (Fig. 1.2). During protrusion, F-actin formation at the leading edge provides the force for displacement, while timed contractions of the trailing edge, or uropod, contribute to cell displacement by squeezing cytoplasm and the nucleus through narrow pores [34]. How is this remarkable motility achieved? Chemokines and integrins induce T cell polarization and the formation of a leading edge and uropod [35, 36]. Chemokines bind to chemokine receptors that belong to the family of G-protein-coupled receptors (GPCRs), such as CCR7 mentioned above. GPCRs are 7-transmembrane domain proteins of

approximately 40 kDa, which are associated at the intracellular domains with a heterotrimeric complex of α, β and γ G proteins. Under steady-state conditions the Gα subunit is bound to GDP and associated with Gβγ. Ligand binding to GPCRs induces the replacement of GDP by GTP. Gα-GTP dissociates from Gβγ dimers and both molecules initiate downstream signaling. The intrinsic GTPase activity of Gα hydrolyses GTP to GDP, leading to a reassembly of the Gαβγ complex, which terminates signaling [37]. Gα proteins consist of four major families: $G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, all of which induce signaling cascades in a variety of biological functions. In the context of chemokine signaling, the $G\alpha_{i/o}$ family is the most relevant as most ligand-induced signaling events are blocked by pertussis toxin (PTX) [38]. PTX ribosylates a specific cysteine residue of the $G\alpha_{i}$ subunits, which leads to its uncoupling from the GPCR. PTX treatment results in the loss of chemokine induced migration in T cells, emphasizing the central role of $G\alpha_{i/o}$ family members in mediating T cell motility [39].

While intermediate filaments support cell stability and rigidity [40], the constant remodelling of the actin cytoskeleton is the major regulator of lymphocyte motility. Changes in the cytoskeleton are regulated by small GTPases of the Rho family, which consists of 25 family members including Rac, Cdc42 and RhoA [41]. Cdc42 activity is important for the initial polarization of a round lymphocyte to promote the formation and the stabilization of a single leading edge. This maintains the polarized phenotype intact and is highly relevant for directed migration [42] (Fig. 1.3). Accordingly, $Cdc42^{-/-}$ DCs failed to navigate through complex 3D environments, due to entangling and formation of multiple competing leading edges [43]. Similarly, human T cells expressing a dominant negative form of Cdc42 are unable to form a single lamellipodium and show impaired random and chemotactic motility [44]. Formation of filopodia also depends on Cdc42 activity, as Cdc42 inhibition by Secramine A blocks T cell transmigration under flow by reducing invasive filopodia formation [15]. In parallel, continuous Rac-mediated F-actin assembly at the leading edge provides the protrusion force in 3D environments, even in the absence of integrin ligands. This is in stark contrast to leukocyte adhesion to endothelium under flow or on 2D surfaces, which is strictly integrin-dependent [34]. RhoA activity is actively suppressed at the leading edge [45] and is mostly localized to trailing edge or uropod of the cell (Fig. 1.3). Rho activates ROCK, which induces actin-myosin contractions that are required to detach LFA-1—ICAM-1 adhesive interactions at the uropod [46]. In vitro and in vivo experiments have furthermore shown the importance of uropod contraction in confined environments, when the nucleus has to be pushed through narrow gaps [34]. Accordingly, impaired ROCK activity delays T cell migration through the basement membrane of endothelial cells [47, 48]. In addition, Rho activates formins, actin nucleation factors that produce unbranched F-actin filaments required for Myosin IIA-mediated contractility [49, 50]. Rho GTPases also have overlapping functions, as Rac and RhoA cooperate during integrin mediated adhesion and de-adhesion [51]. Furthermore, GTP-bound Rac and Cdc42 activate Arp2/3 actin nucleation complexes to induce branched F-actin filament at the leading edge through their downstream effector complexes SCAR/WAVE and WASP-WIP [49].

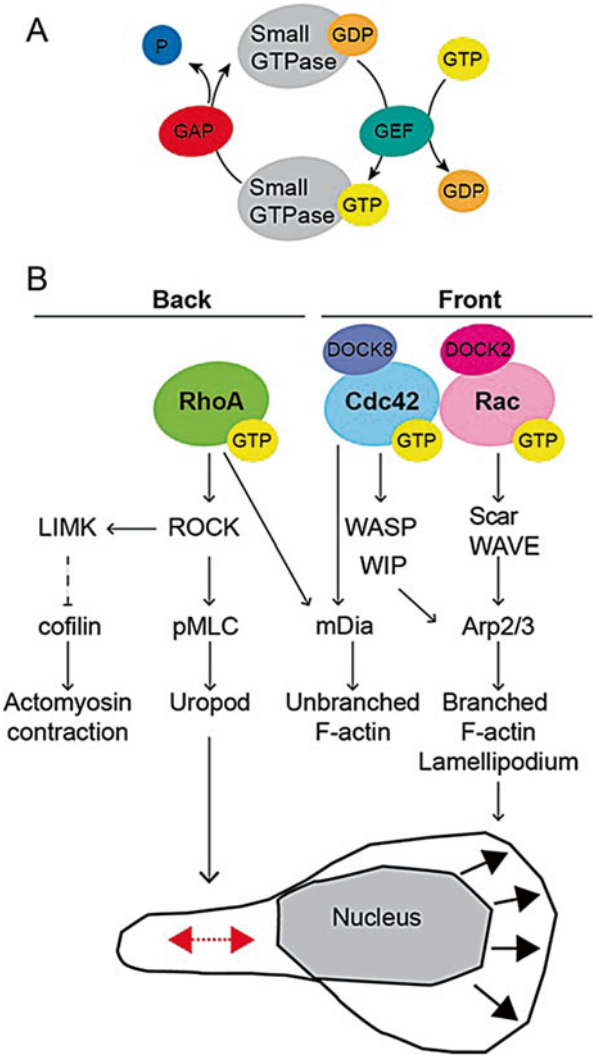

Fig. 1.3 Intracellular signaling in polarized T cells. (**a**) Activation/deactivation cycles of small GTPases. In a GDP-bound state, small GTPases are non-active. Guanine exchange factors (GEFs) including members of the DOCK family promote exchange of GDP for GTP, while GTPase activating factors (GAPs) accelerate the GTPase activity of small GTPases to return to the GDP-bound inactive state. (**b**) Cytoskeletal regulation by downstream effectors of small GTPases. Cdc42 and Rac1/2 lead to the formation of Arp2/3-mediated branched F-actin through the activation of WASP-WIP and Scar-WAVE complexes, respectively, at the leading edge of a polarized cell. RhoA and Cdc42 are involved in unbranched, mDia-driven F-actin polymerization. RhoA is particularly active at the trailing edge, or uropod, of migrating cells, where it contributes to non-muscle Myosin II-mediated actomyosin contractility

1.4 Regulation of Small GTPase Activity

GTPases are controlled by the interplay of activating guanine exchange factors (GEFs) and inhibiting factors including GTPase activating proteins (GAPs) and guanosine dissociation inhibitors (GDI). GEFs induce exchange of GDP with GTP, which promotes local pools of GTP-loaded small GTPases that lead to downstream signaling effects. In turn, GAPs activate the intrinsic GTPase activity of GTPases, which leads to the hydrolysis of GTP to GDP. GDI prevent the GDP-GTP exchange by locking the GDP state and sequestering the GTPase away from membranes, where GEFs and effector molecules are located [52]. Interestingly, it has been shown that even with a strong stimulus, at most 5 % of RhoA, Rac1 or Cdc42 are in their active, GTP-bound state, indicating stringent control mechanisms. Indeed, lymphocytes express 36 of the known 79 Rho GEFs, 38 out of 65 Rho GAPs and 2 of the 3 known GDIs, indicative of a highly regulated network of actin regulation [41, 53]. Recent studies have identified a central role for GEFs of the DOCK family as regulators of actin cytoskeleton rearrangement during migration [54]. DOCK8 is a Cdc42 GEF, which is critically involved in DC migration through extracellular matrix (ECM) networks in vitro and in vivo [55]. Similarly, DOCK8-deficient T cells show impaired scanning and increased cell death in skin [56], which contributes to the immunodeficiency observed in DOCK8-deficient patients [57]. The single most important Rac GEF for T cell migration is DOCK2. T lymphocytes lacking DOCK2 display strongly impaired in vitro motility [58] and, similar to Rac1/2-double-deficient T cells, show virtually no residual migration in the LN parenchyme [59, 60]. The importance of this pathway for host surveillance is underscored by the recent identification of DOCK2-deficient patients, who suffer from early onset severe invasive infections [61]. Interestingly, transendothelial migration is not impaired in the absence of DOCK2-Rac signaling [17, 62], underscoring a central role for Cdc42 in this process.

1.5 Balance Between Motility and Arrest Governs T Cell Activation

The random-guided pattern of naïve T cell migration in lymphoid tissue has evolved to balance two competing requirements. On the one hand, naïve T cells need to scan a maximum number of DCs to find cognate pMHC molecules, which are likely to be rare at the onset of adaptive immune responses. On the other hand, the average T cell dwelling time per DC needs to be long enough to switch their motile behavior to a stationary one. This conversion facilitates prolonged interactions with cognate pMHC-presenting DCs via the establishment of an immunological synapse (IS). Early in vitro evidence suggests that decision making leading to arrest on DCs requires only a few seconds, correlating with the induction of Ca^{2+} influx in responsive T cells [63, 64]. Interestingly, DOCK2—Rac-mediated F-actin assembly at the

leading edge of motile T cells is maintained during interactions with DCs, but in a different spatial arrangement. Thus, TCR signaling and ICAM-1—LFA-1-mediated adhesion convert Rac-driven protrusion activity at the lamellipodium of migrating T cells into an annular F-actin ring with centripetal directionality at the IS interface [65, 66]. Thus, the decision-making of motile T cells to undergo conversion from translocation to arrest requires a threshold pMHC level and LFA-1-mediated firm adhesion. Weak activatory signals because of low pMHC levels, low TCR—pMHC affinity or lack of ICAM-1 on DCs would not suffice to switch F-actin protrusions to a ring-like structure. Consistent with this model, inhibitory receptors of TCR signaling including CTLA-4 and PD-1 prevent T cell adhesion to antigen-presenting cells in lymphoid tissues [9, 67]. Inhibition of the canonical PD-1 ligand PD-1L, but not CTLA-4, prolonged effector T cell arrest at sites of acute inflammation [10], whereas CTLA-4 blockade had the same effect on tumor-infiltrating T cells [68]. While the role of PD1 during T cell interactions in tumors has not been investigated thus far, persistent viral infections lead to a PD1-dependent T cell paralysis [69]. Thus, PD1 signaling may play context-dependent roles, presumably related to the exhaustion state of T cells.

1.6 Imprinting of T Cell Dissemination After Activation

Activation of T cells in lymphoid tissue leads to clonal expansion and changes in their homing behavior. Most effector T cells decrease expression of L-selectin and CCR7, which reduces their re-entry into SLOs. Instead, differentiating T cells start to express high levels of LFA-1, very late antigen 4 (VLA-4, $\alpha_4\beta_1$ integrin) and E- and P-selectin ligands, mostly induced by fucosyltransferase VII-mediated glycosylation of PSGL-1 [70, 71]. Glycosylated PSGL-1 allows T cells to efficiently tether to and roll on activated endothelial cells expressing E- and P-selectins. Inflammation-induced chemokines presented on endothelium activate LFA-1 and VLA-4 to induce firm adhesion on ICAM-1 and VCAM-1, respectively, which are highly expressed on activated but not resting endothelium. Chemokine receptor and adhesion molecule expression on effector T cells is further imprinted by the anatomical localization of SLOs where activation takes place. T cells activated in the presence of Vitamin A-derived retinoic acid, which is provided by DCs of the intestinal immune system, up-regulate the expression of the gut-homing receptors CCR9 and $\alpha_4\beta_7$ integrin, while decreasing expression of skin-homing receptors such as CCR4 [72]. These receptors enable T cells to interact with MAdCAM-1 and CCL25, which are constitutively expressed in the small intestine [73]. In addition to DCs, stromal cells of the mesenteric LN contribute to the generation of $\alpha_4\beta_7$-integrin and CCR9-expressing gut-homing T cells by their capacity to produce retinoic acid [74]. A complementary imprinting mechanism induces skin tropism of T cells: skin-derived DCs process sunlight-induced vitamin D3 to generate an active intermediate, which induces T cells to express cutaneous lymphocyte antigen 4 (CLA), CCR4 and CCR10 while suppressing the gut-homing receptors $\alpha4\beta7$ and CCR9. This

enables T cells to migrate to inflamed skin, where CCL17 and CCL27 are secreted by keratinocytes of the epidermis [75]. Thus, effector T cells are equipped with a receptor repertoire that facilitate homing to regions where DCs have taken up antigen. Imprinting of gut or skin tropism also occurs during T cell activation by tumor antigen-processing DCs [76] and contributes to tumor eradication after vaccination [77].

An additional imprinting of homing patterns has been reported for memory T cells. Central memory T cells (T_{CM}) maintain CCR7 and CD62L expressions and, therefore, keep on re-circulating between blood and SLOs. These cells display little effector function but become activated upon re-encounter of cognate antigen in SLOs, which leads to a fast proliferation and differentiation into effector cells. On the other hand, effector memory T cell (T_{EM}) have lost their CCR7 expression and provide immediate protection by migrating into inflamed peripheral tissue, where they readily exert effector functions upon antigen encounter such as cytokine secretion in case of $CD4^+$ T cells, or target cell killing in case $CD8^+$ T cells [78]. A refinement of this concept has identified stem cell memory T cells (T_{SCM}), which are precursors of T_{CM} and have a high potential in ACT in cancer patients, in part owing to their high proliferative capacity [79].

1.7 T Cell Motility in Non-lymphoid Organs

Effector T cells that extravasated in non-lymphoid tissues show polarized cell shapes, which correlate with continuous migration and scanning of pMHC complexes on tissue-resident stromal and hematopoietic cells. The tissue architecture has a strong impact on T cell motility. Epidermal tissue of the skin with tightly packed keratinocytes imposes a challenging environment for migrating $CD8^+$ T cells, which move with average speeds of only 1–2 μm/min [80, 81]. In contrast, T cell migration speeds in connective tissue-rich dermis is in the range of 5–8 μm/min [81]. Granuloma-associated T cells showed comparable speeds [82]. Thus, outside epithelial tissue, T cell motility is still high and allows for fast recognition of cognate pMHC. Similar to naïve T cell motility in lymphoid tissue, T cell motility is driven by protrusion–contraction sequences, which are likely to be fuelled by DOCK8—Cdc42 and DOCK2—Rac modules, with Rho—ROCK—Myosin IIA contractions for efficient nuclear translocation. What are the driving forces that activate small GTPases in tissue? While chemokines and integrins may contribute to basal immune surveillance [83], activated T cells show spontaneous motility in vitro, in the absence of chemokines. Furthermore, F-actin-mediated protrusions are sufficient to drive leukocyte migration in the absence of integrin-mediated binding in 3D environments [34, 84]. This cell-intrinsic F-actin treadmilling-driven motility, in combination with increased contractility of the trailing edge in confined environments [30, 31], is likely to provide effector T cells a certain degree of autonomy in their tissue surveillance strategies. Yet, chemoattractants still play a significant role for T cells at inflamed sites. The inflammatory chemokine receptor CXCR3

has been recently identified with effective T cell function. CXCR3 expression is increased during T cell activation in an affinity-dependent manner [85] and is important for homing to inflammatory sites, including tumors [86, 87]. Its ligands CXCL9, CXCL10 and CXCL11 are rapidly induced during inflammation and guide T cells into specific microenvironments in lymphoid and non-lymphoid tissues [88]. An important feature for CXCR3 is its role in recruiting activated $CD8^+$ T cells to an epithelial niche [89, 90]. In the absence of CXCR3, $CD8^+$ T cells fail to cross the basement membrane that divides dermis from epidermis, although dermal T cells still display high basal motility. Thus, dense tissue structures constitute a barrier for T cells, which require chemotactic gradients for efficient accumulation. Similarly, CXCR3 contributes to amass T cells at virus-infected sites in skin, while this receptor is not required for baseline T cell motility in tissue [91]. Taken together, chemokine guidance to inflamed sites and through dense ECM structures is required for $CD8^+$ T cell function in non-lymphoid sites.

In LNs, T cells use FRCs as guidance cues for their migration. Similarly, in non-lymphoid tissues, T cells are typically observed in close apposition to substrates, which they use as support. As example, epidermal T cells migrate on top of the basement membrane located at the dermis—epidermis border [92], while granuloma-associated T cells often remain attached to macrophages [82]. Thus, T cells use stromal guidance cues for maneuvering in tissues. While this is an efficient way of dissemination, it can also prevent effective accumulation at tumor sites, as discussed below.

1.8 Challenges for T Cells Infiltrating into Tumor Sites

Since homing and migration of effector T cells into and within tumors is covered in other chapters, we will only briefly delineate the major challenges T cells need to overcome during tumor infiltration for recognition and elimination of neoplastic cells. It is well-described that $CD8^+$ tumor-infiltrating lymphocytes (TILs) face an immunosuppressant environment owing to a number of reasons, including PD-1L expression on stroma and tumor cells [93]. Numerous lines of evidence further suggest that tolerance to tumors is fostered by presentation of peptide antigens on MHC molecules on the surface of non-activating APCs [94, 95]. Furthermore, broad classes of cell types derived from the mononuclear phagocyte system, such as myeloid-derived suppressor cells and tumor-associated macrophages, have been implicated in promoting tumor growth and metastasis while inhibiting productive immune responses by T cells [96, 97]. In a mouse breast cancer model, it was furthermore shown that dendritic cells located at the margins of tumor islets failed to support T cell activation [98]. Similarly, regulatory T cells in tumors induced dysfunctional APCs in tumors that prevented T cell cytotoxicity [99].

The tumor architecture also imposes limits to TIL accumulation in tumors by effectively preventing T cell elimination of neoplastic cells. Similar to non-tumor

sites, 2PM studies have shown that T cells migrate along collagen fibers or in perivascular space surrounding tumors [100, 101]. Thus, TILs often fail to localize to the tumor-cell containing part of the tumor but remain in surrounding stroma. This was elegantly confirmed in a recent study using slices of human lung tumors overlaid with activated autologous T cells. These experiments provided evidence that dense ECM areas preclude T cell entry into tumor islets [102]. As a consequence, T cell trajectories were determined by aligned ECM fibers surrounding blood vessels and other structures, where also endogenous T lymphocytes were located. Accordingly, active T cell motility was observed in loose fibronectin and collagen regions, whereas T cells migrated poorly in dense matrix areas. Thus, the density and orientation of the ECM play a key role in controlling T cell migration and positioning, and act as a physical barrier for T cell infiltration [102]. Taken together, T cells entry into tumor islets is presumably made difficult by tight apposition of tumor cells, ECM structure, absence of attracting chemokines and spatial arrangement of guidance cues.

Chemotherapy has the potential to induce promigratory chemokines, including CXCR3 ligands and CCL5, which binds CCR3, CCR5 and CCR1 [103]. In addition, secretion of CXCL10 and CCL5 is associated with effector $CD8^+$ T cell recruitment in colorectal cancer microenvironments [104]. Thus, combination therapies of chemo- or radiotherapy with ACT may boost antitumor activity of transferred T cells. In this context, it is interesting to note that a number of intracellular negative regulators of antitumor activity were recently identified in activated T cells [105]. These negative regulators include phosphatases, but also, unexpectedly, modulators of the actin cytoskeleton including the Rho downstream effector ROCK1 and the negative regulator of Rho activity, ArhGAP5. ArhGAP5 belongs to the family of GTPAse-activating proteins, which also includes myosin IXb (Myo9b) known to regulate cell shape in macrophages [41, 45]. How the inhibition of such negative regulators induces efficient tumor eradication remains unclear to date. Yet, based on evidence outlined above, an attractive hypothesis is that regulators of the actin cytoskeleton influence the T cell's ability to migrate into tumor sites for increased identification of cognate target cells for killing. Since cytoskeletal dynamics are governed by upstream regulators and downstream effectors of small GTPases, these molecules may constitute valuable drug targets. An important aim will be to exploit knowledge gained from T cell trafficking to non-tumor tissues for improved T cell infiltration into tumors. Genetic engineering of promotility factors, together with breaking of tumor stromal barriers, induction of promigratory chemokines, improved antigen availability and reduced immunosuppression may expand the scope of patients profiting from adoptive cell therapy.

Acknowledgements The Stein laboratory is funded by Swiss National Foundation grants 31003A_135649, CR23I3_156234 and CRSII3_141918, and Swiss Cancer League grant KFS-3524-08-2014.

No Conflict Statement No potential conflicts of interest were disclosed.

References

1. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JMS, Mahmoud SMA, Rakha E, Ellis IO, Liu S, Gao D, Nielsen TO, Pharoah PDP, Caldas C. Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. Ann Oncol. 2014;25(8):1536–43. doi:10.1093/annonc/mdu191.
2. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc P-H, Trajanoski Z, Fridman W-H, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313:1960–4. doi:10.1126/science.1129139.
3. Braumüller H, Wieder T, Brenner E, Aßmann S, Hahn M, Alkhaled M, Schilbach K, Essmann F, Kneilling M, Griessinger C, Ranta F, Ullrich S, Mocikat R, Braungart K, Mehra T, Fehrenbacher B, Berdel J, Niessner H, Meier F, van den Broek M, Häring H-U, Handgretinger R, Quintanilla-Martinez L, Fend F, Pesic M, Bauer J, Zender L, Schaller M, Schulze-Osthoff K, Röcken M. T-helper-1-cell cytokines drive cancer into senescence. Nature. 2013;494:361–5. doi:10.1038/nature11824.
4. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity. 2013;39:11–26. doi:10.1016/j.immuni.2013.07.008.
5. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol. 2006;6:836–48. doi:10.1038/nri1961.
6. DuPage M, Mazumdar C, Schmidt LM, Cheung AF, Jacks T. Expression of tumour-specific antigens underlies cancer immunoediting. Nature. 2012;482:405–9. doi:10.1038/nature10803.
7. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39:1–10. doi:10.1016/j.immuni.2013.07.012.
8. Couzin-Frankel J. Cancer immunotherapy. Science. 2013;342:1432–3. doi:10.1126/science.342.6165.1432.
9. Schneider H, Downey J, Smith A, Zinselmeyer BH, Rush C, Brewer JM, Wei B, Hogg N, Garside P, Rudd CE. Reversal of the TCR stop signal by CTLA-4. Science. 2006;313:1972–5. doi:10.1126/science.1131078.
10. Honda T, Egen JG, Lämmermann T, Kastenmuller W, Torabi-Parizi P, Germain RN. Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues. Immunity. 2014;40:235–47. doi:10.1016/j.immuni.2013.11.017.
11. Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. Nat Rev Immunol. 2013;13:309–20. doi:10.1038/nri3442.
12. Textor J, Henrickson SE, Mandl JN, Von Andrian UH, Westermann J, De Boer RJ, Beltman JB. Random migration and signal integration promote rapid and robust T cell recruitment. PLoS Comput Biol. 2014;10, e1003752. doi:10.1371/journal.pcbi.1003752.
13. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol. 2005;23:127–59. doi:10.1146/annurev.immunol.23.021704.115628.
14. Von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol. 2003;3:867–78. doi:10.1038/nri1222.
15. Shulman Z, Shinder V, Klein E, Grabovsky V, Yeger O, Geron E, Montresor A, Bolomini-Vittori M, Feigelson SW, Kirchhausen T, Laudanna C, Shakhar G, Alon R. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. Immunity. 2009;30:384–96. doi:10.1016/j.immuni.2008.12.020.
16. Bajénoff M, Egen JG, Koo LY, Laugier JP, Brau F, Glaichenhaus N, Germain RN. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity. 2006;25:989–1001. doi:10.1016/j.immuni.2006.10.011.
17. Boscacci RT, Pfeiffer F, Gollmer K, Sevilla AIC, Martin AM, Soriano SF, Natale D, Henrickson S, Von Andrian UH, Fukui Y, Mellado M, Deutsch U, Engelhardt B, Stein JV. Comprehensive analysis of lymph node stroma-expressed Ig superfamily members

reveals redundant and nonredundant roles for ICAM-1, ICAM-2, and VCAM-1 in lymphocyte homing. Blood. 2010;116:915–25. doi:10.1182/blood-2009-11-254334.
18. Katakai T, Habiro K, Kinashi T. Dendritic cells regulate high-speed interstitial T cell migration in the lymph node via LFA-1/ICAM-1. J Immunol. 2013;191:1188–99. doi:10.4049/jimmunol.1300739.
19. Chang JE, Turley SJ. Stromal infrastructure of the lymph node and coordination of immunity. Trends Immunol. 2015;36:30–9. doi:10.1016/j.it.2014.11.003.
20. Wendland M, Willenzon S, Kocks J, Davalos-Misslitz AC, Hammerschmidt SI, Schumann K, Kremmer E, Sixt M, Hoffmeyer A, Pabst O, Förster R. Lymph node T cell homeostasis relies on steady state homing of dendritic cells. Immunity. 2011;35:945–57. doi:10.1016/j.immuni.2011.10.017.
21. Asperti-Boursin F, Real E, Bismuth G, Trautmann A, Donnadieu E. CCR7 ligands control basal T cell motility within lymph node slices in a phosphoinositide 3-kinase-independent manner. J Exp Med. 2007;204:1167–79. doi:10.1084/jem.20062079.
22. Okada T, Cyster J. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. J Immunol. 2007;178:2973.
23. Worbs T, Mempel TR, Bölter J, Von Andrian UH, Förster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. J Exp Med. 2007;204:489–95. doi:10.1084/jem.20061706.
24. Hwang I, Park C, Kehrl J. Impaired trafficking of Gnai2+/- and Gnai2-/- T lymphocytes: implications for T cell movement within lymph nodes. J Immunol. 2007;179:439.
25. Bai Z, Cai L, Umemoto E, Takeda A, Tohya K, Komai Y, Veeraveedu PT, Hata E, Sugiura Y, Kubo A, Suematsu M, Hayasaka H, Okudaira S, Aoki J, Tanaka T, Albers HMHG, Ovaa H, Miyasaka M. Constitutive lymphocyte transmigration across the basal lamina of high endothelial venules is regulated by the autotaxin/lysophosphatidic acid axis. J Immunol. 2013;190:2036–48. doi:10.4049/jimmunol.1202025.
26. Kanda H, Newton R, Klein R, Morita Y, Gunn MD, Rosen SD. Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. Nat Immunol. 2008;9:415–23. doi:10.1038/ni1573.
27. Katakai T, Kondo N, Ueda Y, Kinashi T. Autotaxin produced by stromal cells promotes LFA-1-independent and rho-dependent interstitial T cell motility in the lymph node paracortex. J Immunol. 2014;193:617–26. doi:10.4049/jimmunol.1400565.
28. Knowlden SA, Capece T, Popovic M, Chapman TJ, Rezaee F, Kim M, Georas SN. Regulation of T cell motility in vitro and in vivo by LPA and LPA2. PLoS One. 2014;9, e101655. doi:10.1371/journal.pone.0101655.
29. Zhang Y, Chen Y-CM, Krummel MF, Rosen SD. Autotaxin through lysophosphatidic acid stimulates polarization, motility, and transendothelial migration of naive T cells. J Immunol. 2012;189:3914–24. doi:10.4049/jimmunol.1201604.
30. Liu Y-J, Le Berre M, Lautenschlaeger F, Maiuri P, Callan-Jones A, Heuzé M, Takaki T, Voituriez R, Piel M. Confinement and low adhesion induce fast amoeboid migration of slow mesenchymal cells. Cell. 2015;160:659–72. doi:10.1016/j.cell.2015.01.007.
31. Ruprecht V, Wieser S, Callan-Jones A, Smutny M, Morita H, Sako K, Barone V, Ritsch-Marte M, Sixt M, Voituriez R, Heisenberg C-P. Cortical contractility triggers a stochastic switch to fast amoeboid cell motility. Cell. 2015;160:673–85. doi:10.1016/j.cell.2015.01.008.
32. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. Nat Immunol. 2007;8:1295–301. doi:10.1038/ni1545.
33. Shiow LR, Rosen DB, Brdicková N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature. 2006;440:540–4. doi:10.1038/nature04606.
34. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, Keller M, Förster R, Critchley DR, Fässler R, Sixt M. Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature. 2008;453:51–5. doi:10.1038/nature06887.
35. Krummel MF, Macara I. Maintenance and modulation of T cell polarity. Nat Immunol. 2006;7:1143–9. doi:10.1038/ni1404.
36. Real E, Faure S, Donnadieu E, Delon J. Cutting edge: atypical PKCs regulate T lymphocyte polarity and scanning behavior. J Immunol. 2007;179:5649–52.

37. Latek D, Modzelewska A, Trzaskowski B, Palczewski K, Filipek S. G protein-coupled receptors—recent advances. Acta Biochim Pol. 2012;59:515–29. doi:10.1016/j.jhazmat.2004.10.008.
38. Thelen M, Stein J. How chemokines invite leukocytes to dance. Nat Immunol. 2008;9:953–9.
39. Kehrl JH. Chemoattractant receptor signaling and the control of lymphocyte migration. Immunol Res. 2006;34:211–27. doi:10.1385/IR:34:3:211.
40. Barberis L, Pasquali C, Bertschy-Meier D, Cuccurullo A, Costa C, Ambrogio C, Vilbois F, Chiarle R, Wymann M, Altruda F, Rommel C, Hirsch E. Leukocyte transmigration is modulated by chemokine-mediated PI3Kγ-dependent phosphorylation of vimentin. Eur J Immunol. 2009;39:1136–46. doi:10.1002/eji.200838884.
41. Tybulewicz VLJ, Henderson RB. Rho family GTPases and their regulators in lymphocytes. Nat Rev Immunol. 2009;9:630–44. doi:10.1038/nri2606.
42. del Pozo MA, Vicente-Manzanares M, Tejedor R, Serrador JM, Sánchez-Madrid F. Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. Eur J Immunol. 1999;29:3609–20. doi:10.1002/(SICI)1521-4141(199911)29:11<3609::AID-IMMU3609>3.0.CO;2-S.
43. Lammermann T, Renkawitz J, Wu X, Hirsch K, Brakebusch C, Sixt M. Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration. Blood. 2009;113:5703–10. doi:10.1182/blood-2008-11-191882.
44. Ratner S, Piechocki MP, Galy A. Role of Rho-family GTPase Cdc42 in polarized expression of lymphocyte appendages. J Leukoc Biol. 2003;73:830–40. doi:10.1189/jlb.1001894.
45. Hanley PJ, Xu Y, Kronlage M, Grobe K, Schon P, Song J, Sorokin L, Schwab A, Bahler M. Motorized RhoGAP myosin IXb (Myo9b) controls cell shape and motility. Proc Natl Acad Sci. 2010;107:12145–50. doi:10.1073/pnas.0911986107.
46. Morin NA, Oakes PW, Hyun Y-M, Lee D, Chin YE, Chin EY, King MR, Springer TA, Shimaoka M, Tang JX, Reichner JS, Kim M. Nonmuscle myosin heavy chain IIA mediates integrin LFA-1 de-adhesion during T lymphocyte migration. J Exp Med. 2008;205:195–205. doi:10.1084/jem.20071543.
47. Jacobelli J, Estin Matthews M, Chen S, Krummel MF. Activated T cell trans-endothelial migration relies on myosin-IIA contractility for squeezing the cell nucleus through endothelial cell barriers. PLoS One. 2013;8, e75151. doi:10.1371/journal.pone.0075151.
48. Soriano SF, Hons M, Schumann K, Kumar V, Dennier TJ, Lyck R, Sixt M, Stein JV. In vivo analysis of uropod function during physiological T cell trafficking. J Immunol. 2011;187:2356–64. doi:10.4049/jimmunol.1100935.
49. Goley ED, Welch MD. The ARP2/3 complex: an actin nucleator comes of age. Nat Rev Mol Cell Biol. 2006;7:713–26. doi:10.1038/nrm2026.
50. Krummel MF, Friedman RS, Jacobelli J. Modes and mechanisms of T cell motility: roles for confinement and Myosin-IIA. Curr Opin Cell Biol. 2014;30C:9–16. doi:10.1016/j.ceb.2014.05.003.
51. Vicente-Manzanares M, Sánchez-Madrid F. Role of the cytoskeleton during leukocyte responses. Nat Rev Immunol. 2004;4:110–22. doi:10.1038/nri1268.
52. DerMardirossian C, Bokoch GM. GDIs: central regulatory molecules in Rho GTPase activation. Trends Cell Biol. 2005;15:356–63. doi:10.1016/j.tcb.2005.05.001.
53. Rougerie P, Delon J. Rho GTPases: masters of T lymphocyte migration and activation. Immunol Lett. 2012;142:1–13. doi:10.1016/j.imlet.2011.12.003.
54. Nishikimi A, Kukimoto-Niino M, Yokoyama S, Fukui Y. Immune regulatory functions of DOCK family proteins in health and disease. Exp Cell Res. 2013;319:2343–9. doi:10.1016/j.yexcr.2013.07.024.
55. Harada Y, Tanaka Y, Terasawa M, Pieczyk M, Habiro K, Katakai T, Hanawa-Suetsugu K, Kukimoto-Niino M, Nishizaki T, Shirouzu M, Duan X, Uruno T, Nishikimi A, Sanematsu F, Yokoyama S, Stein JV, Kinashi T, Fukui Y. DOCK8 is a Cdc42 activator critical for interstitial dendritic cell migration during immune responses. Blood. 2012;119:4451–61. doi:10.1182/blood-2012-01-407098.
56. Zhang Q, Dove CG, Hor JL, Murdock HM, Strauss-Albee DM, Garcia JA, Mandl JN, Grodick RA, Jing H, Chandler-Brown DB, Lenardo TE, Crawford G, Matthews HF, Freeman

AF, Cornall RJ, Germain RN, Mueller SN, Su HC. DOCK8 regulates lymphocyte shape integrity for skin antiviral immunity. J Exp Med. 2014;211(13):2549–66. doi:10.1084/jem.20141307.
57. Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, Matthews HF, Davis J, Turner ML, Uzel G, Holland SM, Su HC. Combined immunodeficiency associated with DOCK8 mutations. N Engl J Med. 2009;361:2046–55. doi:10.1056/NEJMoa0905506.
58. Fukui Y, Hashimoto O, Sanui T, Oono T, Koga H, Abe M, Inayoshi A, Noda M, Oike M, Shirai T. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature. 2001;412:826–31.
59. Faroudi M, Hons M, Zachacz A, Dumont C, Lyck R, Stein JV, Tybulewicz VLJ. Critical roles for Rac GTPases in T-cell migration to and within lymph nodes. Blood. 2010;116:5536–47. doi:10.1182/blood-2010-08-299438.
60. Nombela-Arrieta C, Mempel TR, Soriano SF, Mazo I, Wymann MP, Hirsch E, Martínez-A C, Fukui Y, Von Andrian UH, Stein JV. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. J Exp Med. 2007;204:497–510. doi:10.1084/jem.20061780.
61. Dobbs K, Domínguez Conde C, Zhang S-Y, Parolini S, Audry M, Chou J, Haapaniemi E, Keles S, Bilic I, Okada S, Massaad MJ, Rounioja S, Alwahadneh AM, Serwas NK, Capuder K, Çiftçi E, Felgentreff K, Ohsumi TK, Pedergnana V, Boisson B, Haskoloğlu Ş, Ensari A, Schuster M, Moretta A, Itan Y, Patrizi O, Rozenberg F, Lebon P, Saarela J, Knip M, Petrovski S, Goldstein DB, Parrott RE, Savas B, Schambach A, Tabellini G, Bock C, Chatila TA, Comeau AM, Geha RS, Abel L, Buckley RH, İkincioğulları A, Al-Herz W, Helminen M, Doğu F, Casanova J-L, Boztuğ K, Notarangelo LD. Inherited DOCK2 deficiency in patients with early-onset invasive infections. N Engl J Med. 2015;372:2409–22. doi:10.1056/NEJMoa1413462.
62. Shulman Z, Pasvolsky R, Woolf E, Grabovsky V, Feigelson SW, Erez N, Fukui Y, Alon R. DOCK2 regulates chemokine-triggered lateral lymphocyte motility but not transendothelial migration. Blood. 2006;108:2150–8. doi:10.1182/blood-2006-04-017608.
63. Donnadieu E, Bismuth G, Trautmann A. Antigen recognition by helper T cells elicits a sequence of distinct changes of their shape and intracellular calcium. Curr Biol. 1994;4:584–95.
64. Negulescu PA, Krasieva TB, Khan A, Kerschbaum HH, Cahalan MD. Polarity of T cell shape, motility, and sensitivity to antigen. Immunity. 1996;4:421–30.
65. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. The immunological synapse: a molecular machine controlling T cell activation. Science. 1999;285:221–7.
66. Le Floc'h A, Tanaka Y, Bantilan NS, Voisinne G, Altan-Bonnet G, Fukui Y, Huse M. Annular PIP3 accumulation controls actin architecture and modulates cytotoxicity at the immunological synapse. J Exp Med. 2013;210:2721–37. doi:10.1016/j.cub.2005.12.024.
67. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, Azuma M, Krummel MF, Bluestone JA. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol. 2009;10:1185–92. doi:10.1038/ni.1790.
68. Pentcheva-Hoang T, Simpson TR, Montalvo-Ortiz W, Allison JP. Cytotoxic T lymphocyte antigen-4 blockade enhances antitumor immunity by stimulating melanoma-specific T-cell motility. Cancer Immunol Res. 2014;2:970–80. doi:10.1158/2326-6066.CIR-14-0104.
69. Zinselmeyer BH, Heydari S, Sacristan C, Nayak D, Cammer M, Herz J, Cheng X, Davis SJ, Dustin ML, McGavern DB. PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. J Exp Med. 2013;210(4):757–74. doi:10.1084/jem.20121416.
70. Kupper TS, Fuhlbrigge RC, Kieffer JD, Armerding D. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. Nature. 1997;389:978–81. doi:10.1038/40166.
71. Smithson G, Rogers CE, Smith PL, Scheidegger EP, Petryniak B, Myers JT, Kim DSL, Homeister JW, Lowe JB. Fuc-Tvii is required for T helper 1 and T cytotoxic 1 lymphocyte selectin ligand expression and recruitment in inflammation, and together with Fuc-Tiv regulates naive T cell trafficking to lymph nodes. J Exp Med. 2001;194:601–14. doi:10.1016/1074-7613(94)90041-8.

72. Mora JR, Iwata M, Von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008;8:685–98. doi:10.1038/nri2378.
73. Mora JR, Von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. 2006;27:235–43. doi:10.1016/j.it.2006.03.007.
74. Hammerschmidt SI, Ahrendt M, Bode U, Wahl B, Kremmer E, Forster R, Pabst O. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. J Exp Med. 2008;205:2483–90. doi:10.1084/jem.20080039.
75. Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, Butcher EC. DCs metabolize sunlight-induced vitamin D3 to "program" T cell attraction to the epidermal chemokine CCL27. Nat Immunol. 2007;8:285–93. doi:10.1038/ni1433.
76. Calzascia T, Masson F, Di Berardino-Besson W, Contassot E, Wilmotte R, Aurrand-Lions M, Rüegg C, Dietrich P-Y, Walker PR. Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. Immunity. 2005;22:175–84. doi:10.1016/j.immuni.2004.12.008.
77. Sandoval F, Terme M, Nizard M, Badoual C, Bureau M-F, Freyburger L, Clement O, Marcheteau E, Gey A, Fraisse G, Bouguin C, Merillon N, Dransart E, Tran T, Quintin-Colonna F, Autret G, Thiebaud M, Suleman M, Riffault S, Wu T-C, Launay O, Danel C, Taieb J, Richardson J, Zitvogel L, Fridman WH, Johannes L, Tartour E. Mucosal imprinting of vaccine-induced CD8+ T cells is crucial to inhibit the growth of mucosal tumors. Sci Transl Med. 2013;5:172ra20. doi:10.1126/scitranslmed.3004888.
78. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004;22:745–63. doi:10.1146/annurev.immunol.22.012703.104702.
79. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol. 2014;14:24–35. doi:10.1038/nri3567.
80. Ariotti S, Beltman JB, Chodaczek G, Hoekstra ME, van Beek AE, Gomez-Eerland R, Ritsma L, van Rheenen J, Marée AFM, Zal T, De Boer RJ, Haanen JBAG, Schumacher TN. Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly recognize local antigen. Proc Natl Acad Sci. 2012;109:19739–44. doi:10.1073/pnas.1208927109.
81. Gebhardt T, Whitney PG, Zaid A, Mackay LK, Brooks AG, Heath WR, Carbone FR, Mueller SN. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. Nature. 2011;477:216–9. doi:10.1038/nature10339.
82. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. Immunity. 2008;28:271–84. doi:10.1016/j.immuni.2007.12.010.
83. Overstreet MG, Gaylo A, Angermann BR, Hughson A, Hyun Y-M, Lambert K, Acharya M, Billroth-MacLurg AC, Rosenberg AF, Topham DJ, Yagita H, Kim M, Lacy-Hulbert A, Meier-Schellersheim M, Fowell DJ. Inflammation-induced interstitial migration of effector CD4+ T cells is dependent on integrin αV. Nat Immunol. 2013;14:949–58. doi:10.1038/ni.2682.
84. Friedl P, Zänker K, Bröcker E. Cell migration strategies in 3-D extracellular matrix: differences in morphology, cell matrix interactions, and integrin function. Microsc Res Tech. 1998;43:369–78.
85. Preston GC, Feijoo-Carnero C, Schurch N, Cowling VH, Cantrell DA. The impact of KLF2 modulation on the transcriptional program and function of CD8 T cells. PLoS One. 2013;8, e77537. doi:10.1371/journal.pone.0077537.
86. Mikucki ME, Fisher DT, Matsuzaki J, Skitzki JJ, Gaulin NB, Muhitch JB, Ku AW, Frelinger JG, Odunsi K, Gajewski TF, Luster AD, Evans SS. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. Nat Commun. 2015;6:1–14. doi:10.1038/ncomms8458.
87. Slaney CY, Kershaw MH, Darcy PK. Trafficking of T cells into tumors. Cancer Res. 2014;74(24):7168–74. doi:10.1158/0008-5472.CAN-14-2458.
88. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32:659–702. doi:10.1146/annurev-immunol-032713-120145.

89. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, Vega-Ramos J, Lauzurica P, Mueller SN, Stefanovic T, Tscharke DC, Heath WR, Inouye M, Carbone FR, Gebhardt T. The developmental pathway for CD103+CD8+ tissue-resident memory T cells of skin. Nat Immunol. 2013;14:1294–301. doi:10.1038/ni.2744.
90. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. Nature. 2012;491:463–7. doi:10.1038/nature11522.
91. Hickman HD, Reynoso GV, Ngudiankama BF, Cush SS, Gibbs J, Bennink JR, Yewdell JW. CXCR3 chemokine receptor enables local CD8(+) T cell migration for the destruction of virus-infected cells. Immunity. 2015;42:524–37. doi:10.1016/j.immuni.2015.02.009.
92. Zaid A, Mackay LK, Rahimpour A, Braun A, Veldhoen M, Carbone FR, Manton JH, Heath WR, Mueller SN. Persistence of skin-resident memory T cells within an epidermal niche. Proc Natl Acad Sci. 2014;111:5307–12. doi:10.1073/pnas.1322292111.
93. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015;36:265–76. doi:10.1016/j.it.2015.02.008.
94. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. J Immunol. 2005;175:4583–92. doi:10.4049/jimmunol.175.7.4583.
95. Sotomayor EM, Borrello I, Rattis FM, Cuenca AG, Abrams J, Staveley-O'Carroll K, Levitsky HI. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. Blood. 2001;98:1070–7.
96. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9:162–74. doi:10.1038/nri2506.
97. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol. 2009;9:259–70. doi:10.1038/nri2528.
98. Engelhardt JJ, Boldajipour B, Beemiller P, Pandurangi P, Sorensen C, Werb Z, Egeblad M, Krummel MF. Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. Cancer Cell. 2012;21:402–17. doi:10.1016/j.ccr.2012.01.008.
99. Bauer CA, Kim EY, Marangoni F, Carrizosa E, Claudio NM, Mempel TR. Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. J Clin Invest. 2014;124(6):2425–40. doi:10.1172/JCI66375.
100. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J Exp Med. 2007;204:345–56. doi:10.1084/jem.20061890.
101. Mrass P, Takano H, Ng LG, Daxini S, Lasaro MO, Iparraguirre A, Cavanagh LL, Von Andrian UH, Ertl HCJ, Haydon PG, Weninger W. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006;203:2749–61. doi:10.1084/jem.20060710.
102. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean M-C, Validire P, Trautmann A, Mami-Chouaib F, Donnadieu E. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 2012;122:899–910. doi:10.1172/JCI45817DS1.
103. Hong M, Puaux A-L, Huang C, Loumagne L, Tow C, Mackay C, Kato M, Prévost-Blondel A, Avril M-F, Nardin A, Abastado J-P. Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T-cell infiltration and tumor control. Cancer Res. 2011;71:6997–7009. doi:10.1158/0008-5472.CAN-11-1466.
104. Zumwalt TJ, Arnold M, Goel A, Boland CR. Active secretion of CXCL10 and CCL5 from colorectal cancer microenvironments associates with GranzymeB+ CD8+ T-cell infiltration. Oncotarget. 2015;6:2981–91.
105. Zhou P, Shaffer DR, Alvarez Arias DA, Nakazaki Y, Pos W, Torres AJ, Cremasco V, Dougan SK, Cowley GS, Elpek K, Brogdon J, Lamb J, Turley SJ, Ploegh HL, Root DE, Love JC, Dranoff G, Hacohen N, Cantor H, Wucherpfennig KW. In vivo discovery of immunotherapy targets in the tumour microenvironment. Nature. 2014;506:52–7. doi:10.1038/nature12988.

Chapter 2
Regulation of Anti-tumor T Cell Migration and Function: Contribution of Real-Time Imaging

Pierre-Louis Loyher, Christophe Combadière, and Alexandre Boissonnas

Abstract Mounting a protective immune response is critically dependent on the orchestrated movement of leucocytes throughout the body. Effector T cells represent a major cell type in the antitumor immune response as they can specifically recognize and target transformed cells. Immunotherapies based on enhancing antitumor T cell functions are being actively developed with many clinical trials underway. Yet, the definition of basic migratory patterns of lymphocytes in various physiological and pathophysiological contexts has only been enabled recently with the use of intravital imaging (IVM) at high resolution. This technology allows to directly visualize the key events of the T cell-mediated immune response in situ including activation, trafficking, interactions with stromal and immune cell components, cell killing but also immune mechanisms suppressing the T cell response. With information on the spatiotemporal contexts of these events it is possible to determine the relative contribution of different cell types during an antitumor T cell response and the major hurdles to successful tumor immune rejection. This chapter will focus on different points for which IVM contributed to our understanding of antitumor T cell migration and function during an endogenous response or after T cell targeted immunotherapies.

Keywords Intravital Imaging • Cell migration • Cell interactions • Cancer immunotherapy • Tumor antigens • Immunosuppression • Tumor microenvironment • Immune checkpoint

P.-L. Loyher • C. Combadière • A. Boissonnas (✉)
Sorbonne Universités, UPMC Université Paris 06 UMR_S1135, IUC, Inserm U1135, CNRS ERL8255, Centre d'Immunologie et des Maladies Infectieuses,
91 Boulevard de l'Hôpital, Paris F-75013, France
e-mail: alexandre.boissonnas@upmc.fr

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_2

Abbreviations

APCs	Antigen presenting cells
CARS	Coherent anti-stokes raman scattering spectroscopy
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ECM	Extra-cellular matrix
GFP	Green fluorescent protein
HIF-1	Hypoxia inductible factor-1
HLA	Human leucocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon gamma
IL-10	Interleukin 10
IVM	Intra-vital microscopy
LSCM	Laser scanning microscopy
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NK	Natural killer cell
NKT	Natural killer T cell
OPO	Optical parameter oscillator
OTI	Ova-specific CD8 T cell
PD-1	Programmed cell death 1
PD-L1	Programmed cell death-ligand 1
RAE-1γ	Ribonucleic acid export protein 1 gamma
Rag	Recombination-activating genes
SCID	Severe combined immunodeficiency
SHG	Second harmonic generation
TAAs	Tumor-associated antigens
TAMs	Tumor-associated macrophages
TCR	T cell receptor
TDLNs	Tumor draining lymph nodes
Teff	T effector lymphocyte
Th	T helper
THG	Third harmonic generation
TILs	Tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobulin mucin receptor 3
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
TPLSM	Two-photon laser scanning microscopy
TRAIL	Tumor-necrosis-factor related apoptosis inducing ligand
TuDCs	Tumor dendritic cells

VEGF-A	Vascular endothelial growth factor-A
WT	Wild type
YFP	Yellow fluorescent protein

2.1 Introduction

The implication of T lymphocytes in the control of tumor outcome was a matter of controversy. Until recently, evidences that endogenous T cell could help control tumor growth were in large part restricted to preclinical mouse models. Mice deficient for T, B and NKT cellular compartments (Rag1-/-, Rag2-/-, SCID mice, Athymic nude mice) display an increased sensitivity to carcinogen-induced sarcomas [1–3]. Moreover, IFNγ which is secreted by both CD4+ T helper cells (Th) and CD8+ cytotoxic T lymphocyte (CTL) effector cells was shown to play a critical function in cancer immunosurveillance [4]. Subsequently, experiments from Schreiber's group further highlighted the importance of an intact and functional lymphocyte compartment for the shaping of tumors. In these experiments, transplantation of tumor-derived from host depleted of CD4+ and CD8+ T cells into WT recipients leads to rejection whereas tumor transplanted from WT to WT recipient grew readily. This demonstrated that tumors derived from an immunodeficient host are more immunogenic than tumors derived from an immunocompetent host. It was the first demonstration that components of the adaptive immune system could naturally select tumor cells (expressing weaker antigens or incapable of expressing antigens), by destroying only those expressing strong tumor-specific neoantigens, a process known as cancer immunoediting [5].

The immune response depends on the recognition of foreign antigens. This concept is critical in tumor immunity as cancer cells result from neoplastic modifications of the self which may generate tumor-associated antigens (TAAs). TAAs can be formed either from non-mutated proteins that are overexpressed regarding normal tissue patterns or peptides that are entirely absent from the human genome (neoantigens) [6]. Neoantigens are created from novel protein sequence because of tumor-specific DNA alterations or from the viral genome in case of viral-induced cancers. They are particularly relevant for studying anti-tumor T cell functions. Effector T cells that can specifically recognize and target transformed cells may play a crucial role in the immune surveillance of cancer [7]. CTL and Th cells have the potential to kill or control abnormal cells and are also essential for the activation of other components of the immune machinery. However, the precise tumor killing pathways displayed by these effectors are quite elusive so far. The identification of tumor neoantigens and the isolation of tumor-specific T cells have led to a great effort in developing therapeutic strategies focusing on T cell mediated antitumor immune response. The observation that adoptive transfer of ex-vivo expanded tumor-infiltrating lymphocytes (TILs) can induce a clinical response in melanoma patient has given a direct evidence that the T cell compartment could contribute to

the control of tumor growth [8]. Vaccination with tumor antigens were also developed and tested in different cancer types. A key information from these trials is that despite the high level of vaccine-induced circulating T cells and no proof of antigen loss by the cancer cells, they provided little clinical efficacy with evidences of disease recurrence. Collectively, these observations indicate that generating antigen-specific T cells is not sufficient to induce a durable control of tumor growth [9]. One explanation could be that the generated T cells failed to be recruited into the tumor and/or their functional activities were dampened by the tumor microenvironment (TME). Indeed, immuno-histological analysis of human cancers that did not respond to immunotherapies revealed that T cell infiltration was lacking in the tumor core [10]. Subsequent studies have shown that the intra-tumoral location, density and activation status of endogenous or transferred anti-tumor T cells strongly correlate with the long-term outcome in patients with colorectal and ovarian cancers [11, 12].

A series of stepwise events are required for T cells to be activated and to migrate to the tumor site for a productive immune response to occur, although tumor cells and the TME are able to interfere with virtually any of these steps. As a result, the study of the regulation of T cell function should include, in addition to tumor cells, the other cells composing the TME. These stromal cells can either favor or limit tumor growth depending on the cell types and physiological situations [13].

The development of intra-vital microscopy (IVM) has enabled a better understanding of the migration patterns in the physiological environment, interactions with different cell types and function of T cells during an antitumor immune response. The molecular and structural architecture of the TME, including vessels, extracellular matrix and macrophage networks are key emerging factors affecting TIL trafficking and fitness [14]. Distant sites, such as the thymus or tumor-draining lymph nodes (TDLNs), can also be regulated by the upstream tumor and this can have a profound impact on the outcome of the immune response [15].

This chapter will present how real time live imaging contributed to our knowledge of the different steps of antitumor T cell activation and migration, highlighting the mechanisms that tumor cells may utilize to evade immunity.

2.2 Basic Principles of Intravital Imaging

The rejection of nascent and established tumors by leukocytes requires distinct phases which are precisely coordinated both temporally and anatomically. These steps include (i) the homing into tissues via the bloodstream or lymphatics (ii) the ability to navigate within the interstitial space and (iii) the recognition of specific antigens determinants displayed at the surface of target cells or on the surface of antigen presenting cells (APCs) by cell-to-cell contact. Understanding these immune response processes necessitates the study of immune cells in situ, as they interact and adapt uniquely with the microenvironment and the tissue architecture they encounter. Fortunately, exciting advances in IVM technology allow the study of immune cells in their natural environment in real time [16].

The two main forms of optical imaging techniques used to study the dynamic of leukocytes in real-time are laser scanning (or spinning-disk) confocal microscopy (LSCM) and two-photon laser scanning microscopy (TPLSM). Single-photon confocal microscopy allows high resolution optical sectioning through a specimen to produce 3D reconstruction of the sample. Beside the superior resolution, confocal microscopy also benefits from excitation multiplexing which enhances spectral separation. Although modifications can be made to standard single photon confocal microscopes, it generally does not allow imaging at depths greater than 100 μm with important risk of photoxicity due to light scattering and slow speed of data acquisition [17]. TPLSM is a variation of conventional LSCM that has many clear advantages. Compared to conventional fluorescent imaging, TPLSM relies on the excitation of the fluorescent molecule through the simultaneous absorption of two photons of half the energy and thus twice the wavelength. Based on this principle, TPLSM uses near-infrared wavelength pulsed laser sending very dense packages of photons with a femtosecond time frame resolution. The near-infrared wavelengths used permit superior tissue penetration (>300 μm) and high quality images deep inside tissues. Near-infrared excitation also causes less tissue autofluorescence, which improves signal specificity and brightness. Moreover, multiphoton excitation is confined only to the focal plane (where the photon density is sufficient to generate this rare event) which avoids parasite signal from the out-of focus optical pathway and strongly minimizes photobleaching and phototoxicity, thereby allowing longer recording episodes [18, 19] (Fig. 2.1). TPLSM can also be used to visualize non-centrosymmetric structures such as collagen bundle through a non-linear optical effect called second harmonic generation (SHG) [20]. This is particularly useful to provide structural reference within the tissue imaged. Disadvantages of this technique might be related to the cost of the required hardware and the availability of fluorescent reporter mice or fluorescent probes with minimal spectral overlap. Indeed, one major limitation of TPLSM concerns the range of laser wavelengths (around 680 nm–1080 nm classically) which limits considerably spectral separation and, therefore, multi-parameter acquisition. Recent technical development of laserists have rendered much more accessible the coupling of optical parametric oscillator (OPO) to classical pulsed femto laser, increasing the possibility of laser tuning up to 1600 nm with independent rays to perform excitation multiplexing. This improvement opens the door to a larger panel of fluorochromes and also to the development of more sophisticated non-linear optical effects such as third harmonic generation (THG) and Coherent Anti-stokes Raman Scattering (CARS) to study biological structures [21].

Two different approaches are commonly used, either “in vivo”, on anesthetized animals in which the tissue of interest is surgically exposed to the objective or “explanted” for which tumors, as well as other, lymphoid and non-lymphoid tissues can be carefully explanted and immobilized in a heated imaging chamber perfused with oxygenated media. In vivo imaging is possible using a minimally invasive surgical procedure to maintain a reasonable homeostatic equilibrium in the operated animal. In contrast, explanted techniques offer the possibility to study tissues barely inaccessible by in vivo procedures and allow for a better stability, avoiding breathes and muscular-related drifting troubles from the tissue. Imaging is possible over a

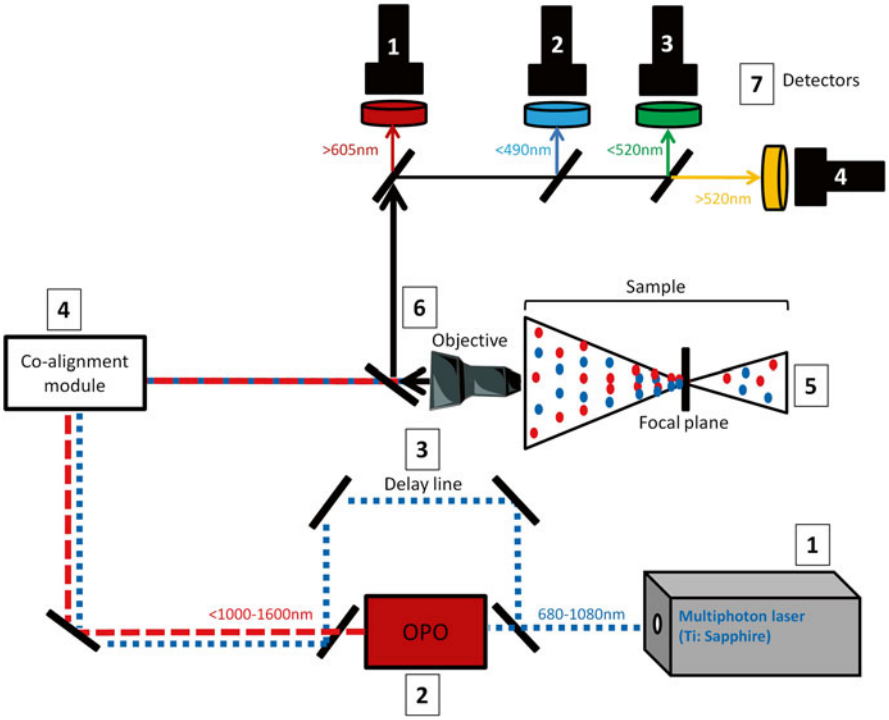

Fig. 2.1 Example of Two-photon instrument design. (1) Multiphoton excitation laser (TI:Saphire) generates femtosecond near-infrared (680–1080 nm) pulses. (2) Optical parametric oscillator (OPO) generates additional beam between (1000–1600 nm). Ti:saphire and OPO beams are temporally synchronized using a delay line (3) and spatially co-aligned (4). (5) Spatial confinement of photons results in multiphoton excitation only in the focal plane. (6) Reflected fluorescence emission is re-directed toward photomultiplier tubes (PMTs) detectors. (7) Emitted light is splitted according to the wavelength using a set of dichroic mirrors and specific filters toward each detector. Schematic configuration shows: Far red fluorescence (e.g., m-cherry; Cy5), in detector 1. Ultra-violet/blue fluorescences (e.g., Hoechst, ECFP), SHG and THG in detector 2. Yellow/Green fluorescences (e.g., EGFP; YFP) in detector 3 and Orange/red fluorescences (e.g., PE, Dsred, rfp) in detector 4

relatively long period of time (~4 h) during which the temperature is more easily maintained and the immune cells dynamics have been shown to be preserved [22]. Unfortunately, explanted organs lack lymphatic and blood flow which might result in inadequate oxygen supply in certain deep regions. On the other hand, surgical techniques that are employed to expose the tissue of interest may be responsible of a side effect inflammation inducing the recruitment of leukocytes independently of the process studied. Alternatively, skin-fold window devices in which tumor cells can be implemented, have allowed stable positioning for longitudinal intravital imaging studies of skin tumors without recent surgical causing inflammation [23, 24].

A major and common limiting point of IVM is that beyond the theoretical good penetrance of the infrared light in biological tissues, the reality is that multiphoton

excitation allows imaging of the first 500 μm deep in most cases. Thus, only a very small fraction of the tissue is really accessible to our knowledge. This reality depends on the intrinsic properties of the tissue (presence of multiple biological layers, density, composition) and on the quality of the fluorescent reporters used. Alternatively, cell dynamic can also be studied using culture of tissue freshly embedded in an agarose matrix and cut with a microtome into thick slices (~400 μm thick). This process preserves tissue architecture and offers the possibility to study deeper regions of the tissue [25, 26].

Obviously there is no optimal procedure and the choice of a suitable approach will depend on the biological question to be answered.

2.3 Induction of Anti-tumor T Cell Activation and Recruitment

There is now doubt that T cells have a clinical relevance in the control of diverse sets of human cancers. The co-existence of cancer cells and T cells that recognize them, rarely leads to complete tumor eradication. The main hypothesis is that the T cells were not generated in sufficient number to induce tumor regression. Indeed, to evade immunity, tumor can interfere with the first steps of T cell generation and priming in order to induce, respectively, central or peripheral tolerance against tumor antigens. The nature of the antigen that is recognized by T cells is also a very important factor regulating the outcome of the immune response [6]. Extensive knowledge has been obtained from the use of preclinical models which are often based on the adoptive transfer of tumor specific T cells. Indeed, adoptive transfer can induce tumor rejection in both mouse models and melanoma patients [8]. It should be kept in mind that the transfer of several million of tumor-antigen-specific T cells in these settings is far from reflecting any spontaneous immune response. Nonetheless, this approach has been useful for IVM studies as it allows the pre-labeling of T cells with vital fluorescent probes which are stable over time and cell divisions, in order to accurately track them.

2.3.1 Tumor Antigen Recognition by T Cells

The presence of TILs has been associated with an increased survival in many cancer patients, however, the relative specificity for self-antigen versus neoantigens in these TILs has been difficult to assess and is likely to vary between patients and tumor types. The principle of immunoediting is that tumor cells may escape the immune response by selecting tumor variants with low immunogenicity. The mechanisms of tumor escape are different for tumors in which antigens are either from self or from neoantigens. T cells having a TCR with high affinity for self-antigens are likely to be deleted in the thymus by negative selection. On the other hand,

neoantigens are seen by the immune system as 'foreign' and as result seem to have superior capacity to induce T cell immunoreactivity [27]. Unfortunately, the process of central tolerance that usually occurs for self-antigens can also occur for tumor-specific neoantigens. Dendritic cells (DCs) that have captured tumor-antigens can contribute to central tolerance by inducing Treg generation or negative selection of tumor-antigen specific T cells [28].

For tumor neoantigens, loss of immunogenicity is frequently achieved through down regulation or loss of HLA Class I molecules. Tumors might also present a defect in the antigen-processing machinery or might lose expression of antigens [27]. The mode of targeting and timing of exposure of neoantigens are nonetheless important factors that can affect the induction of immunity or tolerance [29].

In a recent study, in which analyzes of mutations of different tumor types were performed, a correlation between the expression of immunogenic neoantigens by the tumor and the patient response to immunotherapies was established [30]. This further confirmed that neoantigens are superior targets for induction of an antitumor immune response.

Whole exome sequence data mining of tumors, combined with major histocompatibility complex-binding algorithms allowed the identification of several mutated tumor antigens recognized by T cells that are associated with the patient survival [31] and response to immunotherapies [32] in several cancer types . The presence of both MHC class I and MHC class II restricted epitopes was observed in these contexts, indicating that they might both present clinical relevance. Further identification of potential neoantigens by sequencing and MHC binding prediction might allow the development of new therapeutic strategies and a better targeting of tumors neoantigens repertoires.

2.3.2 Regulation of Anti-tumor T Cell Priming

It is clear from clinical studies that endogenous CD4+ and CD8+ T cells are able to recognize tumor epitopes. The prerequisite step for an adaptive immune response is priming of naïve T cells through antigen encountering.

It is unlikely that tumor cells are capable of CD4+ T cell priming by themselves, as most tumors are MHCII negative. Moreover, in MHCII positive tumor models, priming by professional host APCs seemed to be required, as no rejection occurs in hosts lacking MHCII molecules [33].

CD8+ T cell activation can be direct if the tumors express MHC class I or indirect after capture and cross presentation of tumor antigens by APCs. Cross-presentation of tumor antigens bound to MHC class I is a process occurring during many tumor growth [34]. As a result, it is expected that the TDLNs play a significant role in the activation and proliferation of naive antitumor T cells.

Indeed, the study of tumor antigen presentation has been largely restricted to event occurring in the TDLN, although we will see that it can also occur within the tumor itself. The TDLN is considered to be the site where tumor antigens first drain.

It has a great capacity for antigen collection and for selective migration of naive T cells. IVM of lymph nodes has been intensively exploited to describe the different steps of T cell priming. The encounter of T cells with antigen bearing APCs relies on efficient CCR7-dependent navigation of T cells inside the lymph nodes [26, 35, 36]. In addition, CD4+ and CD8+ T cells Ag-specific engagements with DCs release CCR5 ligands to attract additional naïve Ag-specific CD8 T cells towards the conjugates [37, 38].

Schematically, an efficient immune response relies on the induction of a transient "stop signal" with a stable T/APC interaction. T cell motility subsequently resumes during the expansion phase [39] (Fig. 2.2). Similar reduction in motility were

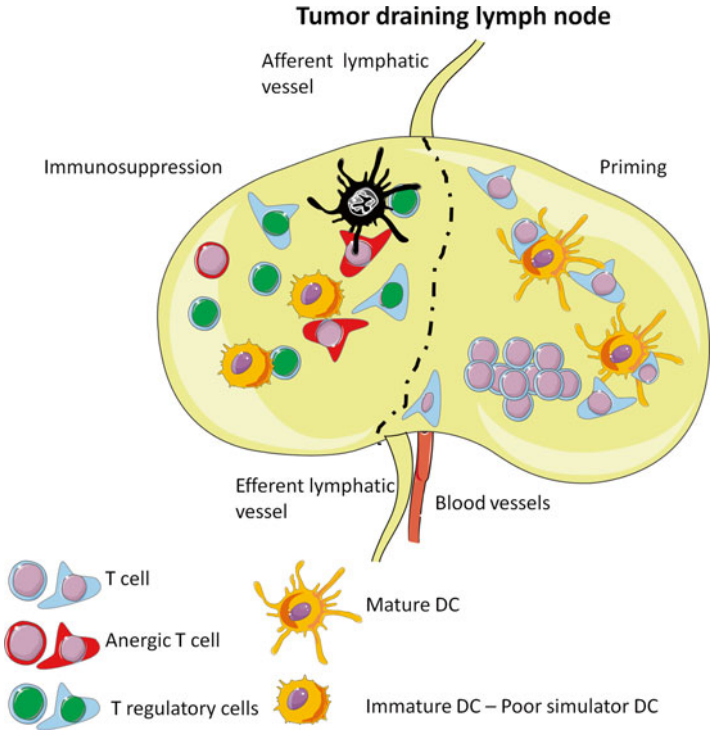

Fig. 2.2 Dynamic cellular interactions during antitumor T cell priming. IVM of the tumor draining lymph nodes allows to visualize the cellular interactions during T cell priming and activation but also the processes of immunosuppression. Efficient priming is characterized by the stable interaction of naïve T cells with tumor antigen bearing dendritic cells (stop signal phase). These interactions can attract additional T cells to the congugate via secretion of chemokines by dendritic cells. After priming T cell motility is regained during the expansion phase (Right side). During tumor development various secreted factors will alter the lymph nodes environment to promote tolerance of tumor antigens. Dendritic cells adopt an immature phenotype with downregulation of stimulatory molecules and secretion of immunosuppressive factors. This interferes with the stop signal phase and favors anergy of T cells and Treg generation. Tregs can in turn further promote immunosuppression partly by interfering with the T effector-DC interactions or by direct killing of DCs (Left side)

observed in tumor draining lymph node using adoptively transferred OVA-specific naïve CD8 T cells (OTI) in EG7 tumor-bearing mice (which is an OVA-expressing thymoma cell line) [40]. In this model, the adoptive transfer leads to efficient tumor rejection, reflecting a strong immunogenic environment with high level of tumor antigens. The fundamental immunological concepts that high levels of antigen presentation and expression of co-stimulatory molecules by APCs are a prerequisite for efficient T cell priming has been associated with the dynamics of T cell during the priming phase [41]. Not only is the number of APCs critical [42] but also the MHC-peptide potency [43]. Agonist of the CD40/CD40L pathways in combination with DEC205 specific targeting of dendritic cells confers the immunogenic-associated stop signal [44, 45]. Genetic deletion of the intercellular adhesion molecule 1 (ICAM-1) impaired the interaction of tumor-specific T cells with APCs in the TDLNs and resulting in a defective memory response to tumor antigens [43].

In a tolerizing context, the stop signal phase is absent and only a transient interaction occurs between T cells and DCs resulting in clonal deletion or anergy [44]. The improvement of intravital imaging toward molecular-level imaging provided novel insights in the links between the cell dynamic and the functional activity and revealed that T cell motility does not preclude TCR internalization and signaling [46, 47], confirming that even transient interactions leads to T cell activation. In human studies a defect in the number and functional properties of DCs in the TDLN was observed. For instance, the spatial-organization of DCs within TDLNs (change in number, maturity and T cell co-localization) was shown to have an impact on the clinical outcome of breast cancer patients [48]. Different mechanisms have been described to explain this process of active immunosuppression. DCs isolated from TDLNs are phenotypically immature and poor stimulatory of T cells. The use of conventional maturation stimuli can, in some cases, overcome this defect and licence CD8+ T cells for tumor eradication in murine models [49]. Tumor cells can secrete sterol metabolites which downregulate the expression of CCR7 by DCs, thereby disrupting DC migration to the lymph nodes [50]. Some tumor derived factors may also induce the scavenger receptor A expression on DCs, leading to enhanced lipid uptake while reducing their capacity to process antigens [51]. DCs expressing the immunoregulatory enzyme IDO are present in both murine and human TDLNs [52]. DCs from TDLN can also secrete TGF-β, thereby, enhancing Treg cell proliferation [53] (Fig. 2.2 see comments).

Accumulating evidence suggests that the TDLN environment is altered such that tumor antigens are presented in a fashion that favors tolerance [15]. An increase in Treg number and suppressive activity in TDLN has been described in most cancers. These cells may substantially contribute to the induction of an immunosuppressive TDLN via various cell-to-cell or soluble factors [54]. In several experimental conditions, IVM showed that Tregs are also able to interfere with the stable interaction of effector T cells with APCs [55] (Fig. 2.2). This mechanism is necessary to prevent auto-immune responses but also contribute to the selection of CD8+ T cells with higher avidity and promote memory [56]. In an immunosuppressive experimental tumor model expressing OVA, Tregs inhibition using anti-CD25 antibodies or specific deletion using the DEREG transgenic mouse strain leads to reduced motil-

ity of OTI T cells in the TDLN. This study revealed that Tregs can directly kill tumor antigen expressing APCs in the draining lymph node via perforin, thereby, limiting CTL expansion and differentiation [57] (Fig. 2.2).

The encounter with cognate antigen in the context of appropriate co-stimulation triggers T cell activation and differentiation into effector T cells (Teff). These cells move out of the lymph nodes via efferent lymphatic vessels into the blood circulation to reach the tumor site and physically engage their target. Teff cells can typically upregulate adhesion and chemokine receptors that are required for homing to the tumor site, while downregulating the receptors that retain them in the draining lymph node [58]. Genetic expression of chemokine receptors that are specific for chemokines overexpressed in the tumor in adoptively transferred T cells could favor their migration to the tumor site [59]. The role of chemokine receptors in the regulation of T cell egress from lymphoid organs and homing toward the tumors will undoubtedly be a source of interest for future IVM studies.

Little is known, however, about the process of T cell cross priming at the tumor site itself. In tumors, cells capable of phagocytic activity, and thus to present tumor-antigen, include tumor associated macrophages (TAMs), tumor dendritic cells (TuDCs), immature myeloid derived suppressor cells (MDSCs) and monocytes [60]. Recently, these cells have always been implicated in dampening the T cells response during tumor progression. In an elegant set of experiments, Broz et al. used extensive flow cytometric phenotyping of APCs from different human tumors and mouse models to discover that one rare population of intratumoral DCs is capable of robust activation and induction of CD8+ T cell priming. These APCs are very low in number but are capable of physically engage T cells in tumor distal regions and to a lesser extent in the tumor-proximal regions; as shown by in vivo imaging. These DCs express CD103 and are required for T cell mediated tumor rejection. Moreover, the expression of CD103+ DC related transcripts in human tumors predicts survival [61].

Overall T cell priming results from a complex temporal and qualitative cumulation of signals. IVM provided a better understanding of how these parameters regulate the probability and the duration of T cell interaction with APCs and how their maturation state defines the outcome of the priming [62].

2.4 Regulation of the Intra-tumoral Localization and Trafficking of T Cells

The intra-tumoral localization and ability of T cells to infiltrate tumors have a major impact on their antitumoral functions. Activated T cell recruitment to the tumor site depends on the expression of appropriate chemokine receptors and adhesion molecules to first egress from the priming site and secondly to extravasate to the vicinity of the tumor. Next, the accumulation of T lymphocytes within the tumor depends on their retention (presence of the antigen, downregulation of chemokine receptors for instances) and their ability to survive. Following extravasation, T cells must face the

TME which displays complex cellular and architectural properties. The TME is suspected to generate immature neovascular structures and secrete soluble factors that limit T cell infiltration [63]. Some of the properties of the stroma are indisputably shared between most tumors; these include the presence of TAMs or TuDC, fibroblasts and mesenchymal cells, fibrilar ECM along with abnormal vessels structure. All of these factors can have profound impact on the intra-tumoral migration and localization of T cells [14]. Due to its complexity, it is difficult to reproduce in vitro a 3D environment that will replicate the actual features of the tumor stoma to study its impact on T cell trafficking. Again, the development of minimally invasive IVM has allowed the visualization of T cell behavior in intact living tumors. Moreover, the combination with novel fluorescent cell labeling techniques allows the study of T cell interactions with the different components of the TME. Finally, the study of tumors that are modified to express a particular antigen combined with the adoptive transfer of transgenic T cells specific for this antigen allows to decipher the impact of the spatiotemporal distribution of TILs.

2.4.1 Antigen Specificity, T Cell Recruitment and Retention Within Tumors

Live imaging has highly contributed to the understanding of tumor infiltration through the real-time visualization of intratumor distribution and migration of lymphocytes. Although there is compelling experimental and clinical evidences that CD4+ T cells play a crucial role in rejecting solid tumors [33], most studies on T cell trafficking so far have focused on CD8+ T cells, because they are considered to be the most potent cytotoxic effectors (CTL). The majority of these studies rely on the use of tumors expressing a neoantigen combined with transgenic CD8+ T cell having a TCR specific for the antigen. Using these tools, it has been possible to assess the importance of the presence of cognate antigen for T cell accumulation within the tumor parenchyma. After activation, CD8+ T cells can infiltrate tumors independently of the expression of the cognate antigen by tumor cells, though preferential accumulation of antigen specific CTL in the tumor expressing the cognate antigen was observed [64]. One hypothesis could be that specific T cells are retained in the tissue through antigen dependent stable interactions with antigen presenting cells, whereas non specific one may leave. Studies that used similar approaches were in accordance with this hypothesis, as antigen specific T cells made stable, long lasting contacts with antigen expressing-tumor cells, while the tumor-T cell contacts were brief and did not cause arrest when the antigen was not expressed [65, 66]. These stable contacts with tumor cells may extend the residence time, and thereby retain T cells within the tumor. The motility-based model of tumor infiltration by CTL suggest that after destruction of tumor cells, CTL motility resume to reach the remaining live tumor cells through a series of “stop” and “go” phases (Fig. 2.3). In addition it was observed in real time, that CTL proliferation persisted in the tumor nest even after their massive clonal expansion in the tumor draining

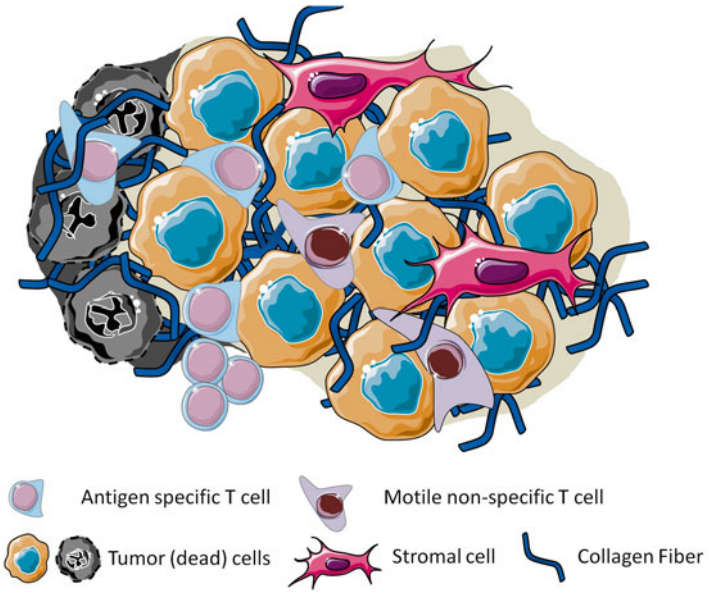

Fig. 2.3 Regulation of CTLs retention by antigen specificity. Both antigen specific and non-specific CTLs may infiltrate the tumors but only antigen specific ones make long lasting interactions with tumor cells. These interactions are thought to extend the time of residency of the antigen-specific CTLs and can also lead to clonal expansion of T cells inside the tumor nest. After tumor cell destruction, CTLs motility is regained to reach the remaining live tumor cells

lymph node [66]. Mathematical extrapolation of the frequency of these events suggested that the proportion of dividing tumor specific CTLs could be very high (up to 40 % within a 24 h time frame). Another hypothesis which is not exclusive, would consider that the presence of antigen is required to prolong CTL life span within the tumor.

T cell retention may also be favored by upregulation of chemokine receptors to local chemokines or loss of sensitivity to external chemoatractants. Likely due to the heterogeneity of tumor cells and the diversity of stromal components of the TME, no specific chemokine can be used as a signature of cancer development. Tumor antigen specific CTLs downregulated the mRNA levels of several chemokine receptors that may likely contribute to desensitization and local retention [64]. Mrass et al. implemented the E7 expressing TC-1 tumors cell in DPE-GFP mice, in which GFP is expressed in both CD4+ and CD8+ T cells. In these settings, vaccination with replication defective adenovirus expressing the E7 protein stimulated the anti-E7 effector T cells to reject TC-1 tumors. Intravital imaging of these tumors revealed that T cells become highly motile and migrated randomly within the tumor parenchyma arguing for an absence of a chemokine gradient [65]. Alternatively, upon antigen recognition, T cells can polarize the chemokine receptor CCR5 toward the immune synapse in order to sequestrate the receptor and stabilize the interaction [67].

In conclusion, tumor-antigens favor the retention and accumulation of tumor-specific T cells by increasing their engagement with tumor cells, thereby, enhancing their effector functions.

2.4.2 *Control of T Cell Infiltration by Vessels Architecture*

T cell infiltration is usually visible in the periphery of solid tumors [68]. The reason why extravasation is reduced in the center mass is unclear so far. One explanation would be that T cells stop and extravasate immediately once reaching the tumor-associated endothelium, thus accumulating first in the periphery of the tumor. Intravenous injection of fluorescent dextran in mice defines the vasculature and allows to directly image the behavior of TILs in the vicinity of the tumor vessels by IVM [66]. This approach revealed that in the tumor parenchyma, TILs are densely packed around the peripheral tumor vessels, which can serve as a route to guide T cell infiltration. TILs stay in close contact with the vessels and migrate with an elongated shape, different than the typical ameboid-like morphology described in 3D collagen matrix [66] (Fig. 2.4). Another explanation for the peripheral

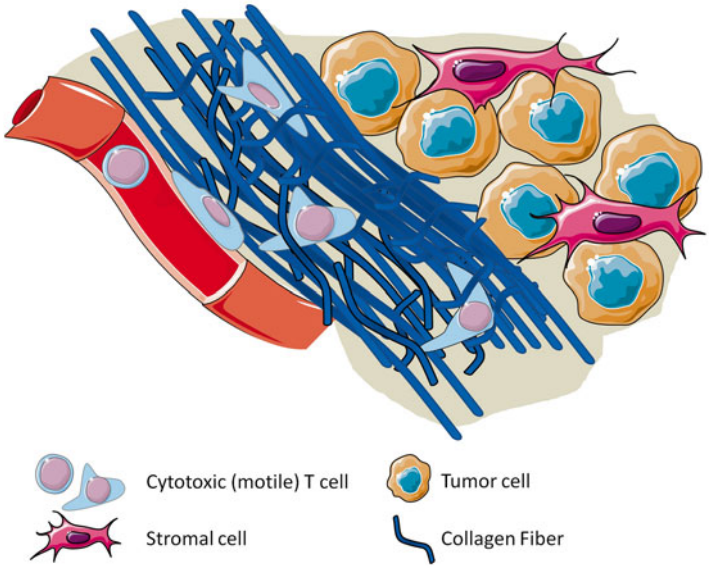

Fig. 2.4 Control of T cell migration in the interstitial space of tumors. IVM of T cell behavior in tumor has allowed to determine the components of TME that control T cell migration. After extravasation, T cells were shown to migrate along peripheral tumor vessels that can serve as a route in the interstitial space. Another well described route that T cell can use is the collagen fibers composing the ECM. There ECM fibers dictate T cells trajectories but have mainly been implicated in preventing direct contact with tumor cells

colonization of the tumor would be that the vasculature from healthy tissue surrounding the tumor is more permeable to T cells compared to deeper tumor vessels. The morphological and architectural abnormalities of the tumor vasculature are known to represent a barrier to efficient T cell extravasation and infiltration [63]. Indeed, ongoing production of factors such as the vascular endothelial growth factor A (VEGF-A) or endothelin-1, by both malignant and stromal cells, lead to enhanced angiogenesis but is accompanied with a decreased inflammatory response of the tumor-associated endothelium. This can results in a decreased influx of immune effector cells into the tumor parenchyma because of the down regulation of adhesion molecules that are required for normal homing, adhesion and transendothelial migration [69]. This process underlying the tumor's resistance to lymphocytic infiltration and immune surveillance has been termed tumor endothelial cell anergy. One pioneer evidence for this phenomenon came from a study that used intravital microscopy to monitor vessels phenotype, microcirculation and leucocytes adhesion during tumor development. In this study, the author used genetically modified mice that develop spontaneous pancreatic islet carcinoma. Excessive angiogenesis in the pancreatic islet was associated with increased frequency of vessels with irregular diameters. In vivo staining of leucocytes revealed a dramatic decreased in adhesion and leucocyte-endothelium interaction that correlated with the morphological alterations of the vasculature [70]. Signaling mechanisms leading to abnormal tumor vascular morphology have been identified, and will be discussed in Chap. 3.

2.4.3 Control of Intratumoral T Cell Migration by the ECM

Two main modes of migration have been characterized, thanks to imaging studies of cell behavior in three-dimensional extracellular matrix [71]. A slow integrin-dependent one coupled to matrix degradation and remodeling capacities that usually concerns tumor cells, mesenchymal cells and fibroblasts. The second, integrin-independent, concerns mainly T cells, NK cells and some APCs displaying ameboid-like structure with much faster displacement [72]. During this migration, T cells adopt a highly polarized morphology (with a uropod, a central compartment and a leading edge) with strict organization and location of the membrane and the cytoskeleton molecules [73]. A similar behavior of CTLs is reported in the different studies that took advantage of IVM. Visualization of the different components of the stroma confirmed that the tumor architecture dictates T cell infiltration. Beyond the density of the collagen matrix, tumor cell density, antigen presenting stromal cells and blood vessels influence the deepness and speediness of TILs.

Successful interstitial navigation of T cells is required for efficient antitumor immunity. The extracellular matrix (ECM) of the tumor stroma is rich in type I collagen and fibronectin secreted by cancer-associated fibroblasts. Because of their particular structural sequence, collagen fibers can be made visible through second harmonic generation. In the previous studies analyzing CTL migration inside a tumor using two-photon IVM, CTLs were found to be in close contact with collagen

fibers and fibronectin rich perivascular region, crawling along them. This suggested that the ECM may influence T cell functions and migration [65, 66]. In the course of tumor destruction, tumor cells are progressively eliminated leaving a loose network of collagen fibers that may correspond to the residual ECM, but active fibrosis could also contribute to remodeling of the ECM. TILs migrate through this network from the extravasation points to the regressing tumor front [66, 74]. This migration was shown to be dependent on the expression of the receptor for extracellular matrix proteins and glycosaminoglycans CD44. CTLs deficient for CD44 migrated at a lower velocity and were unable to sustain a polarized amoeboid-like shape, regardless of the presence of the cognate antigen [75]. Live imaging of fluorescent T cells in viable slices of human tumors revealed that ECM may also represent an obstacle to T cell infiltration. In these experiments, T cells migrated poorly in dense ECM areas near he tumor nests. T cell trajectories were dictated by collagen orientation and density, preventing the direct contact with neoplasic cells. T cell motility could be regained in area loose of fibronectin and collagen or by adding collagenase to reduce matrix rigidity. ECM fibers surrounding perivascular regions and around tumor epithelial regions were shown to restrict T cells from contacting tumor cells, while collagenase enhanced the number of T cells in contact with tumor cells [25]. In conclusion, the density of ECM fibers of the tumor stroma strongly influence the localization and migration of T cells, it provides the structural basement necessary for T cell migration but tumors could also take advantage of this to be protected from T cell attack (Fig. 2.4).

2.5 Imaging Antitumor T Cell Effector Functions and Immunosuppression

In principle, the elimination of neoplastic cells is the main expected T cell function. After recognition of MHCI complex at the surface of tumor cells or APCs, T cells can exert either direct or indirect killing pathways or both [76]. Direct killing pathways which mainly concern CD8+ cytotoxic T cells, are based on the polarized liberation of enzymes and cytotoxins containing granules, such as perforin or granzymes, toward the target or from interactions of membrane molecules on the surface of T cell (Fas-TRAIL) with their ligands on the surface of target cells to trigger apoptosis. The indirect killing pathways are more complex. They correspond to the elimination of tumor cells without a direct interaction. For instance, they can rely on the destruction of stromal cells, such as endothelial cells, resulting in necrosis of the tumor environment or on the local secretion of cytotoxic factors. The sensitivity of stromal cells to the secretion of IFNγ by T cells has been shown to be crucial for efficient tumor rejection in several tumor models [76]. CD4+ Th cytokines can directly induce scenecense in tumor cells; however, in most cases the role of CD4+ T cells in tumor rejection is indirect. Before mediating their beneficial effect, T cell must face and survive the local immunosuppression in the TME. As stated in part 2, regulation of the T cell antitumor response can occur through the regulation of

priming, T cell activation or localization/retention. These different steps have been extensively investigated and numerous immune checkpoints pathways are associated with the immune escape mechanisms. In this part, we will describe how the use of IVM has allowed to better determine the contribution of antitumor T cells to tumor rejection, as T cell killing can be seen in real time, and how it has permitted to visualize the effect of certain immune checkpoint blockade therapies on T cell dynamics.

2.5.1 Imaging T Cell Cancer Killing Mechanisms

The killing of cancer cells by CTL through direct cytotoxic mechanisms would require the formation of stable contacts between the CTL and the tumor. The visualization and dynamic analysis of such interactions have been rather well measured in vitro since several decades [77]. In vivo, this process is far from being accurately characterized. IVM studies confirmed that CTLs can stably engage tumor cells during hours, but evidence of direct tumor cell killing resulting from this interaction has been technically difficult to assess. One study revealed that direct CTL engagements could indeed contribute to tumor killing. Capsase3-sensitive Foster resonance energy transfer (FRET) biosensor expressing tumor cells were used to track tumor apoptosis in real time in vivo. This was combined to an adoptive transfer of in vitro primed CTLs that were efficient at inducing tumor rejection. The results shed light on another limitation to efficient antitumor T cell response. The rate of killing evaluated from this study was extremely slow (average of 6 h for the killing of one target), suggesting that the ratio between CTL and tumor cells is crucial to overcome tumor cell expansion and promote the balance toward tumor reduction, even though synergistic involvement of other effectors could not be ruled out [78]. The in vitro studies suggested that one CTL may engage sequentially several targets [79]. In vivo, different behaviors of CTLs have been described. CTLs that make long lasting interactions with a single target, CTLs that make multiple but proximal interactions with neighboring cells, CTLs making multiple distal interactions and finally CTLs fleeting in the tumor mass without making visible interactions. The relative proportion of these different behaviors was related to the density of tumor cells and the distribution of CTLs within the tumor parenchyma and correlated with the tumor cell apoptosis [66]. CD44 deficiency in CTLs was shown to strongly affect the efficacy of target cell screening and thus tumor rejection, without affecting the duration of cell-to-cell interactions or cytoxic functionalities [75]. Disturbances in these abilities to maintain interstitial navigation further emphasized the crucial role of multiple targeting in the tumor-rejecting capability of T cells.

More recently, IVM was used to compare the behavior of NK and CTLs during tumor regression [80]. Compared to CTLs that require antigen recognition, NKs cells require NKG2D ligand expression by the tumor cells [81]. NK cells were highly motile in tumor regions and made short-lasting contacts with their targets, whereas T cells were retained in these regions by long-lasting contacts. The study

also analyzed the calcium elevation in the two cell types, as it has been shown to occur after both T cell stop signal and NKG2D binding. The results show that although killing by both cell types was dependent on calcium availability, NK cells undergo only limited calcium influx compared to T cells. This influx was still sufficient for NK granule exocytosis [80]. Thus, drastic differences exist between CTLs and NKs cells mechanisms of direct killing and this two cell types could act synergistically during tumor rejection.

Stromal cells cross-presenting tumor-antigens can, in some cases, stimulate antigen-specific CTLs and enhance local IFNγ and TNFα release, which were shown to be required for tumor elimination [82–84]. Tumor cell variants that have lost the antigen recognized by CTLs can also be eliminated though a bystander effect dependent on the sensitization of the stroma [85, 86]. This bystander effect is at least dependent on antigen presentation by APCs and was associated with CTLs-APCs interactions [23]. In contrast, using a mixture of antigen-bearing and non-bearing EL4 tumors, Breart et al. showed that OTI T cells specifically eliminated antigen-bearing tumor islets [78]. The frequency of antigen-loss variants and the level of antigen expression may dictate the efficacy of bystander elimination. Another evidence that T cells can attack stromal components of the tumor came from an IVM study in which the authors used a mouse window chamber model. This approach permits the imaging of the same tumor region over several days. Increased vessel damage was observed and coincided with early T cell entry and was followed by cancer regression, suggesting that T cell-endothelial cells engagements might be important for cancer elimination. Nevertheless, no direct visualization of CTLs interaction with endothelial cells was imaged, despite the fact that vessels destruction was antigen dependent [23].

Antigen-dependent interactions with TAMs have been clearly observed in several models as described below. To date, no direct cytotoxicity against this stromal subset has been identified but the interactions can results in an active immunosuppression of CTLs Altogether these observations argue for a direct contribution of CTL in tumor elimination, but evidence for simultaneous indirect bystander effect through stromal cells sensitization also exists. This further emphasizes the fact that, besides the sole killing of tumor cells, complex reactions involving multiple cell types of the tumor microenvironment are occurring during T cell mediated tumor elimination.

2.5.2 Imaging Immune Checkpoints Blockade

Breaking the immune checkpoints mediated-tolerance is among the most promising cancer immunotherapy. These inhibitory molecules include the receptor cytotoxic T lymphocyte antigen 1 (CTLA-4) and the expression of Programmed Death 1 (PD-1) and its ligand PD-L1, which are potential therapeutic targets under investigation [87]. These pathways can directly terminate T effector functions. Evidences supporting a central role of T cells in the control of tumors were provided by Ipilimumab,

a monoclonal antibody directed against CTLA-4, which increased the overall survival of a significant number of patients with advanced melanoma [88]. The B7 ligand CD80 and CD86 expressed by APCs are co-stimulatory signal that bind CD28 receptor on T cells for their full activation and survival. CTLA-4 can compete with CD28 for binding to CD80 and CD86, reducing the level of B7 ligands on APC and thus attenuating T cell activation. These properties of CTLA-4 are believed to contribute the state of immunosuppression and immune evasion in the tumoral context [87].

Anti-CTLA4 antibodies (mAb) are thought to block the effect of CTLA-4 interaction with CD80 and CD86 thereby enhancing CTLs priming and effector functions. These antibodies may also activate anti-apoptotic, pro-adhesion and pro-polarity signaling pathways in T cells [87]. The effect of anti-CTLA-4 on T cell dynamics in the tumor microenvironment have also been revealed by IVM [89, 90]. 4 T1 breast cancer bearing CXCR6$^{+/gfp}$ reporter mice were used to track CTLs cells that infiltrated the tumor, as the majority of CTLs present in tumors that are being rejected have been shown to be CXCR6+. In these setting, CTLA-4 mAb not only increased intra-tumoral CTLs infiltration but also increased their motility and decreased their arrest coefficient as a monotherapy. CTLA-4 mAb also increase the motility of purified pre-activated CD8+ T cells and reverses the TCR stop signal induced by anti-CD3 ligation, suggesting that the antibody delivers a 'go' signal by binding to CTLA-4 but did not reduce growth of 4 T1 [89, 91]. In vivo, the antibody acted synergistically with ionizing radiation therapy to deliver the "stop" signal allowing the CTLs to make more MHC-I dependent contacts with tumor cells and subsequently delayed tumor growth, inhibited metastases formation and gave a survival advantage. Expression of the NKG2D ligand RAE-1γ by these otherwise poorly immunogenic tumor cells was required for these interactions to occur [89]. Clinical data indicate that the NKG2D receptor-ligand system plays an important role in the response of melanoma patients treated with anti-CTLA-4 [92]. Together these data indicate that the success of anti-CTLA-4 therapy might be dependent on the tumor expression of NKG2D ligands and/or strong antigen. Another target of CTLA-4 inhibition are Tregs, which extensively use this inhibitory molecule to mediate their suppressive functions [93]. In a recent study, the impact of CTLA-4 blockade on Tregs, Th and DC dynamics during an antigen-specific response was assessed by two-photon IVM of the lymph nodes. CTLA-4 blockade was shown to reduce Treg-Th interaction times while increasing the volume of DC-Th clusters. These cellular choreography events were followed by an enhanced Th proliferation and might thereby be a major mechanism underlying the effects CTLA-4 blockade [94].

Independently of their suppressive activities in TDLN, tumor-infiltrating Tregs can induce a state of functional hyporesponsiveness in TILs [54]. In another study, the authors implemented CT26 colonic carcinoma cells into dorsal skin-fold chambers to image antigen-dependent Treg activities and impact on the function of adoptively transferred CTLs [24]. In this model, the transfer of Tregs aggravated the state of dysfunction of tumor-infiltrating CTLs, characterized by impaired cytokine secretion and cytotoxic granule release, as well as, co-expression of the co-inhibitory

receptor PD-1 and TIM-3. This effect was dependent on recognition of the antigen by the Tregs in tumor tissue and correlated with down-regulation of co-stimulatory molecules (CD80, CD86) on DCs. In vitro, activation of CTLs with Treg–conditioned APCs was sufficient to induce expression of the coinhibitory receptor on CTLs. In vivo, antigen-specific Tregs were shown to make short interactions (less than 5 min) with APCs in both the tumor parenchyma and the stroma. However, Tregs did not make a direct physical contact with antigen-specific CTLs [24]. These experiments showed that the dynamics of Treg-DC interactions in tumors might explain the induction TILs dysfunction, similarly to Treg-DC interactions in the lymph nodes that led to attenuated T cell functions.

PD-1 is an inhibitory molecule found on the surface of activated B and T cells which has been implicated in inducing T cell anergy. PD-1 ligands are commonly expressed by multiple human tumors and its expression correlatse positively with worse prognosis [88]. Anti-PDL1 treatment amplifies T cell recruitment to tumors by overcoming T cell exhaustion [95], can yield sustained tumor regression in patients with different cancer types [96] and has also been shown to synergize with adoptive T cell therapies [97].

The two photon IVM system was used to image T cell-DC interactions after PD-L1 blockade and adoptive transfer of pancreatic islet-specific transgenic Th in the autoimmune context of non-obese diabetic (NOD) mice. Antibody blockade of PD-L1 decreased Th cell motility and enhanced T cell-DC contacts duration in vivo, thereby causing autoimmune diabetes in NOD mouse [98]. The imaging data were correlated with functional measurements and revealed that PD-L1 blockade simultaneously promoted Erk phosphorylation and IFN-γ production in the tolerised T cells. This finding supports a key role for PD-L1 blockade in restoration T cell-DC interactions and inhibition of anergy during tissue-specific reactivation [98]. Similar investigations in tumoral settings are needed to help understand the signals that maintain tolerance.

Real-time IVM imaging of DCs, T effectors cells and Tregs interactions provided novel insights in the mechanisms of inhibitory checkpoint pathways.

2.5.3 Role of Tumor-Associated Antigen Presenting Cells in T Cell Infiltration and Immunosuppression

Inflammatory cells of the tumor microenvironment influence every aspect of cancer progression, including tumor cell's ability to metastasize [13]. Beyond Tregs, tumor-associated macrophages (TAMs) represent a main protagonists of intratumoral immunosuppression. TAMs are largely represented in the TME, forming a heterogeneous and plastic population of cells which are associated with poor prognosis in 80 % of studies because of their angiogenic, tissue remodeling, growth factor providing and immunosuppressive properties [99]. Although mature TAM usually express MHCII and are capable of tumor cell phagocytosis, they have been extensively implicated in dampening the responsiveness of both CTLs and helper T cells. Moreover, TAM have been shown to induce tolerance in tumor infiltrating CTLs [100].

Secretion of the immunosuppressive cytokine IL-10 by macrophages can stimulate differentiation of CD4+ T cells toward the Th2 phenotype that will reinforce pro-tumor properties of macrophages [101] and can also limit the CD8+ T cell-dependent response to anticancer therapy [102]. Other molecules secreted by TAMs that have been shown to suppress anti-tumor T cell function include the hypoxia inducible factor-1 alpha HIF-1 [103], Arginase 1 [104] and PD-L1 [105].

Because of their heterogeneity and distinct transcriptional programs compared to normal tissue macrophages, TAMs display high surface markers overlaps and are difficult to classify [99]. Moreover, TAMs phenotypes may also vary between tumors or between different areas of the same tumor. Technological advances of tumor model imaging allow to better dissect the TAM compartment according to their in vivo morphology and behavior. The study of interactions between tumor-specific T cells and TAMs allow to characterize subsets according to their functional capacity to induce a strong productive T cell response. The CD11c-YFP reporter mouse has been used to illuminate APCs of the tumor microenvironment and study their function through intravital imaging. CD11c-YFP+ cells of the tumor stroma have been termed tumor DC (TuDC) because they also express high levels of MHC II, although their co-expression of F4/80 and CD11b indicate that they very likely represent a subset of TAMs. In a spontaneous breast cancer model, both CD11c- and CD11c+ are competent to phagocyte tumor cells as shown by uptake of tumor-derived fluorescent particles [106]; it should be noted however that CD11c+ cells localized along the tumor margin were more efficient at ingesting tumor cells, and that their position would ideally place them to interact and activate upcoming antigen specific T cells . Indeed, transferred tumor-specific CTL were shown to make long-lasting interactions (up to 30 min or more) either as clusters or as single cells, preferentially with TuDCs of the tumor proximal region. On the other hand, T cell-TuDC interactions were less frequent and more transient in the distal region of the tumor. These tumor-specific T cell—APC interactions were confirmed in vitro, with isolated TuDC and did corroborate the observations made during intravital imaging, as T cells preferentially coupled with tumor phagocytic DC and less with tumor cells themselves. Nonetheless, these interactions failed to effectively restimulate T cells to control tumors (Fig. 2.5). Tumor specific T cells cultured with TuDC had significantly lower lytic activity against targets compared to those cultured with bone marrow derived dendritic cells [106]. Broz et al. identified a subset of TuDC (CD103+ described previously in part 2.2) that are efficient at cross-priming and reactivation of effector T cells. Unfortunately, these specific APCs are very scarce and total TAMs typically outnumber CD103+ DCs by approximately tenfold. Analysis of the percentage of APCs that were coupled to T cells revealed that most T cells are captured by poor stimulatory APCs at the tumor margin [61].

These observations are consistent with another report focusing on chemotherapeutic failure [74]. By inducing immunogenic cell death, chemotherapeutic agents are known to, in addition to their direct effect on tumor cells, enhance anti-tumor immunity by restoring the function of effector immune cells, while inhibiting the function of regulatory ones [107]. In this study, combination of cyclophosphamide to adoptive transfer of tumor-specific CTLs initially led to an increase infil-

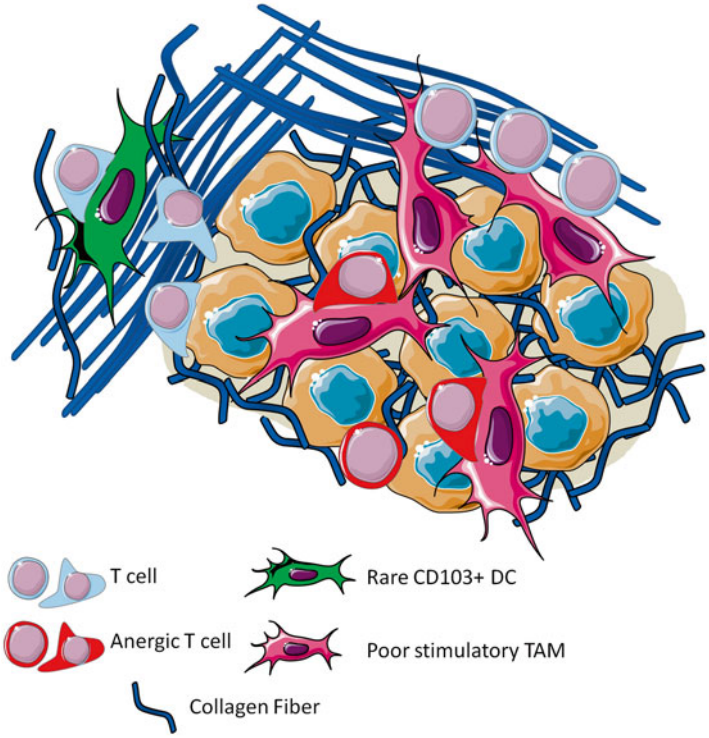

Fig. 2.5 Regulation of T cell activation and infiltration by tumor-associated antigen presenting cells. In most tumors, mature populations of macrophages compose more than 50 % of the CD45+ stroma. These have mainly been associated with dampening the T cell response and promoting tumor growth via various trophic and pro-angiogenic mechanisms. TAMs are organized into a dense network in tumor proximal regions. These networks have been shown to trap tumor-antigen specific CTLs though long-lived contact, preventing deep T cell infiltration of tumors. In contrast to immunosuppressive TAMs, the CD103+ DCs seem to be fully competent for CD8+ T cell priming or CTL reactivation and are in fact required for tumor-cell killing by CTLs in vivo. These DCs lie distal to the tumoral lesion in collagen rich areas. IVM revealed that T cell interactions with TAMs dominate probably because of their higher intra-tumoral abundance compared to CD103 + DCs

tration of CTLs and improved immune control of tumor growth. The synergistic action of both therapies was only transient, between day 4 and 7 after chemotherapy the TILs started to enter in immunosuppressive phase characterized by a significant decrease of the percent of cell secreting IFNγ. At this particular time point, the proportion of CTLs contacting TuDCs increased and these interactions were confirmed to be tumor-antigen specific. Analysis of intratumoral T cell tracks revealed that the CTLs velocity was largely reduced and stopped more frequently in areas rich in TuDC compared to collagen rich areas. The density of TuDC correlated positively with the one of CTLs. TuDC in these models were also shown to be able to cross-prime naïve tumor-specific CTLs in vitro and displayed a similar phenotype to those seen in the breast tumor model, although the

lytic capacity of CTLs that have infiltrated the tumor was not verified. In this case, induction of tolerance in intratumoral CTLs correlated with their trapping by the TuDC network, indicating that these interactions might be involved in the relapse phase that follows chemotherapy-induced anti-tumor immunity [74]. In conclusion, results from real time imaging of T cell dynamics are in accordance to suggest that T cell infiltration is limited by non-productive interactions with tumor APCs which rather impede T cell functions. Thus, trapping of CTLs by TuDC might represent an hurdle to the enhancement of anti-tumor immunity though combined chemo-immunotherapies.

2.6 Concluding Remarks

IVM has allowed to unravel the spatiotemporal regulation of immune cell interactions within the tumor microenvironment and this has highly contributed to our understanding of the mechanisms of tumor clearance. Most studies focused on contact-dependent CTL attack but indirect killing though local secretion of cytokines has been difficult to assess based solely on IVM assays. Although transferred CTLs can successfully mediate tumor rejections if they are in sufficient number, the imaging studies revealed that this was a slow process highly dependent on antigen availability. Given the rapid turnover of tumor cells and that of different cell type exhibiting non-redundant killing mechanism, it is expected that tumor control rather rely on the synergistic action of multiple immune cells [108]. CD8 T cells were also shown to release cytokine to activate and recruit other immune cells at the tumor site. The mechanisms that are required for cancer control may also vary from tumor to tumor and rejection has also been interpreted as a consequence of CD4, NK cells or macrophages depending on the context. Indeed, rejection of solid tumors after CTLs attack is followed by a large infiltration of myeloid cells [109], and these were recently shown to be required for therapeutic peptide vaccine-induced CD8 T cells efficacy [110].

T cells must face many obstacles in the TME. All of these factors such as the tumor vessel phenotype, extracellular matrix, and expression of checkpoint inhibitory molecules can dictate T cell dynamics, infiltration and survival. As a result, combination therapies targeting multiple TME components may improve the overall clinical outcome of immunotherapies [111]. New fluorescent reporters for tracking cell populations that have escaped attention so far and to assay T cell functions in real time will unquestionably be instrumental to in vivo imaging studies of cancer immunology. IVM has great potential to further dissect the mechanisms of anti-tumor immune surveillance.

Acknowledgments The writing of this chapter has been possible thanks to the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 304810—RAIDs, la "Ligue contre le cancer", and "Association pour la Recherche sur le Cancer".

The authors declare that they have no conflict of interest.

References

1. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol. 2001;13(4):459–63.
2. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell. 1992;68(5):855–67.
3. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science. 1974;183(4124):534–6.
4. Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med. 2002;196(1):129–34.
5. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410(6832):1107–11.
6. Heemskerk B, Kvistborg P, Schumacher TN. The cancer antigenome. EMBO J. 2013;32(2):194–203.
7. Gilboa E. The makings of a tumor rejection antigen. Immunity. 1999;11(3):263–70.
8. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. Immunol Rev. 2014;257(1):56–71.
9. Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, Royal RE, Kammula U, Restifo NP, Hughes MS, Schwartzentruber D, Berman DM, Schwarz SL, Ngo LT, Mavroukakis SA, White DE, Steinberg SM. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol. 2005;175(9):6169–76.
10. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res. 1998;58(16):3491–4.
11. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960–4.
12. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348(3):203–13.
13. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423–37.
14. Zal T, Chodaczek G. Intravital imaging of anti-tumor immune response and the tumor microenvironment. Semin Immunopathol. 2010;32(3):305–17.
15. Munn DH, Mellor AL. The tumor-draining lymph node as an immune-privileged site. Immunol Rev. 2006;213:146–58.
16. de Jong M, Essers J, van Weerden WM. Imaging preclinical tumour models: improving translational power. Nat Rev Cancer. 2014;14(7):481–93.
17. Sumen C, Mempel TR, Mazo IB, von Andrian UH. Intravital microscopy: visualizing immunity in context. Immunity. 2004;21(3):315–29.
18. Williams RM, Zipfel WR, Webb WW. Multiphoton microscopy in biological research. Curr Opin Chem Biol. 2001;5(5):603–8.
19. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. Nat Rev Immunol. 2002;2(11):872–80.
20. Nikolenko V, Nemet B, Yuste R. A two-photon and second-harmonic microscope. Methods. 2003;30(1):3–15.
21. Andresen V, Alexander S, Heupel WM, Hirschberg M, Hoffman RM, Friedl P. Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. Curr Opin Biotechnol. 2009;20(1):54–62.

22. Kitano M, Okada T. Four-dimensional tracking of lymphocyte migration and interactions in lymph nodes by two-photon microscopy. Methods Enzymol. 2012;506:437–54.
23. Schietinger A, Arina A, Liu RB, Wells S, Huang J, Engels B, Bindokas V, Bartkowiak T, Lee D, Herrmann A, Piston DW, Pittet MJ, Lin PC, Zal T, Schreiber H. Longitudinal confocal microscopy imaging of solid tumor destruction following adoptive T cell transfer. Oncoimmunology. 2013;2(11), e26677.
24. Bauer CA, Kim EY, Marangoni F, Carrizosa E, Claudio NM, Mempel TR. Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. J Clin Invest. 2014;124(6):2425–40.
25. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, Mami-Chouaib F, Donnadieu E. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 2012;122(3):899–910.
26. Asperti-Boursin F, Real E, Bismuth G, Trautmann A, Donnadieu E. CCR7 ligands control basal T cell motility within lymph node slices in a phosphoinositide 3-kinase-independent manner. J Exp Med. 2007;204(5):1167–79.
27. Blankenstein T, Coulie PG, Gilboa E, Jaffee EM. The determinants of tumour immunogenicity. Nat Rev Cancer. 2012;12(4):307–13.
28. Baba T, Badr Mel S, Tomaru U, Ishizu A, Mukaida N. Novel process of intrathymic tumor-immune tolerance through CCR2-mediated recruitment of Sirpalpha+dendritic cells: a murine model. PLoS One. 2012;7(7), e41154.
29. Zeelenberg IS, van Maren WW, Boissonnas A, Van Hout-Kuijer MA, Den Brok MH, Wagenaars JA, van der Schaaf A, Jansen EJ, Amigorena S, Thery C, Figdor CG, Adema GJ. Antigen localization controls T cell-mediated tumor immunity. J Immunol. 2011;187(3):1281–8.
30. Kreiter S, Vormehr M, van de Roemer N, Diken M, Lower M, Diekmann J, Boegel S, Schrors B, Vascotto F, Castle JC, Tadmor AD, Schoenberger SP, Huber C, Tureci O, Sahin U. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature. 2015;520(7549):692–6.
31. Lu YC, Yao X, Crystal JS, Li YF, El-Gamil M, Gross C, Davis L, Dudley ME, Yang JC, Samuels Y, Rosenberg SA, Robbins PF. Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. Clin Cancer Res. 2014;20(13):3401–10.
32. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med. 2013;19(6):747–52.
33. Perez-Diez A, Joncker NT, Choi K, Chan WF, Anderson CC, Lantz O, Matzinger P. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood. 2007;109(12):5346–54.
34. Platzer B, Stout M, Fiebiger E. Antigen cross-presentation of immune complexes. Front Immunol. 2014;5:140.
35. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature. 2006;440(7086):890–5.
36. Kaiser A, Donnadieu E, Abastado JP, Trautmann A, Nardin A. CC chemokine ligand 19 secreted by mature dendritic cells increases naive T cell scanning behavior and their response to rare cognate antigen. J Immunol. 2005;175(4):2349–56.
37. Hugues S, Scholer A, Boissonnas A, Nussbaum A, Combadiere C, Amigorena S, Fetler L. Dynamic imaging of chemokine-dependent CD8+ T cell help for CD8+ T cell responses. Nat Immunol. 2007;8(9):921–30.
38. Castellino F, Germain RN. Chemokine-guided CD4+ T cell help enhances generation of IL-6R alpha high IL-7R alpha high prememory CD8+ T cells. J Immunol. 2007;178(2):778–87.
39. Dustin ML. Stop and go traffic to tune T cell responses. Immunity. 2004;21(3):305–14.

40. Scholer A, Hugues S, Boissonnas A, Fetler L, Amigorena S. Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. Immunity. 2008;28(2):258–70.
41. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med. 2004;199(12):1607–18.
42. Celli S, Day M, Muller AJ, Molina-Paris C, Lythe G, Bousso P. How many dendritic cells are required to initiate a T-cell response? Blood. 2012;120(19):3945–8.
43. Skokos D, Shakhar G, Varma R, Waite JC, Cameron TO, Lindquist RL, Schwickert T, Nussenzweig MC, Dustin ML. Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. Nat Immunol. 2007;8(8):835–44.
44. Hugues S, Boissonnas A, Amigorena S, Fetler L. The dynamics of dendritic cell-T cell interactions in priming and tolerance. Curr Opin Immunol. 2006;18(4):491–5.
45. Shakhar G, Lindquist RL, Skokos D, Dudziak D, Huang JH, Nussenzweig MC, Dustin ML. Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. Nat Immunol. 2005;6(7):707–14.
46. Azar GA, Lemaitre F, Robey EA, Bousso P. Subcellular dynamics of T cell immunological synapses and kinapses in lymph nodes. Proc Natl Acad Sci U S A. 2010;107(8):3675–80.
47. Friedman RS, Beemiller P, Sorensen CM, Jacobelli J, Krummel MF. Real-time analysis of T cell receptors in naive cells in vitro and in vivo reveals flexibility in synapse and signaling dynamics. J Exp Med. 2010;207(12):2733–49.
48. Chang AY, Bhattacharya N, Mu J, Setiadi AF, Carcamo-Cavazos V, Lee GH, Simons DL, Yadegarynia S, Hemati K, Kapelner A, Ming Z, Krag DN, Schwartz EJ, Chen DZ, Lee PP. Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. J Transl Med. 2013;11:242.
49. van Mierlo GJ, Boonman ZF, Dumortier HM, den Boer AT, Fransen MF, Nouta J, van der Voort EI, Offringa R, Toes RE, Melief CJ. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol. 2004;173(11):6753–9.
50. Villablanca EJ, Raccosta L, Zhou D, Fontana R, Maggioni D, Negro A, Sanvito F, Ponzoni M, Valentinis B, Bregni M, Prinetti A, Steffensen KR, Sonnino S, Gustafsson JA, Doglioni C, Bordignon C, Traversari C, Russo V. Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses. Nat Med. 2010;16(1):98–105.
51. Guo C, Yi H, Yu X, Hu F, Zuo D, Subjeck JR, Wang XY. Absence of scavenger receptor A promotes dendritic cell-mediated cross-presentation of cell-associated antigen and antitumor immune response. Immunol Cell Biol. 2012;90(1):101–8.
52. Munn DH, Sharma MD, Hou D, Baban B, Lee JR, Antonia SJ, Messina JL, Chandler P, Koni PA, Mellor AL. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest. 2004;114(2):280–90.
53. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med. 2005;202(7):919–29.
54. Darrasse-Jeze G, Podsypanina K. How numbers, nature, and immune status of foxp3(+) regulatory T-cells shape the early immunological events in tumor development. Front Immunol. 2013;4:292.
55. Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, Lafaille JJ, Dustin ML. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. J Exp Med. 2006;203(3):505–11.
56. Pace L, Tempez A, Arnold-Schrauf C, Lemaitre F, Bousso P, Fetler L, Sparwasser T, Amigorena S. Regulatory T cells increase the avidity of primary CD8+ T cell responses and promote memory. Science. 2012;338(6106):532–6.
57. Boissonnas A, Scholer-Dahirel A, Simon-Blancal V, Pace L, Valet F, Kissenpfennig A, Sparwasser T, Malissen B, Fetler L, Amigorena S. Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. Immunity. 2010;32(2):266–78.

58. Franciszkiewicz K, Boissonnas A, Boutet M, Combadiere C, Mami-Chouaib F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Res. 2012;72(24):6325–32.
59. Caruana I, Diaconu I, Dotti G. From monoclonal antibodies to chimeric antigen receptors for the treatment of human malignancies. Semin Oncol. 2014;41(5):661–6.
60. Broz ML, Krummel MF. The emerging understanding of myeloid cells as partners and targets in tumor rejection. Cancer Immunol Res. 2015;3(4):313–9.
61. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, Barczak A, Rosenblum MD, Daud A, Barber DL, Amigorena S, Van't Veer LJ, Sperling AI, Wolf DM, Krummel MF. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. Cancer Cell. 2014;26(5):638–52.
62. Moreau HD, Bousso P. Visualizing how T cells collect activation signals in vivo. Curr Opin Immunol. 2014;26:56–62.
63. Hamzah J, Jugold M, Kiessling F, Rigby P, Manzur M, Marti HH, Rabie T, Kaden S, Grone HJ, Hammerling GJ, Arnold B, Ganss R. Vascular normalization in Rgs5-deficient tumours promotes immune destruction. Nature. 2008;453(7193):410–4.
64. Boissonnas A, Combadiere C, Lavergne E, Maho M, Blanc C, Debre P, Combadiere B. Antigen distribution drives programmed antitumor CD8 cell migration and determines its efficiency. J Immunol. 2004;173(1):222–9.
65. Mrass P, Takano H, Ng LG, Daxini S, Lasaro MO, Iparraguirre A, Cavanagh LL, von Andrian UH, Ertl HC, Haydon PG, Weninger W. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006;203(12):2749–61.
66. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J Exp Med. 2007;204(2):345–56.
67. Franciszkiewicz K, Le Floc'h A, Jalil A, Vigant F, Robert T, Vergnon I, Mackiewicz A, Benihoud K, Validire P, Chouaib S, Combadiere C, Mami-Chouaib F. Intratumoral induction of CD103 triggers tumor-specific CTL function and CCR5-dependent T-cell retention. Cancer Res. 2009;69(15):6249–55.
68. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348(6230):74–80.
69. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med. 2008;14(1):28–36.
70. Ali S, Ahmad M, Lynam J, Rees RC, Brown N. Trafficking of tumor peptide-specific cytotoxic T lymphocytes into the tumor microcirculation. Int J Cancer. 2004;110(2):239–44.
71. Friedl P, Brocker EB. The biology of cell locomotion within three-dimensional extracellular matrix. Cell Mol Life Sci. 2000;57(1):41–64.
72. Wolf K, Muller R, Borgmann S, Brocker EB, Friedl P. Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. Blood. 2003;102(9):3262–9.
73. Friedl P, Brocker EB. T cell migration in three-dimensional extracellular matrix: guidance by polarity and sensations. Dev Immunol. 2000;7(2–4):249–66.
74. Boissonnas A, Licata F, Poupel L, Jacquelin S, Fetler L, Krumeich S, Thery C, Amigorena S, Combadiere C. CD8+ tumor-infiltrating T cells are trapped in the tumor-dendritic cell network. Neoplasia. 2013;15(1):85–94.
75. Mrass P, Kinjyo I, Ng LG, Reiner SL, Pure E, Weninger W. CD44 mediates successful interstitial navigation by killer T cells and enables efficient antitumor immunity. Immunity. 2008;29(6):971–85.
76. Boissonnas A, Scholer-Dahire A, Fetler L, Amigorena S. Multiphoton imaging of cytotoxic T lymphocyte-mediated antitumor immune responses. Curr Top Microbiol Immunol. 2009;334:265–87.
77. Huppa JB, Davis MM. T-cell-antigen recognition and the immunological synapse. Nat Rev Immunol. 2003;3(12):973–83.

78. Breart B, Lemaitre F, Celli S, Bousso P. Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. J Clin Invest. 2008;118(4):1390–7.
79. Wiedemann A, Depoil D, Faroudi M, Valitutti S. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. Proc Natl Acad Sci U S A. 2006;103(29):10985–90.
80. Deguine J, Breart B, Lemaitre F, Di Santo JP, Bousso P. Intravital imaging reveals distinct dynamics for natural killer and CD8(+) T cells during tumor regression. Immunity. 2010;33(4):632–44.
81. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol. 2000;1(2):119–26.
82. Zhang B, Karrison T, Rowley DA, Schreiber H. IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. J Clin Invest. 2008;118(4):1398–404.
83. Gerbitz A, Sukumar M, Helm F, Wilke A, Friese C, Fahrenwaldt C, Lehmann FM, Loddenkemper C, Kammertoens T, Mautner J, Schmitt CA, Blankenstein T, Bornkamm GW. Stromal interferon-gamma signaling and cross-presentation are required to eliminate antigen-loss variants of B cell lymphomas in mice. PLoS One. 2012;7(3), e34552.
84. Briesemeister D, Sommermeyer D, Loddenkemper C, Loew R, Uckert W, Blankenstein T, Kammertoens T. Tumor rejection by local interferon gamma induction in established tumors is associated with blood vessel destruction and necrosis. Int J Cancer. 2011;128(2):371–8.
85. Spiotto MT, Rowley DA, Schreiber H. Bystander elimination of antigen loss variants in established tumors. Nat Med. 2004;10(3):294–8.
86. Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, Yu P, Fu YX, Weichselbaum RR, Rowley DA, Kranz DM, Schreiber H. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. J Exp Med. 2007;204(1):49–55.
87. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450–61.
88. Klemm F, Joyce JA. Microenvironmental regulation of therapeutic response in cancer. Trends Cell Biol. 2015;25(4):198–213.
89. Ruocco MG, Pilones KA, Kawashima N, Cammer M, Huang J, Babb JS, Liu M, Formenti SC, Dustin ML, Demaria S. Suppressing T cell motility induced by anti-CTLA-4 monotherapy improves antitumor effects. J Clin Invest. 2012;122(10):3718–30.
90. Pentcheva-Hoang T, Simpson TR, Montalvo-Ortiz W, Allison JP. Cytotoxic T lymphocyte antigen-4 blockade enhances antitumor immunity by stimulating melanoma-specific T-cell motility. Cancer Immunol Res. 2014;2(10):970–80.
91. Schneider H, Downey J, Smith A, Zinselmeyer BH, Rush C, Brewer JM, Wei B, Hogg N, Garside P, Rudd CE. Reversal of the TCR stop signal by CTLA-4. Science. 2006;313(5795):1972–5.
92. Jinushi M, Hodi FS, Dranoff G. Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity. Proc Natl Acad Sci U S A. 2006;103(24):9190–5.
93. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008; 322(5899):271–5.
94. Matheu MP, Othy S, Greenberg ML, Dong TX, Schuijs M, Deswarte K, Hammad H, Lambrecht BN, Parker I, Cahalan MD. Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. Nat Commun. 2015;6:6219.
95. Peng W, Liu C, Xu C, Lou Y, Chen J, Yang Y, Yagita H, Overwijk WW, Lizee G, Radvanyi L, Hwu P. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. Cancer Res. 2012;72(20):5209–18.
96. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H,

Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443–54.
97. Pilon-Thomas S, Mackay A, Vohra N, Mule JJ. Blockade of programmed death ligand 1 enhances the therapeutic efficacy of combination immunotherapy against melanoma. J Immunol. 2010;184(7):3442–9.
98. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, Azuma M, Krummel MF, Bluestone JA. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol. 2009;10(11):1185–92.
99. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141(1):39–51.
100. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. J Immunol. 2005;175(7):4583–92.
101. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell. 2009;16(2):91–102.
102. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, Daniel D, Hwang ES, Rugo HS, Coussens LM. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell. 2014;26(5):623–37.
103. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, Coussens LM, Karin M, Goldrath AW, Johnson RS. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. Cancer Res. 2010;70(19):7465–75.
104. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64(16):5839–49.
105. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. J Exp Med. 2009;206(6):1327–37.
106. Engelhardt JJ, Boldajipour B, Beemiller P, Pandurangi P, Sorensen C, Werb Z, Egeblad M, Krummel MF. Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. Cancer Cell. 2012;21(3):402–17.
107. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov. 2012;11(3):215–33.
108. Bercovici N, Trautmann A. Revisiting the role of T cells in tumor regression. Oncoimmunology. 2012;1(3):346–50.
109. Blohm U, Potthoff D, van der Kogel AJ, Pircher H. Solid tumors "melt" from the inside after successful CD8 T cell attack. Eur J Immunol. 2006;36(2):468–77.
110. van der Sluis TC, Sluijter M, van Duikeren S, West BL, Melief CJ, Arens R, van der Burg SH, van Hall T. Therapeutic peptide vaccine-induced CD8 T cells strongly modulate intratumoral macrophages required for tumor regression. Cancer Immunol Res. 2015;3(9):1042–51.
111. Hodi FS, Lawrence D, Lezcano C, Wu X, Zhou J, Sasada T, Zeng W, Giobbie-Hurder A, Atkins MB, Ibrahim N, Friedlander P, Flaherty KT, Murphy GF, Rodig S, Velazquez EF, Mihm Jr MC, Russell S, DiPiro PJ, Yap JT, Ramaiya N, Van den Abbeele AD, Gargano M, McDermott D. Bevacizumab plus ipilimumab in patients with metastatic melanoma. Cancer Immunol Res. 2014;2(7):632–42.

Chapter 3
Vascular Normalization, T Cell Trafficking and Anti-tumor Immunity

Kellsye L. Fabian and Walter J. Storkus

Abstract Improved understanding of the role of the immune system to limit tumor establishment and progression has led to the development and refinement of targeted immunotherapies. Treatments, such as cancer vaccines, CAR T cells and immune checkpoint inhibitors, aim to activate and harness T cells against cancer. However, tumors induce pathological angiogenesis and, at the same time, condition the tumor blood vessels to form a tumor vascular network that is highly abnormal. As a consequence, activated tumor- and stroma-specific T cells may be impeded from penetrating into the tumor lesions, greatly obstructing the therapeutic efficacy of such therapies. Hence, approaches designed to normalize the tumor vasculature are essential to optimize the activity of effector T cells as well as alternate innate and adaptive immune effector cells. In this chapter, we discuss how T cell trafficking is blunted at the tumor blood vessel interface and how therapies that are effective in normalizing the tumor vasculature such as anti-VEGF, tyrosine kinase inhibitors and vaccines targeting tumor blood vessel-associated antigens, among others, can re-establish T cell infiltration into an inflammatory tumor microenvironment, leading to therapeutic benefits in the cancer setting.

Keywords Tumor vasculature • Tumor microenvironment • T cell trafficking • Anti-angiogenic agents • Immunotherapy

K.L. Fabian
Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

W.J. Storkus (✉)
Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

Department of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA
e-mail: storkuswj@upmc.edu

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_3

Abbreviations

αSMA	α-Smooth muscle actin
ADAM	A disintegrin and metalloproteinase
ACT	Adoptive cell transfer
Ang	Angiopoietin
CAR	Chimeric antigen receptor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DLK1	Delta-like homologue 1
DC	Dendritic cell
ET	Endothelin
ET_BR	Endothelin B receptor
ECM	Extracellular matrix
FGF	Fibroblast growth factor
FDA	Food and Drug Administration
HUVEC	Human umbilical vein endothelial cells
HIF	Hypoxia inducible factor
IGF	Insulin-like growth factor
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon-γ
IL	Interleukin
LFA-1	Lymphocyte function-associated antigen-1
MHC	Major histocompatibility complex
MDSC	Myeloid derived suppressor cell
NRP	Neuropilin
NSCLC	Non-small cell lung cancer
PDGF	Platelet-derived growth factor
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PSGL-1	P-selectin glycoprotein ligand 1
RTK	Receptor-type tyrosine kinase
RGS-5	Regulator of G-protein signaling-5
Treg	Regulatory T cell
RCC	Renal cell carcinoma
SCLC	Small cell lung cancer
TCR	T cell receptor
Th1	T helper type 1
TGF	Transforming growth factor
TBVA	Tumor blood vessel-associated antigen
TEM1	Tumor endothelial marker-1
TIL	Tumor infiltrating lymphocyte
TME	Tumor microenvironment
TNFα	Tumor necrosis factor-α

TAM	Tumor-associated macrophage
TKI	Tyrosine kinase inhibitors
VCAM-1	Vascular adhesion molecule-1
VEC	Vascular endothelial cells
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VLA-4	Very late antigen-4

3.1 Introduction

Type I T lymphocytes play a key role in tumor immune surveillance. In fact, the presence of tumor infiltrating lymphocytes (TILs), specifically $CD8^+$ T cells and $CD4^+$ T helper type 1 (Th1) cells, has been associated with improved patient survival in a number of clinical studies involving various types of cancers, including melanoma, ovarian cancer, head and neck cancer, breast cancer, esophageal cancer, and colorectal cancer, to name just a few [1–5]. T cells recognize tumor cells via specific, clonotypic T cell receptors (TCR) that bind major histocompatibility complexes (MHC) presenting processed tumor antigens (i.e., in the form of short peptides fragments) on the tumor cell surface [6]. Upon TCR:peptide-MHC engagement, $CD8^+$ cytotoxic T lymphocytes (CTLs) are triggered to respond by secreting soluble factors (i.e., cytokines and chemokines) and by directly killing tumor cells via delivery of perforin/granzyme and via FasL/Fas binding [7]. CTL and T helper cell elaborated cytokines and chemokines can also mediate anti-tumor activity and/or can direct the anti-tumor activities of other immune cells [8, 9]. Thus, the ultimate goal of cancer immunotherapy is to enhance the proliferation, migration and activity of tumor-specific T cells. So far, DC-based vaccines [10] and adoptive cell transfer (ACT) [11] have shown promising, but as yet limited, benefits in pre-clinical and clinical settings. In order to improve these therapies, vaccine and ACT strategies have to be optimized and/or administered with "adjuvants" to ensure that the activated tumor-specific T cells are provided with optimal proliferative, survival and homing signals [12–14].

Circulating anti-tumor T cells face a hostile, immunosuppressive environment upon interaction with the tumor bed. Hence, reversal of immunosuppressive mechanisms within the tumor microenvironment (TME) is a key goal of successful cancer immunotherapy [15]. Tumor and stromal cells could impede T cell recognition and functional activity by down-regulating major histocompatibility complex (MHC) molecules [16], upregulating inhibitory receptors like programmed cell death protein ligand 1 (PD-L1) [17, 18] and secreting inhibitory factors such as transforming growth factor (TGF) β, interleukin (IL)-10 and prostaglandin E2 [19]. The tumor stroma also contains large populations of immunosuppressive hematologic progeny, such as regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs) and M2-like tumor-associated macrophages (TAMs) [20, 21]. Despite such therapeutic

concerns, one would be placing the proverbial "cart before the horse", since in most cases, circulating T effector cells fail to gain entry into the TME because they are functionally and physically blocked by unique characteristics in the tumor-associated vasculature. Thus, the "tumor endothelial barrier" must be first broken, before worrying about regulatory mechanisms in place within the TME that may modulate optimal anti-tumor T cell efficacy in vivo.

3.2 Events in T Cell Trafficking

T cell trafficking into peripheral tissues involves a series of sequential adhesion interactions between T cells and tissue-associated vascular endothelial cells (VECs). The T cell initially tethers on the endothelium, followed by rolling, firm adhesion and finally extravasation through the blood vessel wall into sites of inflammation. This multistep cascade requires adhesion molecules such as selectins and integrins, as well as, chemokine receptors [22].

Priming of naïve T cells to become effector T cells results in changes in expression of surface proteins and receptors. Cell surface expression of the molecules CCR7 and CD62L allows naïve T cells to traffic into the lymph nodes via PNAd + high endothelial venules under the chemotactic influence of the CCL19 and CCL21 chemokines produced in secondary lymphoid organs. Both CCR7 and CD62L are down-regulated in their expression of activated T cells. Instead, T effector cells express homing molecules such as Sialyl Lewis^X and P-selectin glycoprotein ligand 1 (PSGL-1) on their surface. Sialyl Lewis^X and PSGL-1 bind E-selectin and P-selectin on the activated endothelial cells, respectively, in weak affinity interactions that result in the tethering and rolling of the effector T cells on the endothelial lumen of a blood vessel. Chemokines CXCL9, CXCL10 and CXCL11 produced within inflamed tissues bind to their CXCR3 receptors on the rolling, activated T cells, causing the spreading and clustering of lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) on the T cell surface. High affinity binding of LFA-1 to intercellular adhesion molecule-1 (ICAM-1) and VLA-4 to vascular adhesion molecule-1 (VCAM-1) on activated VECs mediate the arrest of T cells on the inflamed endothelium, thus facilitating the extravasation of T cells into tissue [22–24].

3.3 The Tumor Vasculature Blocks T Cell Trafficking into Solid Tumors

3.3.1 The Tumor Vasculature is Aberrant

In normal adult tissues, angiogenesis is generally quiescent and the balance of pro- and anti-angiogenic factors is tightly controlled, resulting in mature and stable vessels. The vasculature is composed of VECs, pericytes and the basement membrane.

VECs form a continuous monolayer of cells that lines the blood vessel and acts as a selective barrier that regulates the exchange of substances between the blood and the surrounding tissues [25, 26]. The integrity of the endothelium is maintained by abluminal pericytes. Pericytes form tight associations with the VECs and provide signals via cell-cell contact as well as secreted products that affect VEC proliferation and differentiation, as well as, the contractility, tone, stability, and permeability of intact blood vessels [27–29]. In addition, the pericyte-VEC interaction leads to the deposition of the basement membrane, which is typically composed of Type IV collagen, laminin, fibronectin and heparin sulfate proteoglycans [30]. The basement membrane provides additional queues to maintain normal tissue VECs under steady-state conditions in a state of quiescence, in mature, stable blood vessels [31].

In the TME, on the other hand, factors such as hypoxia, acidosis and epigenetic alterations associated with oncogene activation and tumor-suppressor mutation [32–34] skew towards a pro-angiogenic bias supported by elevated production of factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), TGF, and angiopoietins (Ang) [35]. This turns on the "angiogenic switch" and the imbalance of pro- over anti-angiogenic signals ultimately results in sustained growth of blood vessels in tumors that are phenotypically and transcriptionally distinct from their normal tissue-associated counterparts.

Tumors employ various mechanisms to support blood vessel growth. Tumors may induce vasculogenesis wherein endothelial progenitor cells are recruited from the bone marrow or peripheral blood into the TME to assemble into nascent blood vessels. The tumor cells themselves have the capability to de-differentiate into endothelial-like cells and form tubular conduits that can serve as vessels. Tumors may also induce intussusception, wherein blood vessels are split into daughter vessels by re-organizing the VECs. The most common method utilized by tumors for blood vessel growth, however, is sprouting angiogenesis or the formation of new capillaries from existing ones [36]. The hypoxic environment and gene mutations in the tumor promote the local overproduction of VEGF that causes pre-existing capillaries or post-capillary venules to dilate and become leaky [37]. Plasma proteins extravasate from the leaky vessels and form a provisional matrix in support of VEC migration and proliferation. Ang-2 disrupts the association between VECs and pericytes and, together with matrix metalloproteinases, dissolves the basement membrane and extracellular matrix (ECM) to make way for the growing blood vessel [38]. VEGF, FGF and other pro-angiogenic factors trigger the VECs to invade the surrounding ECM and to proliferate and migrate to form tubular structures [38, 39]. The growing endothelial column secretes PDGF-B that recruits PDGFRβ^+ pericytes to the angiogenic sprout [27]. The pericytes normally form tight associations with the endothelial cells, leading to the eventual production of the basement membrane and resolution of the angiogenic process [40]. In tumor angiogenesis, pericytes remain only loosely attached to the endothelium [41] and this abnormal cell interaction results in a basement membrane that is either too thick or too thin [42] (Fig. 3.1).

The persistent production of pro-angiogenic molecules and the aberrant cell-cell and cell-basement membrane interactions formed as a consequence of tumor angio-

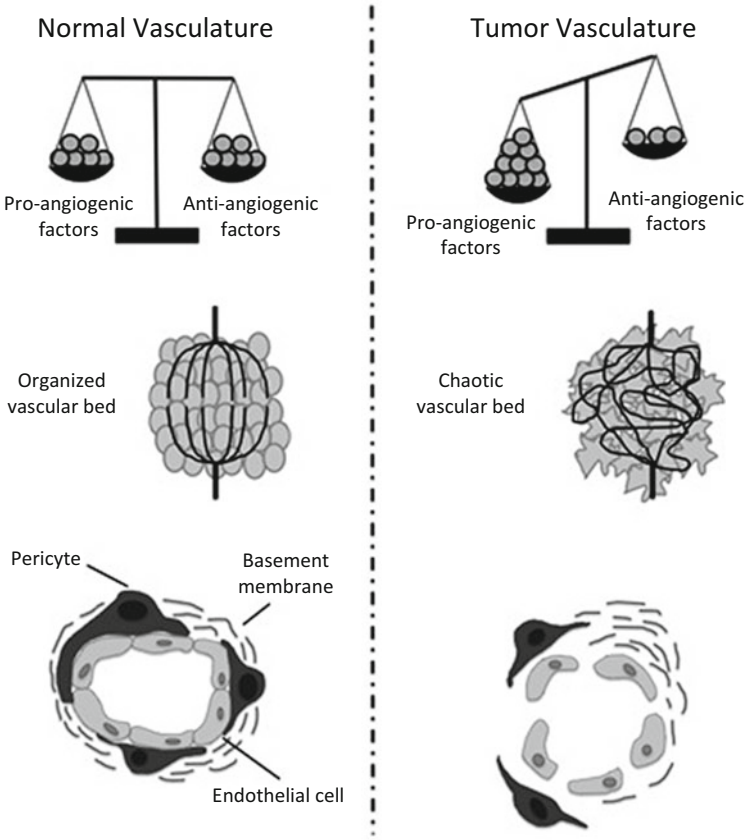

Fig. 3.1 The tumor vasculature is architecturally and functionally aberrant. In contrast to normal, healthy tissues, pro-angiogenic factors are excessively produced within the tumor microenvironment, resulting in a tumor-associated vasculature that is tortuous, hyper-permeable, dilated, leaky, and morphologically and structurally abnormal (i.e., loose physical association between endothelial cells, weak pericyte-endothelial cell interactions, basal membrane modifications); Adapted from Jain et al. [157]

genesis results in a tumor vasculature that is structurally and functionally defective. Unlike the organized vasculature in normal tissues, the tumor vasculature is tortuous, chaotic and does not follow the standard hierarchical arrangement of arterioles, capillaries and venules [43]. In addition, the abundant VEGF produced in the TME dissolves adherens junction complexes that hold VECs together [44] and, hence, the tumor endothelium is filled with intercellular gaps, transendothelial holes and fenestrae (pores) [45] (Fig. 3.1). These structural deficiencies in the endothelium and suboptimal VEC-pericyte interactions make the tumor blood vessels unusually leaky and highly permeable to blood plasma and red blood cells in an unregulated fashion, resulting in increasing interstitial fluid pressure in the TME [45, 46]. The high interstitial fluid pressure and the tensile pressure exerted by the growing tumor

mass leads to reduced blood flow and even blood stagnation/stasis [47, 48], which impedes the trafficking of circulating effector T cells facing a hyperbaric barrier into tumors [49]. Irregular blood flow also leads to decreased oxygen levels in the tumor lesion and the resulting hypoxic environment promotes anaerobic metabolism in the tumor cells, which increases local lactic acid concentrations in the TME [47, 48].

Cellular responses to adapt to low oxygen levels in the TME are regulated by the hypoxia inducible factor (HIF) family of transcription factors [50]. Ultimately, the altered metabolism of tumor cells and immune cells due to the hypoxic TME enables tumors to escape immunosurveillance in number of ways. Hypoxia stimulates the accumulation of extracellular adenosine that inhibits a wide range of T cell activities including proliferation, adhesion to tumor target cells, synthesis of IL-2, interferon-γ (IFNγ) and tumor necrosis factor (TNF) α, upregulation of IL-2 receptor α chain (CD25), expression of FasL and perforin, and CTL granule exocytosis (which is also negated in hyper-baric conditions [51]). In addition, hypoxia promotes the shedding of MHC-I related chain molecules from the tumor cell surface [52]. HIF-1α promotes the expression of "a disintegrin and metalloproteinase" (ADAM)-10 and 17 that cleave MHC class I-related chain molecules A and B [53, 54]. MHC-I related chain molecule shedding renders the tumor cells "less invisible" to CD8+ T cells, natural killer cells and NKT cells [52–54]. HIF-α also promotes the expression of inhibitory molecules PD-L1 on tumor cells [55] and Foxp3 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in Treg cells [56]. Lastly, hypoxia hinders the effector cell response by enhancing the recruitment of Tregs, MDSCs and M2-like TAMs in the TME [57].

3.3.2 Tumor Conditioning of the Endothelial Barrier

Tumor and tumor stroma-derived factors and cellular stressors alter gene programming in VECs and pericytes [58–60] and the resulting altered phenotype of the tumor blood vessel-associated cells hinders T cell trafficking into the tumor lesion. During vasculogenesis and angiogenesis, VEGF receptors (VEGFR) 1 and VEGFR2 are expressed by VECs that allow these cells to proliferate, migrate and form tubular structures in response to VEGF. Once the vessels mature, VECs downregulate VEGFR expression. In tumors, however, VEGFR1, VEGFR2 and even VEGFR3 (which is usually restricted to endothelial cells of the lymphatic vessels, are reactivated and upregulated in VECs) promote the unrestricted growth of tumor blood vessels in response to abundant levels of VEGF in the TME [61–63]. Also, pro-angiogenic factors VEGF and bFGF overproduced in the tumor reduce expression and proper clustering of VCAM-1 and ICAM-1 on the VEC cell surface [64]. The lack of VCAM-1 and ICAM-1 hampers T cell extravasation into the tumor and decreases the efficacy of immunotherapeutic vaccination and adoptive T cell transfer [49].

Neuropilins (NRPs) are non-tyrosine kinase receptors that act as co-receptors for VEGFRs and are important in modulating angiogenesis. It has been reported that while NRP-1 is expressed in the endothelium of healthy donors, it is overexpressed

two to threefolds the normal levels in cancer patients [65]. High neuropilin expression has been associated with abnormal angiogenesis and poor prognosis in patients with different types of cancers [66–69]. Administration of NRP-1 antagonists leads to inhibited tumor angiogenesis, growth and metastasis [70].

Genes aside from growth factor receptors are also upregulated in the tumor vasculature. Most of these genes are also involved in angiogenic pathways but some are overexpressed as a consequence of hypoxia or shear stress due to irregular blood flow [71, 72]. In human ovarian cancers, the expression of endothelin B receptor (ET_BR) on VECs has been associated with the absence of TILs [73]. ET_BR is a G protein-coupled receptor that binds endothelins (ET) 1, 2 and 3 and this ligand-receptor binding is involved in VEC proliferation, invasion and migration during neoangiogenesis [74, 75]. ET-1 is also expressed by ovarian tumors [76] and, hence, this pathway is constitutively activated in the cancer setting. ET_BR activation by ET-1 also causes upregulation of nitric oxide [77], which in turn represses ICAM-1 expression and, therefore, also prevents T cell adhesion to the endothelium [73]. ET_BR blockade in an ovarian mouse model decreased nitric oxide levels resulting in enhanced T cell homing into the tumor in an ICAM-dependent manner. Furthermore, when ET_BR blockade was combined with prophylactic vaccination or adoptive T cell therapy, therapeutic benefits were enhanced [73].

Pericytes in the RIP1-Tag5 model of the pancreatic islet cancer overexpress regulator of G-protein signaling-5 (RGS-5) [78]. These pericytes have immature phenotypes, as indicated by high levels of PDGFRβ and low levels of α-smooth muscle actin (αSMA), and associate preferentially to the growing angiogenic neovasculature [79]. Genetic deletion of RGS-5 in RIP1-Tag5 mice leads to the downregulation of PDGFRβ and upregulation of αSMA in the pericytes. Furthermore, RGS-5 deletion caused the tumor vasculature to become more homogenous such that it resembles the normal vasculature. In RGS-$5^{-/-}$ RIP1-Tag5 mice, immunotherapy (vaccination and adoptive T cell therapy) was also enhanced and prolonged the survival of the tumor bearing mice [80].

3.4 Tumor Vascular Remodeling

Methods to inhibit tumor angiogenesis and to normalize tumor blood vessels for improved immune cell recruitment, infiltration, and anti-tumor function will ultimately be needed to ensure the optimal success of immunotherapeutic intervention. Anti-angiogenic agents were originally developed to prune the excessive neovasculature in tumors in order to cut their supply of nutrients and oxygen, leading to disease regression [81]. This was based on the principle that tumors necessitate the formation of new blood vessels to meet their metabolic requirements, as well as, their need to dispel metabolic waste. Indeed, vascular pruning using high concentrations of anti-angiogenic drugs, caused tumor growth delay and necrosis. However, this effect was only temporary, and eventually, hypoxia was again increased in the TME leading to renewed tumor angiogenesis via drug-refractory pathways [82].

High doses of anti-angiogenic drugs have also been shown to increase tumor invasion and metastasis [83].

One must also consider that the tumor vasculature is required for the delivery of systemically-delivered anti-cancer drugs into the TME. Excessive vascular pruning using anti-angiogenic agents actually impedes the effective delivery of chemotherapeutic drugs and renders radiotherapy ineffective [82, 84].

Hence, instead of using anti-angiogenic therapy to ablate the tumor vasculature, it might be more rational to utilize this approach to instead, normalize it. Optimal doses of anti-angiogenic agents, in fact, prune immature vessel branches and remodel the remaining vessels into more normal architectures. The normalized vasculature is less tortuous, more organized and less leaky. This phenotype potentially alleviates hypoxia and interstitial fluid pressure in the TME and, therefore, could improve chemotherapy delivery into the TME. Furthermore, the normalized vasculature improves the oxygenation of the tumor, which is important for the anti-cancer actions of radiotherapy. Importantly, vascular normalization also boosts tumor infiltration by immune effector cells, including, with the TME becoming more pro-inflammatory as a consequence of decreased hypoxia [82, 84]. The normalized vasculature also acts as a barrier that prevents cancer cell shedding and intravasation, conditional steps for metastasis [85].

3.4.1 TNFα Therapy

TNFα is a vasoactive inflammatory cytokine that has the ability to activate VECs and, hence, if applied to tumors, can potentiate T cell extravasation and infiltration [49]. TNFα therapeutic effects are dose-dependent and associated with VEC activation, increased vascular permeability, VEC apoptosis and hemorrhagic necrosis. To avoid the pathologic effects of high doses of systemic TNFα, low concentrations of the cytokine must be targeted specifically to the tumor vasculature. To achieve this, a Cys-Asn-Gly-Arg-Cys (NGR) sequence serving as a ligand for the CD13 isoform expressed in tumor neovasculature, was fused with TNFα to develop tumor vascular–targeting version of TNF*α* [86]. Therapy using TNF-NGR resulted in transiently enhanced tumor blood vessel permeability, and the increased efficiency of chemotherapy drugs delivery into murine tumors, without the development of systemic toxicities usually observed for administration of native TNFα. Low doses of NGR-TNF also activated the endothelium as evidenced by VEC upregulated expression of VCAM-1 and ICAM-2, and by VEC release of MCP-1/CCL-2, MCP-3/CCL-7, MIP-2, oncostatin-M, and stem cell factor (SCF), factors that serve to attract and activate T cells. This TNF-based therapy also resulted in the loosening of VE-cadherin in the adherens junctions of VECs such that it favors T cell extravasation. Furthermore, this regimen led to reduced hypoxia in the TME, alleviating the immunosuppressive environment and supporting TIL proliferation and survival. When applied with cancer vaccines and ACT, TNF-NGR enhances the efficacy of these immunotherapeutic modalities in the absence of significant toxicity [87].

Moreover, in phase II trials in relapsed patients with ovarian cancer, the combination of NGR-TNF and doxorubicin improved progression-free survival and overall survival [88].

3.4.2 Metronomic Chemotherapy

Most chemotherapeutic agents damage the DNA or inhibit microtubule formation in order to selectively kill rapidly dividing cells, which includes neo-angiogenic endothelial cells. Conventionally, chemotherapeutic drugs are administered at the maximum tolerated dose in single doses or short courses followed by prolonged breaks between each therapeutic cycle [89]. The 2–3-week break between cycles is necessary to allow the patient to recuperate from adverse drug reactions but it also affords the tumor endothelial cells time to recover and continue their pro-angiogenic programming [90].

Metronomic chemotherapy, on the other hand, involves the administration of low doses of chemotherapeutic agents on a frequent or continuous schedule with no drug-free breaks. Metronomic chemotherapy has been shown to have anti-angiogenic effects in vivo. It selectively prevents the proliferation and migration of endothelial cells, while blocking their apoptosis. Metronomic chemotherapy also leads to production of thrombospondin-1 (an anti-angiogenic factor) and reduces the content of bone marrow-derived endothelial cell precursors within the treated TME [90].

Metronomic chemotherapy has also been reported to exert immunomodulatory effects that favor anti-tumor response. Several pre-clinical and clinical studies have shown that metronomic chemotherapy reduces the Treg population and suppresses Treg function. It has also been shown to improve lymphocyte proliferation and T cell memory population expansion [90, 91].

In clinical trials, metronomic chemotherapy as a single modality or in combination with other anti-cancer therapies demonstrated clinical benefits in patients with advanced breast cancer, recurrent ovarian cancer, hormone-resistant prostate cancer, advanced multiple myeloma, recurrent non-Hodgkin's lymphoma, recurrent malignant glioma and glioblastoma, metastatic or locally advanced neuroendocrine carcinoma, among others. Response to metronomic chemotherapy was observed to last for several months and was associated with increased overall survival and progression free-survival [91]. Furthermore, the vascular normalizing and immunomodulatory effects of metronomic chemotherapy make it a potential adjuvant for immunotherapy-based approaches. Indeed, metronomic cyclophosphamide therapy in combination with prophylactic gene-based vaccine targeting mel3 has been reported to inhibit B16.mel3 tumor growth in mice when compared to monotherapy with cyclophosphamide or vaccination [92]. However, several clinical studies have suggested that metronomic chemotherapy does not significantly improve anti-tumor efficacy of immunotherapy although there was a trend towards improved overall survival and even increased antigen-specific $CD8^+$ activity in some cases [93].

Metronomic chemotherapy is generally well-tolerated and, thus, has the potential to improve quality of life with minimal toxicity. However, prolonged metronomic chemotherapy can lead to the accumulation of anti-cancer drugs and to the subsequent development of secondary diseases [94]. Furthermore, resistance to metronomic chemotherapy could develop through "reduced vascular dependence," in which resistant tumor cells grow in hypoxic and malnourished environments without the formation of neovasculature [90].

3.4.3 Anti-VEGF

VEGF is an important driver of angiogenesis and is overexpressed in a number of tumor lesions. Hence, targeting VEGF has been the focus of numerous studies that aims to inhibit tumor angiogenesis. So far, two anti-VEGF agents have been approved by the US Food and Drug Administration (FDA) as anti-angiogenic therapy for cancer: bevacizumab (Avastin, Genetech), which is a humanized anti-VEGF-A monoclonal antibody [95] and aflibercept (Zaltrap, VEGF-Trap, Regeneron and Sanofi Aventis), which is a recombinant fusion protein consisting of the extracellular domains of VEGFR1 and VEGFR2 and the Fc portion of human IgG [96]. Both bevacizumab and aflibercept bind VEGF and prevent its binding to VEGFR (Fig. 3.2). Treatment with these anti-VEGF agents inhibits VEC proliferation, reduces vessel permeability, lowers blood vessel density, and inhibits tumor growth [97–100].

In pre-clinical studies, anti-VEGF treatment pruned immature tumor vessel branches and the vessels that survive were reverted to a more normal phenotype that enhanced blood flow and perfusion and reduced hypoxia in the TME. However, vascular normalization and, therefore, clinical benefit with anti-VEGF treatments is only transient with treatment-refractory disease developing in most cases. Hence, anti-VEGF therapy is typically administered in combination with chemotherapy in the clinic [101, 102].

Bevacizumab, in combination with chemotherapy, has been approved as first- or second-line treatment for metastatic colorectal cancer, non-small cell lung cancer (NSCLC), and metastatic renal cell carcinoma (RCC) and, as a single-agent, second-line treatment option for patients with recurrent glioblastoma [103]. Aflibercept, also in combination with chemotherapy, has been approved as second-line treatment for metastatic colorectal carcinoma [96].

3.4.4 Tyrosine Kinase Inhibitors

VEGF, FGF, PDGF, and other angiogenic factors signal through specific receptor-type tyrosine kinases (RTKs). In general, RTKs consist of an extracellular ligand-binding region, a transmembrane helix, and a cytoplasmic tyrosine kinase domain. Activation of the pro-angiogenic pathway begins with the binding of an angiogenic

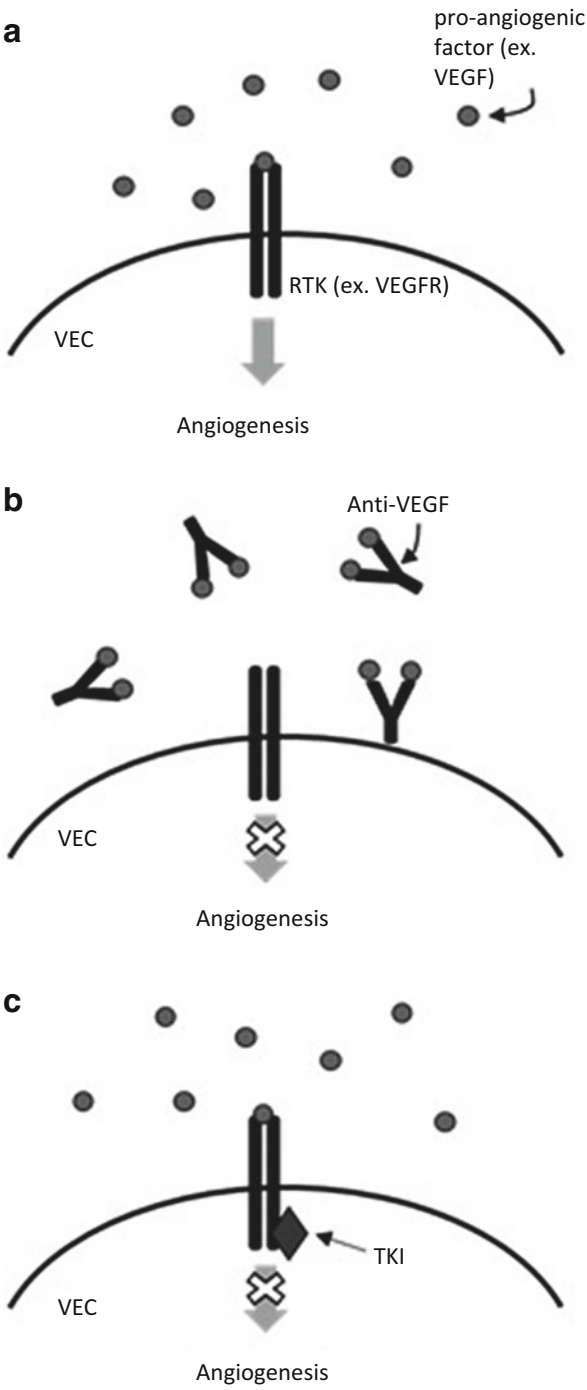

Fig. 3.2 Therapeutic agents that target angiogenesis. (**a**) Angiogenesis is primarily mediated by the interaction of VEGF-A and VEGFR-2 expressed on the surface of endothelial cells. (**b**) Bevacizumab (a monoclonal anti-VEGF antibody) and aflibercept (a recombinant VEGFR-Fc fusion protein), bind VEGF and prevent its interaction with endothelial cell-expressed VEGFR, thereby inhibiting growth of new tumor-associated blood vessels (**c**) TKIs compete with ATP in the catalytic tyrosine kinase domain of VEGFR and other RTKs leading to diminished activation of pro-angiogenic signaling pathways in VEC within the TME

factor to its cognate RTK and the dimerization/oligomerization of the receptor. Assembly of the RTK complex induces a receptor molecule to phosphorylate one or more tyrosines in the adjacent receptor. The phosphorylated receptor then serves as a docking and activation site for other signaling proteins [104]. Tyrosine kinase inhibitors (TKIs) are small molecules that inhibit downstream signaling of RTKs by competing with ATP binding to the catalytic domain of tyrosine kinase [105].

Several TKIs targeting angiogenesis-related RTKs have been developed as anti-cancer treatment. Sunitinib (SU11248, Sutent, Pfizer), sorafenib (BAY43-9006, Nexavar, Bayer, Inc.), pazopanib (GW786034, Votrient, GlaxoSmithKline), and axitinib (Inlyta, AG-013736, Pfizer) target VEGFRs and some of these TKIs also bind PDGFRs, stem cell factor receptor (c-Kit), Flt3 and other RTKs [106–109]. In vitro assays using human umbilical vein endothelial cells (HUVECs) demonstrated that sunitinib, sorafenib, pazopanib and axitinib inhibit downstream VEGFR signaling, leading to decreased proliferation, survival, migration and capillary tube formation of HUVECs [110–113]. In vivo, these TKIs normalize the tumor-associated vasculature by suppressing angiogenesis and decreasing microvessel density. The anti-angiogenic effects of these TKIs are associated with tumor growth inhibition in a range of murine xenograft models [107, 111, 114–116].

Although developed as an anti-angiogenic drug, several pre-clinical studies have shown that sunitinib also has the capacity to reverse immune dysfunction in the TME. In MCA-26 colon and LLC1-Lewis lung carcinomas, treatment with sunitinib led to a reduction in immunosuppressive MDSCs and Tregs, while coordinately promoting $CD8^+$ and $CD4^+$ effector T cell infiltration into the TME. Type-2 immune responses were also blocked as suggested by the decrease in expression of IL-10 and TGFβ. Furthermore, sunitinib treatment decreased PD-L1 expression on MDSCs and DCs, and CTLA-4 and PD-1 expression in $CD8^+$ and $CD4^+$ T cells [117]. Similarly, sunitinib treatment also restored normal T cell function and suppressed MDSCs in the spleens of mice bearing B16, RENCA, CT26 and 4T1 tumors [118, 119]. Clinical results for sunitininb have provided concordant results. After one cycle of sunitinib treatment, the number of IFN-g-producing (type-1) T cells increased, while IL-4-producing type-2T cells were reduced in patients with renal cell carcinoma (RCC) [120]. Although no significant reduction in the Treg cell frequency in patient peripheral blood was observed, there was an inverse correlation noted between increased type-1T cell responses and decreased Treg levels in PBMC after sunitinib treatment. On the other hand, others have reported that sunitinib might have a negative impact on immune cells numbers/function. Sunitinib has been shown to inhibit T cell proliferation and to down-regulate expression of the activation markers CD25 and CD69 on in vitro (mitogen)-activated T cells isolated from healthy volunteers and cancer patients [121]. High doses of sunitinib have also been reported to block IL-12 production from DCs matured in vitro with IFN-α, TNF-α and poly I:C [122]. These data suggest that the dose and schedule of TKIs, such as sunitinib, must be carefully regulated based on possible (high-dose, chronic) immune-toxicities.

Anti-angiogenic TKIs have been FDA-approved as first-line or second-line treatment for different malignancies. Sunitinib is currently being administered as first-

line therapy for patients with RCC and unresectable pancreatic neuroendocrine tumors. It is also approved as second-line therapy for patients with gastrointestinal stromal tumors [123, 124]. Sunitinib is effective in inducing objective clinical responses in patients with small cell lung carcinoma (SCLC), breast cancer, thyroid cancer and chondrosarcoma [123]. Sorafenib has been approved as a therapeutic agent for advanced RCC, unresectable hepatocellular carcinoma, and recurrent or metastatic differentiated thyroid carcinoma [125, 126]. Pazopanib, on the other hand, has been approved as treatment for patients with RCC or soft tissue sarcoma after standard-of-care chemotherapy [127, 128]. Axitinib has been approved as a second-line treatment option for RCC [106].

3.4.5 Tumor Blood Vessel-Associated Antigens (TBVA)-Targeted Vaccines

The FDA approval of the first therapeutic cancer vaccine, sipuleucel-T (Provenge, Dendreon), has confirmed that antigen-specific vaccines can elicit an immune system to attack tumor cells in the clinical setting [129]. Sipuleucel-T is a treatment option for castration-resistant prostate cancer, targeting prostate acid phosphatase expressed on cancer cells. Tumor blood vessel cells are also attractive targets for active, specific immunotherapy, and hypothetically, have several advantages over cancer cells as targets. Cellular stressors in the TME promote unique epigenetic programing, resulting in unique phenotypes that allow for differential recognition of tumor stromal cells, including VECs and pericytes, from their normal counterparts by the adaptive immune response [58–60]. These tumor blood vessel-associated antigens (TBVA) may be targeted immunologically via active, specific vaccination to promote the activation of T effector cells that can selectively target abnormal tumor blood vessel cells, leading to the potential normalization of the tumor-associated vasculature (Fig. 3.3). VECs and pericytes are also strategically more visible to immune surveillance being highly accessible to circulating immune effector cells in the blood. Lastly, VECs and pericytes are more genetically stable than tumor cells and, hence, less prone to adaptive mechanisms associated with immune escape.

Early TBVA-based vaccines targeted VEGFRs, which are highly expressed on the surface of tumor VECs in the neovasculature. In murine tumor models, peptide-based and gene-based VEGFR1- and VEGFR2-targeted vaccines elicited antigen-specific $CD8^+$ T cell responses in association with inhibited tumor angiogenesis and slowed disease progression [130–132]. The success of VEGFR-targeted vaccines in pre-clinical models drove the development of elpamotide, a vaccine against human VEGFR2 containing the HLA-A24-presented peptide epitope RFVPDGNRI [132, 133]. Phase I trials of elpamotide (as a single agent or in combination with other peptide vaccines and/or chemotherapy) demonstrated safety of the treatment and, to some degree, the ability of the approach to induce T cell immune responses associated with reduced angiogenesis in patients with pancreatic cancer, colorectal cancer

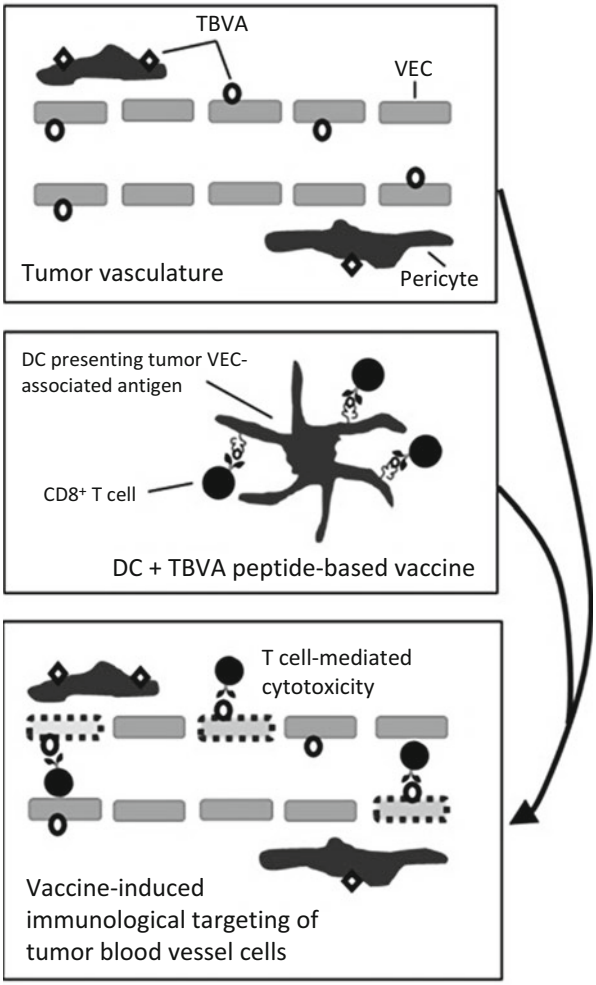

Fig. 3.3 TBVA-targeted vaccines. Tumor-associated pericytes and VECs express unique antigens that differentiate them from their normal counterparts. Active vaccines (such as those implementing patient-derived DCs and TBVA-derived peptides) activate TBVA-specific T effector cells that circulate through the blood system, where they can selectively recognize/target tumor blood vessel cells. Inflammatory/cytotoxic mechanisms mediated by anti-TBVA T cells promote vascular normalization (i.e., reduced vascular arborization and permeability with coordinate decreases in tissue hypoxia and interstitial fluid pressure) and increased T cell trafficking into the tumor (based on vascular activation and locoregional production of T cell-recruiting chemokines)

or NSCLC [134–137]. However, in a Phase II/III clinical trial, elpamotide in combination with the chemotherapeutic agent gemcitabine, did not improve overall survival of patients with advanced-stage pancreatic cancer [133].

Other TBVA that may not be directly involved in angiogenic pathways may also be targeted for use in tumor immunotherapies. Vaccination of HLA-A2 (HHD)

transgenic mice bearing HLA-A2^{neg} B16 melanoma or MC38 colon carcinoma with dendritic-based (DC) 1-based vaccines incorporating HLA-A2-restricted peptide epitopes derived from TBVAs such as delta-like homologue 1 (DLK1), EphA2, hemoglobin-β, NG2, NRP-1, PDGFRβ, RGS5, and tumor endothelial marker (TEM1) were shown to be effective in providing protective and therapeutic anti-tumor immunity [138]. The vaccination strategy resulted in tumor infiltration by CD8$^+$ T cells that differentially recognized tumor VECs and/or pericytes, in association with a reduction in blood vessel density in the TME. Pericytes in murine melanoma, murine RENCA and human RCC overexpress the Notch receptor antagonist DLK1 [138, 139]. DLK1-targeted DC peptide-based and gene-based vaccination of RENCA-bearing mice upregulated the expression of VCAM-1 in VECs and CXCL10 in the TME that improved type-1 CD8$^+$ T effector cell infiltration into tumors and in CD8$^+$ T cell-dependent inhibition of tumor cell growth [139]. The vaccines also resulted in a normalization of the vasculature in the TME, as characterized by treatment-associated reductions in vascular arborization, vascular permeability and hypoxia. Gene-based vaccination against TEM1 (aka CD248) has also been used as a prophylactic or therapeutic strategy against CT26 (colon), TC1 (cervical), and LLC (lung) carcinomas, with the vaccine resulting in CD3$^+$ T cell-dependent tumor growth inhibition [140]. These results suggest that TBVA-targeted vaccination can lead to vascular normalization within the TME as a prelude to the more effective delivery of chemotherapeutic agents and ACT in combination treatment approaches.

3.4.6 Chimeric Antigen Receptor (CAR) T Cells

CAR T cells are genetically-engineered to express antigen-specific receptors that combine antibody-like recognition and T cell receptor activating functions [141]. CARs are composed of antigen-binding region, usually single-chain variable fragments from monoclonal antibodies, a transmembrane domain and an endodomain derived from the CD3 zeta co-receptor.

Recent clinical studies on CAR T cells, the majority of which were designed to treat B cell malignancies, suggest great promise in the clinic [142]. Thus far, however, CAR T cells have displayed only limited effectiveness in solid tumors due to their inability to actively migrate and extravasate into the TME. As a consequence, TBVA-targeting CAR T cells are being developed to circumvent the need for penetration into the TME (or to potentially improve co-transferred anti-tumor CAR T cells based on vascular normalization mechanisms). CAR T cells engineered to target echistatin, which binds to the $\alpha_v\beta_3$ integrin expressed on tumor VECs, have been shown to lyse αvβ3 integrin$^+$ HUVECs in vitro [143]. Treatment of tumor-bearing mice with echistatin-reactive CAR T cells led to the destruction of tumor blood vessels and inhibition of tumor growth. Adoptive transfer of VEGFR-2 CAR T cells co-transduced with cDNA encoding IL-12p70 has also demonstrated anti-tumor efficacy in tumor-bearing mice and prolonged overall survival [144]. Interestingly,

this approach also resulted in a treatment-associated reduction in tumor-associated MDSC (as previously observed for the anti-angiogenic action of TKI as described above). Despite such promising pre-clinical results, TBVA-targeted CAR T cells have yet to be administered to cancer patients in the clinical setting.

3.4.7 Combination Therapy: Vascular Normalizing Agents and Immunotherapy

Improved understanding of the complexities of cancer heterogeneity, genetics and oncogenic signaling pathways associated with acquired resistance to single modality therapies has reinforced the need to develop combination/multi-modality immunotherapies in order to reproducibly achieve durable objective clinical responses in the cancer setting. As single modality approaches (cancer vaccines, immune checkpoint inhibitors, ACT, among others) have each demonstrated promising (sustained) therapeutic benefits in a minority of treated patients [145, 146]. Cancer vaccines are effective in eliciting anti-tumor immune response but one of the main limitations is the inefficient delivery of vaccine-induced T cells into the tumor lesion [147]. A number of studies have suggested that anti-angiogenic agents may be largely immune-dependent in their anti-tumor action, with many suggesting their use as "adjuvants" for integration into cancer vaccine formulations. Anti-angiogenic agents normalize the vasculature, increase blood perfusion and improve delivery of chemotherapeutic drugs and immune effector cells in the tumor bed [148]. In addition, some anti-angiogenic agents also have the capability to reduce hypoxia and immunosuppressive MDSC and T cell populations and promote the upregulation of vascular adhesion molecules and chemokines that enhance T cell recruitment into the TME [147, 149]. Indeed, in murine melanoma models, combination vaccination along with systemic delivery of TKIs axitinib, sunitinib or dasatinib resulted in superior therapeutic benefit when compared to either vaccination alone or TKI treatment alone. Concurrent administration of a DC/peptide vaccine and TKI improved the overall survival of M05 melanoma-bearing mice and was associated with the upregulated expression of VCAM-1 and CXCR3 ligand chemokines in the tumor endothelium and robust type-1 T cell infiltration in the TME [118, 149, 150]. In another study, treatment of MC38 colon carcinoma-bearing mice with the combination of sunitinib pre-conditioning followed by a CEA gene-based vaccine resulted in increased tumor infiltration by CEA-specific $CD8^+$ T cells, decreased Treg and MDSC populations, reduced tumor volumes and prolonged survival [151]. Simultaneous administration of sunitinib and the vaccine or administration of vaccine prior to sunitinib, however, did not result in improved survival [151, 152]. In a phase III clinical trial, the combination of a poxvirus-based vaccine encoding the tumor antigen 5T4 (TroVax) followed by administration of sunitinib was evaluated in renal cancer patients, with no benefit for the combined protocol versus sunitinib monotherapy observed [153]. These translational and clinical data strongly suggest that treatment schedule is likely critical to the success of such combination

therapies. The temporal interval wherein the anti-angiogenic agents (TKIs, TBVA-based vaccines, others) normalize the tumor vasculature and decrease immunosuppressive cell populations in the TME in advance of the arrival of vaccine-induced or ACT anti-tumor T cells must be determined in order to optimize the clinical benefits of such combination vaccines. This is further complicated by the effective window during which the adjuvant qualities of anti-angiogenic inhibitors act in the TME in the absence of off-target toxicities (including some manifest in immune cell populations). Hence, biomarkers to predict the efficacy of anti-angiogenic drugs (i.e., increased systemic levels of CXCR3 ligand chemokines, reduced levels of CXCL12, etc.) or techniques to monitor blood perfusion in patients in a non-invasive manner must be developed to define an optimal combination schedule based on vascular normalization criteria [82, 154, 155].

3.5 Summary

Type-I T cell functions such as IFN-γ secretion and cytolytic activity appear essential to tumor immune surveillance and regulation [156]. Effective immunotherapies (including cancer vaccines, immune checkpoint inhibitors and ACT) have been designed to reinstate, bolster and sustain Type-1 immunity against primary and metastatic cancer cells. These approaches have thus far, yielded sporadic instances of profound clinical efficacy, however, their rate and magnitude of clinical responsiveness may be limited by prohibitory characteristics of the TME that harbor dominant suppressor networks or that curtail T effector cell infiltration from the peripheral blood. These obstacles may be obviated in a coordinate manner by co-therapy approaches that lead to vascular normalization within the TME. Combination protocols integrating TKI or vaccines/CAR T cells targeting TBVAs are currently being developed and tested, bearing in mind the likelihood that vascular normalization should be achieved in advance of eliciting or delivering anti-tumor T cell populations in vivo.

The comparative genetic stability of tumor-associated vascular cells and their expression of a unique antigenic signature borne of TME conditioning supports their targeting as a general strategy for solid cancers in any combination approach. Furthermore, cancer vaccines based on TBVAs have demonstrated the added benefit of expanding the anti-tumor T cell repertoire based on the process of epitope/determinant spreading. The identification of additional novel TBVAs that are broadly shared amongst solid tumor types is of great translational/clinical interest for developing cancer vaccines and targeted therapies. In this regard, genomic and proteomic analyses of isolated cells from tumor versus (patient-matched) normal tissues to define new TBVA may support major advances in these clinical approaches and the future development of increasingly effective combination therapies.

Acknowledgments The authors would like to thank Dr. Ronald Fecek for reviewing this manuscript.

No potential conflicts of interest were disclosed.

References

1. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960–4.
2. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol. 2007;25(18):2586–93.
3. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer. 2011;105(1):93–103.
4. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2010;29(8):1093–102.
5. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348(3):203–13.
6. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat Rev Cancer. 2014;14(2):135–46.
7. Weigelin B, Krause M, Friedl P. Cytotoxic T lymphocyte migration and effector function in the tumor microenvironment. Immunol Lett. 2011;138(1):19–21.
8. Ostrand-Rosenberg S. CD4+ T lymphocytes: a critical component of antitumor immunity. Cancer Invest. 2005;23(5):413–9.
9. Protti MP, Monte LD, Lullo GD. Tumor antigen-specific CD4+ T cells in cancer immunity: from antigen identification to tumor prognosis and development of therapeutic strategies. Tissue Antigens. 2014;83(4):237–46.
10. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: are we making progress? Front Immunol. 2013;4:454.
11. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12(4):269–81.
12. Duong CP, Westwood JA, Berry LJ, Darcy PK, Kershaw MH. Enhancing the specificity of T-cell cultures for adoptive immunotherapy of cancer. Immunotherapy. 2011;3(1):33–48.
13. McIlroy D, Gregoire M. Optimizing dendritic cell-based anticancer immunotherapy: maturation state does have clinical impact. Cancer Immunol Immunother. 2003;52(10):583–91.
14. Zhong S, Malecek K, Johnson LA, Yu Z, Vega-Saenz de Miera E, Darvishian F, McGary K, Huang K, Boyer J, Corse E, Shao Y, Rosenberg SA, Restifo NP, Osman I, Krogsgaard M. T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy. Proc Natl Acad Sci U S A. 2013;110(17):6973–8.
15. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. J Immunother. 1997;20(3):165–77.
16. Leone P, Shin EC, Perosa F, Vacca A, Dammacco F, Racanelli V. MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. J Natl Cancer Inst. 2013;105(16):1172–87.
17. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol. 2004;4(5):336–47.
18. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med. 2002;8(8):793–800.
19. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. 2007;25:267–96.
20. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel). 2014;6(3):1670–90.

21. Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology. 2013;138(2):105–15.
22. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343(14):1020–34.
23. Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol. 1993;11:767–804.
24. Nolz JC. Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions. Immunotherapy. 2011;3(10):1223–33.
25. Eelen G, de Zeeuw P, Simons M, Carmeliet P. Endothelial cell metabolism in normal and diseased vasculature. Circ Res. 2015;116(7):1231–44.
26. Michiels C. Endothelial cell functions. J Cell Physiol. 2003;196(3):430–43.
27. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21(2):193–215.
28. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. 2003;314(1):15–23.
29. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. Exp Cell Res. 2006;312(5):623–9.
30. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer. 2003;3(6):422–33.
31. Form DM, Pratt BM, Madri JA. Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components. Lab Invest. 1986;55(5):521–30.
32. Bottos A, Bardelli A. Oncogenes and angiogenesis: a way to personalize anti-angiogenic therapy? Cell Mol Life Sci. 2013;70(21):4131–40.
33. Dor Y, Porat R, Keshet E. Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. Am J Physiol Cell Physiol. 2001;280(6):C1367–74.
34. Ferrara N, Mass RD, Campa C, Kim R. Targeting VEGF-A to treat cancer and age-related macular degeneration. Annu Rev Med. 2007;58:491–504.
35. Sakurai T, Kudo M. Signaling pathways governing tumor angiogenesis. Oncology. 2011;81 Suppl 1:24–9.
36. Plate KH, Scholz A, Dumont DJ. Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. Acta Neuropathol. 2012;124(6):763–75.
37. Bates D, Hillman N, Williams B, Neal C, Pocock T. Regulation of microvascular permeability by vascular endothelial growth factors. J Anat. 2002;200(6):581–97.
38. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science. 1999;284(5422):1994–8.
39. Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci. 2001;22(4):201–7.
40. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol. 2005;7(4):452–64.
41. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol. 2002;160(3):985–1000.
42. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am J Pathol. 2003;163(5):1801–15.
43. McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. Nat Med. 2003;9(6):713–25.
44. Claesson-Welsh L. Blood vessels as targets in tumor therapy. Ups J Med Sci. 2012;117(2):178–86.
45. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol. 2000;156(4):1363–80.

46. Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. Nat Rev Cancer. 2002;2(4):266–76.
47. Ariffin AB, Forde PF, Jahangeer S, Soden DM, Hinchion J. Releasing pressure in tumors: what do we know so far and where do we go from here? A review. Cancer Res. 2014;74(10):2655–62.
48. Lunt SJ, Fyles A, Hill RP, Milosevic M. Interstitial fluid pressure in tumors: therapeutic barrier and biomarker of angiogenesis. Future Oncol. 2008;4(6):793–802.
49. Bellone M, Calcinotto A. Ways to enhance lymphocyte trafficking into tumors and fitness of tumor infiltrating lymphocytes. Front Oncol. 2013;3:231.
50. Palazon A, Goldrath AW, Nizet V, Johnson RS. HIF transcription factors, inflammation, and immunity. Immunity. 2014;41(4):518–28.
51. Hoskin DW, Mader JS, Furlong SJ, Conrad DM, Blay J. Inhibition of T cell and natural killer cell function by adenosine and its contribution to immune evasion by tumor cells (review). Int J Oncology. 2008;32(3):527–35.
52. Siemens DR, Hu N, Sheikhi AK, Chung E, Frederiksen LJ, Pross H, Graham CH. Hypoxia increases tumor cell shedding of MHC class I chain-related molecule: role of nitric oxide. Cancer Res. 2008;68(12):4746–53.
53. Barsoum IB, Hamilton TK, Li X, Cotechini T, Miles EA, Siemens DR, Graham CH. Hypoxia induces escape from innate immunity in cancer cells via increased expression of ADAM10: role of nitric oxide. Cancer Res. 2011;71(24):7433–41.
54. Chitadze G, Lettau M, Bhat J, Wesch D, Steinle A, Furst D, Mytilineos J, Kalthoff H, Janssen O, Oberg HH, Kabelitz D. Shedding of endogenous MHC class I-related chain molecules A and B from different human tumor entities: heterogeneous involvement of the "a disintegrin and metalloproteases" 10 and 17. Int J Cancer. 2013;133(7):1557–66.
55. Barsoum IB, Smallwood CA, Siemens DR, Graham CH. A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. Cancer Res. 2014;74(3):665–74.
56. Doedens AL, Phan AT, Stradner MH, Fujimoto JK, Nguyen JV, Yang E, Johnson RS, Goldrath AW. Hypoxia-inducible factors enhance the effector responses of CD8(+) T cells to persistent antigen. Nat Immunol. 2013;14(11):1173–82.
57. Kumar V, Gabrilovich DI. Hypoxia-inducible factors in regulation of immune responses in tumour microenvironment. Immunology. 2014;143(4):512–9.
58. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012;74:13–40.
59. Ghilardi C, Chiorino G, Dossi R, Nagy Z, Giavazzi R, Bani M. Identification of novel vascular markers through gene expression profiling of tumor-derived endothelium. BMC Genomics. 2008;9:201.
60. Schaer DA, Lesokhin AM, Wolchok JD. Hiding the road signs that lead to tumor immunity. J Exp Med. 2011;208(10):1937–40.
61. Ruoslahti E. Specialization of tumour vasculature. Nat Rev Cancer. 2002;2(2):83–90.
62. Schwartz JD, Rowinsky EK, Youssoufian H, Pytowski B, Wu Y. Vascular endothelial growth factor receptor-1 in human cancer: concise review and rationale for development of IMC-18 F1 (Human antibody targeting vascular endothelial growth factor receptor-1). Cancer. 2010;116(4 Suppl):1027–32.
63. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A, Jurgensmeier JM, Womack C. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clin Cancer Res. 2010;16(14):3548–61.
64. Bouzin C, Brouet A, De Vriese J, Dewever J, Feron O. Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions: identification of caveolin-1 and nitric oxide as control points of endothelial cell energy. J Immunol. 2007;178(3):1505–11.
65. Xin Y, Li J, Wu J, Kinard R, Weekes CD, Patnaik A, Lorusso P, Brachmann R, Tong RK, Yan Y, Watts R, Bai S, Hegde PS. Pharmacokinetic and pharmacodynamic analysis of circulating biomarkers of anti-NRP1, a novel antiangiogenesis agent, in two phase I trials in patients with advanced solid tumors. Clin Cancer Res. 2012;18(21):6040–8.

66. Ben Q, Zheng J, Fei J, An W, Li P, Li Z, Yuan Y. High neuropilin 1 expression was associated with angiogenesis and poor overall survival in resected pancreatic ductal adenocarcinoma. Pancreas. 2014;43(5):744–9.
67. Cheng W, Fu D, Wei ZF, Xu F, Xu XF, Liu YH, Ge JP, Tian F, Han CH, Zhang ZY, Zhou LM. NRP-1 expression in bladder cancer and its implications for tumor progression. Tumour Biol. 2014;35(6):6089–94.
68. Hong TM, Chen YL, Wu YY, Yuan A, Chao YC, Chung YC, Wu MH, Yang SC, Pan SH, Shih JY, Chan WK, Yang PC. Targeting neuropilin 1 as an antitumor strategy in lung cancer. Clin Cancer Res. 2007;13(16):4759–68.
69. Zhu H, Cai H, Tang M, Tang J. Neuropilin-1 is overexpressed in osteosarcoma and contributes to tumor progression and poor prognosis. Clin Transl Oncol. 2014;16(8):732–8.
70. Geretti E, Klagsbrun M. Neuropilins: novel targets for anti-angiogenesis therapies. Cell Adh Migr. 2007;1(2):56–61.
71. Nanda A, St Croix B. Tumor endothelial markers: new targets for cancer therapy. Curr Opin Oncol. 2004;16(1):44–9.
72. Otsubo T, Hida Y, Ohga N, Sato H, Kai T, Matsuki Y, Takasu H, Akiyama K, Maishi N, Kawamoto T, Shinohara N, Nonomura K, Hida K. Identification of novel targets for antiangiogenic therapy by comparing the gene expressions of tumor and normal endothelial cells. Cancer Sci. 2014;105(5):560–7.
73. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med. 2008;14(1):28–36.
74. Bagnato A, Spinella F. Emerging role of endothelin-1 in tumor angiogenesis. Trends Endocrinol Metab. 2003;14(1):44–50.
75. Rosano L, Spinella F, Bagnato A. Endothelin 1 in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer. 2013;13(9):637–51.
76. Bagnato A, Spinella F, Rosano L. Emerging role of the endothelin axis in ovarian tumor progression. Endocr Relat Cancer. 2005;12(4):761–72.
77. Tsukahara H, Ende H, Magazine HI, Bahou WF, Goligorsky MS. Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. J Biol Chem. 1994;269(34):21778–85.
78. Berger M, Bergers G, Arnold B, Hammerling GJ, Ganss R. Regulator of G-protein signaling-5 induction in pericytes coincides with active vessel remodeling during neovascularization. Blood. 2005;105(3):1094–101.
79. Manzur M, Ganss R. Regulator of G protein signaling 5: a new player in vascular remodeling. Trends Cardiovasc Med. 2009;19(1):26–30.
80. Hamzah J, Jugold M, Kiessling F, Rigby P, Manzur M, Marti HH, Rabie T, Kaden S, Grone HJ, Hammerling GJ, Arnold B, Ganss R. Vascular normalization in Rgs5-deficient tumours promotes immune destruction. Nature. 2008;453(7193):410–4.
81. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007;6(4):273–86.
82. Jain RK. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J Clin Oncol. 2013;31(17):2205–18.
83. Shojaei F. Anti-angiogenesis therapy in cancer: current challenges and future perspectives. Cancer Lett. 2012;320(2):130–7.
84. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005;307(5706):58–62.
85. Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, Tian YM, Lanahan AA, Pollard P, Ruiz de Almodovar C, De Smet F, Vinckier S, Aragones J, Debackere K, Luttun A, Wyns S, Jordan B, Pisacane A, Gallez B, Lampugnani MG, Dejana E, Simons M, Ratcliffe P, Maxwell P, Carmeliet P. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 2009;136(5):839–51.

86. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol. 2000;18(11):1185–90.
87. Bellone M, Calcinotto A, Corti A. Won't you come on in? How to favor lymphocyte infiltration in tumors. Oncoimmunology. 2012;1(6):986–8.
88. Lorusso D, Scambia G, Amadio G, di Legge A, Pietragalla A, De Vincenzo R, Masciullo V, Di Stefano M, Mangili G, Citterio G, Mantori M, Lambiase A, Bordignon C. Phase II study of NGR-hTNF in combination with doxorubicin in relapsed ovarian cancer patients. Br J Cancer. 2012;107(1):37–42.
89. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer. 2004;4(6):423–36.
90. Maiti R. Metronomic chemotherapy. J Pharmacol Pharmacother. 2014;5(3):186–92.
91. Pasquier E, Kavallaris M, Andre N. Metronomic chemotherapy: new rationale for new directions. Nat Rev Clin Oncol. 2010;7(8):455–65.
92. Hermans IF, Chong TW, Palmowski MJ, Harris AL, Cerundolo V. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. Cancer Res. 2003;63(23):8408–13.
93. Chen G, Emens LA. Chemoimmunotherapy: reengineering tumor immunity. Cancer Immunol Immunother. 2013;62(2):203–16.
94. De Vita S, De Matteis S, Laurenti L, Chiusolo P, Reddiconto G, Fiorini A, Leone G, Sica S. Secondary Ph + acute lymphoblastic leukemia after temozolomide. Ann Hematol. 2005;84(11):760–2.
95. Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 1997;57(20):4593–9.
96. Ciombor KK, Berlin J, Chan E. Aflibercept. Clin Cancer Res. 2013;19(8):1920–5.
97. Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, Yancopoulos GD, Jaffe RB. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res. 2003;9(15):5721–8.
98. Rosen LS. VEGF-targeted therapy: therapeutic potential and recent advances. Oncologist. 2005;10(6):382–91.
99. Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther. 2006;28(11):1779–802.
100. Verheul HM, Hammers H, van Erp K, Wei Y, Sanni T, Salumbides B, Qian DZ, Yancopoulos GD, Pili R. Vascular endothelial growth factor trap blocks tumor growth, metastasis formation, and vascular leakage in an orthotopic murine renal cell cancer model. Clin Cancer Res. 2007;13(14):4201–8.
101. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. Mol Cancer Ther. 2008;7(12):3670–84.
102. Tejpar S, Prenen H, Mazzone M. Overcoming resistance to antiangiogenic therapies. Oncologist. 2012;17(8):1039–50.
103. De Falco S. Antiangiogenesis therapy: an update after the first decade. Korean J Intern Med. 2014;29(1):1–11.
104. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141(7):1117–34.
105. Hojjat-Farsangi M. Small-molecule inhibitors of the receptor tyrosine kinases: promising tools for targeted cancer therapies. Int J Mol Sci. 2014;15(8):13768–801.
106. Dabney R, Devine R, Sein N, George B. New agents in renal cell carcinoma. Target Oncol. 2014;9(3):183–93.
107. Iyer R, Fetterly G, Lugade A, Thanavala Y. Sorafenib: a clinical and pharmacologic review. Expert Opin Pharmacother. 2010;11(11):1943–55.

108. Nguewa PA, Calvo A, Pullamsetti SS, Banat GA, Grimminger F, Savai R. Tyrosine kinase inhibitors with antiangiogenic properties for the treatment of non-small cell lung cancer. Expert Opin Investig Drugs. 2011;20(1):61–74.
109. Sonpavde G, Hutson TE, Sternberg CN. Pazopanib, a potent orally administered small-molecule multitargeted tyrosine kinase inhibitor for renal cell carcinoma. Expert Opin Investig Drugs. 2008;17(2):253–61.
110. Harris PA, Boloor A, Cheung M, Kumar R, Crosby RM, Davis-Ward RG, Epperly AH, Hinkle KW, Hunter 3rd RN, Johnson JH, Knick VB, Laudeman CP, Luttrell DK, Mook RA, Nolte RT, Rudolph SK, Szewczyk JR, Truesdale AT, Veal JM, Wang L, Stafford JA. Discovery of 5-[[4-[(2,3-dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a novel and potent vascular endothelial growth factor receptor inhibitor. J Med Chem. 2008;51(15):4632–40.
111. Hu-Lowe DD, Zou HY, Grazzini ML, Hallin ME, Wickman GR, Amundson K, Chen JH, Rewolinski DA, Yamazaki S, Wu EY, McTigue MA, Murray BW, Kania RS, O'Connor P, Shalinsky DR, Bender SL. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. Clin Cancer Res. 2008;14(22):7272–83.
112. Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, Vandenbeldt K, Qian CN, Teh BT. Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. Cancer Res. 2010;70(3):1053–62.
113. Mangiameli DP, Blansfield JA, Kachala S, Lorang D, Schafer PH, Muller GW, Stirling DI, Libutti SK. Combination therapy targeting the tumor microenvironment is effective in a model of human ocular melanoma. J Transl Med. 2007;5:38.
114. Hamberg P, Verweij J, Sleijfer S. (Pre-)clinical pharmacology and activity of pazopanib, a novel multikinase angiogenesis inhibitor. Oncologist. 2010;15(6):539–47.
115. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, Murray LJ, Carver J, Chan E, Moss KG, Haznedar JO, Sukbuntherng J, Blake RA, Sun L, Tang C, Miller T, Shirazian S, McMahon G, Cherrington JM. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res. 2003;9(1):327–37.
116. O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW, Wong LM, Hong W, Lee LB, Town A, Smolich BD, Manning WC, Murray LJ, Heinrich MC, Cherrington JM. SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood. 2003;101(9):3597–605.
117. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, Schwartz M, Divino CM, Pan PY, Chen SH. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. Cancer Res. 2009;69(6):2514–22.
118. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, Ko JS, Cohen PA, Finke JH, Storkus WJ. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. Int J Cancer. 2011;129(9):2158–70.
119. Ko JS, Rayman P, Ireland J, Swaidani S, Li G, Bunting KD, Rini B, Finke JH, Cohen PA. Direct and differential suppression of myeloid-derived suppressor cell subsets by sunitinib is compartmentally constrained. Cancer Res. 2010;70(9):3526–36.
120. Finke JH, Rini B, Ireland J, Rayman P, Richmond A, Golshayan A, Wood L, Elson P, Garcia J, Dreicer R, Bukowski R. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. Clin Cancer Res. 2008;14(20):6674–82.
121. Gu Y, Zhao W, Meng F, Qu B, Zhu X, Sun Y, Shu Y, Xu Q. Sunitinib impairs the proliferation and function of human peripheral T cell and prevents T-cell-mediated immune response in mice. Clin Immunol. 2010;135(1):55–62.
122. Alfaro C, Suarez N, Gonzalez A, Solano S, Erro L, Dubrot J, Palazon A, Hervas-Stubbs S, Gurpide A, Lopez-Picazo JM, Grande-Pulido E, Melero I, Perez-Gracia JL. Influence of

bevacizumab, sunitinib and sorafenib as single agents or in combination on the inhibitory effects of VEGF on human dendritic cell differentiation from monocytes. Br J Cancer. 2009;100(7):1111–9.
123. Kim S, Ding W, Zhang L, Tian W, Chen S. Clinical response to sunitinib as a multitargeted tyrosine-kinase inhibitor (TKI) in solid cancers: a review of clinical trials. Onco Targets Ther. 2014;7:719–28.
124. Vinik AI, Raymond E. Pancreatic neuroendocrine tumors: approach to treatment with focus on sunitinib. Therap Adv Gastroenterol. 2013;6(5):396–411.
125. Ibrahim N, Yu Y, Walsh WR, Yang JL. Molecular targeted therapies for cancer: sorafenib monotherapy and its combination with other therapies (review). Oncol Rep. 2012;27(5):1303–11.
126. Thomas L, Lai SY, Dong W, Feng L, Dadu R, Regone RM, Cabanillas ME. Sorafenib in metastatic thyroid cancer: a systematic review. Oncologist. 2014;19(3):251–8.
127. Deeks ED. Pazopanib: in advanced soft tissue sarcoma. Drugs. 2012;72(16):2129–40.
128. Drabkin HA. Pazopanib and anti-VEGF therapy. Open Access J Urol. 2010;2:35–40.
129. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clin Cancer Res. 2011;17(11):3520–6.
130. Dong Y, Qian J, Ibrahim R, Berzofsky JA, Khleif SN. Identification of H-2Db-specific CD8+ T-cell epitopes from mouse VEGFR2 that can inhibit angiogenesis and tumor growth. J Immunother. 2006;29(1):32–40.
131. Ishizaki H, Tsunoda T, Wada S, Yamauchi M, Shibuya M, Tahara H. Inhibition of tumor growth with antiangiogenic cancer vaccine using epitope peptides derived from human vascular endothelial growth factor receptor 1. Clin Cancer Res. 2006;12(19):5841–9.
132. Wada S, Tsunoda T, Baba T, Primus FJ, Kuwano H, Shibuya M, Tahara H. Rationale for antiangiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2. Cancer Res. 2005;65(11):4939–46.
133. Yamaue H, Tsunoda T, Tani M, Miyazawa M, Yamao K, Mizuno N, Okusaka T, Ueno H, Boku N, Fukutomi A, Ishii H, Ohkawa S, Furukawa M, Maguchi H, Ikeda M, Togashi Y, Nishio K, Ohashi Y. Randomized phase II/III clinical trial of elpamotide for patients with advanced pancreatic cancer. PEGASUS-PC Study Cancer Sci. 2015;106(7):883–90.
134. Hazama S, Nakamura Y, Takenouchi H, Suzuki N, Tsunedomi R, Inoue Y, Tokuhisa Y, Iizuka N, Yoshino S, Takeda K, Shinozaki H, Kamiya A, Furukawa H, Oka M. A phase I study of combination vaccine treatment of five therapeutic epitope-peptides for metastatic colorectal cancer; safety, immunological response, and clinical outcome. J Transl Med. 2014;12:63.
135. Okamoto I, Arao T, Miyazaki M, Satoh T, Okamoto K, Tsunoda T, Nishio K, Nakagawa K. Clinical phase I study of elpamotide, a peptide vaccine for vascular endothelial growth factor receptor 2, in patients with advanced solid tumors. Cancer Sci. 2012;103(12):2135–8.
136. Okuyama R, Aruga A, Hatori T, Takeda K, Yamamoto M. Immunological responses to a multi-peptide vaccine targeting cancer-testis antigens and VEGFRs in advanced pancreatic cancer patients. Oncoimmunology. 2013;2(11), e27010.
137. Suzuki H, Fukuhara M, Yamaura T, Mutoh S, Okabe N, Yaginuma H, Hasegawa T, Yonechi A, Osugi J, Hoshino M, Kimura T, Higuchi M, Shio Y, Ise K, Takeda K, Gotoh M. Multiple therapeutic peptide vaccines consisting of combined novel cancer testis antigens and anti-angiogenic peptides for patients with non-small cell lung cancer. J Transl Med. 2013;11:97.
138. Zhao X, Bose A, Komita H, Taylor JL, Chi N, Lowe DB, Okada H, Cao Y, Mukhopadhyay D, Cohen PA, Storkus WJ. Vaccines targeting tumor blood vessel antigens promote CD8(+) T cell-dependent tumor eradication or dormancy in HLA-A2 transgenic mice. J Immunol. 2012;188(4):1782–8.
139. Chi Sabins N, Taylor JL, Fabian KP, Appleman LJ, Maranchie JK, Stolz DB, Storkus WJ. DLK1: a novel target for immunotherapeutic remodeling of the tumor blood vasculature. Mol Ther. 2013;21(10):1958–68.
140. Facciponte JG, Ugel S, De Sanctis F, Li C, Wang L, Nair G, Sehgal S, Raj A, Matthaiou E, Coukos G, Facciabene A. Tumor endothelial marker 1-specific DNA vaccination targets tumor vasculature. J Clin Invest. 2014;124(4):1497–511.

141. Sharpe M, Mount N. Genetically modified T cells in cancer therapy: opportunities and challenges. Dis Model Mech. 2015;8(4):337–50.
142. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T cell therapy for acute lymphoblastic leukemia. Blood. 2015;125(26):4017–23.
143. Fu X, Rivera A, Tao L, Zhang X. Genetically modified T cells targeting neovasculature efficiently destroy tumor blood vessels, shrink established solid tumors, and increase nanoparticle delivery. Int J Cancer. 2013;133(10):2483–92.
144. Chinnasamy D, Yu Z, Kerkar SP, Zhang L, Morgan RA, Restifo NP, Rosenberg SA. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. Clin Cancer Res. 2012;18(6):1672–83.
145. Vujanovic L, Butterfield LH. Melanoma cancer vaccines and anti-tumor T cell responses. J Cell Biochem. 2007;102(2):301–10.
146. Yu Z, Restifo NP. Cancer vaccines: progress reveals new complexities. J Clin Invest. 2002;110(3):289–94.
147. Shi S, Chen L, Huang G. Antiangiogenic therapy improves the antitumor effect of adoptive cell immunotherapy by normalizing tumor vasculature. Med Oncol. 2013;30(4):698.
148. Griffioen AW, Weiss A, Berndsen RH, Abdul UK, te Winkel MT, Nowak-Sliwinska P. The emerging quest for the optimal angiostatic combination therapy. Biochem Soc Trans. 2014;42(6):1608–15.
149. Bose A, Lowe DB, Rao A, Storkus WJ. Combined vaccine + axitinib therapy yields superior antitumor efficacy in a murine melanoma model. Melanoma Res. 2012;22(3):236–43.
150. Lowe DB, Bose A, Taylor JL, Tawbi H, Lin Y, Kirkwood JM, Storkus WJ. Dasatinib promotes the expansion of a therapeutically superior T-cell repertoire in response to dendritic cell vaccination against melanoma. Oncoimmunology. 2014;3(1), e27589.
151. Farsaci B, Higgins JP, Hodge JW. Consequence of dose scheduling of sunitinib on host immune response elements and vaccine combination therapy. Int J Cancer. 2012;130(8):1948–59.
152. Kwilas AR, Donahue RN, Tsang KY, Hodge JW. Immune consequences of tyrosine kinase inhibitors that synergize with cancer immunotherapy. Cancer Cell Microenviron. 2015;2(1), e677.
153. Amato RJ, Hawkins RE, Kaufman HL, Thompson JA, Tomczak P, Szczylik C, McDonald M, Eastty S, Shingler WH, de Belin J, Goonewardena M, Naylor S, Harrop R. Vaccination of metastatic renal cancer patients with MVA-5 T4: a randomized, double-blind, placebo-controlled phase III study. Clin Cancer Res. 2010;16(22):5539–47.
154. Franco M, Man S, Chen L, Emmenegger U, Shaked Y, Cheung AM, Brown AS, Hicklin DJ, Foster FS, Kerbel RS. Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. Cancer Res. 2006;66(7):3639–48.
155. Moserle L, Jimenez-Valerio G, Casanovas O. Antiangiogenic therapies: going beyond their limits. Cancer Discov. 2014;4(1):31–41.
156. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity. 2004;21(2):137–48.
157. Jain RK, Au P, Tam J, Duda DG, Fukumura D. Engineered vascularized tissue. Nat Biotechnol. 2005;23(7):821–3.

Chapter 4
Disruption of Anti-tumor T Cell Responses by Cancer-Associated Fibroblasts

Arnaud Pommier and Douglas T. Fearon

Abstract Tumors arise from the malignant transformation of normal cells through genetic dysregulation of the cell growth controls. However, cancer cells are not the only component of a tumor and the non-cancer cell part of the tumor has been termed stroma. The tumor stroma comprises cells of at least three different origins: endothelial, mesenchymal and hematopoietic, thereby forming a complex microenvironment. Among these three elements, immune cells have been extensively studied for the last 20 years because of their potential tumoricidal properties. However, immunologists failed to efficiently harness the immune system's anti-tumor properties until very recently, shedding light on a very complex immuno-suppressive network in tumor-bearing hosts. The mesenchymal part of the tumor, the so-called cancer-associated fibroblasts (CAFs), is among those actors of the tumor that have been disregarded despite their crucial role. Indeed, CAFs have been recently shown to be one of the more potent immune-suppressive cells of the tumor microenvironment. This chapter focuses on their roles on immune components of the tumor micro-environment, especially T cells. CAFs can impact T cells in two major ways: Disruption of T cell homeostasis and functions, and exclusion of T cells from the vicinity of tumor cells. These recent advances in the understanding the tumor micro-environment reveals potential new ways for attacking tumor cells.

Keywords Anti-tumor immune response • T cells • Cancer-associated fibroblasts • Immunotherapies

A. Pommier (✉) • D.T. Fearon
Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Weill Cornell Medical College, New York, NY, USA
e-mail: apommier@cshl.edu; dfearon@cshl.edu

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_4

Abbreviations

Ab	Antibody
APCs	Antigen presenting cells
CAFs	Cancer-associated fibroblasts
CSC	Cancer stem cell
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T lymphocyte antigen-4
DCs	Dendritic cells
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
FAP	Fibroblast activation protein
FRCs	Fibroblastic reticular cells
FSP1	Fibroblast specific protein-1
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HO-1	Heme oxidase-1
HSCs	Hepatic stellate cells
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
ITIM	Immunoreceptor tyrosine inhibitory motif
LOX	Lysyl-oxidase
MDSCs	Myeloid derived suppressor cells
MHC	Major hiscompatibility complex
MLR	Mixed lymphocyte reaction
MMPs	Matrix metalloproteases
MSCs	Mesenchymal stem cells
NSCLC	Non-small cell lung carcinoma
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PSCs	Pancreatic stellate cells
ROS	Reactive oxygen species
α-SMA	α-Smooth muscle actin
TAMs	Tumor-associated macrophages
TGFβ	Transforming growth factor β
TLSs	Tertiary lymphoid structures
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin

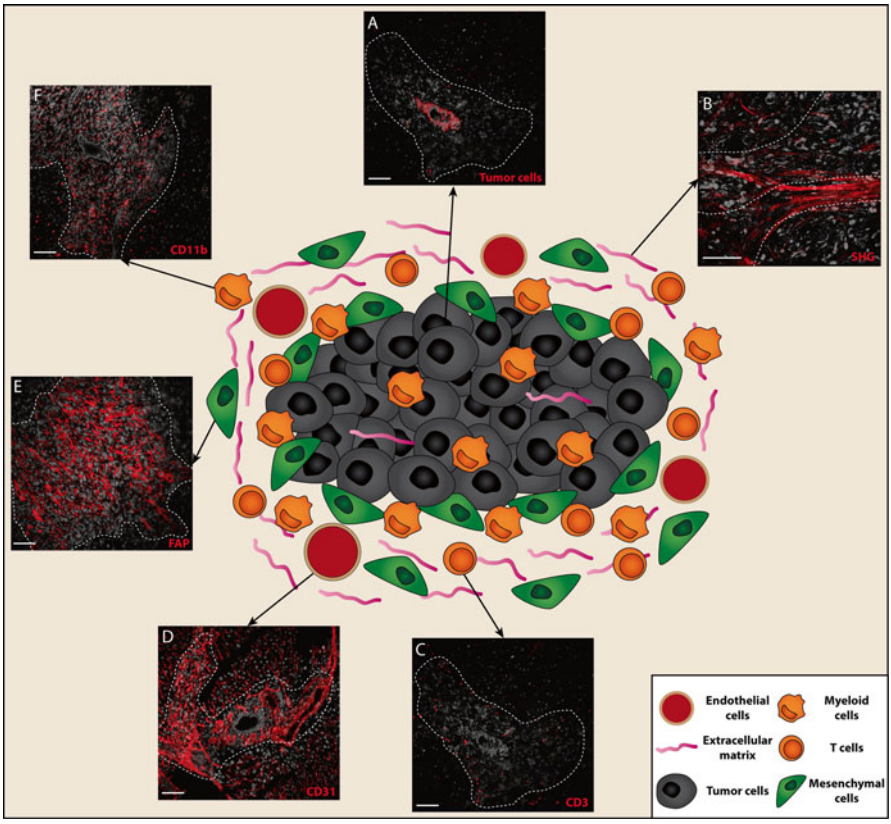

Fig. 4.1 Components of the tumor micro-environment. Immunofluorescence staining of liver metastases sections from KPC mice. (**a**) Tumor cells identified by ErbB2 staining, (**b**) Collagen identified by second harmonic generation (SHG), (**c**) T cells identified by CD3 staining, (**d**) Endothelial cells identified by CD31 staining, (**e**) CAFs identified by FAP staining and (**f**) Myeloid cells identified by CD11b staining. Dashed lines delimitate the desmoplastic region, that include tumor cells and stromal compartment, for each tumor. Scale bar = 100μm

4.1 Introduction

Tumors arise from the malignant transformation of normal cells through genetic dysregulation of the cell growth controls as highlighted by Hanahan and Weinberg in their hallmarks of cancer almost 15 years ago [1]. However, cancer cells are not the only component of a tumor. Although the importance of the tumor stroma, i.e., the non-cancer cell part of the tumor, has been proposed since a very long time through the concept of "seed and soil" [2], it has been only recently that there has been renewed interest in it. The tumor stroma comprises cells of at least three different origins: endothelial, mesenchymal and hematopoietic, thereby forming a complex microenvironment (Fig. 4.1). Among these three elements, immune cells

have been extensively studied for the last 20 years because of their potential tumoricidal properties [3]. However, immunologists failed to efficiently harness the immune system's anti-tumor properties until very recently, shedding light on a very complex immuno-suppressive network in tumor-bearing hosts [4]. Cytotoxic T cells (CTL) are a main component of the adaptive immune system and are the most potent anti-tumor cells [5] yet their activity is inhibited by multiple actors of the tumor microenvironment. Indeed, every cell type in the tumor may contribute to CTL suppression by a variety of mechanisms. Cancer cells on their own have developed multiple mechanisms to avoid CTL-mediated killing either by avoiding recognition through down-regulation of antigen expression [6, 7], resistance to lysis [8] or expression/secretion of immuno-regulatory molecules [9]. Aberrant vasculature in tumor leads to impaired CTL infiltration and localization, thus, limiting their anti-tumor properties [10]. Immune cells in the tumor microenvironment not only have potential roles in immune activation, but also have immuno-suppressive capabilities like tolerogenic dendritic cells (DCs) [11], tumor-associated macrophages (TAMs) [11], myeloid-derived suppressor cells (MDSCs) [11], and regulatory $CD4^+$ T cells (Tregs) [12]. Finally, the mesenchymal part of the tumor, the so-called cancer-associated fibroblasts (CAFs), also is a major component of the suppressive tumor microenvironment [13, 14].

4.2 Definition: From Myofibroblasts to CAFs

Cells of mesenchymal origin, or fibroblasts, are a key component of tissue homeostasis. They model the framework of tissues by their synthesis of extracellular matrix (ECM) constituents such as fibronectin and collagens [15]. Fibroblasts remain in a quiescent state until tissue remodeling becomes necessary during wound-healing and tissue regeneration. These activated fibroblasts have been termed myofibroblasts and were originally described during the wound-healing process. Upon tissue damage, fibroblasts proliferate and differentiate to myofibroblasts, as originally described by Darby and Laverdet [16], which express the contractile stress fibers, spliced variants of fibronectin and α-smooth muscle actin (α-SMA), and produce high levels of ECM components and chemokines. Thus, myofibroblasts acquire contractile capabilities to close the wound, regenerate the ECM and attract epithelial cells to complete the healing process [16]. Once the wound-healing process is completed, the majority of myofibroblasts are cleared from the tissue through apoptosis [16] or nemosis [17]. Tumors share many of the features of the wound-healing process, leading to the proposal that they are "wounds that do not heal" [18]. Therefore, CAFs reasonably share similarities with myofibroblasts found in wounded tissues, including expression of α-SMA and contractile properties [19]. CAFs are found in all solid tumors but their prevalence varies greatly. Many are found in prostate, breast and pancreatic cancers whereas there are fewer CAFs in ovarian, renal and melanoma tumors [15]. The lack of generally accepted specific markers for CAFs suggests that they are a heterogeneous population at the phenotypic level. Besides

α-SMA, many other markers have been used to define CAFs, many of which, however, may also be found in mesenchymal cells from normal tissues, which have made difficult their specific targeting for conditional ablation. Of note, Fibroblast-specific protein-1 (FSP-1) has long been used as a fibroblast marker but it is now known that macrophages express FSP-1 particularly in the inflamed environment [20]. Also, when combined with other markers, FSP-1 expression by CAFs seems rather poor in the Rip1Tag2 transgenic model of pancreatic cancer and transplanted breast tumors [21]. Platelet-derived growth factor (PDGF) receptors α and β, α-SMA and NG2 are frequently expressed on CAFs; however they are also found on pericytes, the smooth-muscle cells coating vasculature [22, 23]. Furthermore, some cancer cells, especially sarcomas, express PDGFRα and PDGFRβ [24]. Fibroblast-activation protein-α (FAP) is expressed by fibroblastic cells of most human adenocarcinomas [25], but is also expressed on mesenchymal cells at sites of chronic inflammation, such as rheumatoid arthritis synovial tissue, cirrhotic lesions of the liver, and atherosclerosis. In the mouse, FAP is expressed by CAFs in the autochthonous model of pancreatic ductal adenocarcinoma (PDAC) KPC ($Kras^{G12D}$; $p53^{R172H}$; Pdx-1Cre) [26] and in transplanted tumors [13, 14]. FAP is also expressed on a small fraction (less than 10 %) of TAMs [27]. Nevertheless, all FAP-expressing cells of the tumor microenvironment express α-SMA and PDGFRα but do not surround the vasculature [14] thus probably making FAP the most accurate marker of CAFs. However, the presence of FAP-expressing cells in many normal tissues excludes it from being a specific marker for CAFs [28].

4.3 On the Origin of CAFs

The origin of the CAF population is still debatable. Indeed, several reports have suggested different, and sometimes contradictory, origins of CAFs. A first source of CAFs is local fibroblasts. Using a mouse model of non-small cell lung cancer (NSCLC), it has been shown that exposure of normal lung fibroblasts to tumor cells induces the gene signature and phenotype found in CAFs [29]. In breast cancer, co-implantation of tumor cells with normal human resident fibroblasts gradually converts them to a CAF phenotype in a TGFβ- and CXCL12-dependent manner [30]. Stellate cells are a specialized population of fibroblasts found in the liver (hepatic stellate cells, HSCs) and pancreas (pancreatic stellate cells, PSCs). Normally they may support organ development and regeneration [31]. However, they are also involved in liver and pancreas fibrosis that precede the occurrence of hepatocellular carcinoma (HCC) and b-cell tumors of the pancreas [31, 32], making them a likely source of CAFs. Two other local sources of CAFs have been suggested: In fibrosis of the kidney, lung and liver, epithelial-to-mesenchymal transition (EMT) has been found to account for a large proportion of myofibroblasts [33]. Also, in models of melanoma and pancreatic carcinoma, transition of endothelial cells to a mesenchymal phenotype has been shown to be an important origin of CAFs [34]. Beyond those local sources, more distant ones have been suggested. In breast and ovarian

murine tumors, it has been proposed that the mesenchymal cells associated with the vasculature originate from the surrounding adipose tissue, whereas the majority of the CAFs not associated with vasculature are recruited from the bone marrow [35]. Indeed, bone marrow mesenchymal stem cells (MSCs) share many features with CAFs [36]. In a mouse model of gastric cancer, 20 % of CAFs originate from bone marrow MSCs [37]. Finally, fibrocytes, which are a circulating population that is recruited to sites of inflammation, have been shown to account for 25 % of CAFs in β-cell tumors of the pancreas [38].

4.4 Role of CAFs on Non-immune Components of the Tumor

CAFs have both a capacity for modulating the tumor microenvironment, and for acting directly on cancer cells to support multiple aspects of tumor growth. Co-culture of CAFs and pre-malignant epithelial cells from human prostate cancer suggested that CAFs support the initiation of tumors. In vivo, co-implantating pre-malignant prostate cells with prostate-cancer derived CAFs markedly enhanced tumor growth while normal prostate fibroblasts showed no sign of malignant transformation of the epithelial cells [39]. Moreover, CAFs support growth of pre-malignant and malignant cells but not normal epithelial cells in vitro as well as in vivo [40]. Furthermore, CAFs have the capacity to remodel ECM leading to a tumor-prone desmoplastic stroma. This is of particular importance in breast [41], lung [42], colorectal [43] and pancreas tumors [44].

CAFs have been shown to increase the numbers of cancer stem cells (CSC) and expression of CSC markers through secretion of IL-6 [45, 46], reactive oxygen species (ROS) [47], hepatocyte growth factor (HGF) [48], CXCL7 [46], IL-17 [49] and prostaglandin E2 (PGE2) [50], leading to more aggressive tumor behavior.

CAFs may further support progression by increasing proliferation of tumor cells through secretion of CXCL12, CXCL14 [51, 52] and TGFβ [53], increasing angiogenesis [54, 55]. Another important role of CAFs is to provide a tumor friendly ECM [56]. Indeed CAFs remodel and regulate the ECM thanks to their expression of multiple Matrix Metallo-Proteases (MMPs) and Lysyl oxidase (LOX), an enzyme responsible for type I collagen cross-linking [15]. Overall, CAFs acquire an inflammatory signature during cancer progression [57] that correlates with a poor clinical outcome [58]. Interestingly, TGFβ is required for the differentiation of normal fibroblasts to CAFs and their maintenance [59] but CAFs are also a major source of TGFβ in the tumor micro-environment [30]. This suggests an autocrine loop where CAFs increase their own activation and therefore pro-tumoral properties.

Finally, CAFs can increase metastatic activity through multiple processes. Secretion of IL-11 in colorectal cancer (CRC) [60] and CCL5 in breast cancer [61] stimulates tumor cells and accelerates metastasis. Degradation of the ECM by CAF-derived MMPs have been shown to promote metastasis in melanoma [62], skin skamous cell carcinoma [63], breast cancer [64] and CRC [65]. EMT has been proposed to be a crucial, if not mandatory, step to metastasis [66], and CAFs may promote

EMT in PDAC [67] and in prostate cancer through secretion of ROS [47]. Beyond promoting primary tumor seeding by influencing the microenvironment, CAFs can support tumor cells that are actually migrating. Indeed, analysis of the efferent blood of tumors showed clusters of epithelial cells and CAFs that are more likely to survive in the blood stream. Furthermore, those clusters seem to colonize target organs more efficiently than solitary tumor cells [68].

Recently, it has been suggested that CAFs may also act to restrain tumor progression, a finding that recalls much earlier work [69]. In the KPC mouse model of PDAC [70], deletion of Shh, a soluble ligand that drive generation of desmoplastic stroma, in tumor cells led to tumors with fewer CAFs (by α-SMA staining), less immune cell infiltration (by CD45 staining) and more vasculature (by CD31 staining) that caused more rapid mortality despite being smaller than control PDACs. Moreover, tumors displayed a less undifferentiated morphology characteristic of EMT. While the mechanism underlying this process remains elusive, one can hypothesize that disruption of the cross-talk between CAFs and epithelial cells could lead to increase of the EMT-inducing program in CAFs. On the other hand, the alteration of the tumor microenvironment by the relative absence of immune cells could have had a major effect on cancer cell proliferation and differentiation.

4.5 Influence of CAFs on Immune Components of Tumors

MSCs in the bone marrow have been shown to have immunoregulating properties contributing to maintenance of peripheral tolerance, autoimmunity, fetal-maternal tolerance and transplantation tolerance [71]. Similarly, beyond influencing the tumor cells on their own, CAFs, which some have proposed may be developmentally related to MSCs, are powerful modulators of the tumor immune environment, acting both on innate and adaptive immunity.

4.5.1 Role of CAFs on Innate Immune Components (Fig. 4.2)

(a) CAFs promote an inflammatory micro-environment

The tumor micro-environment may shape the secretory profile of CAFs. For example, CAFs that are co-cultured with carcinoma cells exhibit a pro-inflammatory secretory profile including the biosynthesis of CCL7, CXCL1, CXCL2 and CXCL8 [26, 72, 73]. Similarly, gene analysis of CAFs from skin tumors has shown that they possess an "inflammatory signature", also secreting high quantities of IL-6, IL-1β and CXCL5 [14, 74]. Mass spectrometry analysis of the secretome of CAFs from CRC further adds CXCL12, CCL5 and CXCL6 [75]. Finally, CAFs also produce IL-1β, CCL20 [76], CCL2 and CCL26 [77]. Of note, CAFs have been suggested to be higher producers of those inflammatory mediators than tumor cells [76].

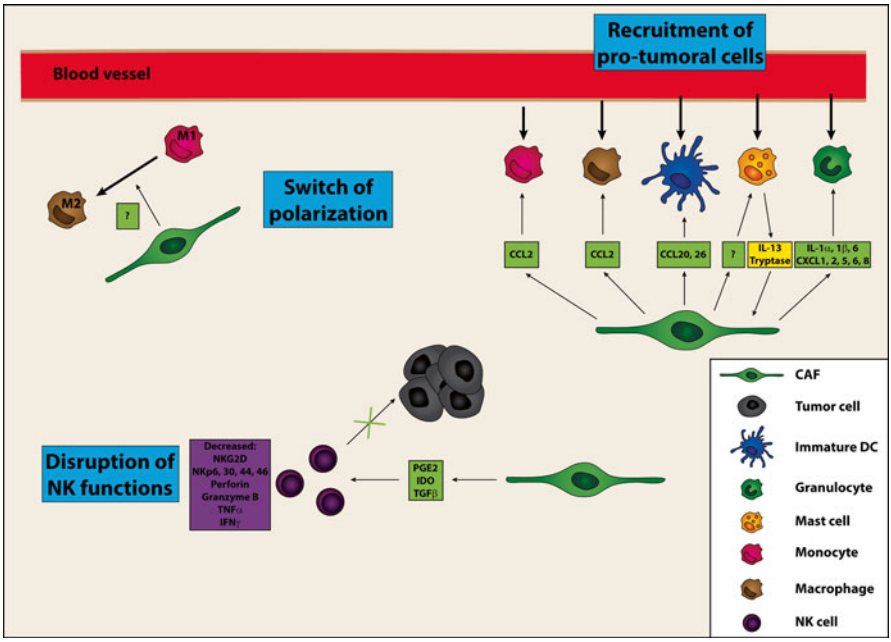

Fig. 4.2 Influence of CAFs on innate immune components of the tumor. CAFs act by three main mechanisms: Recruitment and promotion of survival of pro-tumoral innate immune cells, disruption of NK cell functions, and switch of innate immune cells from anti- to a pro-tumoral functions

This secretion profile shapes the tumor micro-environment to be inflammatory. Indeed, IL-6, IL-1α and β cytokines of granulocytes and their recruitment in the tumor micro-environment but also stimulate invasion and angiogenesis [78]. CXCL1, 2, 5, 6 and 8 are ligands for the receptors CXCR1 and CXCR2 and are also implicated in granulocytes generation and recruitment [79]. Beyond its role on tumor cells, it was suggested that CXCL12 and its receptor CXCR4 play a role on hematopoietic precursors that in turn act on tumor progression and metastasis [80]. CCL5 and CCL7 act widely on immune cells but are of a particular importance in the generation and recruitment of monocytes and macrophages [81]. CCL20 and CCL26 interact with CCR6 and CCR3, respectively, and have been implicated in the recruitment of immature DCs [79] that are inefficient at presenting antigens (Ag) to T cells and can be immune-suppressive [82]. CCL2 has a broad range of targets but is mandatory to the generation, extravasation and recruitment of inflammatory monocytes [81]. While inflammatory monocytes have mainly been associated with tumor progression [83], they have also been shown to exert anti-tumor properties in melanoma metastasis [84]. CCL2 expression in CAFs is controlled by galectin-1 which was also shown to regulate their activation [85] and CCL2 was implicated in inducing monocytes to a M2 macrophages program [86]. This raises the possibility of CAFs playing a role in the switch from anti- to pro-tumoral profile of monocytes. Moreover, depletion of CAFs via a DNA

vaccine targeting FAP has been shown to decrease TAMs and MDSCs numbers in breast cancer, suggesting a role of CAFs in their recruitment and/or differentiation [87]. However, this study fails to directly link CAF depletion to myeloid cell decrease. Indeed, some macrophages express FAP [27]. Thus, in the absence of appropriate verification of the FAP-cell depletion, the disruption of the microenvironment observed may be also due to FAP-expressing macrophages depletion. Nevertheless, CAFs and myeloid cells seems to overall synergize to increase tumor progression, as CAFs recruit monocytes and polarize them into M2 macrophages, and the later in turn enhance activation of CAFs [88]. Of note, CAFs and M2 macrophages biomarkers are associated with the clinical outcome of CRC patients, those whose tumors have high numbers of both cells types having poor long term survival [89].

Mast cells have been implicated in the reorganization of the tumor microenvironment and stimulation of angiogenesis [90]. In PDAC, a complex interaction between CAFs and mast cells has been recently suggested whereby CAFs promote both mast cell recruitment and activation. Thereafter, mast cells reciprocally enhance CAFs proliferation by their secretion of IL-13 and tryptase [91]. Accordingly, blockade of mast cell degranulation in this orthotopic model of PDAC decreased tumor growth.

In conclusion, due to their production of cytokines and chemokines, CAFs shape the inflammatory character of the tumor micro-environment by recruiting innate immune cells, such as granulocytes, monocytes and mast cells. In turn, those inflammatory cells support tumor growth by stimulating angiogenesis and creating an immune-suppressive environment [11].

(b) CAFs disrupt cytotoxic response of NK cells

NK cells are potent cytotoxic lymphocytes that recognize aberrant expression of MHC class I molecules and stress factors. Their potential as an anti-tumor effector cell type due to their high capacity for lysing cancer cells was apparent to early investigators [92]. Therefore, it is relevant that MSCs and skin fibroblasts have been shown to decrease NK cell cytotoxicity in co-culture models [93]. Likewise, recent studies suggested that CAFs also are able to suppress NK cells functions. Co-culture of NK cells with CAFs from HCC decreased expression of the NK activation molecules, NKG2D and NKp46, as well as impaired granzyme B, perforin, TNFα and IFNγ secretion resulting in a decrease of 50 % of the cytolytic activity. This effect was mediated by expression of PGE2 and indolamine-2,3-dioxygenase (IDO) in CAFs [23]. A similar, PGE-dependent effect was observed with CAFs derived from CRC [94] and melanoma [95]. As previously stated, CAFs may be a major source of TGFβ in the tumor micro-environment [30], and TGFβ has been shown to decrease NK cell activation and cytotoxic activities by downregulating the activating receptors, NKG2D, Nkp6, NKp44, and NKp30, and decreasing IFNγ production [96].

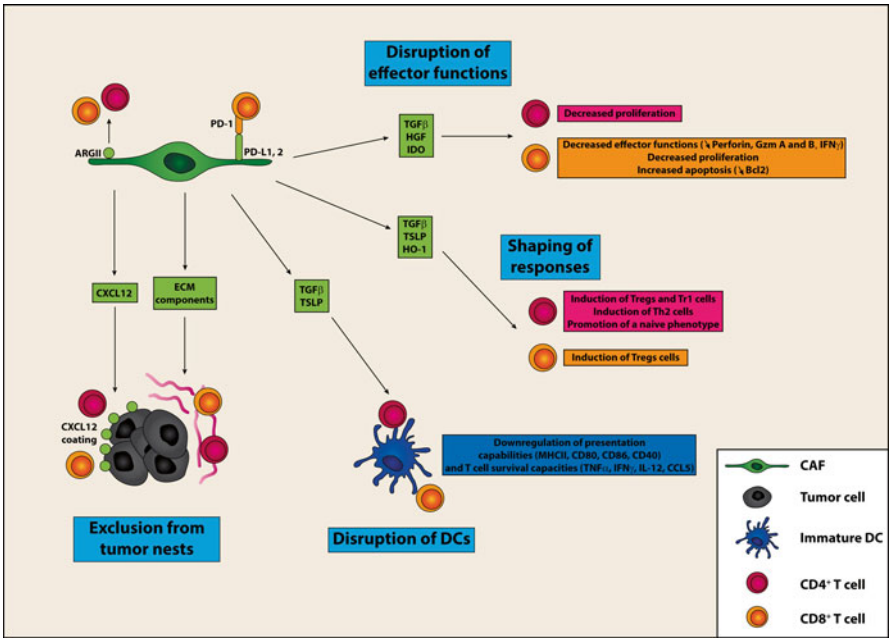

Fig. 4.3 Influence of CAFs on T cells. CAFs impair anti-tumor properties of T cells by directly disrupting their effector functions, shaping T cells response to a pro-tumoral phenotype, disrupt their activation by DCs, and prevent T cells from contacting tumor cells

4.5.2 Role of CAFs on Tumor Infiltrating T Cells

A role for CAFs in regulating tumor-infiltrating T cells has been highlighted by the analysis of mice in which FAP-expressing CAFs can be conditionally depleted. Mice depleted in CAFs show an immediate arrest of the growth of a transplanted Lewis lung carcinoma that was mediated by T cells [13]. There are several mechanisms by which CAFs may disrupt T cell function in the tumor micro-environment (Fig. 4.3).

(a) TGFβ dependent role of CAFs

CAFs may produce large quantities of TGFβ [30], which, in the tumor microenvironment, may act on $CD8^+$ T cells both by decreasing their frequency and also by dampening their functions [97]. TGFβ has been shown to lower Bcl-2 expression in $CD8^+$ T cells leading to apoptosis [98]. TGFβ also inhibits effector functions of $CD8^+$ T cells. When cultured with TGFβ cells. Ag specific $CD8^+$ T cells from melanoma patients display a decrease in effector functions, as demonstrated showed by a decreased production of IFNγ, TNFα and GM-CSF, perhaps through a suppressive effect on T-bet expression. Interestingly, these changes were not reflected by an altered effector memory phenotype of the cells [99]. Cytotoxic functions of $CD8^+$ T cells also are hampered by TGFβ, which downregulates expression of perforin, granzyme A, granzyme B, Fas ligand and IFNγ. This inhibitory outcome was a

direct effect of TFGβ as a direct effect of TGFβ through activation of the Smad pathway and direct binding of pSmads to the granzyme B and INFγ promoters [100].

Finally, TGFβ may contribute to immune suppression within the tumor microenvironment by inducing Foxp3 in naïve $CD8^+$ T cells at sites of T cell priming turning them into $CD8^+$ Tregs capable of inducing new Tregs in the tumor micro-environment [101].

TGFβ also acts on $CD4^+$ T cells, downregulating GATA3 and T-bet to promote naïve over effector programming [102]. Furthermore, TGFβ is a powerful inducer of $CD4^+$ Tregs. TGFβ induces Foxp3 that in turn shuts down Smad7 expression, a protein that normally limits TGFβ signaling. Thus, a positive autoregulatory loop of TGFβ signaling is induced due to the absence of Smad7 [103]. Of note, it has been suggested that Tregs engage a cross-talk with CAFs via their own expression of TGFβ, increasing CAFs activation and pro-tumoral programming. Overall, the presence of high quantities of both CAFs and Tregs in the tumor microenvironment of lung adenocarcinoma correlates with a poor clinical outcome [104].

Finally, TGFβ acts as an indirect inhibitor of T cells via its action on Antigen-Presenting Cells (APCs). TGFβ decreases the number of DCs in the favor of TAMs. Moreover, it downregulates the expression of MHCII, CD80, CD86, CD40 that are necessary to efficient Ag presentation as well as TNFα, IFNy, IL-12 and CCL5 secretion that promote survival and recruitment of T cells. MSCs can inhibit $CD4^+$ and $CD8^+$ T cell proliferation in a dose-dependent manner via their secretion of TGFβ [105], but whether this effect directly targets T cells or works through impairment of DCs presentation properties remains to be elucidated.

While few of the previous effects have been directly linked to TGFβ produced by CAFs they are major producers of that cytokine in the tumor micro-environment. Thus it is very likely that one of their roles on T cells is in part mediated by TGFβ.

(b) CAFs shape T cell responses

CAFs have the ability to influence T cell responses by switching them from an anti-tumor to a pro-tumor program. Naïve $CD4^+$ T cells can differentiate to multiple T helper cell subsets with different functions and cytokine secretion [106]. Th1 secretes IFNy and are the main anti-tumor $CD4^+$ T cell population, compared to Th2 that secrete IL-4 and stimulate antibody (Ab) secretion [107]. In a murine model of breast cancer, depletion of CAFs has been suggested to drastically influence T cells in the tumor micro-environment by reverting them from a Th2 to a Th1 phenotype. Moreover, this was accompanied by a decrease in Tregs and increase in $CD8^+$ cytotoxic T cells proportions. As an indirect influence on T cells, recruitment of DCs was also increased [87]. However, as stated before, in the absence of accurate verification of FAP-cell depletion in this study, more work will be needed to confirm the multiple impacts of CAFs on T cells. Another indirect role of CAFs has been suggested in PDAC patients. CAFs produce Thymic Stromal LymphoPoietin (TSLP) that activates myeloid DCs and makes them secrete Th2-polarizing cytokines. This results in a micro-environment dominated by Th2 as shown by GATA3 expession over Th1 [108].

As stated previously, MSCs may be a source of CAFs [37]. Through expression of Heme Oxygenase-1 (HO-1), MSCs promote differentiation of naïve $CD4^+$ T cells to $Foxp3^+$ Tregs and IL-10 producing $CD4^+$ T cells (Tr1) in Mixed Leukocyte Reaction (MLR). Interestingly, MSCs pre-conditioned in MLR lost expression of HO-1 but depict even higher Treg-promoting functions [109]. This suggests that a cross-talk between MSCs and T cells could lead to increased immunosuppressive environment by upregulation of other mechanisms to be determined. Two other reports support this idea of Tregs induction by MSCs, further suggesting induction of Tregs from $CD8^+$ T cells [110, 111]. Interestingly, a study suggests that local fibroblasts, that are another main source of CAFs, are potent immune-regulatory cells that inhibit $CD8^+$ T cell activation and promote Th2 polarization of naïve $CD4^+$ T cells [112].

Controversially, a recent study suggests an anti-tumoral role of CAFs by restraining Tregs infiltration in the tumor micro-environment [113]. Indeed, this study reports that depletion of α-SMA expressing cells in PDAC leads to a Treg-dominant environment that result in more aggressive tumors and decreased survival. Curiously, despite essentially all α-SMA^+ stromal fibroblasts expressing FAP in the autochthonous murine pancreatic cancer [14], this report found no loss of FAP^+ CAFs following depletion of α-SMA^+ cells. Nonetheless, as stated before, α-SMA is found in pericytes surrounding tumor vasculature [114]. Decreasing of vasculature by CD31 staining was also reported in the same study, thus it is unclear whereas the observed effect was mainly due to CAFs depletion or to a dominant vasculature disruption. Indeed, vascular impairment leads to increase hypoxia that is a major condition for increase recruitment of Tregs through upregulation of HIF1α [115] that is expressed in CAFs from PDAC patients [116].

(c) CAFs directly impair T cell functions

CAFs can directly disrupt the functions of effector T cells using several mechanisms. One study suggests that MSCs can suppress proliferation of $CD8^+$ T cells, although, interestingly, the effect on $CD8^-$ T cell subsets was marginal. Moreover, this effect is likely to be direct on T cells and not on presentation capacities by DCs. Indeed, co-injection of B16 melanoma with MSCs into C3H mice, which are not syngeneic, prevents rejection of the tumor cells that occurs with tumor cells alone [117]. HGF secreted by MSCs also seems to have a role in the inhibition of T cell proliferation independently of DCs [105]. Furthermore, T cell proliferation in vitro may be disrupted by IDO expression in MSCs, and addition of tryptophan to the cell culture partially restores proliferation. Of note, induction of IDO in MSCs may be dependent on IFNγ, implying a possible cross-talk between MSCs and T cells [118]. In PDAC patients, CAFs have been shown to express high levels of Arginase II (ARG2) correlated to a poor clinical outcome. ARG2 expression was mainly observed around necrotic areas and HIF1a was almost always co-expressed suggesting a role of hypoxia in ARG expression [116]. ARG2 is one of the two enzymes catabolizing arginine into ornithine and urea. Thus, ARG2 acts on T cells by depriving the environment of arginine that is mandatory for T cell proliferation and functions [119].

Recently, the T-cell checkpoints, Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) and Programmed Death-1 (PD-1), have attracted much attention because of their capacity to induce immune control of tumors in subsets of patients with melanoma, non-small cell lung cancer and renal cell cancer [120]. PD-1 is expressed by T cells following T-cell Receptor (TcR) signaling, and contains an immunoreceptor tyrosine inhibitory motif (ITIM) that binds the inhibitory phosphatase SHP-2 [121]. Engagement of PD-1 inhibits TcR-mediated effector functions [122] and thereby limits the time of interaction with peptide-MHC complexes therefore impairing activation, differentiation and proliferation [123]. Myofibroblasts from the colon, which are likely to give rise to CAFs in CRC, express the ligands of PD-1, PD-L1 and PD-L2 and regulate proliferative response of $CD4^+$ T cells [124]. PD-L1 and PD-L2 expression has also been reported on CAFs from lung cancer. This expression was induced by IFNγ and disrupts $CD8^+$ T cell activation [125]. Interestingly, in melanoma it has been shown that expression of PD-1 ligands on CAFs is dependent on IL-1α and β secretion by melanoma cells and melanocytes [126]. As mentioned before CAFs are also producers of IL-1α and β in many tumor types. Thus, one may consider an autocrine induction of PD-L1 and PD-L2 on CAFs.

Controversially, a recent study in lung carcinoma suggests that CAFs have the ability to increase T cell cytokine production and proliferation. T cells in culture stimulated by CAFs or their supernatant showed increased IFNγ and IL-17A production, this effect being related to IL-6 production by CAFs [127]. However, the authors did not determine if CAFs were promoting a particular T cell subset that may potentially present pro-tumoral properties. Indeed, IL-6 is critical to the generation of the pro-inflammatory Th17 subset from naïve $CD4^+$ T cells. Th17 are an important pro-tumoral cell type through their high inflammatory profile [128]. This observation of an immuno-stimulatory role of CAFs that seems favorable at first could thus be deleterious. Yet, there is a potential beneficial role of CAFs that remains to be further investigated. Tertiary lymphoid structures (TLSs) are ectopic lymph-node like structures found in inflamed tissues that can serve as a site of antigen presentation and T cell activation without the requirement of secondary lymphoid organs [129]. They are frequently found within solid tumors, especially lung carcinomas [130]. The presence of TLSs has been mainly associated with a positive clinical output in patients with breast carcinoma, CRC, NSCLC and melanoma [130]. The formation of such structures is mainly orchestrated by the chemokines CCL19, CCL21 and CXCL13 [131], similar to the requirements for lymph node formation [132]. In the lymph nodes, those chemokines are mainly produced by fibroblastic reticular cells (FRCs), that can be considered lymph node counterparts of CAFs, and are crucial to T and B cells migration, survival and activation [132]. The exact mechanisms ruling TLSs formation and their implication in anti-tumor immune responses remains to be elucidated, however it is very likely that local fibroblasts play a key role. Thus CAFs may have crucial anti-tumoral properties by allowing formation of functional TLSs.

(d) CAFs impair T cell accumulation in the tumor micro-environment

CAFs are able to influence T cell activation, proliferation and functions. However, depletion of FAP-expressing cells from the tumor micro-environment didn't show

an increase in proportion of tumor infiltrating T cells or difference in T cell subsets despite a drastic T-cell dependent decrease of tumor growth [13, 14]. Moreover, there was no difference in activation (as shown by CD69 staining), cytolytic capabilities (as shown by granzyme B expression) or IFNγ secretion [13]. This result suggests that T cells from the micro-environment are functional. Thus, CAFs may also act by preventing T cells from contacting tumor cells, therefore impairing their function only by lack of recognition. Indeed, T cell exclusion from the vicinity of tumor cells was shown to be a dominant mechanism in multiple cancers [133]. Lack of infiltration of $CD8^+$ T cells predicts a poor outcome in CRC patients, even being the most efficient histopathological method to use [134]. There is also a significant difference in progression-free survival between patients with ovarian cancer showing exclusion of T cells or not [135]. Finally, lack of infiltration of T cells is also found in PDAC patients [14] and lung tumors [136].

CAFs have been shown to impact on T cell localization within the tumor microenvironment by two mechanisms. First, in lung cancer, CAFs restrain T cells in the stroma via their synthesis of extracellular matrix [136]. Very few T cells were found in the tumor nests and their motility was decreased compared to the stroma. This affected $CD4^+$ or $CD8^+$ T cells and resting or activated T cells in the same way. While T cells were more frequent to contact tumor cells engineered to overexpress CCL5, the role of chemokines on the exclusion phenomenon seems to be marginal. Indeed, treatment with pertussis toxin to inhibit G-coupled proteins signaling abrogates motility of T cells in the stroma but doesn't allow accumulation of T cells in the tumor nests, thus chemo-repulsion isn't responsible for T cell exclusion. Adhesion molecules also don't account for this process as blocking of β1 and β2 integrins does not allow T cell infiltration. On the other hand, T cells are accumulating in stromal zones with less collagen and fibronectin density, suggesting a role of matrix proteins in defining localization of T cells [136]. Indeed, T cells show an elongated morphology on the matrix fibers and, even when they are close, are not contacting tumor cells. Treatment with collagenase allowed T cells to accumulate at the boundary of the tumor regions [136]. However, even if T cells were able to contact tumor cells, they remained at the boundary of the nests suggesting other mechanisms involved in T cell exclusion.

In the KPC model of PDAC, depletion of FAP-expressing cells allows a T cell-dependent decrease of tumor growth [14]. Checkpoint antagonist, which were not effective in human PDAC [137] or the KPC mice [14], exhibited their anti-tumor Effects in the mouse model of this cancer [14]. Thus, exclusion seems to be a simple yet dominant mechanism of disruption of the anti-tumor immune response of T cells. The mechanism of T cell exclusion from the vicinity of cancer cells in this setting was attributable to production of CXCL12 by FAP^+ CAFs, which were the only CXCL12 producers of the tumor micro-environment, as inhibition of signaling by its receptor CXCR4 with the antagonist, AMD3100, allows the same decrease in tumor growth and improvement of checkpoint antagonists' efficiency as depletion of CAFs. This response was also associated with accumulation of T cells among tumor nests [14]. Interestingly, there was no difference in the quality of infiltrating T cells, Tregs accumulating the same way as effector T cells do, and also no increase

in T cell proliferation. This suggests that effector T cells in the tumor micro-environment are functional and may be dominant over other immune-suppressive mechanisms. Remarkably, although the CAFs were the producer of CXCL12, the chemokine was found to "coat" the tumor cells. This association of tumor cells with CXCL12, by a mechanism yet to be identified, may be a critical point in the T cell exclusion phenomenon. FAP cells being also recruited to all sites of inflammation, we consider that CXCL12 coating may be a mean by which stressed epithelial cells are protected from immune attack and that tumor cells exploit this homeostasis feature. Interestingly, CXCL12 coating of tumor cells is not a characteristic only in PDAC [138] as it has been reported also in CRC [139] and ovarian cancer [140]. Since these tumors are also unresponsive to anti-PD-1/anti-PD-L1 immunotherapy [137], there is a possibility that these murine findings may be relevant to human epithelial carcinomas.

4.6 Targeting CAFs to Harness the Immune System Against Tumors

The roles of CAFs in disrupting anti-tumor immune responses make them and their functions potential targets for new therapies. Depleting CAFs from the tumor micro-environment is a very seductive concept. The targeting of CAFs with Chimeric Antigen Receptor (CAR) T cells directed against FAP has shown promising results. Decreased tumor growth was found in murine models of cervical cancer (TC1), malignant mesothelioma (AE17), lung cancer (LKR), CRC (C26) and breast cancer (4T1) after infusion with FAP-CAR $CD8^+$ T cells. Moreover, endogenous anti-tumor response of $CD8^+$ T cells was enhanced as shown by IFNγ secretion and CD69 expression [94]. However, FAP cells are present in multiple tissues including skeletal muscles and bone marrow and their depletion was shown to induce cachexia and anemia. Cachexia and anemia are common syndromes associated with cancer and coherently, cachectic KPC or C26 bearing mice were shown to have impaired FAP cells [28]. In another study, injection of FAP-CAR T cells in mice bearing melanoma (B16), CRC (MC38 and C26), fibrosarcoma (MC17-51), breast cancer (4 T1) or kidney cancer (Renca) showed limited impact on tumor growth and lead to severe cachexia and anemia [141]. These studies point forward caution in attempts to target FAP-expressing cells on their own. To circumvent this issue, a recent report showed reprogramming of PSCs in KPC mice. A vitamin D analog, calcipotriol, was able to revert activation of PSCs to a quiescent state, decreasing their inflammatory properties and increasing the effect of chemotherapeutic agent gemcitabine [142].

Targeting the functions of CAFs may be the safest way to apprehend their role, especially regarding T cell exclusion from the tumor nests. Indeed the small molecule inhibitor AMD3100 (or Plerixafor) was originally designed to as an anti-HIV drug [143] and was approved. It is also already approved for mobilization of stem

cells in non-Hodgkin's lymphoma and multiple myeloma [144]. As previously stated, CAFs alter T cell distribution in the tumor micro-environment by remodeling of the ECM. Targeting this function can be promising as inhibition of LOX thanks to β-Aminopropionitrile (BAPN) was shown to decrease tumor growth in a model of breast cancer [145]. More studies are needed in tumors where T cell exclusion is a hallmark.

4.7 Conclusion

After more than 20 years of modest progress, immunotherapies have now emerged as an exciting perspective for future cancer treatments. This is the result of two decades of extensive efforts in understanding fundamental immunology and its applications in cancer. Only very recently the major role of the tumor micro-environment in disrupting anti-cancer therapies was properly apprehended. CAFs are among those actors of the tumor that have been disregarded despite their crucial role. Here we have stressed their potential roles, especially on immune components of the tumor micro-environment, which reveals potential new ways for attacking tumor cells. At the time where "personalized medicine" begins to show promise, it is important to keep in mind that a simple idea, T cell exclusion from tumor nests by CAFs, may be the dominant mechanism impairing anti-tumor immune responses.

Acknowledgements The authors would like to thank Mickael Ludwig, Jia-Yun Li and Zhikai Wang for critical reading.

No potential conflicts of interest were disclosed.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70.
2. Paget G. Remarks on a case of alternate partial anaesthesia. Br Med J. 1889;1(1462):1–3.
3. Steer HJ et al. Harnessing the immune response to treat cancer. Oncogene. 2010;29(48):6301–13.
4. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6(10):715–27.
5. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12(4):269–81.
6. Khong HT, Wang QJ, Rosenberg SA. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother. 2004;27(3):184–90.
7. Restifo NP et al. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. J Natl Cancer Inst. 1996;88(2):100–8.
8. Hinz S et al. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. Oncogene. 2000;19(48):5477–86.
9. Mittal D et al. New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape. Curr Opin Immunol. 2014;27:16–25.

10. Mauge L et al. Control of the adaptive immune response by tumor vasculature. Front Oncol. 2014;4:61.
11. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–68.
12. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Curr Opin Immunol. 2014;27:1–7.
13. Kraman M et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science. 2010;330(6005):827–30.
14. Feig C et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013;110(50):20212–7.
15. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014;211(8):1503–23.
16. Darby IA et al. Fibroblasts and myofibroblasts in wound healing. Clin Cosmet Investig Dermatol. 2014;7:301–11.
17. Vaheri A et al. Nemosis, a novel way of fibroblast activation, in inflammation and cancer. Exp Cell Res. 2009;315(10):1633–8.
18. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315(26):1650–9.
19. Klingberg F, Hinz B, White ES. The myofibroblast matrix: implications for tissue repair and fibrosis. J Pathol. 2013;229(2):298–309.
20. Osterreicher CH et al. Fibroblast-specific protein 1 identifies an inflammatory subpopulation of macrophages in the liver. Proc Natl Acad Sci U S A. 2011;108(1):308–13.
21. Sugimoto H et al. Identification of fibroblast heterogeneity in the tumor microenvironment. Cancer Biol Ther. 2006;5(12):1640–6.
22. Franco M et al. Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. Blood. 2011;118(10):2906–17.
23. Li T et al. Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO. Cancer Lett. 2012;318(2):154–61.
24. Heldin CH. Targeting the PDGF signaling pathway in tumor treatment. Cell Commun Signal. 2013;11:97.
25. Garin-Chesa P, Old LJ, Rettig WJ. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc Natl Acad Sci U S A. 1990;87(18):7235–9.
26. Acharyya S et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell. 2012;150(1):165–78.
27. Arnold JN et al. Tumoral immune suppression by macrophages expressing fibroblast activation protein-alpha and heme oxygenase-1. Cancer Immunol Res. 2014;2(2):121–6.
28. Roberts EW et al. Depletion of stromal cells expressing fibroblast activation protein-alpha from skeletal muscle and bone marrow results in cachexia and anemia. J Exp Med. 2013;210(6):1137–51.
29. Vicent S et al. Cross-species functional analysis of cancer-associated fibroblasts identifies a critical role for CLCF1 and IL-6 in non-small cell lung cancer in vivo. Cancer Res. 2012;72(22):5744–56.
30. Kojima Y et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A. 2010;107(46):20009–14.
31. Yin C et al. Hepatic stellate cells in liver development, regeneration, and cancer. J Clin Invest. 2013;123(5):1902–10.
32. Bachem MG et al. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology. 1998;115(2):421–32.
33. Iwano M et al. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest. 2002;110(3):341–50.

34. Zeisberg EM et al. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res. 2007;67(21):10123–8.
35. Kidd S et al. Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma. PLoS One. 2012;7(2), e30563.
36. Paunescu V et al. Tumour-associated fibroblasts and mesenchymal stem cells: more similarities than differences. J Cell Mol Med. 2011;15(3):635–46.
37. Quante M et al. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. Cancer Cell. 2011;19(2):257–72.
38. Direkze NC et al. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. Cancer Res. 2004;64(23):8492–5.
39. Olumi AF et al. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res. 1999;59(19):5002–11.
40. Krtolica A et al. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A. 2001;98(21):12072–7.
41. Cardone A et al. Prognostic value of desmoplastic reaction and lymphocytic infiltration in the management of breast cancer. Panminerva Med. 1997;39(3):174–7.
42. Maeshima AM et al. Modified scar grade: a prognostic indicator in small peripheral lung adenocarcinoma. Cancer. 2002;95(12):2546–54.
43. Tsujino T et al. Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res. 2007;13(7):2082–90.
44. Erkan M et al. The activated stroma index is a novel and independent prognostic marker in pancreatic ductal adenocarcinoma. Clin Gastroenterol Hepatol. 2008;6(10):1155–61.
45. Giannoni E et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer Res. 2010;70(17):6945–56.
46. Liu S et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. Cancer Res. 2011;71(2):614–24.
47. Giannoni E et al. Cancer associated fibroblasts exploit reactive oxygen species through a proinflammatory signature leading to epithelial mesenchymal transition and stemness. Antioxid Redox Signal. 2011;14(12):2361–71.
48. Vermeulen L et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol. 2010;12(5):468–76.
49. Lotti F et al. Chemotherapy activates cancer-associated fibroblasts to maintain colorectal cancer-initiating cells by IL-17A. J Exp Med. 2013;210(13):2851–72.
50. Li HJ et al. Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. Cancer Discov. 2012;2(9):840–55.
51. Kuperwasser C et al. Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A. 2004;101(14):4966–71.
52. Allinen M et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell. 2004;6(1):17–32.
53. Bhowmick NA et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science. 2004;303(5659):848–51.
54. Orimo A et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121(3):335–48.
55. Rasanen K, Vaheri A. Activation of fibroblasts in cancer stroma. Exp Cell Res. 2010;316(17):2713–22.
56. Levental KR et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891–906.
57. Saadi A et al. Stromal genes discriminate preinvasive from invasive disease, predict outcome, and highlight inflammatory pathways in digestive cancers. Proc Natl Acad Sci U S A. 2010;107(5):2177–82.
58. Herrera M et al. Functional heterogeneity of cancer-associated fibroblasts from human colon tumors shows specific prognostic gene expression signature. Clin Cancer Res. 2013;19(21):5914–26.

59. Shangguan L et al. Inhibition of TGF-beta/Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. Stem Cells. 2012;30(12):2810–9.
60. Calon A et al. Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation. Cancer Cell. 2012;22(5):571–84.
61. Karnoub AE et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature. 2007;449(7162):557–63.
62. Zigrino P et al. Stromal expression of MMP-13 is required for melanoma invasion and metastasis. J Invest Dermatol. 2009;129(11):2686–93.
63. Vosseler S et al. Distinct progression-associated expression of tumor and stromal MMPs in HaCaT skin SCCs correlates with onset of invasion. Int J Cancer. 2009;125(10):2296–306.
64. Sternlicht MD et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell. 1999;98(2):137–46.
65. Poulsom R et al. Stromal expression of 72 kda type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. Am J Pathol. 1992;141(2):389–96.
66. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology. Pathology. 2007;39(3):305–18.
67. Kikuta K et al. Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells. Biochem Biophys Res Commun. 2010;403(3–4):380–4.
68. Duda DG et al. Malignant cells facilitate lung metastasis by bringing their own soil. Proc Natl Acad Sci U S A. 2010;107(50):21677–82.
69. Stoker MG, Shearer M, O'Neill C. Growth inhibition of polyoma-transformed cells by contact with static normal fibroblasts. J Cell Sci. 1966;1(3):297–310.
70. Rhim AD et al. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer Cell. 2014;25(6):735–47.
71. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007;110(10):3499–506.
72. Jung DW et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. Int J Cancer. 2010;127(2):332–44.
73. Anderson IC et al. The angiogenic factor interleukin 8 is induced in non-small cell lung cancer/pulmonary fibroblast cocultures. Cancer Res. 2000;60(2):269–72.
74. Erez N et al. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell. 2010;17(2):135–47.
75. De Boeck A et al. Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression. Proteomics. 2013;13(2):379–88.
76. Tjomsland V et al. Interleukin 1alpha sustains the expression of inflammatory factors in human pancreatic cancer microenvironment by targeting cancer-associated fibroblasts. Neoplasia. 2011;13(8):664–75.
77. Lin ZY, Chuang YH, Chuang WL. Cancer-associated fibroblasts up-regulate CCL2, CCL26, IL6 and LOXL2 genes related to promotion of cancer progression in hepatocellular carcinoma cells. Biomed Pharmacother. 2012;66(7):525–9.
78. Rider P et al. Interleukin-1alpha. Semin Immunol. 2013;25(6):430–8.
79. Singh R, Lillard Jr JW, Singh S. Chemokines: key players in cancer progression and metastasis. Front Biosci (Schol Ed). 2011;3:1569–82.
80. Shi J et al. CXCL12-CXCR4 contributes to the implication of bone marrow in cancer metastasis. Future Oncol. 2014;10(5):749–59.
81. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27:669–92.
82. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol. 2004;4(12):941–52.
83. Kitamura T, Qian BZ, Pollard JW. Immune cell promotion of metastasis. Nat Rev Immunol. 2015;15(2):73–86.

84. Pommier A et al. Inflammatory monocytes are potent antitumor effectors controlled by regulatory CD4+ T cells. Proc Natl Acad Sci U S A. 2013;110(32):13085–90.
85. Wu MH et al. Targeting galectin-1 in carcinoma-associated fibroblasts inhibits oral squamous cell carcinoma metastasis by downregulating MCP-1/CCL2 expression. Clin Cancer Res. 2011;17(6):1306–16.
86. Roca H et al. CCL2 and interleukin-6 promote survival of human CD11b+peripheral blood mononuclear cells and induce M2-type macrophage polarization. J Biol Chem. 2009;284(49):34342–54.
87. Liao D et al. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4 T1 murine breast cancer model. PLoS One. 2009;4(11), e7965.
88. Comito G et al. Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression. Oncogene. 2014;33(19):2423–31.
89. Herrera M et al. Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients. Cancer Sci. 2013;104(4):437–44.
90. Crivellato E, Nico B, Ribatti D. Mast cells and tumour angiogenesis: new insight from experimental carcinogenesis. Cancer Lett. 2008;269(1):1–6.
91. Ma Y, Ullrich SE. Intratumoral mast cells promote the growth of pancreatic cancer. Oncoimmunology. 2013;2(10), e25964.
92. Larsen SK, Gao Y, Basse PH. NK cells in the tumor microenvironment. Crit Rev Oncog. 2014;19(1–2):91–105.
93. Pradier A et al. Human bone marrow stromal cells and skin fibroblasts inhibit natural killer cell proliferation and cytotoxic activity. Cell Transplant. 2011;20(5):681–91.
94. Li T et al. Colorectal carcinoma-derived fibroblasts modulate natural killer cell phenotype and antitumor cytotoxicity. Med Oncol. 2013;30(3):663.
95. Balsamo M et al. Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity. Proc Natl Acad Sci U S A. 2009;106(49):20847–52.
96. Laouar Y et al. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. Nat Immunol. 2005;6(6):600–7.
97. Zhang Q et al. Blockade of transforming growth factor-{beta} signaling in tumor-reactive CD8(+) T cells activates the antitumor immune response cycle. Mol Cancer Ther. 2006;5(7):1733–43.
98. Sanjabi S, Mosaheb MM, Flavell RA. Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. Immunity. 2009;31(1):131–44.
99. Ahmadzadeh M, Rosenberg SA. TGF-beta 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. J Immunol. 2005;174(9):5215–23.
100. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell. 2005;8(5):369–80.
101. Shafer-Weaver KA et al. Cutting edge: tumor-specific CD8+ T cells infiltrating prostatic tumors are induced to become suppressor cells. J Immunol. 2009;183(8):4848–52.
102. Travis MA, Sheppard D. TGF-beta activation and function in immunity. Annu Rev Immunol. 2014;32:51–82.
103. Fantini MC et al. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol. 2004;172(9):5149–53.
104. Kinoshita T et al. Forkhead box P3 regulatory T cells coexisting with cancer associated fibroblasts are correlated with a poor outcome in lung adenocarcinoma. Cancer Sci. 2013;104(4):409–15.
105. Di Nicola M et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002;99(10):3838–43.
106. Martin B et al. Highly self-reactive naive CD4 T cells are prone to differentiate into regulatory T cells. Nat Commun. 2013;4:2209.

107. Geginat J et al. Plasticity of human CD4 T cell subsets. Front Immunol. 2014;5:630.
108. De Monte L et al. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. J Exp Med. 2011;208(3):469–78.
109. Mougiakakos D et al. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cells. Blood. 2011;117(18):4826–35.
110. Prevosto C et al. Generation of CD4+ or CD8+ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. Haematologica. 2007;92(7):881–8.
111. Maccario R et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005;90(4):516–25.
112. Haniffa MA et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol. 2007;179(3):1595–604.
113. Ozdemir BC et al. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell. 2014;25(6):719–34.
114. Xue Y et al. PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells. Nat Med. 2012;18(1):100–10.
115. Kumar V, Gabrilovich DI. Hypoxia-inducible factors in regulation of immune responses in tumour microenvironment. Immunology. 2014;143(4):512–9.
116. Ino Y et al. Arginase II expressed in cancer-associated fibroblasts indicates tissue hypoxia and predicts poor outcome in patients with pancreatic cancer. PLoS One. 2013;8(2), e55146.
117. Djouad F et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood. 2003;102(10):3837–44.
118. Meisel R et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood. 2004;103(12):4619–21.
119. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158(3):638–51.
120. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell. 2015;161(2):205–14.
121. Okazaki T et al. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc Natl Acad Sci U S A. 2001;98(24):13866–71.
122. Parry RV et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol. 2005;25(21):9543–53.
123. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450–61.
124. Pinchuk IV et al. PD-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates CD4+ T-cell activity. Gastroenterology. 2008;135(4):1228–37. 1237 e1-2.
125. Nazareth MR et al. Characterization of human lung tumor-associated fibroblasts and their ability to modulate the activation of tumor-associated T cells. J Immunol. 2007;178(9):5552–62.
126. Khalili JS et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. Clin Cancer Res. 2012;18(19):5329–40.
127. Barnas JL et al. Reciprocal functional modulation of the activation of T lymphocytes and fibroblasts derived from human solid tumors. J Immunol. 2010;185(5):2681–92.
128. Bailey SR et al. Th17 cells in cancer: the ultimate identity crisis. Front Immunol. 2014;5:276.
129. Neyt K et al. Tertiary lymphoid organs in infection and autoimmunity. Trends Immunol. 2012;33(6):297–305.
130. Dieu-Nosjean MC et al. Tertiary lymphoid structures in cancer and beyond. Trends Immunol. 2014;35(11):571–80.

131. Goc J et al. Characteristics of tertiary lymphoid structures in primary cancers. Oncoimmunology. 2013;2(12), e26836.
132. Fletcher AL, Acton SE, Knoblich K. Lymph node fibroblastic reticular cells in health and disease. Nat Rev Immunol. 2015;15(6):350–61.
133. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348(6230):74–80.
134. Galon J et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960–4.
135. Zhang L et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348(3):203–13.
136. Salmon H et al. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 2012;122(3):899–910.
137. Brahmer JR et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366(26):2455–65.
138. Liang JJ et al. High levels of expression of human stromal cell-derived factor-1 are associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Cancer Epidemiol Biomarkers Prev. 2010;19(10):2598–604.
139. Akishima-Fukasawa Y et al. Prognostic significance of CXCL12 expression in patients with colorectal carcinoma. Am J Clin Pathol. 2009;132(2):202–10. quiz 307.
140. Scotton CJ et al. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res. 2002;62(20):5930–8.
141. Tran E et al. Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia. J Exp Med. 2013;210(6):1125–35.
142. Sherman MH et al. Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. Cell. 2014;159(1):80–93.
143. Schols D et al. Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res. 1997;35(3):147–56.
144. Keating GM. Plerixafor: a review of its use in stem-cell mobilization in patients with lymphoma or multiple myeloma. Drugs. 2011;71(12):1623–47.
145. Bondareva A et al. The lysyl oxidase inhibitor, beta-aminopropionitrile, diminishes the metastatic colonization potential of circulating breast cancer cells. PLoS One. 2009;4(5), e5620.

Chapter 5
Cancer-Associated Tertiary Lymphoid Structures, from Basic Knowledge Toward Therapeutic Target in Clinic

Bertrand Dubois, Hélène Kaplon, Coline Couillault, Christophe Caux, and Marie-Caroline Dieu-Nosjean

Abstract The tumor growth is under the control of the immune system, and this represents a significant challenge for the development of new therapeutic strategies. It is now recognized that not only the density, but also the organization of tumor-infiltrating immune cells is critical for the shaping of the tumor microenvironment. In human cancers, several levels of structuration of lymphoid aggregates have been observed, including Tertiary Lymphoid Structures (TLS) displaying strong similarities with secondary lymphoid organs. In this review, we discuss about the role of immune cells homing selectively in the T-cell or B-cell zone of TLS with putative consequences on the initiation of efficient cellular and humoral immune responses against tumor, and ultimately on the clinical outcome of cancer patients. However, immunoregulatory cells may also infiltrate TLS and it is thus crucial to determine the circumstances in which TLS might be a site for the development of a suppressive and detrimental immune responses. We also discuss how TLS could be a useful

B. Dubois • C. Couillault
Lyon University, Lyon, France

INSERM U1052 CNRS UMR5286, Centre de Recherche en Cancérologie de Lyon, Lyon, France

H. Kaplon • M.-C. Dieu-Nosjean (✉)
Laboratory "Cancer, Immune Control, and Escape", INSERM UMRS1138, Cordeliers Research Center, 15, rue de l'Ecole de Médecine, F-75270, Paris, Cedex 06, France

Sorbonne University, Pierre and Marie Curie University, UMRS1138, Paris, France

Sorbonne Paris Cité University, Paris Descartes University, UMRS1138, Paris, France
e-mail: mc.dieu-nosjean@crc.jussieu.fr

C. Caux
Lyon University, Lyon, France

INSERM U1052 CNRS UMR5286, Centre de Recherche en Cancérologie de Lyon, Lyon, France

Centre Léon Bérard, Lyon, France

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_5

marker of efficient immunotherapy, and raise the question of the capacity of immune-based vaccinations along with immune checkpoint blockade to induce TLS neogenesis. As TLS may represent the best place to induce or amplify protective immunity targeting neoantigens, TLS may be a promising target in order to boost anti-tumoral immunity in cancer patients.

Keywords Tumor microenvironment • Tertiary lymphoid structure • Dendritic cell • T cell • Antibody • Immune checkpoint • Biomarker • Immunointervention • High endothelial venule • Neoantigen

Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
Ag	Antigen
AID	Activation-Induced cytidine Deaminase
APC	Antigen-Presenting Cell
BCR	B-Cell Receptor
ccRCC	Clear cell Renal Cell Carcinoma
CDC	Complement-Dependent Cytotoxicity
CIN	Cervical Intraepithelial Neoplasia
CRC	Colorectal Cancer
CSR	Class Switch Recombination
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
FcR	Fc Receptor
FcRn	Neonatal Fc Receptor
GALT	Gut-Associated Lymphoid Tissue
GC	Germinal Center
G-VAX	GM-CSF transfected tumor cell vaccine
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HCV	Hepatitis C Virus
HEV	High Endothelial Venule
HPV	Human Papilloma Virus
ICP	Immune CheckPoint
Ig	Immunoglobulin
LN	Lymph Node
MHC	Major Histocompatibility Complex
NK	Natural Killer cell
NSCLC	Non-Small Cell Lung Cancer

PAP	Prostate Acid Phosphatase
PB	Plasmablast
PC	Plasma Cell
PDAC	Pancreatic Ductal Adenocarcinoma
RCC	Renal Cell Carcinoma
SCC	Squamous Cell Carcinoma
SHM	Somatic HyperMutation
SLO	Secondary Lymphoid Organ
TAA	Tumor-Associated Antigen
T_{CM}	Central-Memory T cell
TCR	T-Cell Receptor
T_{EM}	Effector-memory T cell
T_{FH}	Follicular Helper T cell
TLS	Tertiary Lymphoid Structure
T_{Reg}	Regulatory T cell

5.1 Introduction

During the evolution of animal species, the adaptive immune system has evolved from ectopic lymphoid organization with the presence of Gut-Associated Lymphoid Tissues (GALT) since lower vertebrates to a highly sophisticated system with the appearance of fully dedicated organs like lymph nodes (LN) in mammals and rare birds. Thus, many different lymphoid organizations exist in the body (i.e., spleen, LN, Peyer's patches, cryptopatches, isolated lymphoid follicles, tonsils, adenoids, tertiary lymphoid structures (TLS), and milky spots in the omentum), which differ in their anatomical localization, duration (constitutive versus transient), cellular composition, time of appearance (during embryogenesis or later on) and, as a consequence of all these characteristics, by their immune function. The role of canonical secondary lymphoid organs (SLO) in the development of antigen (Ag)-specific immunity has been extensively studied. Yet, it is now clearly established that adaptive immune responses can be induced independently of SLO [1, 2]. Thus, many efforts have been concentrated on TLS in different physiopathological disorders among which cancers. TLS are ectopic lymphoid structures that can arise in every inflamed tissue and then turn off after resorption of the inflammation. Initially described in autoimmune diseases, TLS have been reported to have a deleterious effect in autoimmunity and transplantation, whereas they have been associated with a beneficial effect in infectious diseases [3, 4].

This review will highlight recent data from the literature on tumor-associated TLS with a particular focus on their local impact on T- and B-cell immune responses and ultimately on patient's outcome. We will also discuss how TLS could represent a putative target in the next generation of immunotherapies and/or could be used as biomarkers of therapeutic efficacy of current cancer treatments.

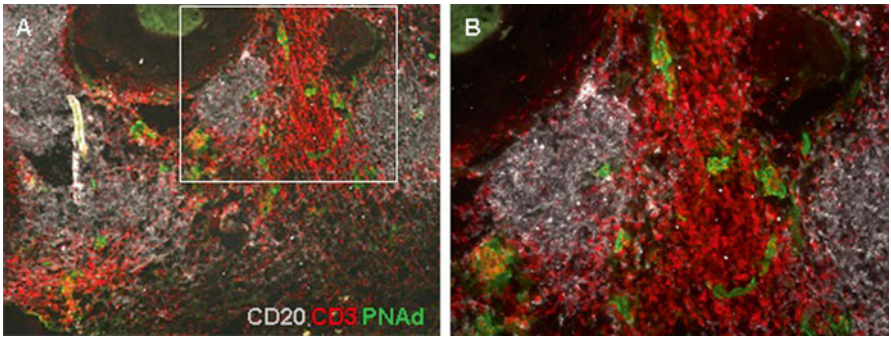

Fig. 5.1 Colocalization between TLS and HEV. Triple immunofluorescent stainings on breast cancer-associated TLS. (**a**) Presence of PNAd$^+$ HEV (*green*) within TLS segregated into two distinct areas, a CD3$^+$ T-cell rich zone (*red*) adjacent to a CD20$^+$ B-cell follicle (*grey*) in the tumor stroma. (**b**) Higher magnification of insert in (**a**). Original magnification: (**a**), ×10; (**b**), ×20

5.2 Relationship Between Tumor-Associated TLS and T Cell Infiltrate

5.2.1 From Rudimental Lymphoid Aggregates to Fully Organized TLS

Different levels of organization of infiltrating immune cells have been reported in cancer, ranging from rudimental clusters of lymphocytes, dense T and/or B cell aggregates to fully organized LN-like structures containing High Endothelial Venules (HEV) (Fig. 5.1) [5–7]. The presence of such lymphoid organizations is highly heterogeneous from one tumor to another, even if they are of the same histologic type. For instance, in infiltrated breast carcinoma, lymphocyte organization varied from simple aggregates in some tumors [8] to fully organized ectopic lymphoid structures, which exhibit strong analogy with LN [6, 9]. Using a retrospective cohort of 290 breast cancer patients, Figenschau et al. reported that TLS negative tumors were mainly estrogen and progesterone receptors positive and HER2 negative [10]. These data indicate that the tumor microenvironment along with the tumor cell itself may dictate permissiveness for TLS neogenesis. In Non-Small Cell Lung Cancer (NSCLC), melanoma or colorectal carcinoma (CRC), most of lymphoid organizations are TLS with the selective homing of mature dendritic cells (DC), the presence of proliferating lymphocytes and reactive germinal centers (GC) [5, 11–13]. The situation is much more complex in Renal Cell Carcinoma (RCC) where mature DC can also be detected outside TLS [14]. This raises the possibility of a differential function and behavior of these antigen-presenting cells (APC) depending on their localization inside versus outside TLS.

5.2.2 *Cellular Composition of the TLS T-Cell Rich Areas*

As in LN, tumor-associated TLS are composed of infiltrating T and B cells, which segregate into two distinct areas, a T-cell rich zone adjacent to a B-cell follicle. The B-cell zone will be described below. Like in SLO, the T-cell zone mainly consists of T cells and, to a lesser extend, of mature DC and few B cells (Fig. 5.2) [5, 15, 16]. However, no NK cells (based on the expression of NKp46) were observed in lung tumor-associated TLS in contrast to LN [17]. Interestingly, NK cells have not been reported so far in TLS whatever the pathology concerned (other cancers, transplantation, infectious or autoimmune diseases) indicating that it is a common characteristic of TLS. As for mature DC, naïve and central-memory T cells (T_{CM}), CD62L and the CCL19/CCL21 axis are critical for the extravasation of circulating NK cells through HEV and their migration to the T-cell zone of LN. As the expression of CD62L and lymphoid chemokines have been reported in TLS of many cancers, the absence of TLS NK cells could rather be a consequence of an inappropriate expression of chemokine receptors (i.e., CCR7) by NK cells and/or the presence of distinct subsets of NK cells infiltrating the tumor compared to the canonical SLO.

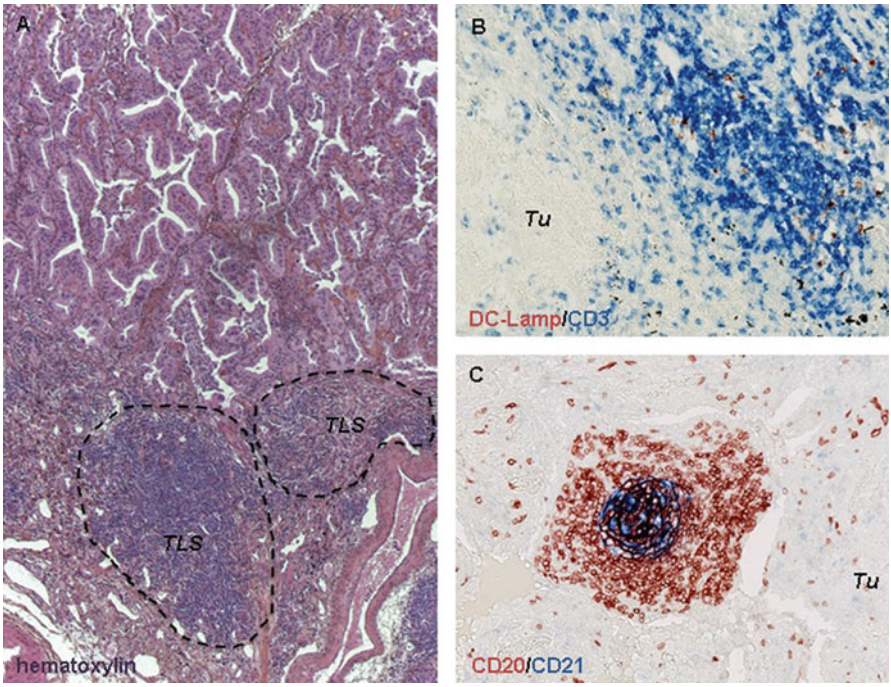

Fig. 5.2 Characterization of tumor-associated TLS. (**a**) Characterization of TLS (*dashed line*) in lung tumor section counterstained with hematoxylin. TLS are composed of a T-cell rich areas which comprised DC-Lamp$^+$ mature DC (*red*) and CD3$^+$ T cells (*blue*), and a CD20$^+$ B cell follicle (*red*) characterized by the presence of a network of CD21$^+$ follicular DC (*blue*). Original magnification: (**a**), ×200; (**b**), (**c**), ×400. *Abbreviations*: TLS, Tertiary Lymphoid Structure; Tu, tumor cells

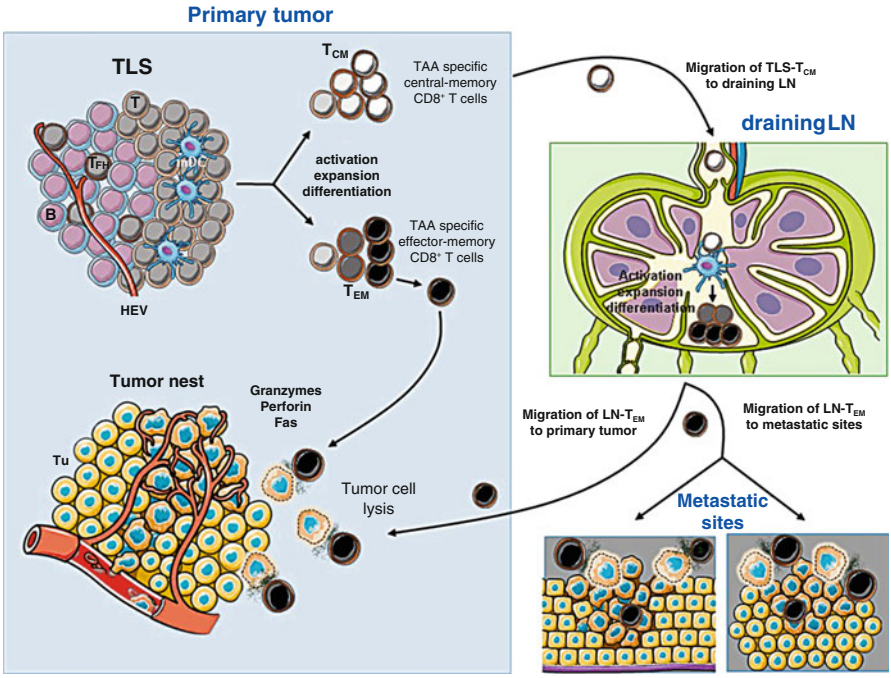

Fig. 5.3 Shaping of the cellular immune response by cancer-associated TLS. TLS may represent a privileged site for the priming and activation of naïve specific T cells by mature DC. This encounter will give rise to an active proliferation and differentiation of cells into either T_{EM} or T_{CM}. T_{EM} can migrate to tumor cells in order to exert their cytotoxic function whereas T_{CM} can migrate to the draining LN in order to develop a systemic anti-tumoral immunity at the primary and metastatic sites via new T_{EM} differentiation. TLS may be also a major site for the reactivation and differentiation of TAA-specific T_{CM} cells into T_{EM} which can then kill tumor cells present in the microenvironment. Abbreviations: B, B cell; LN, Lymph Node; mDC, mature Dendritic Cell; HEV, High Endothelial Venule; T, T cell; TAA, Tumor-Associated Antigen; T_{CM}, central-memory T cell; T_{EM}, effector-memory T cell; T_{FH}, follicular helper T cell; TLS, Tertiary Lymphoid Structure; Tu, tumor cell

The presence of HEV and expression of lymphoid chemokines in both TLS and LN strongly suggests that HEV may represent a major gateway for tumor entrance by immune cells. This hypothesis is supported by two major observations. First, HEV are positive for PNAd and its receptor, CD62L is exclusively expressed by TLS-T and B cells (except GC-B cells), as previously described in LN [6, 18]. Secondly, the density of HEV in tumors positively correlates with the lymphocyte infiltrate in human melanoma [12, 19], breast cancer [6], CRC [7] as well as in a mouse model of methylcholanthrene-induced fibrosarcoma [20]. This privileged access of immune cells from the blood stream to TLS can present many advantages, starting with the direct extravasation of circulating cells into TLS prone to the induction or amplification of a protective immunity (Fig. 5.3). In other words, myeloid and lymphoid cells infiltrating the tumor do not need to traffic into the immunosuppressive tumor microenvironment before reaching TLS. This may explain why HEV are the unique entity among whole blood vessels that are associ-

ated with a favorable clinical outcome in cancer patients [6]. It is known that naïve and T_{CM} enter LN through HEV. In NSCLC, rare naïve T cells infiltrate the tumor but all of them home in TLS [18]. T_{CM} are also present in TLS whereas the majority of effector-memory T cells (T_{EM}) are detected in the stroma (except for TLS) and tumor nests. The selective homing of the different T cell subsets is in accordance with the expression of CCR7 by only naïve and T_{CM}, and the secretion of its two lymphoid ligands CCL19 and CCL21 in TLS. Regulatory T cells (T_{reg}) expressing CCR7 and CCR4 can migrate in response to their ligands (CCL19/CCL21 and CCL17/CCL22, respectively) produced in TLS [8, 18, 21, 22] indicating that putative suppressive mechanisms can operate in TLS (see below).

5.2.3 Impact of Tumor-Associated TLS on the Local Immune Microenvironment

Tumor infiltration by T cells—especially Th1, memory and cytotoxic T cells—has been correlated to a positive clinical outcome of patients in many solid cancers [23]. Nevertheless, whether the activation and differentiation of tumor-specific T cells takes place in the draining LN or in the tumor site still remains enigmatic. Thus, it is becoming critical to address this question in the context of tumor-associated TLS. One limitation for this kind of investigation is the limited relevance of currently used animal models in face of the complexity and heterogeneity of human tumors. However, some indirect responses and arguments came from human studies. In NSCLC, the presence of TLS-mature DC was strongly correlated with a specific gene signature related to Th1 polarization, T-cell activation and cytotoxic effector functions [24, 25], as well as to the density of intra-tumoral T-bet$^+$ Th1 cells, some of which being in contact with tumor cells [5]. In addition, all these genes co-regulate together indicating that a coordination of the immune response occurs in tumors. By contrast, the huge density of mature DC was not associated with immune genes related to Th2 orientation, immunosuppression, inflammation, or angiogenesis. The correlation between a high expression level of cytotoxic genes and presence of TLS has also been reported in CRC and breast carcinoma [9, 21]. Thus, the presence of TLS seems to influence the shaping of the intra-tumoral immune microenvironment by inducing a bias toward a Th1 orientation, T-cell activation and cytotoxic effector functions, in at least NSCLC and CRC. This is consistent with several mouse studies demonstrating that circulating naïve CD8$^+$ T cells can migrate through HEV in the tumor, and undergo activation, proliferation and differentiation into effector cells to promote tumor eradication [26], even in the absence of any SLO [27, 28]. The same demonstration has been published in infectious diseases [1] indicating that initiation of specific immunity can occur independently of canonical lymphoid organs. It is tempting to postulate that the presence of TLS mature DC may be a prerequisite for optimal processing and presentation of tumor-associated antigens (TAA)—emerging as a consequence of somatic mutations in tumor cells—and coordination of local adaptive immune response (Fig. 5.3).

Thus, TLS could play a key role in anti-tumoral immunity by allowing activation of naïve T cells and their differentiation into anti-tumor effector cells and/or reactivation of tumor-specific memory T cells at the tumor site.

In LN, many cross-talks have been reported between NK cells and other immune cells including DC and T cell subsets. Therefore, the absence of NK cells in TLS may have functional consequences on the local shaping of both innate and adaptive immune responses such as deficient IFN-γ production, incomplete T and dendritic cell activation and biased T-cell polarization towards a non-Th1 pathway. Several studies also demonstrated that tumor-infiltrating NK cells display a profound functional defect and an altered phenotype characterized by the downregulation of activation receptors and upregulation of inhibitory receptors [17]. Thus, the absence of functionally impaired NK cells in intra-tumoral TLS may favor an optimal T cell activation. It remains to be determined whether there is a causal link between the preferential localization of NK cells outside TLS and their dysfunction in situ.

In conclusion, TLS represent a privileged site for T cell recruitment, differentiation and activation in tumors, and support a key role of these lymphoid structures in the shaping of a local anti-tumor immunity.

5.2.4 Prognostic Value of Cells Homing in the TLS T Cell-Areas

During the last decade, the association between the presence of immune infiltrate and the clinical outcome of cancer patients has been extensively documented, but this is only recently that the organization of immune cells is taken into account. Indeed, the prognostic value of cancer-associated TLS has been considered through different approaches including immunohistochemistry and gene expression (Table 5.1). Whatever the strategy used, results are consistent meaning that either strategy can be used for retrospective and prospective studies depending on the biological material available.

Among the first studies, Miyagawa et al. reported that high infiltration of $CD83^+$ cells, which supposedly correspond to mature DC, is associated with long-term survival of patients with colorectal liver metastasis [29] (Table 5.1). Yet, it is of note that CD83 does not solely identify mature DC but also some activated B and T cells. DC-Lamp appears as a better specific marker of human mature DC whatever the DC lineage considered (except in lung, where this molecule is also expressed by type II pneumocytes which can however be distinguished from DC by their distinct cellular morphology). Two studies showed that a high DC-$Lamp^+$ mature DC density correlates with a favorable clinical outcome of patients with primary melanoma [30] or NSCLC [5], the last study achieved a complete cellular characterization of TLS. Since this pioneer work, many other publications showed that a high frequency of TLS (detection of lymphoid aggregates, mature DC, T, and B cells, and/or HEV) has a good prognostic value in primary breast, colorectal and lung

Table 5.1 Prognostic value of TLS-associated biomarkers in naïve and vaccinated cancer patients

Criteria	Cancer type	Stages of the disease	No. of patients	TLS detection by IHC	TLS detection by gene expression	Prognostic value	References
Primary tumors	Breast carcinoma	I to III	146	HEV	–	Positive	[6]
		I to III	146	mature DC	–	Positive	[113]
		I to III	794	–	T_{FH}, Th1, CXCL13	Positive	[9]
	CRC	I to IV	350	Lymphoid aggr.	–	Positive	[114]
		ND	25	mature DC	–	Positive	[31]
		I to IV	40	mature DC and T	–	Positive	[115]
		II	185	T	–	Positive	[13]
		III	166	T	–	No value	[13]
		0 to IV-A	21	–	12-chemokine genes	Positive	[21]
		I to IV	125	–	CXCL13 and CD20	Positive	[37]
	Gastric cancer	all without chemo	82	B	–	Positive	[77]
		I to III	365	–	both Th1 and B	Positive	[77]
	NSCLC	I to II	74	mature DC	–	Positive	[5]
		I to IV	362	mature DC	–	Positive	[24]
		III with neo-adj. chemo	122	mature DC	–	Positive	[11]
		III with neo-adj. chemo	122	mature DC and B	–	Positive	[11]
		III with neo-adj. chemo	122	B	–	Positive	[11]
	Melanoma	I-A to III-A	82	mature DC	–	Positive	[30]
		IV	21	–	12-chemokine genes	Positive	[22]
	Oral SCC	all	80	B, T, GC-B, HEV, FDC	–	Positive	[116]
	Pancreatic cancer	all	308 + 226	Lymphoid aggr.	–	Positive	[117]
	RCC	all	135	mature DC	–	Positive	[14]

(continued)

Table 5.1 (continued)

Criteria	Cancer type	Stages of the disease	No. of patients	TLS detection by IHC	TLS detection by gene expression	Prognostic value	References
Metastatic tumors	CRC (lung)	ND	140	mature DC	–	Positive	[31]
	RCC (lung)	ND	51	mature DC	–	Positive	[14]
Post-vaccination	CIN + HPV16 DNA vaccine	CIN2/3	12	B, T, HEV	–	TLS neogenesis	[101]
	PDAC + G-VAX	ND	54	B, T, $T_{reg,}$, FDC, HEV, mature DC, myeloid cells, CCL21	–	TLS neogenesis	[103]
	PDAC + G-VAX	ND	39	–	T_{reg}^{Low}, $Th17^{High}$	Positive	[103]

Abbreviations: *chemo* chemotherapy, *CIN* cervical intraepithelial neoplasia, *CRC* ColoRectal cancer, *DC* dendritic cell, *FDC* follicular dendritic cell, *G-VAX* GM-CSF transfected tumor cell vaccine, *GC* germinal center, *HEV* high endothelial venule, *HPV* human papilloma virus, *ND* not done, *neo-adj* neo-adjuvant, *NSCLC* non-small cell lung cancer, *PDAC* pancreatic ductal adenocarcinoma, *RCC* renal cell carcinoma, *SCC* squamous cell carcinoma, T_{FH} follicular helper T cell, *TLS* tertiary lymphoid structure, T_{reg} regulatory T cell.

carcinomas, gastric cancers, melanomas as well as in metastatic CRC (Table 5.1). Despite the fact that DC-Lamp expression is considered as a hallmark of TLS in at least NSCLC, high mature DC infiltrate was unexpectedly shown to correlate with short-term survival in RCC [31]. Further analysis revealed that TLS DC-Lamp$^+$ cells are associated with a favorable clinical outcome whereas non-TLS DC-Lamp$^+$ cells are correlated with poor prognosis in ccRCC patients [14].

High density of tumor-infiltrating CD8$^+$ T cells positively influences the outcome of NSCLC patients [24, 32–34]. Interestingly, Goc et al. showed that the presence of TLS conditions the prognostic value of intra-tumoral CD8$^+$ T cells [24]. Indeed, among NSCLC patients with high density of CD8$^+$ T cells and overall favorable clinical outcome, those having a low density of mature DC have the worst prognosis (comparable to patients having low CD8$^+$ T cell infiltrate) indicating that TLS mature DC are required to license the positive prognostic value of infiltrating CD8$^+$ T cells (Fig. 5.3). The mechanism underlying the imprinting of CD8$^+$ T cell function remains to be elucidated.

Altogether, it is tempting to speculate that TLS could potentiate anti-tumor immunity mediated by CD8$^+$ T cells through different mechanisms. Firstly, infiltrating immature DC could take up and process TAA and migrate to TLS in order to present processed tumor peptides to specific T cells (Fig. 5.3). This could favor the continuous differentiation of tumor-specific T cells through the activation of naïve T cells and/or reactivation of specific memory T cells. Such local immune responses initiated in TLS could be more adaptable to the shift of tumor antigen expression occurring during tumor progression. Secondly, CD4$^+$ T cells are enriched in TLS and may exert a key helper function on CD8$^+$ T cells by promoting the recruitment, activation, and differentiation of naïve cells into effector cells, as demonstrated in a model of pancreatic tumors [35]. This is consistent with the strongly coordinated expression of genes related to Th1, activation and cytotoxicity, and their association with high density of mature DC reported in NSCLC [24]. Thirdly, because their prognostic impact is always observed after resection of the tumor, TLS might allow the establishment of systemic immunity against the tumors i.e., through the recirculation of anti-tumor memory T cells (T_{CM} and/or T_{EM}) previously generated in TLS.

5.3 Relationship Between TLS and B Cell Infiltrate

The existence of intra-tumor lymphoid-like structures with segregated T and B cell zones argues for the existence of a local Ag-driven humoral immune response and suggests potential roles of antibodies (Ab) and B cell subsets in anti-tumor immunity.

5.3.1 TLS as Sites for Development of Tumor Specific Humoral Immunity

Intra-tumor TLS have a LN-like organization with the presence of typical B cell follicles containing naïve B cells and GC, and more dispersed plasmablasts (PB)/plasma cells (PC) [5, 9, 16, 21, 36, 37]. As in SLO, GC contain highly proliferating B cells expressing AID—an enzyme involved in somatic hypermutation and Ig class switching—as well as mesenchymal follicular DC and follicular helper T cells (T_{FH}) (Fig. 5.2), a specialized subset of T helper cells critical for GC formation and development of high-affinity Ab, memory B cells and PC. TLS also contain cells producing the chemokine CXCL13, which governs the recruitment of circulating CXCR5 expressing B and T cells and structuration of B cell follicles. Importantly, this chemokine appears as one of the best predictive marker for prognosis in breast [9] and colon [21] cancers (Table 5.1), further pointing to an essential role of TLS in anti-tumor immunity. Studies in BC and NSCLC revealed that highly infiltrated tumors contained nearly all stages of B cell differentiation [11] (Couillault et al., unpublished data) arguing for an in situ Ag-driven humoral immune response resulting in the formation of memory B cells and Ab-producing PC (Fig. 5.4). Through a comprehensive molecular profiling of tumor-infiltrating $CD4^+$ T cells, Gu-Trantien et al. recently revealed that the presence of a T_{FH} signature robustly predicts breast cancer patient survival and response to chemotherapy, suggesting that local T_{FH}-driven B cell differentiation is essential for anti-tumor immunity [9].

Evidence of an in situ Ag-driven humoral response in intra-tumor TLS has been revealed by a restricted immunoglobulin (Ig) variable gene usage by tumor-infiltrating B cells, isotype switching and somatic hypermutation (SHM) in various cancers including breast medullary carcinoma [38–42], breast ductal carcinoma [16, 36, 40] and metastatic melanoma [12]. More recently, using the Cancer Genome Atlas mRNA-seq data, Iglesia et al. identified, in a subset of tumors from basal like breast cancers, specific BCR segments, which expression correlates with better patient survival, supporting that the presence of a productive and potentially restricted anti-tumor B cell response may directly impact patient clinical outcome [43].

TLS thus exhibit many features of an ongoing T-cell-dependent humoral response to protein Ag and analysis of the specificity of Ab produced in the tumor microenvironment provides evidence that TLS may be elicited by TAA [16] or autoAg [39, 44]. More recently, Germain et al. demonstrated that about half of the TLS rich NSCLC tumors contained cells producting Ab directed against one or several shared lung TAA, such as LAGE-1, MAGEA1, MAGEA2, TP53 and NY-ESO-1, known to elicit spontaneous immune responses in cancer patients [11]. Whether the Ab produced by tumor specific PC infiltrating the tumor versus those localized in peripheral tissues target similar Ag is so far poorly understood and remains an important issue to address in order to better appreciate the still obscure role of humoral immunity in cancer. In this respect, besides the shared TAA analyzed so far, it will be important to study Ab reactivity to patient specific neoantigens emerging as a result of mutations in tumor cells, as this is now being done for T cells [45].

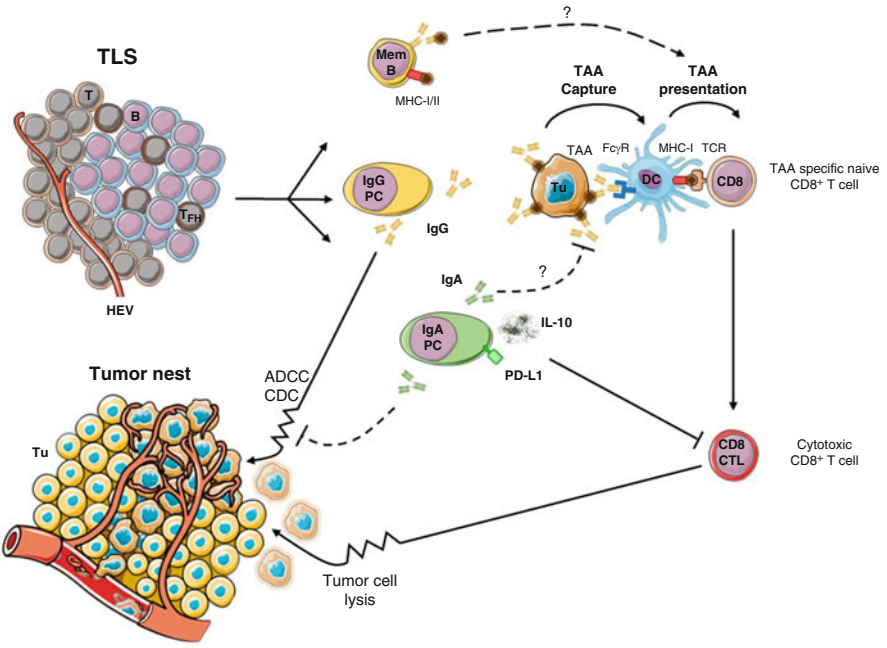

Fig. 5.4 Opposing functions of IgG and IgG produced/expressed in the tumor microenvironment. TLS allow expansion of naïve B cells, activation of CSR and SHM machineries, and differentiation into memory B cells and plasma cells producing IgG or IgA, depending on the nature of the cytokines produced by T_{FH} and other immune infiltrating cells. IgG released in the tumor microenvironment may contribute to the elimination of tumor cells by CDC and ADCC, while IgA may antagonize these processes by competition. Opsonisation of tumor cells by IgG favors the transfer of tumor Ag to dendritic cells through engagement of the FcγR for cross-presentation to $CD8^+$ T cells and induction of anti-tumor cytotoxic T cells. After capture of TAA through their membrane Ig and accommodation into MHC molecules, memory B cells can also impact anti-tumor T cell immunity. IgA PC can express IL10 and PDL1 and impair anti-tumor T cell immunity. *Abbreviations*: ADCC, antibody-dependent cell-mediated cytotoxicity; B, B cell; CDC, complement-dependent cytotoxicity; CSR; Class Switch Recombination; CTL, Cytotoxic T Lymphocyte; DC, Dendritic Cell; HEV, High Endothelial Venule; mem B, memory B cell; PC, Plasma Cell; SHM: Somatic HyperMutation; T, T cell; TAA, Tumor-Associated Antigen; T_{FH}, follicular helper T cell; TLS, Tertiary Lymphoid Structure; Tu, tumor cell

5.3.2 The Various Ways TLS B Cells May Impact Anti-tumor Immunity

5.3.2.1 By the Production of Ab

In many cancers, tumor-infiltrating B cells have been shown to produce class-switched affinity-matured anti-tumor Ab [46], most often IgG, and some anti-tumor effects of these Ab have been demonstrated [47–49]. This is in accordance with the fact that the presence of PC or IgG in the tumor is associated with a better prognosis in breast cancer, NSCLC, CRC, and metastatic melanoma [43, 50–53]. Anti-TAA

IgG may contribute to the elimination of tumor cells, either directly after fixation of complement or indirectly by activating accessory cells expressing FcγR to exert cytotoxic function (i.e., antibody-dependent cell-mediated cytotoxicity, ADCC). Tumor mouse models have indeed revealed that administration of anti-TAA IgG2 Ab can induce ADCC and prevent tumor metastasis by a process involving FcγR [54], but there is so far no formal proof in humans that a naturally occurring anti-tumor IgG response can exert such a role. Besides these effector roles, Ab can also contribute to the efficient priming of anti-tumor immunity (Fig. 5.4), which requires the capture of TAA and their presentation in association with MHC-class-I molecules to $CD8^+$ T cells. This process, known as Ag cross-presentation, is greatly enhanced in the case of Ag/Ab immune complexes, which can engage FcγR on multiple DC subsets to shuttle exogenous Ag into the cross-presentation pathway [55]. IgG deposition on tumor cells has been documented in several cancers and two recent studies in mouse models point to an essential role of Ab/TAA immune complexes for priming anti-tumor $CD8^+$ T cells through DC [56, 57]. This is reminiscent to a previous observation in a mouse model of CRC showing that administration of anti-TAA IgG potentiates the efficacy of anti-cancer drugs and results in enhanced anti-tumor $CD8^+$ T cell response [58].

The function of Ab inside the tumor microenvironment likely varies with the class of Ig, which is known to dictate its effector functions. In this respect, one should distinguish pro-inflammatory complement fixing and ADCC-prone Ab (IgG1-3) from non-inflammatory IgG_4 and IgA, which emerge in situations of chronic antigen exposure, have poor ability to bind complement and Fc receptors and to activate effector cells, and can actively contribute to immune tolerance. B cell isotype switching to IgA and IgG4 are induced by TGF-β and IL10, two suppressive cytokines that are often expressed in the tumor microenvironment, and there is now some evidence that these two classes of Ig may contribute to immune suppression in cancer patients. By analyzing rearranged immunoglobulin genes from micro-dissected TLS B cells, Cipponi et al. recently revealed that IgA and IgG isotype switching occurred nearly at a similar frequency in TLS of metastatic melanoma patients, indicating that TLS may favor differentiation of IgA producing PC [12] in addition to IgG^+ PC. Indeed, serum anti-TAA IgA or tumor-infiltrating IgA^+ PC can be detected in a proportion of tumor patients [11, 16, 59–64]. Importantly, tumor-infiltrating IgA PC were recently shown to strongly dampen anti-tumor CTL responses induced by immunogenic chemotherapy in mouse models of prostate cancer [65]. This suggests that IgG PC and IgA PC may exert opposite functions in anti-tumor immunity [66] (Fig. 5.4). IgA^+ PC were also detected in human prostate tumors near lymphoid-like structures and patients with a high density of IgA^+ PC displayed lower CD8/B cell ratios suggesting that, also in humans, the presence of IgA^+ PC within the tumor microenvironment may be associated with tumor immune evasion [65]. Regarding IgG, a differential role of subclasses in anti-tumor immunity has been recently highlighted in melanoma. PC-producing tumor reactive IgG4 were detected in melanoma tumors and elevated serum IgG4 levels inversely correlated with patient survival [67]. Mechanistically, IgG4 antibodies were ineffective in triggering effector cell-mediated tumor killing and even blocked IgG1-mediated

tumoricidal functions through reduction of FcγRI activation. IgG4 PC have also been reported in extra-hepatic cholangiosarcoma and their presence was associated with a lower survival rate and positively and negatively correlated with tumor infiltration by FoxP3$^+$ T_{reg} and CD8$^+$ T cells, respectively [68]. This indicates that IgG4 responses promoted by Th2-biased inflammation may restrict effector cell function and contribute to immune escape.

Altogether, these recent important advances have revealed some of the so far neglected essential roles of Ab in modulating anti-tumor immunity within the tumor microenvironment (Fig. 5.4). These include induction or modulation of tumor cell death (via ADCC and/or CDC), modulation of the biology of targeted tumor molecules, induction of anti-tumor immunity by facilitating DC-mediated TAA presentation to T cells and modulation of DC functions by engagement of FcR or C-type lectins [69]. The anti- versus pro-tumoral functions of Ab depends on several factors including the isotype of Ab and IgG subclasses, their degree of syalilation [70], the targeted Ag and the nature of infiltrating immune cells and pattern of expression of Ig receptors.

5.3.2.2 By Presenting TAA to T Cells

As sites of Ag-driven oligoclonal B cell expansion, TLS are most likely enriched in TAA specific B cells, which may become the dominant APC within tumors with the capacity to efficiently capture tumor Ag through their BCR. Compatible with such a role, TLS B cells and activated T cells were found in close contacts in melanoma and the presence of both cell types correlates with a better survival of patients [71]. B cell antigen presentation to T cells is critical for the establishment and recall of antigen-specific memory CD4$^+$ T cells [72]. Not only memory B cells, which can express costimulatory molecules, but also IgM$^+$ and IgA$^+$ PC, which retain surface expression of Ig [73] and are able to exert Ag-presenting functions towards CD4$^+$ T cells [74], could present TAA inside tumor-associated TLS. B cells and PC may thus, through their APC function, modulate T cell fates within the tumor microenvironment.

5.3.2.3 By Producing Molecules Modulating the T Cell Response

It is noteworthy that the beneficial impact of TLS B cells or T_{FH} in cancer is often associated with increased Th1 and/or CD8 responses [9], suggesting that B cells may support anti-tumoral T cell responses and/or Th1-T_{FH}- dominant immunity. It is indeed noteworthy that tumor infiltration by both CD20$^+$ B cells and Th1/CD8$^+$ T cells often shows the best survival benefit is several cancer types, as compared to infiltration by either population alone [37, 75–77]. At the same line, it was recently demonstrated in NSCLC that a high density of TLS B cells was associated with increased clonality of tumor-infiltrating CD4$^+$ and CD8$^+$ T cells [78]. Besides their already discussed capacity to promote TAA presentation

though Ab production, B cells could shape T cell differentiation and functions by releasing immuno-modulatory type 1 (IL12, IFN-γ), type 2 (IL4, IL13), and suppressive (IL10, TGF-β, IL35) cytokines [79]. As for example, tumor-infiltrating B cells have been shown to produce IFN-γ and IL12 in hepatocellular carcinoma [76], suggesting their potential involvement in inducing or amplifying cellular immunity. On the opposite, so-called regulatory B cells producing IL10, TGF-β or Granzyme B have been shown to dampen anti-tumor immunity and/or expand T_{reg} and to promote tumor progression [80–82]. Likewise, B cells and PC can express PD1 ligands at high levels [83, 84] and tumor-infiltrating PDL1-expressing IgA^+ PC were recently shown to dampen $CD8^+$ T cell-mediated immunity [65] (Fig. 5.4). Altogether, through their capacity to produce Th cell polarizing cytokines, such as IFN-γ, IL12, IL4 or IL13 or immunosuppressive cytokines like IL10 and TGFβ [85] and express of ligands for stimulatory or inhibitory immune check points (ICP), tumor-infiltrating B cells can modulate T cell functions and increase or dampen anti-tumor immunity.

5.3.3 Regulation of TLS Immune Function

Although TLS have been associated with a better clinical outcome in most types of cancers (Table 5.1), their exact role remains debated and it is not excluded that particular types of TLS might be detrimental and favor immune suppression. Such notion of "immunosuppressive TLS" came from the field of transplantation, where TLS have been observed in tolerated lung or kidney grafts [86, 87]. Indeed, long-term lung graft acceptance is associated with induction of bronchus-associated lymphoid tissues, where $FoxP3^+$ T_{reg} accumulate and control the anti-donor immune response [87]. Such TLS have not been described so far in cancer. Nevertheless, in similarly, Gobert et al. have shown in breast cancer that patients with a high density of T_{reg} in lymphoid aggregates have a worse prognosis [8]. Others have shown in mice that the transgenic expression of the TLS-inducing cytokine LTαβ in hepatocytes leads to recruitment and activation of immune cells and development of hepatocellular carcinoma [88]. In likewise, LTαβ expression in B cells drives the rapid re-emergence of prostate cancer following androgen deprivation [89]. Just as LN, TLS may thus act by amplifying the prevailing immune response in tumor-bearing patients, should it be a suppressive and detrimental immune response or an anti-tumoral Tc1/Th1 beneficial immune response. What differentiates these two putative types of TLS is so far poorly understood, but might rely in the functional properties of their B cells, including the nature of the cytokines and the class of Ig they produce, the pattern of expressed ICP ligands and the presence of immunosuppressive cells such as T_{reg}.

5.4 Immunotherapies and TLS Development

5.4.1 TLS Neogenesis

The mechanism of lymphoid organogenesis are now quite well known and involved many molecules including lymphotoxin (LT)-α and LT-β. TNF-α the lymphoid chemokines CCL19, CCL21, CZCL13, and RANK-L (for more details, see review by [90]). Since the growing interest in the field of TLS in autoimmunity, infectious diseases and cancers, many laboratories studied whether the neogenesis of TLS can occur via the same set of molecules involved in lymphoid organogenesis. Strikingly, the answer is yes, meaning that there is no apparent specificity within the two developmental programs (reviewed by [4]). In particular, the concomitant expression of the lymphoid chemokines is (i) mandatory for the completion of lymphoid neogenesis program, and (ii) critical for the maintenance of lymphoid organization via a self-amplification loop.

The knowledge on TLS is very recent compared to LN but it is worth noting that during the evolution of vertebrates, TLS and other lymphoid organizations like Gut-associated lymphoid tissues (GALT), emerged since lower vertebrates whereas LN appeared very recently in mammals (also in few birds). This indicates that LN organogenesis has evolved through the recapitulation of the TLS neogenesis, and thus can explain the redundancy of the molecules implicated in each of their formation.

Nonetheless, it is important to underline that one major difference exists between LN and TLS formations. During fetal development, LN organogenesis is initiated by interactions between lymphoid tissue inducer (LTi) cells and stromal organizer cells, and this key cellular cross-talk is LTα1β2 (membrane form of LTβ)-LTβ dependent. Several studies have shown that TLS can be induced upon inflammatory stimuli in mice devoid of any LTi cells (i.e., ROR$\gamma^{-/-}$ and Id2$^{-/-}$ mice [91]), suggesting that other inducer cells can trigger TLS neogenesis [92, 93]. By using different mouse models, several immune cell types have been shown to promote TLS induction, along with the presence of DC that seems to play a central role. In breast cancer, mature DC are the major source of the membrane-bound LTβ [94] and thus this immune population play a key role in the differentiation and maintenance of mature HEV, which are essential for the extravasation of peripheral blood immune cells into TLS [94]. In a model of ectopic expression of CCL21 in the thyroid, DC-CD4^{+} T cell interaction initiates the formation of HEV in a CCR7- and LTβR-dependent mechanism, and ultimately TLS neogenesis [92]. In addition, the presence of DC has been shown to be essential for the maintenance of TLS in the lung after virus infection (influenza virus) [95] and modified vaccinia virus Ankara [96]. Interestingly, a proactive role for neutrophils in TLS neogenesis has been recently highlighted in mice in a model of acute LPS instillations [97]. Finally, TLS formation can also be negatively regulated. Indeed, regulatory T cells (Tregs) can interfere with this lymphoid neogenesis program by dampening the neutrophilic inflammation induced

by acute LPS exposure [97]. Very recently, Joshi et al. have shown that depletion of Tregs results in an increase number of cancer-associated TLS and the promotion of anti-tumor immunity in established lung adenocarcinoma [98].

Altogether, the mechanisms of LN and TLS formation share many similarities with the particularity that several LTi-like cells can promote TLS development at the inflammatory site.

5.4.2 Vaccination

The vaccination was discovered in 1796 by Edward Jenner who first inoculated cowpox pus, an agent of mild disease infecting cows, and then injected the smallpox to James Phipps. This pioneering experiment opened a field of intense investigation on strategies to manipulate the immune system to protect the host [99]. Thereby, anti-infectious vaccination has permitted to eradicate or at least to greatly reduce morbidity of many pathologies, notably poliomyelitis, smallpox, measles, and mumps. In the field of cancer, vaccination against human papilloma virus (HPV) serotypes 16 and 18 (Cervarix®, Gardasil®) prevents 70 % of cervical cancer [100]. Despite the recent introduction of these preventative HPV vaccines, the frequency of high-grade cervical intraepithelial neoplasia (CIN) in women remains too high worldwide. In this setting, Maldonado et al. investigated the immune infiltrate of lesional mucosa after therapeutic vaccination against HPV16 [101]. In post-vaccination, a huge modification of the immune microenvironment is observed with a marked increase of T cell infiltrate with an activated effector memory phenotype and oligoclonal expansion. In contrast to unvaccinated subjects, the immune infiltrate is organized in TLS adjacent to HEV in target lesions of treated patients indicating that TLS are a marker of efficient immunotherapy. CIN-associated TLS have features of an ongoing immune response, and their presence correlates with increased Th1 responses and immune cell activation-related genes, as previously observed in some primary tumors such as NSCLC. Thus, we can speculate that TLS may provide a local niche for the differentiation and maintenance of memory cells and consequently, may participate in the generation of a long-term protection against HPV.

Several immune-based cancer therapies aim to target DC subsets to induce anti-tumor immunity. The two main strategies of DC-based therapies were to pulse ex vivo generated DC with TAA and to reinject them to cancer patients or to directly mobilize endogenous DC using cytokines like Flt3 ligand or GM-CSF. Despite the limited adverse effects, the objective responses using either approach were quite disappointing with regards to the central role of DC as bridges between the innate and adaptive immunity. It is of note nowadays that only two anti-tumor vaccines, both targeting GM-CSF, have been approved by the US Food and Drug Administration: GVAX for pancreatic cancer and Sipuleucel-T for treatment of metastatic castrate-resistant prostate cancer [102]. GVAX is an allogeneic GM-CSF gene-transfected tumor cell vaccine designed to elicit T cell immunity to cancer

antigens including mesothelin. Recently, Lutz et al. observed TLS neogenesis in most pancreatic tumors following G-VAX vaccination [103]. TLS-T cells exhibit signs of activation and effector functions, and a Th17 orientation. Importantly, even in the presence of T_{reg} and expression of inhibitory ICPs i.e., PD1/PD-L1 in TLS, the effector T cells/T_{reg} ratio is highly in favor of an anti-tumor response in vaccinated patients and the enhanced Th1/Th17 axis in TLS correlates with decreased T_{reg} density and long-term survival after vaccine therapy. Thus, these studies showed that immune-based vaccinations, which were initially designed to boost the adaptive immunity, are able to reprogram the tumor microenvironment from an unfavorable to a favorable milieu for TLS neogenesis.

It is of note that Sipuleucel-T is a GM-CSF-prostate acid phosphatase (PAP, a tumor antigen) fusion molecule contraindicated in patients with history of metastatic castrate-resistant prostate cancer. In this context, it will be interesting to evaluate the shaping of the local immune microenvironment post-vaccination, and to correlate the putative presence of TLS with the objective response to the treatment.

Altogether, it will be interesting to reanalyze data from previous vaccine clinical trials, according to TLS stratification, density of tumor-infiltrating effector T cells and outcome of vaccinated cancer patients in order to characterize therapies that best promote *de novo* formation of TLS with anti-tumoral activity.

5.4.3 Immune Checkpoints

The undeniable success of ICP inhibitors with anti-CTLA-4 (Ipilimumab) and anti-PD1 (Pembrolizumab and Nivolumab) antibodies is considered as a revolutionary therapeutic advance in cancer [104–108]. First introduced in unresectable or metastatic melanoma and now in chemo-resistant NSCLC, anti-PD1/PD-L1 therapies remarkably enhanced the survival of a fraction of patients. Recently, it has been demonstrated that the mutational landscape of tumor cells could predict response to anti-PD1 treatment in NSCLC patients [109]. Numerous somatic mutations are indeed found in smokers compared to never-smokers, leading to an increased number of neoantigens and promote the efficacy of anti-PD1 treatment. In many human cancers, PD1 and its ligands have been shown to be expressed by tumor-infiltrating immune cells among which T cells [110–112] and TLS-T cells [9]. Thus, it will be very interesting to correlate the presence of TLS with response to ICP inhibitory antibodies in order to evaluate the putative impact of these immunotherapies in patients with an already well organized immune microenvironment.

Taking into account the role of TLS in the shaping of the local immune microenvironment, we can speculate that immunotherapies based on ICP blockade may act as a powerful booster of immune responses initiated in TLS already present in the tumor, TLS being the best place to induce or amplify protective immunity targeting neoantigens.

5.5 Conclusions

The recent discovery of tumor-associated TLS adds a new variable to be integrated into the current efforts to better understand the role played by the immune system in human cancers and identify efficient immunotherapies aimed to harness anti-tumor immunity. That tumor TLS display many similarities with canonical SLO and most often correlate with long-term survival of patients, argue for a protective role of TLS in cancer. These structures, which are enriched in DC and organized in T and B cell zones, may indeed constitute preferential sites for the presentation of TAA allowing the priming of tumor-reactive naïve T cells and/or the reactivation of specific memory T cells. In addition, thanks to the presence of T_{FH} and GC, TLS are also sites for initiation and/or amplification of humoral responses, which role in cancer just start to be appreciated. From a therapeutic point of view, pre-existing intra-tumor TLS may constitute a very useful biomarker for selecting patients who could best beneficiate of immunotherapy aimed to reactivate tumor-specific T cells. In addition, neo-induction of TLS might be beneficial in cancer patients to generate efficient anti-tumor immunity, as suggested in animal models. Yet, several critical questions need to be addressed before exploiting TLS in therapy. One of these concerns the possible existence of detrimental TLS favoring tumor progression and the identification of the key factors that distinguish good TLS from bad TLS. Another important issue is to identify the mechanisms allowing TLS neogenesis in the tumor milieu in order to define strategies that could be used in patients. Finally, determining how TLS-based strategies could best be combined with currently used targeted- or immunotherapies is of utmost importance. In particular, combining inhibitory ICP blockade and TLS induction may represent a promising strategy to promote efficient cellular and humoral immune responses against tumor.

Acknowledgments We wish to acknowledge the participation of members of the laboratories among whom Prs. C. Sautès-Fridman and W. H. Fridman, clinicians, and pathologists who participated in the articles referenced in our review.

Conflict statement: No potential conflicts of interest were disclosed.

Grants: This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), University Paris Descartes, University Pierre et Marie Curie, SIRIC Cancer Research and Personalized Medicine (CARPEM), the LabeX Immuno-Oncology, Institut National du Cancer (INCa) and Cancerop̂le Ile-de-France, Fondation ARC pour la Recherche sur le Cancer, MedImmune, the LABEX DEVWECAN (ANR-10-LABX-0061) of University of Lyon, the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR), and the SIRIC project (LYRIC, Grant No. INCa-4664). Hélène Kaplon was supported by a grant from La Ligue contre le Cancer.

References

1. Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, Hartson L, Sprague F, Goodrich S, Woodland DL, Lund FE, Randall TD. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat Med. 2004;10(9):927–34.

2. Moyron-Quiroz JE, Rangel-Moreno J, Hartson L, Kusser K, Tighe MP, Klonowski KD, Lefrançois L, Cauley LS, Harmsen AG, Lund FE, Randall TD. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. Immunity. 2006;25(4):643–54.
3. Carragher DM, Rangel-Moreno J, Randall TD. Ectopic lymphoid tissues and local immunity. Semin Immunol. 2008;20(1):26–42.
4. Dieu-Nosjean MC, Goc J, Giraldo NA, Sautès-Fridman C, Fridman WH. Tertiary lymphoid structures in cancer and beyond. Trends Immunol. 2014;35(11):571–80.
5. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L, Lebecque S, Fridman WH, Cadranel J. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. J Clin Oncol. 2008;26(27):4410–7.
6. Martinet L, Garrido I, Filleron T, Le Guellec S, Bellard E, Fournie JJ, Rochaix P, Girard JP. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. Cancer Res. 2011;71(17):5678–87.
7. Bento DC, Jones E, Junaid S, Tull J, Williams GT, Godkin A, Ager A, Gallimore A. High endothelial venules are rare in colorectal cancers but accumulate in extra-tumoral areas with disease progression. Oncoimmunology. 2015;4(3), e974374.
8. Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, Biota C, Doffin AC, Durand I, Olive D, Perez S, Pasqual N, Faure C, Ray-Coquard I, Puisieux A, Caux C, Blay JY, Ménétrier-Caux C. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res. 2009;69(5):2000–9.
9. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, Ravoet M, Le Buanec H, Sibille C, Manfouo-Foutsop G, Veys I, Haibe-Kains B, Singhal SK, Michiels S, Rothé F, Salgado R, Duvillier H, Ignatiadis M, Desmedt C, Bron D, Larsimont D, Piccart M, Sotiriou C, Willard-Gallo K. CD4+ follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest. 2013;123(7):2873–92.
10. Figenschau SL, Fismen S, Fenton KA, Fenton C, Mortensen ES. Tertiary lymphoid structures are associated with higher tumor grade in primary operable breast cancer patients. BMC Cancer. 2015;15:101.
11. Germain C, Gnjatic S, Tamzalit F, Knockaert S, Remark R, Goc J, Lepelley A, Becht E, Katsahian S, Bizouard G, Validire P, Damotte D, Alifano M, Magdeleinat P, Cremer I, Teillaud JL, Fridman WH, Sautès-Fridman C, Dieu-Nosjean MC. Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer. Am J Respir Crit Care Med. 2014;189(7):832–44.
12. Cipponi A, Mercier M, Seremet T, Baurain JF, Théate I, van den Oord J, Stas M, Boon T, Coulie PG, van Baren N. Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. Cancer Res. 2012;72(16):3997–4007.
13. Di Caro G, Marchesi F. Tertiary lymphoid tissue: a gateway for T cells in the tumor microenvironment. Oncoimmunology. 2014;3, e28850.
14. Giraldo NA, Becht E, Pagès F, Skliris G, Verkarre V, Vano Y, Mejean A, Saint-Aubert N, Lacroix L, Natario I, Lupo A, Alifano M, Damotte D, Cazes A, Triebel F, Freeman GJ, Dieu-Nosjean MC, Oudard S, Fridman WH, Sautès-Fridman C. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. Clin Cancer Res. 2015;21(13):3031–40.
15. Suzuki A, Masuda A, Nagata H, Kameoka S, Kikawada Y, Yamakawa M, Kasajima T. Mature dendritic cells make clusters with T cells in the invasive margin of colorectal carcinoma. J Pathol. 2002;196(1):37–43.
16. Coronella JA, Spier C, Welch M, Trevor KT, Stopeck AT, Villar H, Hersh EM. Antigen-driven oligoclonal expansion of tumor-infiltrating B cells in infiltrating ductal carcinoma of the breast. J Immunol. 2002;169(4):1829–36.

17. Platonova S, Cherfils-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, André P, Dieu-Nosjean MC, Alifano M, Régnard JF, Fridman WH, Sautès-Fridman C, Cremer I. Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. Cancer Res. 2011;71(16):5412–22.
18. de Chaisemartin L, Goc J, Damotte D, Validire P, Magdeleinat P, Alifano M, Cremer I, Fridman WH, Sautès-Fridman C, Dieu-Nosjean MC. Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Res. 2011;71(20):6391–9.
19. Martinet L, Le Guellec S, Filleron T, Lamant L, Meyer N, Rochaix P, Garrido I, Girard JP. High endothelial venules (HEVs) in human melanoma lesions: Major gateways for tumor-infiltrating lymphocytes. Oncoimmunology. 2012;1(6):829–39.
20. Hindley JP, Jones E, Smart K, Bridgeman H, Lauder SN, Ondondo B, Cutting S, Ladell K, Wynn KK, Withers D, Price DA, Ager A, Godkin AJ, Gallimore AM. T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. Cancer Res. 2012;72(21):5473–82.
21. Coppola D, Nebozhyn M, Khalil F, Dai H, Yeatman T, Loboda A, Mulé JJ. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. Am J Pathol. 2011;179(1):37–45.
22. Messina JL, Fenstermacher DA, Eschrich S, Qu X, Berglund AE, Lloyd MC, Schell MJ, Sondak VK, Weber JS, Mulé JJ. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? Sci Rep. 2012;2:765.
23. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298–306.
24. Goc J, Germain C, Vo-Bourgais TK, Lupo A, Klein C, Knockaert S, de Chaisemartin L, Ouakrim H, Becht E, Alifano M, Validire P, Remark R, Hammond SA, Cremer I, Damotte D, Fridman WH, Sautès-Fridman C, Dieu-Nosjean MC. Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating CD8+ T cells. Cancer Res. 2014;74(3):705–15.
25. Goc J, Fridman WH, Sautès-Fridman C, Dieu-Nosjean MC. Characteristics of tertiary lymphoid structures in primary cancers. Oncoimmunology. 2013;2(12), e26836.
26. Yu P, Lee Y, Liu W, Chin RK, Wang J, Wang Y, Schietinger A, Philip M, Schreiber H, Fu YX. Priming of naive T cells inside tumors leads to eradication of established tumors. Nat Immunol. 2004;5(2):141–9.
27. Thompson ED, Enriquez HL, Fu YX, Engelhard VH. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. J Exp Med. 2010;207(8):1791–804.
28. Peske JD, Thompson ED, Gemta L, Baylis RA, Fu YX, Engelhard VH. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. Nat Commun. 2015;6:7114.
29. Miyagawa S, Soeda J, Takagi S, Miwa S, Ichikawa E, Noike T. Prognostic significance of mature dendritic cells and factors associated with their accumulation in metastatic liver tumors from colorectal cancer. Hum Pathol. 2004;35(11):1392–6.
30. Ladányi A, Kiss J, Somlai B, Gilde K, Fejos Z, Mohos A, Gaudi I, Tímár J. Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. Cancer Immunol Immunother. 2007;56(9):1459–69.
31. Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC, Riquet M, Crozet L, Ouakrim H, Goc J, Cazes A, Fléjou JF, Gibault L, Verkarre V, Régnard JF, Pagès ON, Oudard S, Mlecnik B, Sautès-Fridman C, Fridman WH, Damotte D. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. Clin Cancer Res. 2013;19(15):4079–91.
32. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. Clin Cancer Res. 2008;14(16):5220–7.

33. Ruffini E, Asioli S, Filosso PL, Lyberis P, Bruna MC, Macrì L, Daniele L, Oliaro A. Clinical significance of tumor-infiltrating lymphocytes in lung neoplasms. Ann Thorac Surg. 2009;87(2):365–71.
34. Suzuki K, Kachala SS, Kadota K, Shen R, Mo Q, Beer DG, Rusch VW, Travis WD, Adusumilli PS. Prognostic immune markers in non-small cell lung cancer. Clin Cancer Res. 2011;17(16):5247–56.
35. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer Res. 2010;70(21):8368–77.
36. Nzula S, Going JJ, Stott DI. Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas. Cancer Res. 2003;63(12):3275–80.
37. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pagès F, Speicher MR, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39(4):782–95.
38. Coronella JA, Telleman P, Kingsbury GA, Truong TD, Hays S, Junghans RP. Evidence for an antigen-driven humoral immune response in medullary ductal breast cancer. Cancer Res. 2001;61(21):7889–99.
39. Hansen MH, Nielsen H, Ditzel HJ. The tumor-infiltrating B cell response in medullary breast cancer is oligoclonal and directed against the autoantigen actin exposed on the surface of apoptotic cancer cells. Proc Natl Acad Sci U S A. 2001;98(22):12659–64.
40. Wang Y, Ylera F, Boston M, Kang SG, Kutok JL, Klein-Szanto AJ, Junghans RP. Focused antibody response in plasma cell-infiltrated non-medullary (NOS) breast cancers. Breast Cancer Res Treat. 2007;104(2):129–44.
41. Kotlan B, Gruel N, Zafrani B, Füredi G, Foldi J, Petranyi GG, Fridman WH, Teillaud JL. Immunoglobulin variable regions usage by B-lymphocytes infiltrating a human breast medullary carcinoma. Immunol Lett. 1999;65(3):143–51.
42. Kotlan B, Simsa P, Foldi J, Fridman WH, Glassy M, McKnight M, Teillaud JL. Immunoglobulin repertoire of B lymphocytes infiltrating breast medullary carcinoma. Hum Antibodies. 2003;12(4):113–21.
43. Iglesia MD, Vincent BG, Parker JS, Hoadley KA, Carey LA, Perou CM, Serody JS. Prognostic B-cell signatures using mRNA-seq in patients with subtype-specific breast and ovarian cancer. Clin Cancer Res. 2014;20(14):3818–29.
44. Hansen MH, Nielsen HV, Ditzel HJ. Translocation of an intracellular antigen to the surface of medullary breast cancer cells early in apoptosis allows for an antigen-driven antibody response elicited by tumor-infiltrating B cells. J Immunol. 2002;169(5):2701–11.
45. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348(6230):69–74.
46. Siliņa K, Rulle U, Kalniņa Z, Linē A. Manipulation of tumour-infiltrating B cells and tertiary lymphoid structures: a novel anti-cancer treatment avenue? Cancer Immunol Immunother. 2014;63(7):643–62.
47. Yasuda M, Mizukami M, Hanagiri T, Shigematsu Y, Fukuyama T, Nagata Y, So T, Ichiki Y, Sugaya M, Takenoyama M, Sugio K, Yasumoto K. Antigens recognized by IgG derived from tumor-infiltrating B lymphocytes in human lung cancer. Anticancer Res. 2006;26(5A):3607–11.
48. Mizukami M, Hanagiri T, Shigematsu Y, Baba T, Fukuyama T, Nagata Y, So T, Ichiki Y, Sugaya M, Yasuda M, So T, Takenoyama M, Sugio K, Yasumoto K. Effect of IgG produced by tumor-infiltrating B lymphocytes on lung tumor growth. Anticancer Res. 2006;26(3A):1827–31.
49. Mizukami M, Hanagiri T, Yasuda M, Kuroda K, Shigematsu Y, Baba T, Fukuyama T, Nagata Y, So T, Ichiki Y, Sugaya M, So T, Takenoyama M, Sugio K, Yasumoto K. Antitumor effect of antibody against a SEREX-defined antigen (UOEH-LC-1) on lung cancer xenotransplanted into severe combined immunodeficiency mice. Cancer Res. 2007;67(17):8351–7.
50. Schmidt M, Hellwig B, Hammad S, Othman A, Lohr M, Chen Z, Boehm D, Gebhard S, Petry I, Lebrecht A, Cadenas C, Marchan R, Stewart JD, Solbach C, Holmberg L, Edlund K, Kultima HG, Rody A, Berglund A, Lambe M, Isaksson A, Botling J, Karn T, Müller V,

Gerhold-Ay A, Cotarelo C, Sebastian M, Kronenwett R, Bojar H, Lehr HA, Sahin U, Koelbl H, Gehrmann M, Micke P, Rahnenführer J, Hengstler JG. A comprehensive analysis of human gene expression profiles identifies stromal immunoglobulin k C as a compatible prognostic marker in human solid tumors. Clin Cancer Res. 2012;18(9):2695–703.
51. Nagalla S, Chou JW, Willingham MC, Ruiz J, Vaughn JP, Dubey P, Lash TL, Hamilton-Dutoit SJ, Bergh J, Sotiriou C, Black MA, Miller LD. Interactions between immunity, proliferation and molecular subtype in breast cancer prognosis. Genome Biol. 2013;14(4):R34.
52. Lohr M, Edlund K, Botling J, Hammad S, Hellwig B, Othman A, Berglund A, Lambe M, Holmberg L, Ekman S, Bergqvist M, Pontén F, Cadenas C, Marchan R, Hengstler JG, Rahnenführer J, Micke P. The prognostic relevance of tumour-infiltrating plasma cells and immunoglobulin kappa C indicates an important role of the humoral immune response in non-small cell lung cancer. Cancer Lett. 2013;333(2):222–8.
53. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, Patterson JW, Slingluff Jr CL. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res. 2012;72(5):1070–80.
54. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science. 2005;310(5753):1510–2.
55. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Théry C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med. 1999;189(2):371–80.
56. Baker K, Rath T, Flak MB, Arthur JC, Chen Z, Glickman JN, Zlobec I, Karamitopoulou E, Stachler MD, Odze RD, Lencer WI, Jobin C, Blumberg RS. Neonatal Fc receptor expression in dendritic cells mediates protective immunity against colorectal cancer. Immunity. 2013;39(6):1095–107.
57. Carmi Y, Spitzer MH, Linde IL, Burt BM, Prestwood TR, Perlman N, Davidson MG, Kenkel JA, Segal E, Pusapati GV, Bhattacharya N, Engleman EG. Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. Nature. 2015;521(7550):99–104.
58. Noguchi T, Kato T, Wang L, Maeda Y, Ikeda H, Sato E, Knuth A, Gnjatic S, Ritter G, Sakaguchi S, Old LJ, Shiku H, Nishikawa H. Intracellular tumor-associated antigens represent effective targets for passive immunotherapy. Cancer Res. 2012;72(7):1672–82.
59. Ito T, Saga S, Nagayoshi S, Imai M, Aoyama A, Yokoi T, Hoshino M. Class distribution of immunoglobulin-containing plasma cells in the stroma of medullary carcinoma of breast. Breast Cancer Res Treat. 1986;7(2):97–103.
60. Sieinski W. Immunohistological patterns of immunoglobulins in dysplasias, benign neoplasms and carcinomas of the breast. Tumori. 1980;66(6):699–711.
61. Pekáriková A, Sánchez D, Palová-Jelínková L, Simsová M, Benes Z, Hoffmanová I, Drastich P, Janatková I, Mothes T, Tlaskalová-Hogenová H, Tucková L. Calreticulin is a B cell molecular target in some gastrointestinal malignancies. Clin Exp Immunol. 2010;160(2):215–22.
62. Erić-Nikolić A, Milovanović Z, Sánchez D, Pekáriková A, Džodić R, Matić IZ, Tučková L, Jevrić M, Buta M, Rašković S, Juranić Z. Overexpression of calreticulin in malignant and benign breast tumors: relationship with humoral immunity. Oncology. 2012;82(1):48–55.
63. Suzuki H, Graziano DF, McKolanis J, Finn OJ. T cell-dependent antibody responses against aberrantly expressed cyclin B1 protein in patients with cancer and premalignant disease. Clin Cancer Res. 2005;11(4):1521–6.
64. Hannani D, Locher C, Yamazaki T, Colin-Minard V, Vetizou M, Aymeric L, Viaud S, Sanchez D, Smyth MJ, Bruhns P, Kroemer G, Zitvogel L. Contribution of humoral immune responses to the antitumor effects mediated by anthracyclines. Cell Death Differ. 2014;21(1):50–8.
65. Shalapour S, Font-Burgada J, Di Caro G, Zhong Z, Sanchez-Lopez E, Dhar D, Willimsky G, Ammirante M, Strasner A, Hansel DE, Jamieson C, Kane CJ, Klatte T, Birner P, Kenner L, Karin M. Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. Nature. 2015;521(7550):94–8.
66. Zitvogel L, Kroemer G. Cancer: antibodies regulate antitumour immunity. Nature. 2015;521(7550):35–7.

67. Karagiannis P, Gilbert AE, Josephs DH, Ali N, Dodev T, Saul L, Correa I, Roberts L, Beddowes E, Koers A, Hobbs C, Ferreira S, Geh JL, Healy C, Harries M, Acland KM, Blower PJ, Mitchell T, Fear DJ, Spicer JF, Lacy KE, Nestle FO, Karagiannis SN. IgG4 subclass antibodies impair antitumor immunity in melanoma. J Clin Invest. 2013;123(4):1457–74.
68. Kimura Y, Harada K, Nakanuma Y. Pathologic significance of immunoglobulin G4-positive plasma cells in extrahepatic cholangiocarcinoma. Hum Pathol. 2012;43(12):2149–56.
69. Ziętara N, Łyszkiewicz M, Krueger A, Weiss S. B-cell modulation of dendritic-cell function: signals from the far side. Eur J Immunol. 2014;44(1):23–32.
70. Oaks M, Taylor S, Shaffer J. Autoantibodies targeting tumor-associated antigens in metastatic cancer: Sialylated IgGs as candidate anti-inflammatory antibodies. Oncoimmunology. 2013;2(6), e24841.
71. Ladányi A, Kiss J, Mohos A, Somlai B, Liszkay G, Gilde K, Fejös Z, Gaudi I, Dobos J, Tímár J. Prognostic impact of B-cell density in cutaneous melanoma. Cancer Immunol Immunother. 2011;60(12):1729–38.
72. Linton PJ, Harbertson J, Bradley LM. A critical role for B cells in the development of memory CD4 cells. J Immunol. 2000;165(10):5558–65.
73. Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, Jarrossay D. A functional BCR in human IgA and IgM plasma cells. Blood. 2013;121(20):4110–4.
74. Pelletier N, McHeyzer-Williams LJ, Wong KA, Urich E, Fazilleau N, McHeyzer-Williams MG. Plasma cells negatively regulate the follicular helper T cell program. Nat Immunol. 2010;11(12):1110–8.
75. Nielsen JS, Sahota RA, Milne K, Kost SE, Nesslinger NJ, Watson PH, Nelson BH. CD20+ tumor-infiltrating lymphocytes have an atypical CD27-memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. Clin Cancer Res. 2012;18(12):3281–92.
76. Shi JY, Gao Q, Wang ZC, Zhou J, Wang XY, Min ZH, Shi YH, Shi GM, Ding ZB, Ke AW, Dai Z, Qiu SJ, Song K, Fan J. Margin-infiltrating CD20(+) B cells display an atypical memory phenotype and correlate with favorable prognosis in hepatocellular carcinoma. Clin Cancer Res. 2013;19(21):5994–6005.
77. Hennequin A, Derangère V, Boidot R, Apetoh L, Vincent J, Orry D, Fraisse J, Causeret S, Martin F, Arnould L, Beltjens F, Ghiringhelli F, Ladoire S. Tumor infiltration by Tbet + effector T cells and CD20+ B cells is associated with survival in gastric cancer patients. Oncoimmunology. 2015;5(2), e1054598.
78. Zhu W, Germain C, Liu Z, Sebastian Y, Devi P, Knockaert S, Brohawn P, Lehmann K, Damotte D, Validire P, Yao Y, Valge-Archer V, Hammond S, Dieu-Nosjean MC, Higgs BW. A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased $CD4^+$ T cell receptor repertoire clonality. Oncoimmunology. 2015;4(12), e1051922.
79. Shen P, Fillatreau S. Antibody-independent functions of B cells: a focus on cytokines. Nat Rev Immunol. 2015;15(7):441–51.
80. Inoue S, Leitner WW, Golding B, Scott D. Inhibitory effects of B cells on antitumor immunity. Cancer Res. 2006;66(15):7741–7.
81. Lindner S, Dahlke K, Sontheimer K, Hagn M, Kaltenmeier C, Barth TF, Beyer T, Reister F, Fabricius D, Lotfi R, Lunov O, Nienhaus GU, Simmet T, Kreienberg R, Möller P, Schrezenmeier H, Jahrsdörfer B. Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells. Cancer Res. 2013;73(8):2468–79.
82. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, Malchinkhuu E, Wersto RP, Biragyn A. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting $CD4^+$ T cells to T-regulatory cells. Cancer Res. 2011;71(10):3505–15.
83. Doi T, Kanai T, Mikami Y, Sujino T, Jun L, Ono Y, Hayashi A, Hibi T. IgA plasma cells express the negative regulatory co-stimulatory molecule programmed cell death 1 ligand and have a potential tolerogenic role in the intestine. Biochem Biophys Res Commun. 2012;425(4):918–23.
84. Khan AR, Hams E, Floudas A, Sparwasser T, Weaver CT, Fallon PG. PD-L1hi B cells are critical regulators of humoral immunity. Nat Commun. 2015;6:5997.
85. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000;1(6):475–82.

86. Brown K, Sacks SH, Wong W. Tertiary lymphoid organs in renal allografts can be associated with donor-specific tolerance rather than rejection. Eur J Immunol. 2011;41(1):89–96.
87. Li W, Bribriesco AC, Nava RG, Brescia AA, Ibricevic A, Spahn JH, Brody SL, Ritter JH, Gelman AE, Krupnick AS, Miller MJ, Kreisel D. Lung transplant acceptance is facilitated by early events in the graft and is associated with lymphoid neogenesis. Mucosal Immunol. 2012;5(5):544–54.
88. Haybaeck J, Zeller N, Wolf MJ, Weber A, Wagner U, Kurrer MO, Bremer J, Iezzi G, Graf R, Clavien PA, Thimme R, Blum H, Nedospasov SA, Zatloukal K, Ramzan M, Ciesek S, Pietschmann T, Marche PN, Karin M, Kopf M, Browning JL, Aguzzi A, Heikenwalder M. A lymphotoxin-driven pathway to hepatocellular carcinoma. Cancer Cell. 2009;16(4):295–308.
89. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature. 2010;464(7286):302–5.
90. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol. 2006;7(4):344–53.
91. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J Exp Med. 2012;209(4):729–40.
92. Marinkovic T, Garin A, Yokota Y, Fu YX, Ruddle NH, Furtado GC, Lira SA. Interaction of mature CD3+CD4+ T cells with dendritic cells triggers the development of tertiary lymphoid structures in the thyroid. J Clin Invest. 2006;116(10):2622–32.
93. Furtado GC, Pacer ME, Bongers G, Bénézech C, He Z, Chen L, Berin MC, Kollias G, Caamaño JH, Lira SA. TNFα-dependent development of lymphoid tissue in the absence of RORγt$^+$ lymphoid tissue inducer cells. Mucosal Immunol. 2014;7(3):602–14.
94. Martinet L, Girard JP. Regulation of tumor-associated high-endothelial venules by dendritic cells: A new opportunity to promote lymphocyte infiltration into breast cancer? Oncoimmunology. 2013;2(11), e26470.
95. Geurts van Kessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, Osterhaus AD, Hendriks R, Rimmelzwaan GF, Lambrecht BN. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. J Exp Med. 2009;206(11):2339–49.
96. Halle S, Dujardin HC, Bakocevic N, Fleige H, Danzer H, Willenzon S, Suezer Y, Hämmerling G, Garbi N, Sutter G, Worbs T, Förster R. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. J Exp Med. 2009;206(12):2593–601.
97. Foo SY, Zhang V, Lalwani A, Lynch JP, Zhuang A, Lam CE, Foster PS, King C, Steptoe RJ, Mazzone SB, Sly PD, Phipps S. Regulatory T cells prevent inducible BALT formation by dampening neutrophilic inflammation. J Immunol. 2015;194(9):4567–76.
98. Joshi NS, Akama-Garren EH, Lu Y, Lee DY, Chang GP, Li A, DuPage M, Tammela T, Kerper NR, Farago AF, Robbins R, Crowley DM, Bronson RT, Jacks T. Regulatory T cells in tumor-associated tertiary lymphoid structures suppress anti-tumor T cell responses. Immunity. 2015;43(3):579–90.
99. Riedel S. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent). 2005;18(1):21–5.
100. Winer RL, Hughes JP, Feng Q, O'Reilly S, Kiviat NB, Holmes KK, Koutsky LA. Condom use and the risk of genital human papillomavirus infection in young women. N Engl J Med. 2006;354(25):2645–54.
101. Maldonado L, Teague JE, Morrow MP, Jotova I, Wu TC, Wang C, Desmarais C, Boyer JD, Tycko B, Robins HS, Clark RA, Trimble CL. Intramuscular therapeutic vaccination targeting HPV16 induces T cell responses that localize in mucosal lesions. Sci Transl Med. 2014;6(221):221ra13.
102. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clin Cancer Res. 2011;17(11):3520–6.
103. Lutz ER, Wu AA, Bigelow E, Sharma R, Mo G, Soares K, Solt S, Dorman A, Wamwea A, Yager A, Laheru D, Wolfgang CL, Wang J, Hruban RH, Anders RA, Jaffee EM, Zheng

L. Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. Cancer Immunol Res. 2014;2(7):616–31.
104. Weber J, Thompson JA, Hamid O, Minor D, Amin A, Ron I, Ridolfi R, Assi H, Maraveyas A, Berman D, Siegel J, O'Day SJ. A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma. Clin Cancer Res. 2009;15(17):5591–8.
105. Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, Thomas L, Waterfield W, Schadendorf D, Smylie M, Guthrie Jr T, Grob JJ, Chesney J, Chin K, Chen K, Hoos A, O'Day SJ, Lebbé C. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. Lancet Oncol. 2010;11(2):155–64.
106. O'Day SJ, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, Bondarenko IN, Queirolo P, Lundgren L, Mikhailov S, Roman L, Verschraegen C, Humphrey R, Ibrahim R, de Pril V, Hoos A, Wolchok JD. Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Ann Oncol. 2010;21(8):1712–7.
107. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, Linette GP, Meyer N, Giguere JK, Agarwala SS, Shaheen M, Ernstoff MS, Minor D, Salama AK, Taylor M, Ott PA, Rollin LM, Horak C, Gagnier P, Wolchok JD, Hodi FS. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. N Engl J Med. 2015;372(21):2006–17.
108. Gettinger SN, Horn L, Gandhi L, Spigel DR, Antonia SJ, Rizvi NA, Powderly JD, Heist RS, Carvajal RD, Jackman DM, Sequist LV, Smith DC, Leming P, Carbone DP, Pinder-Schenck MC, Topalian SL, Hodi FS, Sosman JA, Sznol M, McDermott DF, Pardoll DM, Sankar V, Ahlers CM, Salvati M, Wigginton JM, Hellmann MD, Kollia GD, Gupta AK, Brahmer JR. Overall survival and long-term safety of Nivolumab (anti-programmed death 1 antibody, BMS-936558, ONO-4538) in patients with previously treated advanced non-small-cell lung cancer. J Clin Oncol. 2015;33(18):2004–12.
109. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015;348(6230):124–8.
110. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3—potential mechanisms of action. Nat Rev Immunol. 2015;15(1):45–56.
111. McDermott DF, Atkins MB. PD-1 as a potential target in cancer therapy. Cancer Med. 2013;2(5):662–73.
112. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252–64.
113. Martinet L, Filleron T, Le Guellec S, Rochaix P, Garrido I, Girard JP. High endothelial venule blood vessels for tumor-infiltrating lymphocytes are associated with lymphotoxin β-producing dendritic cells in human breast cancer. J Immunol. 2013;191(4):2001–8.
114. Väyrynen JP, Sajanti SA, Klintrup K, Mäkelä J, Herzig KH, Karttunen TJ, Tuomisto A, Mäkinen MJ. Characteristics and significance of colorectal cancer associated lymphoid reaction. Int J Cancer. 2014;134(9):2126–35.
115. McMullen TP, Lai R, Dabbagh L, Wallace TM, de Gara CJ. Survival in rectal cancer is predicted by T cell infiltration of tumour-associated lymphoid nodules. Clin Exp Immunol. 2010;161(1):81–8.
116. Wirsing AM, Rikardsen OG, Steigen SE, Uhlin-Hansen L, Hadler-Olsen E. Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma. BMC Clin Pathol. 2014;14:38.
117. Hiraoka N, Ino Y, Yamazaki-Itoh R, Kanai Y, Kosuge T, Shimada K. Intratumoral tertiary lymphoid organ is a favourable prognosticator in patients with pancreatic cancer. Br J Cancer. 2015;112(11):1782–90.

Chapter 6
Homing Improvement: Boosting T Cell Trafficking for Cancer Immunotherapy

Joseph M. Cantor

Abstract Advances in T cell tumor immunotherapy have raised hopes for this approach to become a significant treatment for a variety of cancers. Recent successes in leukemia using CAR-modified T cells and in metastatic melanoma using tumor-infiltrating lymphocytes have provided impetus to expand adoptive cellular immunotherapy into treatment of solid tumors. In this setting, adoptively-transferred T cells face a hostile tumor environment that suppresses their anti-tumor functions. In addition, T cells activated and expanded outside of the lymph node lack naturally imprinted homing cues and often exhibit poor homing to most sites of tumor growth. This significant problem has limited the application of cellular tumor immunotherapy to a select few malignancies. However, new ideas to improve the migration of transferred T cells have been generated and tested in preclinical models. Supercharging inflammatory migration of T cells is possible by modulating any number of components in the leukocyte migration machinery, from chemo-attractants, to integrins, to extracellular matrix adhesion ligands. Promising results suggest that the homing problem can indeed be overcome to remove a major barrier in allowing cellular tumor immunotherapy to achieve its full potential as a cancer treatment.

Keywords T cell • Immunotherapy • Homing • Trafficking • Integrin • Chemokine • Migration

J.M. Cantor (✉)
Department of Medicine, University of California San Diego, MC 0726, 9500 Gilman Drive, La Jolla, CA 92093-0726, USA
e-mail: jmcantor@ucsd.edu

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_6

Abbreviations

ACT	Adoptive cellular tumor immunotherapy
AML	Acute myeloid leukemia
CAR	Chimeric antigen receptor
CCR9	Chemokine receptor 9
CML	Chronic myeloid leukemia
CRISPR	Clustered regularly-interspaced short palindromic repeats
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
ICAM-1	Intercellular adhesion molecule 1
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IVIG	Intravenous immunoglobulin
LFA-1	Leukocyte functional adhesion molecule 1
NK	Natural killer cell
PD-1	Programmed cell death protein 1
PKA-I	Protein kinase A, Type I
SDF-1α	Stem cell-derived factor 1 alpha
TAA	Tumor-associated antigen(s)
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TIL(s)	Tumor-infiltrating lymphocyte(s)
TLS	Tumor lysis syndrome
TNF-α	Tumor necrosis factor-alpha
TSA	Tumor-specific antigen(s)
VCAM-1	Vascular cell adhesion molecule 1
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very late activation antigen 4

6.1 Introduction

Harnessing the potential of the immune system to fight tumors has long been a goal of the immunology and oncology research fields. Immune-related therapies for cancer have been evolving for 50 years [1], but in the last decade, the promise of tumor immunotherapy has rapidly changed from potential to possibility as exciting clinical trial results have identified several successful approaches [2–9]. At the same time,

significant barriers have come to light that must be overcome before tumor immunotherapy can make a broad contribution to cancer treatment [10–13]. Poor homing of T cells to sights of tumor growth is one of the major difficulties in applying adoptive cellular tumor immunotherapy to solid cancers [7, 12–14]. This review will (1) briefly describe the field of cancer immunotherapy, (2) review current T cell adoptive transfer therapies, (3) define the problem of T cell migration to tumors, (4) outline strategies to improve migration, and (5) propose future directions for research in this area.

6.1.1 *Passive Immunotherapy: Therapeutic Antibodies with Direct Effects on Tumor Cells*

Paralleling the state of immunologic knowledge, early immunotherapies utilized soluble immune-proteins: antibodies. Antibodies were raised in mice to human tumor-specific antigens and developed for infusion to take advantage of antibody effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement fixation, and innate immune cell sensitization. In addition to these "naked" monoclonal antibodies, immunoglobulins have been conjugated to toxins, radioisotopes, and even used as bi-specific reagents to bring relevant immune cells or cytotoxic molecules into close proximity to the tumor (Fig. 6.1) [15]. Anti-tumor

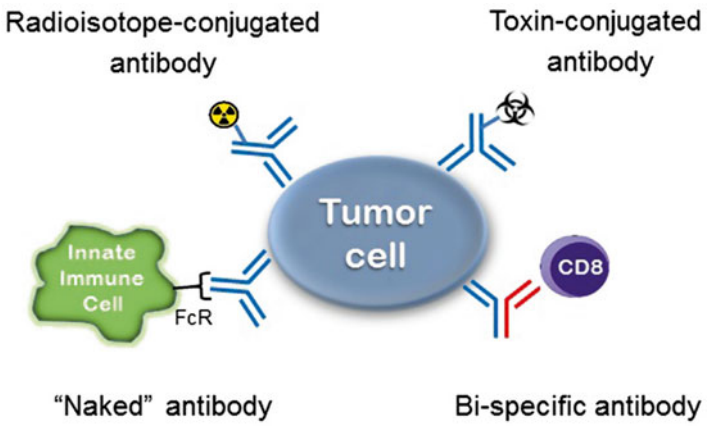

Fig. 6.1 *Immunotherapy using anti-tumor antibodies.* Several forms of monoclonal antibodies have been used for cancer immunotherapy. Each major type depicted uses the specific of the antibody to bring destruction from a different source to the tumor cell. Radioisotope-conjugated antibodies expose rapidly dividing tumor cells to radiation-induced DNA damage. Toxin-conjugated antibodies deliver cytotoxic molecules to the cancerous cell, whereas "naked", or unconjugated, antibodies utilize natural immunoglobulin effector functions to kill tumor cells, including ADCC, complement fixation, opsonization, and sensitization of Fc receptor-bearing immune cells. Bi-specific antibodies bring activate immune cells into close proximity to target tumor cells for recognition and killing

Table 6.1 Anti-tumor antibodies approved for use in the United States

Target	Malignancy
CD20	B cell lymphomas
GD2	Neuroblastoma
EGFR	Colorectal Cancer
VEGFR2	Gastric Cancer
CD52	CML
Her2	Breast Cancer
CD30	Hodgkins' Lymphoma
CD33	AML
CTLA4	**Metastatic Melanoma**
PD-1	**Melanoma**

therapeutic antibodies (typically IgG) generally diffuse easily into inflamed tissues and have not faced significant difficulties getting to the tumor. Rather, the challenges of antibody therapy are finding the ever-elusive tumor-specific antigens (TSA's), early problems with clearance of foreign IgG, and tumor immune evasion via antigen downregulation. To overcome xenogeneic antibody clearance, current generations of therapeutic monoclonal antibodies for tumor treatment are extensively "humanized" or comprised of fully human sequences. Broadly speaking, tumor-specific antibodies aim to either (1) deplete tumor cells through immune mechanisms, (2) block division of tumor cells by downregulating or inhibiting function of critical cell surface proteins, or (3) prevent adhesion/metastasis of tumor cells [16]. Rituximab is the most widely-used anti-tumor therapeutic antibody and has been a highly successful treatment for many B cell malignancies [17]. Since malignant cells evolve resistance and often escape single-antigen blockades, there is a current push to develop individual antibodies that kill cancer cells using multiple mechanisms in a so-called "multipronged" attack, or to use combinations of multiple anti-tumor antibodies on the same disease [2, 18]. Table 6.1 summarizes targets for FDA-approved therapeutic anti-tumor antibodies in use today.

6.1.2 Immunomodulatory Therapy: Antibodies that Modify Immune Responses

Therapeutic antibodies represent a type of immunotherapy that utilizes the remarkable specificity and affinity of the B cell receptor directly; they could thus be considered "passive" immunotherapy. However, the next major goal of oncologists and immunologists was to modulate the natural immune system to actively attack tumors. In most cancers, the immune system is sufficiently present, but fails to mount an aggressive response to the tumor. Various checkpoints on natural T cell-dependent immune responses are hijacked by tumors to inappropriately suppress anti-cancer immunity [11, 19–21]. Following up on this knowledge, several antibodies have targeted immune checkpoints (summarized in [22]). Anti-CTLA-4 removes a "brake"

on initial T cell activation to allow full co-stimulation of antigen-specific T cells. PD-1 is a co-inhibitory receptor that is expressed on "exhausted" effector T cells in a suppressive environment after constant antigen exposure and failed clearance. The success of these two reagents alone, or in combination, for treatment of metastatic melanoma, has highlighted the potential of "checkpoint blockade" as a way to modulate effector immune cells to clear tumors [2]. Depleting immunoregulatory cells (e.g., Treg, Breg) that suppress effector T cells could also become a useful immunomodulatory strategy [23–25]. One more way that antibodies are used as immunomodulatory therapy for cancer is to bring immune cells or cytotoxins into close proximity with tumor cells. These so-called bi-specific antibodies are difficult to produce, but have shown some promise [26, 27]. Overall however, harnessing the specificity of immune receptors using antibody treatment will continue to be a major mode of anti-cancer therapy for the near future since the methods are simple and well-established. The barriers to realizing the full potential of these agents are (1) the cost of producing large quantities of biologics for longer-term treatment (2) the limited number of known tumor-specific antigens, and (3) the loss of antigenic targets within the time frame required for efficacy of passive immunotherapy.

6.1.3 Active (Cellular) Tumor Immunotherapy

Despite early successes in passive tumor immunotherapy, cell-mediated immune responses, both innate and adaptive, have nevertheless been considered the crucial route for immune surveillance and defense against aberrant cell growth (cancer). Harnessing active cellular immunity to prevent and/or eliminate tumors is thus a continuing quest in immunology and oncology [1]. Cell-mediated immunity is initiated by tissue-resident cells that have an "innate" capacity to be activated by pathogen-like features (in the case of dendritic cells, or DC) or missing/altered self (in the case of "natural" killer, or NK, cells). After antigen uptake, DC prime T lymphocytes to mount a full adaptive immune response. Cancerous cells can be recognized in this indirect fashion by T cells (through DC) or be recognized directly by NK cells. In a healthy individual, this system prevents growth of tumors. When cancer does appear, it is thought to represent a breakdown in the ability of DC, T cells, or NK cells to successfully recognize and eliminate the neoplastic growth. Efforts to harness active cellular immunity to cancer have thus focused on boosting activation and function of DC, T, or NK cells [1].

6.1.4 Dendritic Cell Tumor Immunotherapy

Some of the earliest cellular tumor immunotherapies attempted to produce fully activated DC, loaded with tumor antigen and competent to prime efficient anti-tumor T cell responses. DC-based tumor immunotherapy approaches have been most thoroughly tested in melanoma, renal cell carcinoma, brain, and prostate

cancers [28]. Since DC shape the adaptive immune response by the quality of their initial activation, vaccination strategies represent the furthest development of these cells for cancer immunotherapy [9, 28]. Common protocols begin by isolating DC from a patient, activating/maturing them extensively in vitro with pro-inflammatory cytokines, and loading them with tumor peptide antigens in the form of soluble peptides, recombinant protein, whole tumor cells, or peptide-expressing virus or bacteria [29]. The antigen-loaded, mature DC are then re-infused by intravenous, intra-dermal, intra-nodal, subcutaneous, or intra-lymphatic injection [29]. An in-depth discussion of this topic is outside the focus of this chapter; excellent reviews on this topic can be found elsewhere [9, 28, 30, 31]. With a few exceptions, most notably the FDA-approved Sipuleucel-T (Provenge) for prostate cancer [32], the primary emphasis of cellular tumor immunotherapy research has now shifted toward direct manipulation of T cell activity.

6.1.5 T Cell Tumor Immunotherapy: Boosting Endogenous Responses

CD8+ T cells are often considered the primary effector cell of the natural adaptive immune response to cancer. The generation of large numbers of functionally competent CD8 T cells is thus a critical determinant for the successful defense against tumor growth. In the cancer patient, it is assumed that there is a defect in CD8 T cell function that prevents their clearance of tumor cells. One remedial approach is to strongly prime anti-tumor T cell immunity by vaccination with a tumor-specific antigen and a strong adjuvant to activate lethargic endogenous naïve and effector anti-tumor T cells and convert them into efficient killers [33, 34]. Compared to stimulating production of high-affinity antigen-specific antibody, it is less clear how to most efficiently elicit a strong T cell response to a given antigen in humans. The most successful efforts have indirectly boosted T cell responses using DC vaccines (described briefly above). Another way to prime endogenous anti-tumor T cell responses is to deliver an immunogenic form of a relevant tumor-specific antigen (TSA) into the tumor itself [35]. In this way, the immunogenicity of viral vectors in vivo could be a benefit rather than a liability, providing a natural adjuvant. An alternative to optimizing priming signals for effector T cells, endogenous T cells can be expanded by infusion of interleukin-2 (IL-2), an important growth cytokine for T cells and NK cells. Some success was observed in metastatic melanoma with various doses of recombinant IL-2, although side effects were significant and response rates were remained less than 15 % [36]. Other than checkpoint blockade, overall attempts to boost the generation and function of endogenous T cell immunity through vaccination and growth factor treatment have fallen short of expectations. It is possible that this is due to immunosuppressive conditions present both in tumors and lymphoid tissue after tumors have progressed, resulting in insurmountably lethargic endogenous T cells [36]. As a result of this disappointing efficacy, recent attempts have turned toward ex vivo re-programming of T cells under controlled conditions.

6.1.6 T Cell Tumor Immunotherapy: Adoptive Cellular Immunotherapy (ACT)

In adoptive cellular immunotherapy, T cells (or NK in rare cases) are isolated from the affected patient, activated, expanded, and re-infused [3, 37, 38]. These methods have several advantages. First, lymphocytes can be "reprogrammed" in vitro in the absence of suppressive signals present in the cancer patient and, instead, in the presence of growth and inflammatory cytokines. Expansion and cytotoxic activity of tumor-infiltrating T cells can often by recovered after they are removed from hypoxia, inhibitory ligands, immunosuppressive cytokines, barren extracellular matrix, and regulatory T cells that characterize the tumor environment. In the place of this immunosuppression, T cells are strongly activated using anti-CD3/28 stimulation and expanded in the presence of large amounts of IL-2. Within these protocols, T cells can be genetically altered in vitro with a nearly unlimited array of stimulatory or cytotoxic molecules, specifically increasing the potency of these cells without collateral damage to sensitive tissues [39, 40]. By way of contrast, modification of endogenous T cells in the host has largely ground to a halt due to the potential of unpredictable immune reactions to some viral vectors. Finally, in ACT protocols, harvest of autologous T cells and expansion ex vivo provides a window of time during which the remaining endogenous immune system can be therapeutically modulated to provide a receptive setting for re-infused effector T cells. For example, non-myeloablative short-term chemotherapy or total body irradiation has greatly increased response rates to ACT using TIL for treatment of metastatic melanoma [3, 36].

6.1.7 Adoptive Cellular Immunotherapy Using TILs

Two main sources of anti-tumor cells have been investigated for autologous re-infusion: (1) tumor-infiltrating lymphocytes (TIL) or (2) peripheral T cells modified with tumor-specific antigen receptors. In the first approach, TILs are isolated from surgically resected tumors, expanded, and re-injected [6, 8]. When tumor fragments are grown in IL-2, the surviving/proliferating cells represent a nearly pure population of tumor-infiltrating CD4+ and CD8+ T cells. Tumor-specific T cells can then be identified by reactivity to tumor cell targets. A portion is typically frozen as a cellular back-up, and the remainder is expanded in IL-2 and re-injected into the patient 5–6 weeks later. For reasons that are not entirely clear, the ability to grow out tumor-specific TIL for immunotherapy has been largely limited to melanoma [3]. One possible explanation for the unique sensitivity of metastatic melanoma to immunotherapy may have to do with its rather high mutation frequency, providing a large pool of tumor-specific antigens along with infiltrating T cells that are specific for these antigens. There is now a large effort to identify the less frequent mutations in other solid cancers and construct corresponding peptides to expand

mutation-specific TIL that are quite scarce in these tumors. Exome sequencing of normal vs. tumor tissue is a newer technology that may allow the identification of possible mutant antigens for expansion in an individual patient, bringing the concept of personalized medicine to cellular tumor immunotherapy [3]. For metastatic melanoma, however, there has been steady progress in the efficacy of TIL-based ACT. Since the addition of pre-conditioning chemotherapy and/or total body irradiation (TBI), 40–50 % Overall Response (OR) rates are now observed at some of the most successful centers in treating highly advanced melanoma and ~20 % of patients experienced durable complete regression. Although checkpoint blockade has already made a major impact on treatment of advanced melanoma, TIL–based cell infusion seems likely to join this rank in the near future.

6.1.8 Adoptive Cellular Immunotherapy Using CAR-Modified T Cells

An alternative cellular immunotherapy strategy re-directs polyclonal circulating peripheral T cells by introducing high affinity tumor-specific antigen receptors and co-stimulatory molecules before re-infusion [7, 38, 40]. These synthetic chimeric antigen receptors (CARs) are typically composed of single-chain variable regions (scFv) of a proven tumor-specific antibody, fused to portions of a T cell receptor signaling complex. They can thus recognize a tumor-specific antigen in an HLA-independent fashion, which theoretically sidesteps tumor cell immune evasion caused by antigen processing variation. Lack of MHC restriction also raises the possibility of universal (allogeneic) CAR T cells if the endogenous lymphoid compartment is depleted prior to T cell infusion, although this may require silencing of the potentially alloreactive original TCR in the CAR T cell. CARs are now in their 3rd or 4th iteration of composition, mostly for receptor length and presence of co-stimulatory signaling sequences [38–42]. As noted above, it is possible to introduce other proteins to the T cells in the same setting that CAR's are added, in efforts to boost effector function. Alone these lines, "armored" CAR T cells engineered to co-express IL-12 show evidence of increased resistance to suppression/exhaustion in pre-clinical models [39, 40]. This demonstrates that modification of tumor-specific T cells with carefully chosen genes can overcome some of the weaknesses in anti-tumor T cells and turn them into more effective tumor killers. Delivery of genes encoding CARs and other important proteins into T cells in vitro using lentivirus or gammaherpes viruses is now fairly safe and efficient [43], although alternative modes exist [44], including electroporation, and more recently, zinc-finger nucleases or Cas/CRISPR gene editing. After polyclonal T cells are isolated from circulation, stimulated with anti-CD3/CD28 beads, and modified with CAR (and genes for other proteins of interest), they are expanded in IL-2 and re-infused. Adoptive transfer of chimeric antigen receptor-modified T cells has received much recent attention due to a number of encouraging results in clinical trials for leukemia/lymphoma [4, 5, 45–48]. Durable remissions have been seen in a number of patients, and transferred CAR T cells have persisted for many years [43]. The choice of which T cell subset(s)

to modify and re-infuse is a relevant question with the CAR approach, and recent work seems to have identified the need for a blend of central memory CD8+ T cells (T_{CM}) with naïve CD4+ cells for maximum effectiveness [49–51]. This knowledge, and the remarkable capacity of CAR T cells to expand, persist, and continue to express CAR after adoptive transfer for >10 years [43], has allowed the T cell number needed for transfer to be lowered from 5×10^9 to 5×10^6 cells/patient [49, 52]. Thus, tumor immunotherapy with CAR T cells is likely to become an effective option for non-melanoma tumors where antigenic targets exist that are either tumor-specific or are expressed on tumors and non-essential tissues (e.g., CD19).

6.2 Barriers to Cellular Tumor Immunotherapy

Given the recent explosion in the development and initial success of ACT, it seems poised to become a major therapeutic, or even curative, treatment for some cancers. However, as this therapeutic strategy has been developed and implemented, several significant barriers have become clear that must be overcome for ACT to realize its full potential (Table 6.2). Some of these challenges are inherent in the approach, others have been partially addressed, but several major hurdles remain that require creative solutions.

6.2.1 Identification and Prioritization of Tumor-Specific Antigens (TSA)

An inherent challenge to tumor immunotherapy is the same immense task that the immune system faces daily: distinguishing self from non-self (or altered self). As we attempt to re-program and guide immune molecules and cells, we face the same

Table 6.2 Barriers to effective ACT

Cell immunotherapy barrier	Affects	Solutions (realized or potential)	Refs
Lack of Tumor-specific Antigens	CAR, TIL	Exome sequencing to ID mutant self-antigens in tumors	[3]
Insufficient tumor-specific T cells	TIL	IL-2, pre-conditioning/lymphodepletion	[36]
Integration-related transformation	CAR	Newer viral vectors, Non-viral delivery methods	[10, 43]
Excessive persistence of infused T cells	CAR	Incorporate suicide genes; use short lived cells (NK)	[10, 43, 53]
On-target toxicity	CAR	Delete endogenous TCR; use dual CAR	[10]
Tumor Lysis Syndrome	CAR	Anti-IL-6, Anti-TNF treatment; use minimal CAR T cells	[51, 54, 55]
Suppressive tumor microenvironment	TIL, CAR	Combine w/ checkpoint blockade, use "Armored CARs"	[39, 40]
Poor migration of T cells to solid tumors	CAR, TIL	(See strategies for improving tumor homing below)	[13, 56–72]

dilemma: how do we identify antigens that are unique to tumors, and whose targeting would not harm essential tissue? This problem has hampered antibody-based immunotherapy and similarly limits the repertoire of effective antigen receptors that can be used in CAR T cell therapy. Cutting edge sequencing technologies may be used to identify mutated self-antigens or antigens non-essential to normal tissues [3]. In cancers where identification of TSAs is impossible or impractical, forms of immunotherapy may need to be used that leave this to endogenous immune mechanisms, such as checkpoint blockade, or TIL-based cellular immunotherapy.

6.2.2 Generation and Persistence of Sufficient Tumor-Reactive T Cells

A difficulty encountered early on in cellular immunotherapy was an inability to generate large numbers of tumor-reactive effector T cells from tumors for re-infusion. This is partly due to central and peripheral tolerance mechanisms that protect from autoimmunity but can also weaken responses to tumor cells that represent altered self. IL-2 seemed to largely address the problem of low numbers since it can stimulate such a large expansion of activated T cells [36]. Although tumor-reactive T cells are a rare cell within tumors, culture in IL-2 allows the CD4 and CD8 T cells present to outgrow and kill the tumor cells in primary culture and permits expansion of tumor-reactive lines in the second culture step of TIL-based immunotherapy. Injection of recombinant IL-2 can even boost expansion of tumor-reactive TILs after infusion. Unfortunately, generating enough tumor-reactive TIL from cancers other than melanoma has been limiting and will require more creative solutions, such as the exome sequencing for antigens mentioned above [3]. For CAR immunotherapy, tumor-specific T cells are not limiting as their specificity is generated artificially, and these, too, can be further expanded in IL-2 before re-infusion [7, 38, 40]. Maintenance of transferred T cell numbers was also a significant problem, at least in the case of TIL therapy. However, provision of IL-2 and inclusion of lympho-depleting treatment prior to T cell infusion creates a niche and an available cytokine pool for survival and expansion [36]. Transferred CAR T cells do not seem to suffer poor in vivo persistence, perhaps because they are stimulated with so many targets when used as a treatment for leukemias [43]. On the contrary, the outstanding survival and expansion in vivo is itself a potential challenge for CAR T cell therapy to provide an optimal long-term outcome for the patient (see below). It will be interesting to see if CAR T cells persist and expand as dramatically when CAR T cell therapy is applied to solid tumor cancers with scarcer and less accessible antigens.

6.2.3 Safety: Integration-Related Transformation

Since retro- and lenti-viruses are commonly used for gene delivery in creating CAR T cells, insertional mutagenesis is a concern. This worry is partly based on leukemias observed following early attempts at viral gene therapy for SCID-X1 [73].

However, no suspected retrovirus-related leukemias have been observed in >200 patients now infused with CAR T cells [43]. These recipients were further analyzed for significant clonal expansions within the transferred CAR T cell population using two methods and no evidence of such expansions was found [43]. While the potential for transformation always exists, it appears that modern retroviral vectors have largely overcome the risk of insertional mutagenesis. Nevertheless, this risk may be gene-dependent and could thus vary as additional genes are delivered in conjunction with chimeric receptors. It is therefore prudent to continue developing targeted gene delivery strategies using zinc-finger nucleases or Cas/CRISPR technology, along with the inclusion of suicide genes as outlined below [10].

6.2.4 Safety: Excessive Persistence

Following the incorporation of co-stimulation, IL-2, and lymphodepleting preconditioning into protocols, CAR T cells were observed to persist long-term, and may do so indefinitely [43]. In some patients, the transferred T cells occupied the majority of the CD8 subset. After tumor clearance, long-term persistence of CAR T cells at high levels might present some problems. First, this level of "clonal" dominance is likely to lessen the efficiency of cell-mediated immunity, as oligoclonal T cell repertoires in the elderly correlate with, and may be the cause of, declining immune function [74]. Second, the long-term presence of effective CAR T cells prevents recovery of healthy cells/tissues that were targeted by virtue of shared antigen expression. For example, successful CD19 CAR T cell therapy generally results in a long-term loss of most B cells; however, monthly injections of IVIG can mostly compensate for protective B cell immunity [10]. Third, the long-term presence of dual-TCR cells raises the risk of possible autoimmunity due to activation/differentiation signals provided by the CAR when a possibly autoreactive 2nd TCR could still be present and available for targeting sensitive self-antigen.

Two possible remedies have been proposed to address these concerns. One is to use a more transient cell population such as effector-memory T cells as CAR T cells; the other idea is to develop CAR NK cells, which are potent tumor-killing cells, but do not survive long-term [53]. Both of these approaches may hold promise, but are in the early stages of development and may limit the effectiveness of CAR T therapy. The most attractive strategy to address over-persistence (and long-term safety) is to introduce a suicide gene into CAR T cells. Following this idea, when the tumor cells are clearly gone, a specific reagent could be given that the CAR T cells would respond to and be eliminated. HSV-TK was the first suicide gene tested for CAR T cell removal in response to ganciclovir; unfortunately HSV-TK may suffer from immunogenicity due to recognition as a foreign protein [10]. Other options include: (1) iCasp9 (activated by a small molecule drug), (2) a chimeric protein containing anti-CD34 and anti-CD20 antibody epitopes (for selection with anti CD34 and deletion with rituximab), or more recently, (3) a truncated EGF receptor (EGFR) used with approved anti-EGFR antibody [48]. With

these options in hand, new protocols will soon offer a patient the choice to remove CAR T cells if exhibiting unequivocal absence of disease for a pre-determined period of time.

6.2.5 *Safety: Off-Target Damage*

Damage to essential tissue due to shared antigens ("on-target toxicity") is another safety concern in adoptive cellular immunotherapy, particularly in using CAR T cells [75–78]. This is partly related to the paucity of tumor-specific antigens; when tumor-associated antigens (TAA) are the only choice, collateral damage can be anticipated. Continued efforts to identify the best TAA, or if possible TSA, using new molecular technologies should gradually lessen this problem. Another clever approach is to introduce 2 CARs [10]. A CAR specific for one TAA could contain the co-stimulatory moieties, while another CAR specific for another TAA transmits the antigen receptor signal (TCR ζ chain). Full activation would only be given in the presence of both antigens, a less likely scenario in normal tissue. A second version of this concept combines two antigen-binding domains in one CAR that would only be fully activated when both antigens are bound. A further way to utilize dual CARs to avoid on-target toxicity is to use one regular full-activation CAR specific for TAA in conjunction with an inhibitory CAR (iCAR) specific for an antigen expressed in normal tissue that the TAA is frequently expressed in. One of these possible solutions seems likely to lessen the problem of CAR on-target toxicity. However, the endogenous TCR present in CAR-modified T cells can also cause "second-target" toxicity if it is self-reactive. In this scenario the CAR T cell is activated/differentiated in vivo due to the CAR receptor signal. Thereafter, if it encounters the natural TCR self-antigen, it may precipitate autoimmunity. So far this has not been a major problem and the option to delete endogenous TCR while adding CAR is always an option [10].

6.2.6 *Safety: Tumor Lysis Syndrome*

A final and serious safety concern is tumor lysis syndrome, primarily seen in CAR therapy for leukemia with heavy tumor burden. Tumor lysis syndrome (TLS) is a life-threatening shock-like condition caused by the release of pro-inflammatory cytokines when CAR T cells rapidly kill a large number of (circulating) target tumor cells [51]. The sudden release of intracellular ions and metabolic byproducts apparently prompts a chaotic response from the liver as it processes the debris. Although it is a critical complication, TLS seems to occur in a predictable time window after CAR T cell therapy, and careful monitoring for metabolic and renal symptoms, combined with appropriate and immediate cytokine blockade has lessened the dangers of TLS [51, 54, 55]. Moreover, recent CAR protocols that use up to 1000-fold

fewer T cells should allow a more gradual tumor cell elimination, less bystander activation, and ultimately less cytokine release [79].

6.2.7 *Suppressive Tumor Microenvironment*

A large barrier to both natural anti-tumor immunity and to therapeutic immunotherapy is the hostile extracellular environment of the tumor [12]. Partly an immune evasion strategy and partly a result of tumor cell metabolism, the "suppressive tumor microenvironment" refers to conditions within a tumor that dampen or shutdown protective T cell immunity. This occurs through a number of mechanisms, the details of which are beyond the scope of this chapter; rather, a brief summary will be offered [80, 81]. First, the unusual cellular metabolism of tumor cells creates a hypoxic environment, which is particularly challenging for effector T cells and inhibits their anti-tumor function. Second, the recruitment and activity of immunosuppressive leukocyte subsets such as regulatory T cells (Treg) and/or myeloid-derived suppressor cells (MDSC) dampen cell-mediated immunity [23, 24]. The roles of these subsets in effector T cell proliferation have been well-described and are an important natural counterbalance against unrestrained inflammation or autoimmunity. However, they are commandeered by tumors in their defense against anti-tumor immunity [80, 81]. Similarly, a wide range of co-inhibitory receptors govern natural cell-mediated immunity and function as natural "check-points" for T cell activation and function. CTLA-4 directly blocks co-stimulation during early T cell activation, while PD-1 and many other proteins provide negative regulation during later stages of T cell differentiation and effector function. Blockade of CTLA-4 or PD-1 signals appears to partly overcome the "exhausted" phenotype of tumor-specific T cells seen in cancer patients, particularly within tumors [2]. Anti-CTLA-4 and/or anti-PD-1 antibodies have indeed become valuable therapeutics for the treatment of advanced melanoma and represent a weapon to battle tumor-mediated immunosuppression [2, 22]. A major way that tumors co-opt immune checkpoints is through expression of ligands for T cell co-inhibitory receptors, and blockade of these ligands may also release T cell anti-tumor immunity [2, 11, 20, 21]. Avenues to specifically inhibit Treg and MDSC are also in current high demand [23, 24], since they represent the main leukocyte sources for immunosuppression within the tumor. Re-programming the extracellular tumor environment may also be possible. CAR T cells that secrete pro-inflammatory cytokines (IL-12 or IFN-γ) under the control of CAR signaling have been engineered and are currently in testing. These "Armored CAR's" [40] or "Trucks" [39] carry their anti-suppressive products with them to be released in the tumor environment and away from sensitive tissues. IL-12 secretion within the tumor also recruits pro-inflammatory macrophages and activates their anti-tumor functions [82]. Even while much progress is being made to reverse tumor immunosuppressive mechanisms, circumspection is required, as unregulated immune activation may result in auto-immune or pathological inflammatory reactions [83].

6.2.8 Poor Homing to Tumors

Since ex vivo-modified T cells have been activated and expanded outside the lymph node environment, they lack naturally-imprinted homing cues and exhibit relatively poor homing to sites of solid tumor growth [7, 14, 84, 85]. This is a particularly significant problem for CAR T cell immunotherapy and to date has largely confined its application to hematological malignancies [7, 12, 86]. In order to understand why this barrier exists and begin to formulate strategies to surmount this obstacle, a brief overview of T cell trafficking to inflamed tissue is necessary.

6.2.9 T Cell Trafficking During Inflammation

The general circulation pattern of central memory (T_{CM}) and naïve T cells throughout the body is from blood, across high endothelial venules (HEV) into lymph nodes, through T cell zones, out via efferent lymphatics, and eventually back into the blood through the thoracic duct [87–89]. If circulating T_{CM} and naïve T cells encounter their cognate antigenic peptide presented on a mature dendritic cell as they pass through lymphoid T cell zones, they stop circulating and begin the transition to effector T cells. During this activation and differentiation period (2–5 days), antigen-specific T cells are essentially trapped in the lymph node and guided by signals from the antigen-presenting cell (APC, usually DC for naïve T cells) for cues as to (1) what effector subset to become, and (2) what tissue(s) to migrate to when they leave [89]. Once the responding T cell (CD4+ or CD8+) is activated, it undergoes rapid clonal expansion (up to 100,000-fold), and differentiates into an army of the appropriate effector cells [90]. The activated, antigen-specific effector T cells then exit the lymph node via the efferent lymph and re-enter the bloodstream, this time with a different adhesion molecule pattern that enables them to extravasate into inflamed tissues [91]. Once they have left the bloodstream, they follow gradients of chemoattractants to the site of infection where they should re-encounter antigen in the form of infected or altered cells [92]. Once they are re-activated by antigen, they kill or secrete cytokines until antigen is cleared, which signals clonal contraction [93]. A small fraction of responding antigen-specific cells remain as memory cells, either in local tissues as "effector memory" cells (T_{EM}) capable of short-term immediate protection, or as a circulating "central memory" subset (T_{CM}) that must be re-activated, differentiated, and expanded for efficient immunity [93].

Adhesion molecules are critical for nearly every stage of the T cell immune response. A wide array of chemoattractants and adhesion receptors control the circulation of naïve T and T_{CM} cells, positioning/cell contact/retention within lymph nodes, exit from nodes, trafficking to inflamed endothelium, extravasation, and migration within tissues [94]. We will primarily consider chemokine signaling and integrin-dependent migration. Chemokines/receptors and integrins act in concert with a complex signaling network and structural proteins to provide firm extracellular attachment,

traction, and directional migration [95, 96]. Chemokines are small (~10 kD) soluble proteins that bind to specific G-protein-coupled receptors and stimulate localized activation of integrins through the activity of small GTPases (such as Rap1) that promote binding of talin to integrin cytoplasmic tails [94, 96, 97]. Integrins are transmembrane heterodimeric proteins comprised of α and β chains that connect extracellular ligands to the cytoskeleton [98]. When integrins are in an inactive conformation, they cannot bind ligand with significant affinity. After intracellular signals are received from chemokine receptors, integrin β chains are re-positioned via the binding of talin to the cytoplasmic tail of the integrin β chain, allowing efficient binding of extracellular ligand [99]. This is often termed "inside-out" signaling, or "integrin activation," [100, 101] which can be contrasted with "outside-in" signals [102] transduced from integrins after ligand binding that result in cell attachment, survival, proliferation, migration, and even T cell effector function [103, 104].

Dendritic cells "imprint" different combinations of chemokine receptors and integrins on T cells during activation to provide specific guidance cues that localize effector T cells to appropriate tissues [105–108]. After exit via lymph to the bloodstream, effector T cells encounter corresponding chemokines and integrin ligands that provide additional specificity and directional cues for homing to T cell targets [94]. The provision of retinoic acid (Vitamin A) from DC prompts expression of α4β7 integrin and the chemokine receptor CCR9 on effector T cells, which results in homing to mucosal tissues [109]. Activated T cells are directed to most other tissues via expression of α4β1 (VLA-4). The presence of Vitamin D during activation instructs T cells to home to the skin through expression of CLA, E-selectin, and the chemokine receptor CCR10 [110, 111]. Other adhesion molecules have been shown to be important for migration to different tissues in specific conditions [112], but the exact chemokine receptor + integrin "zip codes" for every tissue have not been fully deciphered [113].

As they pass near inflamed tissues, effector T cells encounter immobilized chemokines and other adhesion molecules on activated endothelium that capture the cells from circulation via the well-described steps of the "adhesion cascade," comprised of rolling, tethering, firm adhesion, and extravasation [94, 95]. After they pass between or through endothelial cells, T cells must transit the pericyte layer that surrounds blood vessels on the baso-lateral surface. Once in the tissues, effector T cells may migrate via classical integrin-dependent chemotaxis in response to complex chemokine gradient vectors or by using amoeboid migration. Many chemoattractants, both classical chemokine proteins and lipid chemoattractants, contribute to extravasation and intra-tissue migration, in addition to an ever-growing list of other adhesion molecules [94]. In the case of chemotaxis, it appears that migrating T cells must integrate complex vectors of chemokine gradients to home within tissues [114]. It should be noted that in comparison to the complexity of chemokine receptors the migration of effector T cells depends heavily on comparatively few integrin heterodimers: α4β7 (LPAM-1), α4β1 (VLA-4), and $\alpha_L\beta2$ (LFA-1) [95]. This suggests that control of individual chemokine receptors could govern migration to very specific destinations, while changes in the function of particular integrins would be predicted to have broader effects.

6.2.10 T Cell Trafficking to Tumors

T cell migration to sites of neoplastic growth could be expected to follow that of homing to tissues inflamed via infection or other tissue pathology. However, tumors present unique challenges to the migrating T cell [115]. First, there may be a disorganization of normal "danger" signals that an invading pathogen normally provides to provoke the release of chemoattractants and upregulation of adhesion molecules on tissue-resident leukocytes and endothelial cells [116]. Secondly in normal infections, neo-angiogenesis and lymphangiogenesis provide highways for rapid flow of antigens and APC to lymph nodes and efficient homing of effector cells to sites of inflammation; tumors have instead hijacked this route for metastasis [117, 118]. Moreover, tumor growth results in excess VEGF and abnormal blood vessels that express sparse adhesion molecules [115]. Third, tumors can directly degrade chemoattractants, blocking the recruitment of effector T cells [56]. Many other aspects of the tumor environment [80, 81] affect T cell homing, such as hypoxia and the presence of anti-inflammatory cytokines that antagonize the effects of TNF-α, etc. on endothelium. Finally, the migration of leukocytes to inflamed tissues is often tuned to occur in waves of different immune cell subsets, with the function of initial waves helping recruit secondary assaults. When primary infiltrating immune cells (myeloid or lymphoid) are suppressed or polarized away from an "inflammatory" phenotype, further infiltration suffers [119]. There are also likely, as of yet unknown, ways that cancers inhibit migration of anti-tumor T cells to growing tumors, but overall it is clear that poor migration of effector T cells is a major barrier for the immune response to cancer [7, 13, 14, 84, 85, 120]. This significant challenge is amplified in adoptive cellular tumor immunotherapy when T cells are activated and differentiated/modified apart from natural migrational imprinting. Thus, improving the migration of adoptively transferred tumor-specific T cells should be a major goal for optimizing cellular tumor immunotherapy.

6.3 Strategies to Overcome Poor T Cell Migration to Tumors

Since T cell inflammatory migration is governed at so many levels, there are numerous ways to potentially enhance tumor homing. Some approaches involve acute systemic delivery of an agent shortly before or after adoptive T cell transfer, others seek to inflame or otherwise modify the tumor to improve T cell recruitment, and still others take advantage of the opportunity to upgrade the homing capacity of T cells ex vivo during their activation and expansion. Table 6.3 summarizes current strategies under development or clinical testing to improve T cell tumor homing.

Table 6.3 Strategies for improving T cell tumor homing

Approach	Strengths	Weaknesses	Refs
Inhibit chemokine degradation	Simple when drug is available	Limited applicability; side effects?	[56]
Administer hyperthermia	Simple technology	Limited data; need to control	[60–62]
Guide inflammatory cytokines	Simple, multiple benefits	Effects on other tissues	[13, 59]
Chemotherapy/local irradition	Treatments already approved	Limited data; radiation risks	[58, 63, 64]
Express chemokine in tumors	Highly specific	Difficult to achieve	[65, 66]
Express chemokine receptor in T cells	Promising results	May require customization	[57, 67–71]
Boost integrin function	More universal treatment	Limited data; other tissue infiltration?	[72]

6.3.1 Systemic Treatments

Non-cellular systemic approaches to improve tumor homing of transferred T cells have practical appeal and are easily combined with other migration enhancements. One strategy is to counter one of the tumor's T cell infiltration blockade mechanisms. Along these lines, a recent study reported that some tumors use dipeptidyl-peptidase-4 (DPPT-4) to degrade CXCL10 and prevent CXCR3-dependent infiltration of lymphocytes [56]. Blocking this degradation using a drug approved for another disease increased lymphocyte infiltration and decreased tumor growth in pre-clinical models for melanoma and colon carcinoma. The authors also found that this therapy could synergize with checkpoint blockade, adoptive cellular immunotherapy, and adjuvant-based immunotherapy, which raises its appeal [56]. It is, however, currently unclear how many different types of tumors use DPPT-4. These is also some evidence that checkpoint blockade itself [57], and certain chemotherapies [58] can delivery pro-inflammatory signals that increase recruitment of T cells to tumors. Along these lines, injection of a pro-inflammatory cytokine, TNF-α, modified for tumor vasculature selectivity, shows evidence of increasing lymphocyte migration across the tumor vasculature and combines with anti-CTLA-4 to reduce the growth of ovarian cancer in a mouse model [13, 59]. Another systemic approach to improve T cell tumor homing is to utilize the febrile response. For many years, the immune-biological purpose of fever was unclear. Recently, however, it was demonstrated in animal models that fever-range hyperthermia increases the density of adhesion molecules and vastly increases T cell migration to sites of tissue injury or infection [60]. There are now efforts to translate this finding into the cancer setting [61]. In the mouse B16 melanoma model, adoptively transferred tumor-specific T cells home to tumors *much* more efficiently when the mice are subjected to

fever-range hyperthermia prior to transfer [62]. It is currently unknown whether this type of therapy would need to be applied systemically, or whether the cancerous organ/tissue could be heated locally to obtain the same result. If local hyperthermia could be applied simultaneous to TIL or CAR T cell infusion, it is possible that more efficient homing could result. If any of these systemic strategies proves successful, it is likely to be combined with other strategies outlined below.

6.3.2 Tumor Modifications

Directly altering tumors to elicit greater effector T cell infiltration is an attractive strategy to avoid systemic side effects that could cause inflammation of other important tissues. The difficulty in this setting is the genetic instability and multiple suppressive mechanisms that tumors employ to resist protective inflammation. However, local irradiation and certain chemotherapies can cause local release of pro-inflammatory cytokines and chemokines that raise the rate of extravasation and tissue migration of T cells [58, 63, 64]. Radiation therapy has its own drawbacks though, and the existence of multiple metastases makes application difficult. Nevertheless, it is possible that increasing infiltration of a primary tumor could "prime the pump" and serve to stimulate a greater tumor-specific response throughout the body due to cell death and release of more tumor antigens to be carried to other lymph nodes. Another possibility is to use an oncolytic virus (suc as Ad5Δ24) to express cytokines and chemokines in tumor cells to draw in and permit the survival of CAR T cells in tumors [65]. The benefit of this technique is that the location of chemokine gradients should precisely match up with actively-dividing tumor cells. The challenges to viral chemokine expression in tumors may lie with choice of virus for tropism, robust infection, and safety. As with any other tumor modification, genetically unstable tumor cells may mutate and be selected for low chemokine expression. An interesting investigation examined the relative importance of various chemokines secreted by different tumors in eliciting infiltration of T cells [66]. This suggests that the chemokine to be expressed in tumors needs to be carefully chosen for maximum impact on T cell tumor homing.

6.3.3 Expressing Chemokine Receptors on T Cells

Modifying T cells with the receptors for chemoattractants secreted within tumors has attracted investigation as a reasonable way to direct the migration of tumor-specific T cells to sites of tumor growth. A study in 2002, years before the modern explosion of cellular immunotherapy development, established a model for expressing chemokine receptors in T cells to improve tumor-specific homing [67]. The investigators carefully selected a receptor lacking in most activated human T cells for a chemokine that was present in a variety of tumors (Gro-α, CXCL1) and was

not secreted by T cells themselves. The study showed that expression of CXCR2 in human T cells conferred responsiveness to CXCL1, in terms of signaling, cytokine secretion, and chemotaxis. No investigation of in vivo activity was performed in this study, but it showed that ectopically expressed chemokine receptors could function normally in human T cells and could increase their chemotaxis and effector function(s).

Others have since reported improving adoptive cellular immunotherapy in preclinical models by expressing various chemokine receptors in adoptively-transferred tumor-specific T cells [57, 68, 71]. Di Stasi and colleagues were first to show an increase in tumor homing and anti-tumor activity by adding a chemokine receptor to a CAR-modified T cell. Using T cells directed toward CD30 for Hodgkin's Lymphoma (HL), they showed that CCR4 expression enhanced effector function in vitro and controlled tumor growth in a xenograft model using human HL [70]. Once again, a chemokine receptor was selected that was lacking in effector CD8+ T cells, but whose ligand was present in lymphoma tumors. Of note was the fact that the most dramatic migration and tumor protection results were seen when the relevant chemokine, TARC (CCL17), was transgenically expressed in tumor cells. Two studies in 2010 reported similar improvements in tumor homing and tumor protection, the first by adding CCR2b with a GD2-specific CAR to target a neuroblastoma cell line tumor [69]. The second utilized two melanoma models to show that CXCR2 expression increased the homing of TCR transgenic T cells to tumors expressing the cognate CAR antigen [121]. A further step forward was achieved when Moon and colleagues expressed CCR2b in CAR mesothelin-specific T cells and showed that a single injection of CCR2b+mesothelin-CAR T cells had much better homing to large established pleural mesothelioma tumors [71]. The CCR2b+mesothelin-CAR T cells also demonstrated significantly greater control of tumor growth than those lacking CCR2b. A more recent study also introduced CCR2 into T cells, this time in conjunction with a novel retroviral delivery of a WT1 peptide-specific TCR [68]. These authors also observed increased migration and anti-tumor activity, and documented that transgenic CCR2 could boost TCR-dependent T cell functions. This highlights a possible benefit to upgrading chemokine signaling beyond chemotaxis. Interestingly, there are recent efforts to utilize NK cells for shorter-term CAR immunotherapy, and their migration and anti-tumor function can also benefit from the introduction of chemokine receptors [122], signifying the broad appeal of chemokine receptor provision to improve tumor homing of adoptively transferred cells.

The above studies, over several years from multiple labs using different tumor models, demonstrate that chemokine receptor expression can indeed be a useful approach to improving tumor homing. Use of xenograft models in some of the studies has the benefit of modeling the chemokines secreted by a human tumor, and the effects on human T cells. However, migration of these cells on mouse extracellular integrin ligands can be confusing, as some (e.g., ICAM-1) are species-specific. A common theme in these studies is the careful selection of chemokine and receptor pair based on chemokine concentrations and chemokine receptor levels. The complexity of the chemokine/receptor system may dictate a custom approach dependent on the specific cancer. Even more importantly, the in vitro activation protocol used can determine the

expression, or lack thereof, of critical chemokine receptors [71]. Nevertheless, adding chemokine receptors to CAR T cells is likely to augment tumor immunotherapy going forward, particularly when critical chemokine/receptor pairs are identified for common cancers, or when chemokine production is instigated within the tumor.

6.3.4 Boosting Integrin Function on T Cells

Chemokines affect cell migration by increasing the affinity of integrins for extracellular ligand [97]. After ligand binding, integrins control cell migration by reorganizing the cytoskeleton and providing a forceful connection with the extracellular environment such that traction and movement may occur [98, 104]. As noted previously, a large variety of chemokine receptors can receive signals as combinatorial vectors of chemokine gradients within peripheral and lymphoid tissues [114]. These signals are integrated to control the function of comparatively few integrins. Thus modulating integrin function may have applicability to broader tissues and tumors. Altering one integrin "node" could thus prove more potent than altering one chemokine receptor. Historically, the common therapeutic integrin intervention strategy has been to block ligand binding. In particular, anti-α4 integrin antibody is an approved therapy to block leukocyte recruitment in multiple sclerosis [123, 124], and more recently an antibody specific for the α4β7 integrin has been approved for inflammatory bowel disease [125]. α4 integrin blockade has proven quite powerful, but unwanted side effects have limited its application [126, 127]. A more subtle and specific way to alter integrin activity may be to intervene in distinct integrin signaling pathways [128, 129]. For example, we have investigated the importance of the dynamic binding and release of the adaptor protein paxillin with the cytoplasmic domain of α4 integrin (Fig. 6.2, left panel). This binding is spatially regulated through phosphorylation of α4 integrin by Type I PKA (PKA-I) to direct α4-dependent directional migration of leukocytes [130–132]. Paxillin and α4 integrin mutants that prevent binding or release of paxillin from integrin cytoplasmic tail have proven valuable in investigating outside-in α4 integrin signaling in leukocytes in vitro [133–136]. In addition, small-molecule inhibitors can be used to block the paxillin-α4 integrin interaction in vitro and in vivo [137, 138].

6.3.4.1 Integrin Transregulation in T Cells

To probe the in vivo role of the dynamic paxillin-α4, two knock-in mice were engineered (Fig. 6.2, right panels) [72, 139, 140]. In the α4(Y991A) mouse, paxillin is prevented from binding to the α4 tail. This mouse showed impaired recruitment of most leukocytes to inflammatory sites and defective chemotaxis in vitro on α4 integrin substrates (e.g., VCAM-1), consistent with the role of α4-dependent migration in hematopoietic cells [139]. A mutation that blocks phosphorylation of α4 integrin at Ser988 *prevents release* of paxillin and was predicted to cause similar migration defects to α4(Y991A). However, α4(S988A) mice exhibited a

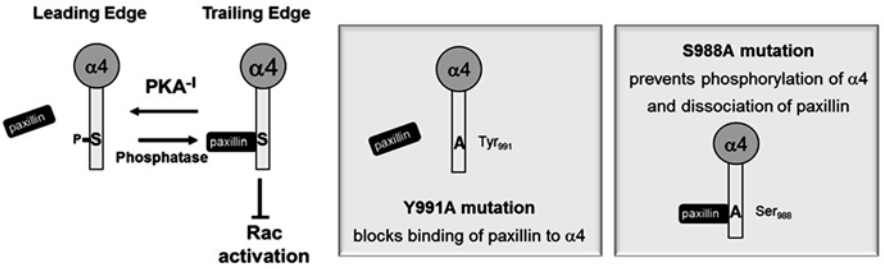

Fig. 6.2 *Dynamic Interaction of paxillin and α4 integrin.* Spatial restriction of α4 integrin phosphorylation by PKA-I regulates the dynamic dissociation and association of paxillin. At the leading edge of a migrating cell, α4 integrin is phosphorylated at Ser988, preventing paxillin binding and allowing Rac activation and stable cell protrusions. α4 integrin is de-phosphorylated at the trailing edge, blocking Rac function and promoting cell retraction. Two α4 integrin mutants interfere with this process; Y991A precludes paxillin binding and S988A prevents its release

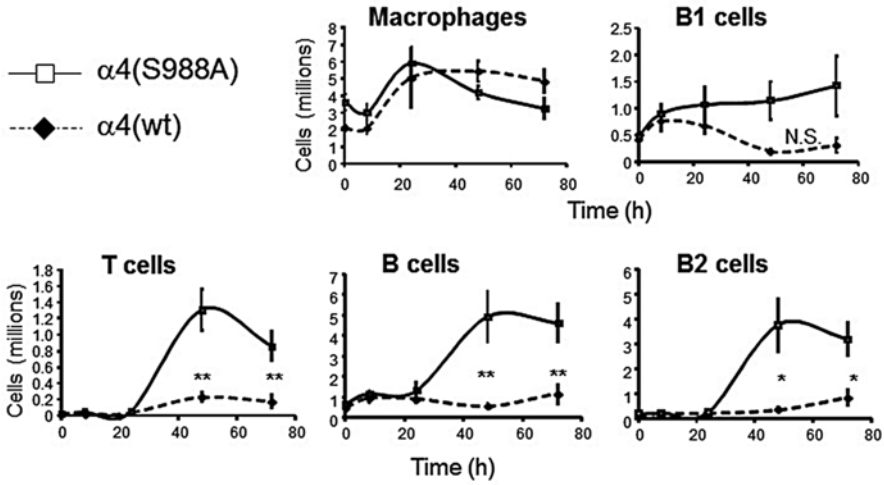

Fig. 6.3 *Selectively increased lymphocyte migration in α4(S988A) mice.* Adult α4(S988A) or wild type mice were injected with 1 mL thioglycollate medium i.p. At the indicated time points (12, 24, 48, and 72 h), mice were sacrificed and analyzed for peritoneal cells by cytospin/DiffQuick for differential cell analysis, and stained with antibodies to identify T cells and B cell subsets by flow cytometry. Error bars are S.E.M. of n = 5 for each group. $*p < 0.03$, $**p < 0.02$; N.S. = not statistically significant

selective *increase* in migration of lymphocytes to inflamed peritoneum, whereas myeloid subsets appeared unaffected (Fig. 6.3). Further evidence indicated that this advantage in inflammatory migration was intrinsic to the lymphocytes [72]. The in vivo peritonitis experiment requires migration on mixed substrates and is dependent on both α4 and β2 integrins [141], conditions in which the α4(S988A) mutation could actually *increase* migration of lymphocytes [135]. To explore this

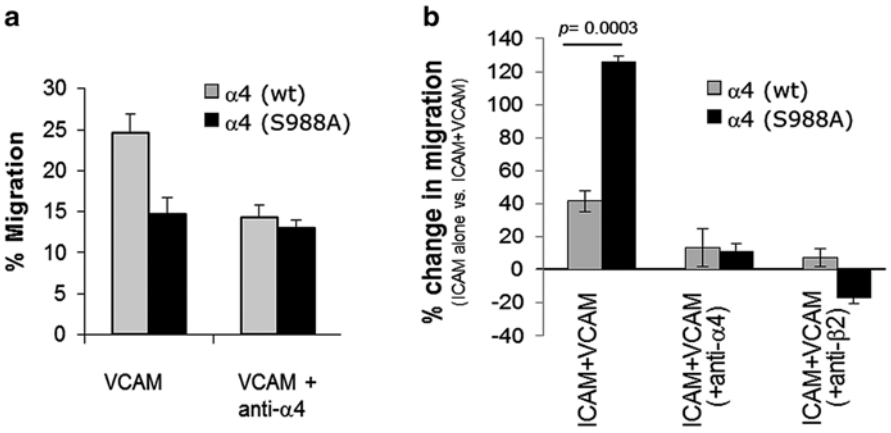

Fig. 6.4 *Increased integrin trans-regulation in α4(S988A) T cells.* T cells were purified from α4(S988A) or control α4(wt). Migration towards SDF-1α (15 ng/mL) was assessed using a modified Boyden Chamber assay in (**a**) wells coated with VCAM-1 alone (2 μg/mL), or (**b**) ICAM-1 (5 μg/mL) +/− VCAM-1 (0.02 μg/mL). For anti-integrin antibody blocking studies, cells were treated with 10 μg/mL of either anti-α4 or anti-β2 integrin for 30 min prior to the assay. Part (**b**) is the % increase in migration on ICAM-1 + VCAM-1 compared to ICAM-1 alone

possibility in a controlled setting, purified T cells from α4(S988A) mice were measured for their ability to migrate in vitro on purified α4 ligand (VCAM-1), purified β2 ligand (ICAM-1), or mixed substrates (VCAM-1 + ICAM-1) in response to the chemokine SDF-1α. Using a modified Boyden chamber assay, we confirmed that α4(S988A) lymphocytes exhibited reduced migration on a purified α4 integrin ligand: VCAM-1 (Fig. 6.4). In contrast, when plated on substrates containing predominantly ICAM-1 and small amounts of VCAM-1, T cells from α4(S988A) mice displayed enhanced migration that was dependent on both α4 and β2 integrins (Fig. 6.4b). These data indicate that the α4(S988A) mutation provides an increase in β2 integrin-dependent migration, i.e., "integrin trans-regulation." Paxillin interaction with α4 integrin cytoplasmic tail enhances $\alpha_L\beta 2$ integrin-mediated migration "in trans" by accelerating FAK or Pyk2 activation [135], perhaps through recruiting these kinases to α4 integrin adhesions. Maintaining the paxillin-α4 interaction thus boosts integrin transregulation, as seen in α4(S988A) lymphocytes.

6.3.5 Integrin Transregulation in Tumor Homing

We thus hypothesized that α4(S988A) mice may have increased ability to resist tumors due to selective migration of lymphocytes to a tumor site. We tested this idea using the well-established B16 melanoma model [142] and found that α4(S988A)

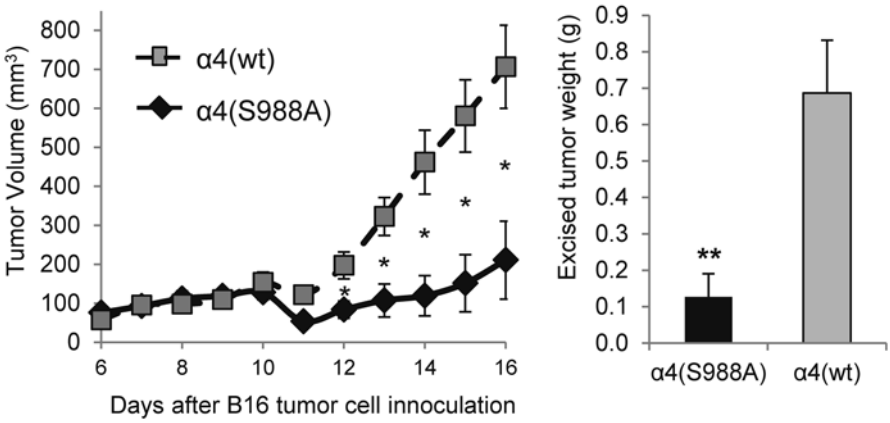

Fig. 6.5 *Reduced tumor growth in α4 (S988A) mice.* B16 melanoma cells (3×10^5) were injected subcutaneously in the hind flank of α4(S988A) or control α4(wt) mice. Tumor area was measured daily and converted to an ellipsoid volume: (length × width^2)/2. On day 15, tumors were excised and weighed. *$p < 0.015$, **$p < 0.002$

mice had ~4-fold smaller tumors than wild type BL6 controls (Fig. 6.5), indicating that blocking α4 integrin phosphorylation on Ser988 increased tumor protection. Similar protection was observed when mice were followed for 21 days, or when using a Lewis Lung Carcinoma model [72].

We next asked whether α4(S988A) resistance to tumor growth was associated with increased T cell homing. Whereas α4(S988A) mice have normal numbers of resting lymphocytes in blood and lymphoid tissue [72], B16 tumors in α4(S988A) mice had greater concentrations of T cells than those grown in wild type mice (Fig. 6.6), while containing similar numbers of macrophages, supporting the idea that the decreased tumor growth is a result of selective homing of lymphocytes vs. macrophages.

The differential requirement of β2 integrins for lymphocytes or myeloid cells in vivo can account for the remarkable leukocyte specificity of this form of trans-regulation. Whereas αLβ2 plays a major role in the migration of T-cells to inflammatory sites [141, 143–145], macrophage migration to inflamed peritoneum is not dependent on β2 integrins, and is reported to be solely dependent on α4β1 [146, 147]. Thus trans-regulation of migration would be absent in macrophages since β2 integrins are not required; indeed we were unable to observe integrin transregulation in α4(S988A) or control α4(wt) macrophages [72]. Among lymphoid cells, tumors in α4(S988A) mice had greater numbers of CD4+, CD8+, and regulatory T cells, whereas NK cell abundance was similar to controls (Fig. 6.6, right panel). These increases could not be explained by mere differences in intrinsic clonal expansion or CD8+ T cell cytotoxic capacity as these functions were similar between α4(S988A) and control mice [72]. The data thus indicate that blocking phosphorylation of α4 integrin by Type I PKA is a promising strategy to improving the homing of T cells to sites of solid tumor growth.

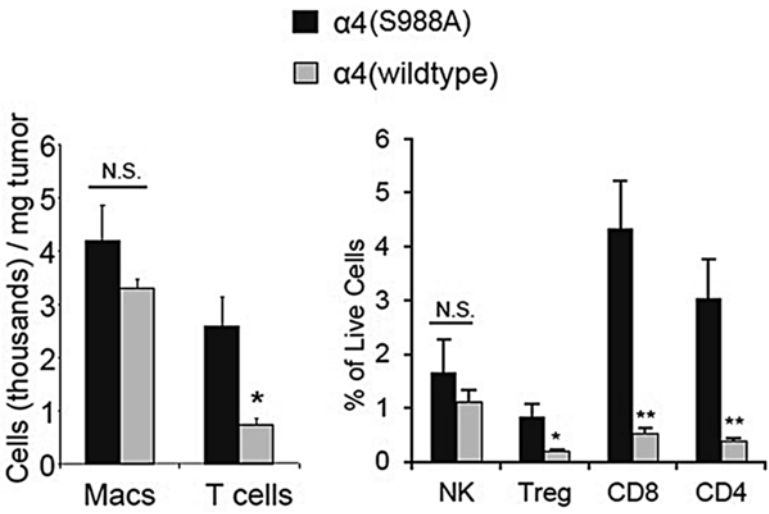

Fig. 6.6 *Tumor-infiltrating leukocytes in α4 (S988A) mice.* B16 melanoma cells (3×10^5) were grown in α4(S988A) or control α4(wt) mice. On day 15, mice were sacrificed and excised tumors were weighed, digested with collagenase, and stained for (CD45+) CD11b+macrophages, CD3+ T cells, CD4+ T-cells, CD8+ T-cells, Foxp3+(CD4+) Treg, and NK1.1+ NK cells. Error bars are S.E.M. of $n \geq 10$ mice per group. $*p < 0.015$, $**p < 0.002$

6.3.6 *Introducing Integrin Transregulation to Adoptive Cellular Immunotherapy*

There are several possible ways to utilize integrin transregulation to improve adoptive cellular immunotherapy for solid cancers. Along with CAR genes, sequences encoding α4(S988A) or similar mutants could be introduced using viral vectors. The transregulation effect of α4(S988A) should be dominant in the presence of α4(wt) protein, since it will compete for pairing with the integrin β chain. Expressing a paxillin-α4 fusion protein results in a similar phenotype to α4(S988A) in T cells [134], so this represents another in vitro modification that should improve the tumor homing of adoptively transferred T cells. Another way to mimic the integrin transregulation effect of α4(S988A) is to interfere with the function of PKA-I to prevent its phosphorylation of α4 integrin. A novel strategy to accomplish this is to sequester PKA-I to the mitochondria, away from α4 integrin on the plasma membrane. A peptide specific for the PKA R1 subunit has been identified and its fusion with a mitochondrial targeting sequence produces a reagent that prevents α4 phosphorylation by PKA-I and results in a cellular phenotype similar to the α4(S988A) mutant [152]. This use of a Type I PKA "trap" requires introduction of a much shorter genetic construct, making its delivery by viral vectors more efficient. An even more practical method would be to inhibit PKA-I pharmacologically. Chemical inhibitors have been reported that are specific for Type I PKA (Rp-8-Br-cAMPS and

Rp-8-Cl-cAMPS) [148]. If treatment of primary human T cells with this reagent boosts integrin transregulation in similar fashion to blocking α4 Ser988 phosphorylation, then this could be a convenient therapeutic possibility.

Integrin transregulation could thus be combined with CAR immunotherapy, perhaps even with "armored CAR's" to produce "guided armored CAR's." One other interesting benefit of this and other homing improvement strategies is the possible upgrade of T cell effector function. As noted previously, several of the studies using chemokine receptors to improve cellular immunotherapy reported enhanced cytokine secretion, cytotoxicity, and TCR signaling. Indeed, we have unpublished preliminary data hinting that integrin transregulation may improve T cell resistance to suppression in the tumor environment. If this proves to be the case, then integrin-based modifications (and those targeting chemokine signaling) could address *two* of the major barriers to adoptive cellular tumor immunotherapy.

6.3.7 Considerations and Future Questions for Improving Homing

This chapter has described several major parts of the natural T cell trafficking machinery that could be utilized to improve tumor homing. Interventions specific for several of these migration components are close to clinical testing and others are not far behind. There will thus be a choice of *which homing mechanism to stimulate*: Inhibition of chemokine degradation? Localized irradiation or hyperthermia? Enforced chemokine expression in the tumor? Incorporation of chemokine receptors of trans-regulated integrins into T cells before transfer?

Finding the most productive method to execute each of these strategies is the first step. For example, *what is the best timeframe* for systemic treatments (e.g., DPPT-4, local irradiation, hyperthermia, checkpoint blockade, IL-2 treatment) in relation to T cell adoptive transfer? Timing the effect of these interventions to maximize their influence on transferred T cells will be crucial to their success. Another important key to successfully implement each of these approaches will be *how to effect the desired change*. In general, small molecule inhibitors are the simplest way to do this, such as in the case of DPPT-4. However, in order to *boost* a mechanism using an inhibitor, relevant natural regulators of the pathway would need to be inhibited (e.g., inhibiting PKA-I to boost integrin transregulation). Otherwise, gene delivery or stimulation of a pathway may be the only choice. Thus an important choice will be whether to stimulate or inhibit a negative regulator of a given pathway (often easier). Another major question will be which anti-tumor cells to modify: CD8+ effectors? CD4+ effectors? NK cells? For CAR T cell therapy, it appears that transfer of a combination of CD8+ T_{CM} and naïve CD4+ T_{conv} offers the greatest benefit [49–51]. We will need to test which of these subsets to boost migration of; perhaps one approach will work best for CD8+ T_{CM} and another for CD4+ T_{conv}. Adding chemokine receptors to CAR NK appears to benefit their tumor homing [122]; what about integrin transregulation in NK subsets?

The third major question in how to apply homing improvement is whether the strategies will need to be custom tailored for specific cancers (more feasible) or for each individual patient (more difficult). Chemokine receptor modification and integrin transregulation may need to be optimized for cancers of different tissues. Even with our incomplete understanding of the details that dictate migration in each tissue, it is clear that they differ greatly. In particular the adhesion molecules and signaling pathways to home to brain, liver, lung, intestinal, and skin tumors are each distinct due to anatomical constraints, composition of extracellular matrix, endothelial characteristics, and other features [107, 112, 149–151]. Given these tissue differences, would integrin transregulation work through α4β7 integrin to boost migration to mucosal sites? For stabilizing chemokines, which cancers degrade which chemokines using which enzymes? The chemokine receptor studies in CAR T cells have laid down a pattern of initially characterizing the tumor chemokines and identifying the corresponding receptors that are lacking in CAR T cells before transfer. Finding similar ways to predict success for other homing improvement strategies in individual cancers may also be needed. For example, it may be possible to construct an in vitro migration assay that could predict whether T cells with trans-regulated integrins could migrate better to a given tumor fragment or cell suspension across a transwell. Once these questions are answered for given cancers, we may need to determine if truly patient-tailored strategies are needed; this would only become feasible with rapid in vitro assays predictive of homing improvement in vivo. A more realistic hope is that one or two universal methods to improve tumor homing will be adopted, and could then be enhanced with a more disease-specific, or patient-specific component, when necessary.

A fourth consideration for improving T cell tumor homing is the possibility of cross-talk between signaling pathways controlling T cell activation/effector functions and those pathways governing cell migration. Hints of this connection were observed in several studies when TCR signaling, cytokine secretion, and cytotoxic efficiency were altered after chemokine receptors were added to CAR T cells [68, 70]. There is also the possibility that blocking PKA-I phosphorylation of α4 integrin may offer increased resistance to T cell exhaustion in the tumor microenvironment. These findings highlight the prospect of using one approach to possibly overcome multiple barriers that impede widespread application of cellular tumor immunotherapy.

6.4 Summary

As a result of current advances in adoptive cellular tumor immunotherapy, a growing understanding of T cell migration, and development of effective gene and drug delivery technology, there is reason to believe that we can overcome the barrier of poor T cell trafficking to tumors for cellular immunotherapy. Moreover, there are several promising approaches that have been tested in animal models, some quite extensively. Combining improvements in homing with methods to overcome tumor-mediated immunosuppression will likely make the greatest impact on tumor immunotherapy. The side effects of super-homing T cells are unknown, but combinations of subtle, yet specifically targeted

methods should address this concern. In conclusion, the promise of infusing T cells that charge efficiently into a solid tumor should focus efforts on conquering the challenge of poor tumor homing in T cell immunotherapy for solid cancers.

Acknowledgements The author would like to thank Dr. Mark H. Ginsberg and Dr. David M. Rose for their contributions to the integrin transregulation studies.

No potential conflicts of interest were disclosed.

References

1. Miller JF, Sadelain M. The journey from discoveries in fundamental immunology to cancer immunotherapy. Cancer Cell. 2015;27(4):439–49. doi:10.1016/j.ccell.2015.03.007.
2. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell. 2015;161(2):205–14. doi:10.1016/j.cell.2015.03.030.
3. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348(6230):62–8. doi:10.1126/science.aaa4967.
4. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, Fry TJ, Orentas R, Sabatino M, Shah NN, Steinberg SM, Stroncek D, Tschernia N, Yuan C, Zhang H, Zhang L, Rosenberg SA, Wayne AS, Mackall CL. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet. 2015;385(9967):517–28. doi:10.1016/S0140-6736(14)61403-3.
5. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF, Mahnke YD, Melenhorst JJ, Rheingold SR, Shen A, Teachey DT, Levine BL, June CH, Porter DL, Grupp SA. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014;371(16):1507–17. doi:10.1056/NEJMoa1407222.
6. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. Immunol Rev. 2014;257(1):56–71. doi:10.1111/imr.12132.
7. Kalos M, June CH. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. Immunity. 2013;39(1):49–60. doi:10.1016/j.immuni.2013.07.002.
8. Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy. Science Transl Med. 2012;4(127):127–8. doi:10.1126/scitranslmed.3003634.
9. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12(4):265–77. doi:10.1038/nrc3258.
10. Minagawa K, Zhou X, Mineishi S, Di Stasi A. Seatbelts in CAR therapy: how safe are CARS? Pharmaceuticals (Basel). 2015;8(2):230–49. doi:10.3390/ph8020230.
11. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor microenvironment. Cell Death Dis. 2015;6, e1792. doi:10.1038/cddis.2015.162.
12. Abken H. Adoptive therapy with CAR redirected T cells: the challenges in targeting solid tumors. Immunotherapy. 2015;7(5):535–44. doi:10.2217/imt.15.15.
13. Bellone M, Calcinotto A, Corti A. Won't you come on in? How to favor lymphocyte infiltration in tumors. Oncoimmunology. 2012;1(6):986–8. doi:10.4161/onci.20213.
14. Fisher DT, Chen Q, Appenheimer MM, Skitzki J, Wang WC, Odunsi K, Evans SS. Hurdles to lymphocyte trafficking in the tumor microenvironment: implications for effective immunotherapy. Immunol Invest. 2006;35(3–4):251–77. doi:10.1080/08820130600745430.
15. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12(4):278–87. doi:10.1038/nrc3236.
16. Redman JM, Hill EM, AlDeghaither D, Weiner LM. Mechanisms of action of therapeutic antibodies for cancer. Mol Immunol. 2015;67:28–45. doi:10.1016/j.molimm.2015.04.002.
17. Lim SH, Levy R. Translational medicine in action: anti-CD20 therapy in lymphoma. J Immunol. 2014;193(4):1519–24. doi:10.4049/jimmunol.1490027.

18. Hayes GM, Chinn L, Cantor JM, Cairns B, Levashova Z, Tran H, Velilla T, Duey D, Lippincott J, Zachwieja J, Ginsberg MH, HvdH E. Antitumor activity of an anti-CD98 antibody. Int J Cancer. 2015;137(3):710–20. doi:10.1002/ijc.29415.
19. Pentcheva-Hoang T, Corse E, Allison JP. Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. Immunol Rev. 2009;229(1):67–87. doi:10.1111/j.1600-065X.2009.00763.x.
20. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015;36(4):265–76. doi:10.1016/j.it.2015.02.008.
21. Mittal R, Chen CW, Lyons JD, Margoles LM, Liang Z, Coopersmith CM, Ford ML. Murine lung cancer induces generalized T-cell exhaustion. J Surg Res. 2015;195(2):541–9. doi:10.1016/j.jss.2015.02.004.
22. Sharma P, Allison JP. The future of immune checkpoint therapy. Science. 2015;348(6230):56–61. doi:10.1126/science.aaa8172.
23. Curiel TJ. Regulatory T cells and treatment of cancer. Curr Opin Immunol. 2008;20(2):241–6. doi:10.1016/j.coi.2008.04.008.
24. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? Lancet Oncol. 2012;13(1):e32–42. doi:10.1016/S1470-2045(11)70155-3.
25. Zhang Y, Gallastegui N, Rosenblatt JD. Regulatory B cells in anti-tumor immunity. Int Immunol. 2015;27:521–30. doi:10.1093/intimm/dxv034.
26. Fournier P, Schirrmacher V. Bispecific antibodies and trispecific immunocytokines for targeting the immune system against cancer: preparing for the future. BioDrugs. 2013;27(1):35–53. doi:10.1007/s40259-012-0008-z.
27. Hoffman LM, Gore L. Blinatumomab, a bi-specific anti-CD19/CD3 BiTE((R)) antibody for the treatment of acute lymphoblastic leukemia: perspectives and current pediatric applications. Front Oncol. 2014;4:63. doi:10.3389/fonc.2014.00063.
28. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. Lancet Oncol. 2014;15(7):e257–67. doi:10.1016/S1470-2045(13)70585-0.
29. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: are we making progress? Front Immunol. 2013;4:454. doi:10.3389/fimmu.2013.00454.
30. Cohn L, Delamarre L. Dendritic cell-targeted vaccines. Front Immunol. 2014;5:255. doi:10.3389/fimmu.2014.00255.
31. Pizzurro GA, Barrio MM. Dendritic cell-based vaccine efficacy: aiming for hot spots. Front Immunol. 2015;6:91. doi:10.3389/fimmu.2015.00091.
32. Graff JN, Chamberlain ED. Sipuleucel-T in the treatment of prostate cancer: an evidence-based review of its place in therapy. Core Evidence. 2015;10:1–10. doi:10.2147/CE.S54712.
33. Yang B, Jeang J, Yang A, Wu TC, Hung CF. DNA vaccine for cancer immunotherapy. Hum Vaccin Immunother. 2014;10(11):3153–64. doi:10.4161/21645515.2014.980686.
34. Liao S, Zhang W, Hu X, Wang W, Deng D, Wang H, Wang C, Zhou J, Wang S, Zhang H, Ma D. A novel "priming-boosting" strategy for immune interventions in cervical cancer. Mol Immunol. 2015;64(2):295–305. doi:10.1016/j.molimm.2014.12.007.
35. Fend L, Gatard-Scheikl T, Kintz J, Gantzer M, Schaedler E, Rittner K, Cochin S, Fournel S, Preville X. Intravenous injection of MVA virus targets CD8+ lymphocytes to tumors to control tumor growth upon combinatorial treatment with a TLR9 agonist. Cancer Immunol Res. 2014;2(12):1163–74. doi:10.1158/2326-6066.CIR-14-0050.
36. Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. J Immunol. 2014;192(12):5451–8. doi:10.4049/jimmunol.1490019.
37. Ramos CA, Dotti G. Chimeric antigen receptor (CAR)-engineered lymphocytes for cancer therapy. Expert Opin Biol Ther. 2011;11(7):855–73. doi:10.1517/14712598.2011.573476.
38. Maus MV, Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. Blood. 2014;123(17):2625–35. doi:10.1182/blood-2013-11-492231.
39. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. Expert Opin Biol Ther. 2015;15(8):1145–54. doi:10.1517/14712598.2015.1046430.

40. Pegram HJ, Park JH, Brentjens RJ. CD28z CARs and armored CARs. Cancer J. 2014;20(2):127–33. doi:10.1097/PPO.0000000000000034.
41. Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. Cancer Discov. 2013;3(4):388–98. doi:10.1158/2159-8290.CD-12-0548.
42. Srivastava S, Riddell SR. Engineering CAR-T cells: design concepts. Trends Immunol. 2015;36:494–502. doi:10.1016/j.it.2015.06.004.
43. Scholler J, Brady TL, Binder-Scholl G, Hwang WT, Plesa G, Hege KM, Vogel AN, Kalos M, Riley JL, Deeks SG, Mitsuyasu RT, Bernstein WB, Aronson NE, Levine BL, Bushman FD, June CH. Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. Sci Transl Med. 2012;4(132):132ra153. doi:10.1126/scitranslmed.3003761.
44. Gilham DE, Debets R, Pule M, Hawkins RE, Abken H. CAR-T cells and solid tumors: tuning T cells to challenge an inveterate foe. Trends Mol Med. 2012;18(7):377–84. doi:10.1016/j.molmed.2012.04.009.
45. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O, Olszewska M, Bernal Y, Pegram H, Przybylowski M, Hollyman D, Usachenko Y, Pirraglia D, Hosey J, Santos E, Halton E, Maslak P, Scheinberg D, Jurcic J, Heaney M, Heller G, Frattini M, Sadelain M. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood. 2011;118(18):4817–28. doi:10.1182/blood-2011-04-348540.
46. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med. 2011;3(95):95ra73. doi:10.1126/scitranslmed.3002842.
47. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011;365(8):725–33. doi:10.1056/NEJMoa1103849.
48. Xu XJ, Zhao HZ, Tang YM. Efficacy and safety of adoptive immunotherapy using anti-CD19 chimeric antigen receptor transduced T-cells: a systematic review of phase I clinical trials. Leuk Lymphoma. 2013;54(2):255–60. doi:10.3109/10428194.2012.715350.
49. Maus MV, Kovacs B, Kwok WW, Nepom GT, Schlienger K, Riley JL, Allman D, Finkel TH, June CH. Extensive replicative capacity of human central memory T cells. J Immunol. 2004;172(11):6675–83.
50. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest. 2008;118(1):294–305. doi:10.1172/JCI32103.
51. Barrett DM, Grupp SA, June CH. Chimeric antigen receptor- and TCR-modified T cells enter main street and wall street. J Immunol. 2015;195(3):755–61. doi:10.4049/jimmunol.1500751.
52. Davila ML, Bouhassira DC, Park JH, Curran KJ, Smith EL, Pegram HJ, Brentjens R. Chimeric antigen receptors for the adoptive T cell therapy of hematologic malignancies. Int J Hematol. 2014;99(4):361–71. doi:10.1007/s12185-013-1479-5.
53. Klingemann H. Are natural killer cells superior CAR drivers? Oncoimmunology. 2014;3, e28147. doi:10.4161/onci.28147.
54. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, Grupp SA, Mackall CL. Current concepts in the diagnosis and management of cytokine release syndrome. Blood. 2014;124(2):188–95. doi:10.1182/blood-2014-05-552729.
55. Maude SL, Barrett D, Teachey DT, Grupp SA. Managing cytokine release syndrome associated with novel T cell-engaging therapies. Cancer J. 2014;20(2):119–22. doi:10.1097/PPO.0000000000000035.
56. Barreira da Silva R, Laird ME, Yatim N, Fiette L, Ingersoll MA, Albert ML. Dipeptidylpeptidase 4 inhibition enhances lymphocyte trafficking, improving both naturally occurring tumor immunity and immunotherapy. Nat Immunol. 2015;16(8):850–8. doi:10.1038/ni.3201.
57. Peng W, Liu C, Xu C, Lou Y, Chen J, Yang Y, Yagita H, Overwijk WW, Lizee G, Radvanyi L, Hwu P. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. Cancer Res. 2012;72(20):5209–18. doi:10.1158/0008-5472.CAN-12-1187.

58. Wang L, Amoozgar Z, Huang J, Saleh MH, Xing D, Orsulic S, Goldberg MS. Decitabine enhances lymphocyte migration and function and synergizes with CTLA-4 blockade in a murine ovarian cancer model. Cancer Immunol Res. 2015;3(9):1030–41. doi:10.1158/2326-6066.CIR-15-0073.
59. Calcinotto A, Grioni M, Jachetti E, Curnis F, Mondino A, Parmiani G, Corti A, Bellone M. Targeting TNF-alpha to neoangiogenic vessels enhances lymphocyte infiltration in tumors and increases the therapeutic potential of immunotherapy. J Immunol. 2012;188(6):2687–94. doi:10.4049/jimmunol.1101877.
60. Chen Q, Fisher DT, Clancy KA, Gauguet JM, Wang WC, Unger E, Rose-John S, von Andrian UH, Baumann H, Evans SS. Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism. Nat Immunol. 2006;7(12):1299–308. doi:10.1038/ni1406.
61. Mikucki ME, Fisher DT, Ku AW, Appenheimer MM, Muhitch JB, Evans SS. Preconditioning thermal therapy: flipping the switch on IL-6 for anti-tumour immunity. Int J Hyperthermia. 2013;29(5):464–73. doi:10.3109/02656736.2013.807440.
62. Fisher DT, Chen Q, Skitzki JJ, Muhitch JB, Zhou L, Appenheimer MM, Vardam TD, Weis EL, Passanese J, Wang WC, Gollnick SO, Dewhirst MW, Rose-John S, Repasky EA, Baumann H, Evans SS. IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. J Clin Invest. 2011;121(10):3846–59. doi:10.1172/JCI44952.
63. Vanpouille-Box C, Pilones KA, Wennerberg E, Formenti SC, Demaria S. In situ vaccination by radiotherapy to improve responses to anti-CTLA-4 treatment. Vaccine. 2015;33(51):7415–22. doi:10.1016/j.vaccine.2015.05.105.
64. Zheng Y, Dou Y, Duan L, Cong C, Gao A, Lai Q, Sun Y. Using chemo-drugs or irradiation to break immune tolerance and facilitate immunotherapy in solid cancer. Cell Immunol. 2015;294(1):54–9. doi:10.1016/j.cellimm.2015.02.003.
65. Nishio N, Diaconu I, Liu H, Cerullo V, Caruana I, Hoyos V, Bouchier-Hayes L, Savoldo B, Dotti G. Armed oncolytic virus enhances immune functions of chimeric antigen receptor-modified T cells in solid tumors. Cancer Res. 2014;74(18):5195–205. doi:10.1158/0008-5472.CAN-14-0697.
66. Brown CE, Vishwanath RP, Aguilar B, Starr R, Najbauer J, Aboody KS, Jensen MC. Tumor-derived chemokine MCP-1/CCL2 is sufficient for mediating tumor tropism of adoptively transferred T cells. J Immunol. 2007;179(5):3332–41.
67. Kershaw MH, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, Wang E, Young HA, Murphy PM, Hwu P. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum Gene Ther. 2002;13(16):1971–80. doi:10.1089/10430340260355374.
68. Asai H, Fujiwara H, An J, Ochi T, Miyazaki Y, Nagai K, Okamoto S, Mineno J, Kuzushima K, Shiku H, Inoue H, Yasukawa M. Co-introduced functional CCR2 potentiates in vivo anti-lung cancer functionality mediated by T cells double gene-modified to express WT1-specific T-cell receptor. PLoS One. 2013;8(2), e56820. doi:10.1371/journal.pone.0056820.
69. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM, Foster AE. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother. 2010;33(8):780–8. doi:10.1097/CJI.0b013e3181ee6675.
70. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, Foster AE, Heslop HE, Brenner MK, Dotti G, Savoldo B. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. Blood. 2009;113(25):6392–402. doi:10.1182/blood-2009-03-209650.
71. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, Powell Jr DJ, Riley JL, June CH, Albelda SM. Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res. 2011;17(14):4719–30. doi:10.1158/1078-0432.CCR-11-0351.
72. Cantor JM, Rose DM, Slepak M, Ginsberg MH. Fine-tuning tumor immunity with integrin trans-regulation. Cancer Immunol Res. 2015;3(6):661–7. doi:10.1158/2326-6066.CIR-13-0226.

73. Mukherjee S, Thrasher AJ. Gene therapy for PIDs: progress, pitfalls and prospects. Gene. 2013;525(2):174–81. doi:10.1016/j.gene.2013.03.098.
74. Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. J Exp Med. 2008;205(3):711–23. doi:10.1084/jem.20071140.
75. Palmer DC, Chan CC, Gattinoni L, Wrzesinski C, Paulos CM, Hinrichs CS, Powell Jr DJ, Klebanoff CA, Finkelstein SE, Fariss RN, Yu Z, Nussenblatt RB, Rosenberg SA, Restifo NP. Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. Proc Natl Acad Sci U S A. 2008;105(23):8061–6. doi:10.1073/pnas.0710929105.
76. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18(4):843–51. doi:10.1038/mt.2010.24.
77. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM, Rosenberg SA. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther. 2011;19(3):620–6. doi:10.1038/mt.2010.272.
78. Lamers CH, Sleijfer S, van Steenbergen S, van Elzakker P, van Krimpen B, Groot C, Vulto A, den Bakker M, Oosterwijk E, Debets R, Gratama JW. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. Mol Ther. 2013;21(4):904–12. doi:10.1038/mt.2013.17.
79. Xu XJ, Tang YM. Cytokine release syndrome in cancer immunotherapy with chimeric antigen receptor engineered T cells. Cancer Lett. 2014;343(2):172–8. doi:10.1016/j.canlet.2013.10.004.
80. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1–10. doi:10.1016/j.immuni.2013.07.012.
81. Motz GT, Coukos G. Deciphering and reversing tumor immune suppression. Immunity. 2013;39(1):61–73. doi:10.1016/j.immuni.2013.07.005.
82. Chmielewski M, Kopecky C, Hombach AA, Abken H. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. Cancer Res. 2011;71(17):5697–706. doi:10.1158/0008-5472.CAN-11-0103.
83. Haanen JB, Thienen H, Blank CU. Toxicity patterns with immunomodulating antibodies and their combinations. Semin Oncol. 2015;42(3):423–8. doi:10.1053/j.seminoncol.2015.02.011.
84. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, McKee M, Gajewski TF. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res. 2009;69(7):3077–85. doi:10.1158/0008-5472.CAN-08-2281.
85. Huang H, Langenkamp E, Georganaki M, Loskog A, Fuchs PF, Dieterich LC, Kreuger J, Dimberg A. VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-kappaB-induced endothelial activation. FASEB J. 2015;29(1):227–38. doi:10.1096/fj.14-250985.
86. Lizee G, Cantu MA, Hwu P. Less yin, more yang: confronting the barriers to cancer immunotherapy. Clin Cancer Res. 2007;13(18 Pt 1):5250–5. doi:10.1158/1078-0432.CCR-07-1722.
87. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science. 1996;272(5258):60–6.
88. Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naive, effector, and memory CD8(+) T cells. J Exp Med. 2001;194(7):953–66.
89. Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. Nat Rev Immunol. 2013;13(5):309–20. doi:10.1038/nri3442.
90. Fearon DT. The expansion and maintenance of antigen-selected CD8(+) T cell clones. Adv Immunol. 2007;96:103–39. doi:10.1016/S0065-2776(07)96003-4.

91. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343(14):1020–34. doi:10.1056/NEJM200010053431407.
92. Fu H, Wang A, Mauro C, Marelli-Berg F. T lymphocyte trafficking: molecules and mechanisms. Front Biosci (Landmark Ed). 2013;18:422–40.
93. D'Cruz LM, Rubinstein MP, Goldrath AW. Surviving the crash: transitioning from effector to memory CD8+ T cell. Semin Immunol. 2009;21(2):92–8. doi:10.1016/j.smim.2009.02.002.
94. Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. Immunity. 2014;41(5):694–707. doi:10.1016/j.immuni.2014.10.008.
95. Rose DM, Alon R, Ginsberg MH. Integrin modulation and signaling in leukocyte adhesion and migration. Immunol Rev. 2007;218:126–34. doi:10.1111/j.1600-065X.2007.00536.x.
96. Alon R, Shulman Z. Chemokine triggered integrin activation and actin remodeling events guiding lymphocyte migration across vascular barriers. Exp Cell Res. 2011;317(5):632–41. doi:10.1016/j.yexcr.2010.12.007.
97. Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuente EM, Boussiotis VA, Shattil SJ, Ginsberg MH. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol. 2006;16(18):1796–806. doi:10.1016/j.cub.2006.08.035.
98. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673–87.
99. Kim C, Ye F, Hu X, Ginsberg MH. Talin activates integrins by altering the topology of the beta transmembrane domain. J Cell Biol. 2012;197(5):605–11. doi:10.1083/jcb.201112141.
100. Ye F, Kim C, Ginsberg MH. Reconstruction of integrin activation. Blood. 2012;119(1):26–33. doi:10.1182/blood-2011-04-292128.
101. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol. 2010;11(4):288–300. doi:10.1038/nrm2871.
102. Shen B, Delaney MK, Du X. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. Curr Opin Cell Biol. 2012;24(5):600–6. doi:10.1016/j.ceb.2012.08.011.
103. Zhang Y, Wang H. Integrin signalling and function in immune cells. Immunology. 2012;135(4):268–75. doi:10.1111/j.1365-2567.2011.03549.x.
104. Iwamoto DV, Calderwood DA. Regulation of integrin-mediated adhesions. Curr Opin Cell Biol. 2015;36:41–7. doi:10.1016/j.ceb.2015.06.009.
105. Tufail S, Badrealam KF, Sherwani A, Gupta UD, Owais M. Tissue specific heterogeneity in effector immune cell response. Front Immunol. 2013;4:254. doi:10.3389/fimmu.2013.00254.
106. Stock A, Napolitani G, Cerundolo V. Intestinal DC in migrational imprinting of immune cells. Immunol Cell Biol. 2013;91(3):240–9. doi:10.1038/icb.2012.73.
107. Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. J Exp Med. 2013;210(9):1855–69. doi:10.1084/jem.20130091.
108. Brinkman CC, Peske JD, Engelhard VH. Peripheral tissue homing receptor control of naive, effector, and memory CD8 T cell localization in lymphoid and non-lymphoid tissues. Front Immunol. 2013;4:241. doi:10.3389/fimmu.2013.00241.
109. Guo Y, Brown C, Ortiz C, Noelle RJ. Leukocyte homing, fate, and function are controlled by retinoic acid. Physiol Rev. 2015;95(1):125–48. doi:10.1152/physrev.00032.2013.
110. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008;8(9):685–98. doi:10.1038/nri2378.
111. Edele F, Molenaar R, Gutle D, Dudda JC, Jakob T, Homey B, Mebius R, Hornef M, Martin SF. Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. J Immunol. 2008;181(6):3745–9.
112. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. Trends Immunol. 2012;33(12):579–89. doi:10.1016/j.it.2012.07.004.
113. Marelli-Berg FM, Cannella L, Dazzi F, Mirenda V. The highway code of T cell trafficking. J Pathol. 2008;214(2):179–89. doi:10.1002/path.2269.

114. Heit B, Tavener S, Raharjo E, Kubes P. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol. 2002;159(1):91–102. doi:10.1083/jcb.200202114.
115. Slaney CY, Kershaw MH, Darcy PK. Trafficking of T cells into tumors. Cancer Res. 2014;74(24):7168–74. doi:10.1158/0008-5472.CAN-14-2458.
116. Pradere JP, Dapito DH, Schwabe RF. The Yin and Yang of Toll-like receptors in cancer. Oncogene. 2014;33(27):3485–95. doi:10.1038/onc.2013.302.
117. Garmy-Susini B, Varner JA. Roles of integrins in tumor angiogenesis and lymphangiogenesis. Lymphat Res Biol. 2008;6(3–4):155–63. doi:10.1089/lrb.2008.1011.
118. Mumprecht V, Detmar M. Lymphangiogenesis and cancer metastasis. J Cell Mol Med. 2009;13(8A):1405–16. doi:10.1111/j.1582-4934.2009.00834.x.
119. Kilinc MO, Gu T, Harden JL, Virtuoso LP, Egilmez NK. Central role of tumor-associated CD8+ T effector/memory cells in restoring systemic antitumor immunity. J Immunol. 2009;182(7):4217–25. doi:10.4049/jimmunol.0802793.
120. Abastado JP. The next challenge in cancer immunotherapy: controlling T-cell traffic to the tumor. Cancer Res. 2012;72(9):2159–61. doi:10.1158/0008-5472.CAN-11-3538.
121. Peng W, Ye Y, Rabinovich BA, Liu C, Lou Y, Zhang M, Whittington M, Yang Y, Overwijk WW, Lizee G, Hwu P. Transduction of tumor-specific T cells with CXCR2 chemokine receptor improves migration to tumor and antitumor immune responses. Clin Cancer Res. 2010;16(22):5458–68. doi:10.1158/1078-0432.CCR-10-0712.
122. Muller N, Michen S, Tietze S, Topfer K, Schulte A, Lamszus K, Schmitz M, Schackert G, Pastan I, Temme A. Engineering NK cells modified with an EGFRvIII-specific chimeric antigen receptor to overexpress CXCR4 improves immunotherapy of CXCL12/SDF-1alpha-secreting Glioblastoma. J Immunother. 2015;38(5):197–210. doi:10.1097/CJI.0000000000000082.
123. Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue EW, Lublin FD, Weinstock-Guttman B, Wynn DR, Lynn F, Panzara MA, Sandrock AW. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. N Engl J Med. 2006;354(9):911–23. doi:10.1056/NEJMoa044396.
124. Romme Christensen J, Ratzer R, Bornsen L, Lyksborg M, Garde E, Dyrby TB, Siebner HR, Sorensen PS, Sellebjerg F. Natalizumab in progressive MS: results of an open-label, phase 2A, proof-of-concept trial. Neurology. 2014;82(17):1499–507. doi:10.1212/WNL.0000000000000361.
125. Bryant RV, Sandborn WJ, Travis SP. Introducing vedolizumab to clinical practice: who, when, and how? J Crohns Colitis. 2015;9(4):356–66. doi:10.1093/ecco-jcc/jjv033.
126. Kivisakk P, Healy BC, Viglietta V, Quintana FJ, Hootstein MA, Weiner HL, Khoury SJ. Natalizumab treatment is associated with peripheral sequestration of proinflammatory T cells. Neurology. 2009;72(22):1922–30. doi:10.1212/WNL.0b013e3181a8266f.
127. Berger JR, Koralnik IJ. Progressive multifocal leukoencephalopathy and natalizumab—unforeseen consequences. N Engl J Med. 2005;353(4):414–6. doi:10.1056/NEJMe058122.
128. Kummer C, Ginsberg MH. New approaches to blockade of alpha4-integrins, proven therapeutic targets in chronic inflammation. Biochem Pharmacol. 2006;72(11):1460–8. doi:10.1016/j.bcp.2006.06.014.
129. Cantor JM, Ginsberg MH, Rose DM. Integrin-associated proteins as potential therapeutic targets. Immunol Rev. 2008;223:236–51. doi:10.1111/j.1600-065X.2008.00640.x.
130. Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M, Ginsberg MH. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature. 1999;402(6762):676–81.
131. Goldfinger LE, Han J, Kiosses WB, Howe AK, Ginsberg MH. Spatial restriction of alpha4 integrin phosphorylation regulates lamellipodial stability and alpha4beta1-dependent cell migration. J Cell Biol. 2003;162(4):731–41.
132. Nishiya N, Kiosses WB, Han J, Ginsberg MH. An alpha4 integrin-paxillin-Arf-GAP complex restricts Rac activation to the leading edge of migrating cells. Nat Cell Biol. 2005;7(4):343–52.

133. Liu S, Kiosses WB, Rose DM, Slepak M, Salgia R, Griffin JD, Turner CE, Schwartz MA, Ginsberg MH. A fragment of paxillin binds the alpha 4 integrin cytoplasmic domain (tail) and selectively inhibits alpha 4-mediated cell migration. J Biol Chem. 2002;277(23):20887–94. doi:10.1074/jbc.M110928200.
134. Han J, Rose DM, Woodside DG, Goldfinger LE, Ginsberg MH. Integrin alpha 4 beta 1-dependent T cell migration requires both phosphorylation and dephosphorylation of the alpha 4 cytoplasmic domain to regulate the reversible binding of paxillin. J Biol Chem. 2003;278(37):34845–53.
135. Rose DM, Liu S, Woodside DG, Han J, Schlaepfer DD, Ginsberg MH. Paxillin binding to the alpha 4 integrin subunit stimulates LFA-1 (integrin alpha L beta 2)-dependent T cell migration by augmenting the activation of focal adhesion kinase/proline-rich tyrosine kinase-2. J Immunol. 2003;170(12):5912–8.
136. Alon R, Feigelson SW, Manevich E, Rose DM, Schmitz J, Overby DR, Winter E, Grabovsky V, Shinder V, Matthews BD, Sokolovsky-Eisenberg M, Ingber DE, Benoit M, Ginsberg MH. Alpha4beta1-dependent adhesion strengthening under mechanical strain is regulated by paxillin association with the alpha4-cytoplasmic domain. J Cell Biol. 2005;171(6):1073–84. doi:10.1083/jcb.200503155.
137. Ambroise Y, Yaspan B, Ginsberg MH, Boger DL. Inhibitors of cell migration that inhibit intracellular paxillin/alpha4 binding: a well-documented use of positional scanning libraries. Chem Biol. 2002;9(11):1219–26.
138. Kummer C, Petrich BG, Rose DM, Ginsberg MH. A small molecule that inhibits the interaction of paxillin and alpha 4 integrin inhibits accumulation of mononuclear leukocytes at a site of inflammation. J Biol Chem. 2010;285(13):9462–9. doi:10.1074/jbc.M109.066993.
139. Feral CC, Rose DM, Han J, Fox N, Silverman GJ, Kaushansky K, Ginsberg MH. Blocking the alpha 4 integrin-paxillin interaction selectively impairs mononuclear leukocyte recruitment to an inflammatory site. J Clin Invest. 2006;116(3):715–23.
140. Goldfinger LE, Tzima E, Stockton R, Kiosses WB, Kinbara K, Tkachenko E, Gutierrez E, Groisman A, Nguyen P, Chien S, Ginsberg MH. Localized alpha4 integrin phosphorylation directs shear stress-induced endothelial cell alignment. Circ Res. 2008;103(2):177–85.
141. Ulyanova T, Priestley GV, Banerjee ER, Papayannopoulou T. Unique and redundant roles of alpha4 and beta2 integrins in kinetics of recruitment of lymphoid vs myeloid cell subsets to the inflamed peritoneum revealed by studies of genetically deficient mice. Exp Hematol. 2007;35(8):1256–65. doi:10.1016/j.exphem.2007.04.015.
142. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. Curr Protoc Immunol. 2001;Chapter 20:Unit 20 21.
143. Engelhardt B. Molecular mechanisms involved in T cell migration across the blood-brain barrier. J Neural Transm. 2006;113(4):477–85. doi:10.1007/s00702-005-0409-y.
144. Koboziev I, Karlsson F, Ostanin DV, Gray L, Davidson M, Zhang S, Grisham MB. Role of LFA-1 in the activation and trafficking of T cells: implications in the induction of chronic colitis. Inflamm Bowel Dis. 2012;18(12):2360–70. doi:10.1002/ibd.22947.
145. Takeichi T, Mocevicius P, Deduchovas O, Salnikova O, Castro-Santa E, Buchler MW, Schmidt J, Ryschich E. alphaL beta2 integrin is indispensable for CD8+ T-cell recruitment in experimental pancreatic and hepatocellular cancer. Int J Cancer. 2012;130(9):2067–76. doi:10.1002/ijc.26223.
146. Henderson RB, Hobbs JA, Mathies M, Hogg N. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. Blood. 2003;102(1):328–35. doi:10.1182/blood-2002-10-3228.
147. Imhof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. Nat Rev Immunol. 2004;4(6):432–44. doi:10.1038/nri1375.
148. Gjertsen BT, Mellgren G, Otten A, Maronde E, Genieser HG, Jastorff B, Vintermyr OK, McKnight GS, Doskeland SO. Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 beta action. J Biol Chem. 1995;270(35):20599–607.

149. Grabbe S, Varga G, Beissert S, Steinert M, Pendl G, Seeliger S, Bloch W, Peters T, Schwarz T, Sunderkotter C, Scharffetter-Kochanek K. Beta2 integrins are required for skin homing of primed T cells but not for priming naive T cells. J Clin Invest. 2002;109(2):183–92. doi:10.1172/JCI11703.
150. Reinhardt RL, Bullard DC, Weaver CT, Jenkins MK. Preferential accumulation of antigen-specific effector CD4 T cells at an antigen injection site involves CD62E-dependent migration but not local proliferation. J Exp Med. 2003;197(6):751–62. doi:10.1084/jem.20021690.
151. Lee WY, Kubes P. Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. J Hepatol. 2008;48(3):504–12. doi:10.1016/j.jhep.2007.12.005.
152. Lim CJ, Han J, Yousefi N, Ma Y, Amieux PS, McKnight GS, Taylor SS, Ginsberg MH. Alpha4 integrins are type I cAMP-dependent protein kinase-anchoring proteins. Nat Cell Biol. 2007;9(4):415–21. doi: 10.1038/ncb1561.

Chapter 7
Chemokines and T Cell Trafficking into Tumors: Strategies to Enhance Recruitment of T Cells into Tumors

Stefano Garetto, Claudia Sardi, Diego Morone, and Marinos Kallikourdis

Abstract Chemokines are small proteins used by the cells of the immune system in order to orchestrate their movement in the body during physiological and pathological conditions. Yet they are also expressed in tumors and their metastases. There, they mediate a variety of tumor-specific functions, including the recruitment of different immune cell populations to the tumor site. These cells may have a pro- or anti-tumoral function. Yet the cells mediating the latter are often prevented from infiltrating the tumor mass, due to functional or physical barriers. This is a major obstacle for successful tumor immunotherapy based on cytotoxic T cell administration. Genetic and other pre-clinical studies have provided insights into the mechanisms that regulate these barriers, such as the peri-tumoral fibrotic capsule. Recent novel strategies involving modification of the chemokine receptors expressed in the transferred cytotoxic T cells are providing a possible means of overcoming such obstacles. Integration of such strategies in immunotherapy protocols may hopefully pave the way to a more successful clinical application of T cell immunotherapy.

Keywords Tumor • T cells • Adoptive Cell Therapy • Recruitment • Chemokines • Chemokine receptors

S. Garetto • C. Sardi
Adaptive Immunity Laboratory, Humanitas Clinical and Research Center, Via Manzoni 56, Rozzano (Milano), Italy

D. Morone
Humanitas Clinical and Research Center, Via Manzoni 56, Rozzano (Milano), Italy

M. Kallikourdis (✉)
Adaptive Immunity Laboratory, Humanitas Clinical and Research Center, Via Manzoni 56, Rozzano (Milano), Italy

Humanitas University, Via Manzoni 113, Rozzano (Milano), Italy
e-mail: marinos.kallikourdis@humanitasresearch.it

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_7

Abbreviations

ACT	Adoptive cell therapy
CAF	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
ECM	Extra cellular matrix
FAP	Fibroblast Activation Protein-α
GPCR	G-protein-coupled receptors
MDSC	Myeloid-derived suppressor cells
NK	Natural Killer
SHG	Second harmonic generation
TAM	Tumor-associated macrophages
TCR	T cell antigen receptor
TIL	Tumor infiltrating Lymphocytes
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

7.1 Chemokines and Their Receptors

Chemokines (**chemo**tactic cytok**ines**) are small proteins divided into four subfamilies (CC, CXC, CX3C or XC), according to the position of conserved cysteine residues in their amino acid sequence [1, 2]. Chemokines are expressed and secreted by diverse cell types and each chemokine can bind to a cognate chemokine receptor, albeit with a certain degree of promiscuity. The chemokine receptors are seven-transmembrane G-protein-coupled receptors (GPCRs). The cells of the immune system express different chemokine receptors according to their developmental stage and activation status. This enables the immune cells to utilize chemokine gradients released by different tissues in order to move around the body in physiological conditions, as well as during an immune response. The chemokines involved in the migration of immune cells to sites of infection are termed "inflammatory" chemokines (e.g., CCL3, CCL4, CXCL9, CXCL10). They mediate both the extravasation of immune cells into the inflamed tissue—together with the action of integrins-, as well as the movement within the tissue itself. On the other hand, chemokines involved in the orchestration of cell recruitment and retention in physiological conditions are termed "homeostatic" (e.g., CXCL4, CXCL12) [1, 2]. The mechanism of chemokine-mediated cell migration is thought to be based on the chemotaxis of chemokine receptor-expressing cells along gradients of their cognate chemokine ligand. Yet, at least two additional parameters also come into play: (i) atypical chemokine receptors, such as ACKR2, function as decoys and remove circulating chemokines, affecting the formation of chemokine gradients [3]; (ii) cell membrane-bound heparan sulfate proteoglycans can bind chemokines, thus creating cell surface-bound gradients [4].

7.2 Chemokine and Chemokine Receptor Expression in Tumors

Chemokines and chemokine receptors are expressed in tumor and metastatic sites. The chemokines may recruit different immune cell subpopulations to the tumor, whilst the receptors may also be used by the tumor cells to guide their metastatic spreading (discussed in detail below). A very large number of studies have elucidated the identity of the chemokines released by human tumors. Breast cancer induces the expression of CCL2, CCL5, CCL19, CCL20, CXCL16, CXCL5, CXCL12 and CCL20 [1, 2]. Pancreatic cancer is associated with CCL2 and CCL20, whilst prostate carcinoma with CCL2, CCL5, CCL22, CXCL12 and CXCL16 [5–8]. Indeed, CCL2 has been proposed as a potential biomarker for prostate cancer [9]. CCL22 is also found in lung cancer, several lymphomas and ovarian cancer, whilst melanoma drives the expression of CCL5 and CXCL1. Colorectal carcinoma is characterized by CXCL9, CXCL10, CXCL16, and CX3CL1 expression [1]. The examples listed here are by no means exhaustive, though they do demonstrate the very wide range of chemokines detected in human tumors.

Likewise, a wide range of chemokine receptors are expressed by malignant cells including CCR4, CCR7, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR7 and CX3CR1 [2]. It is worth discussing additionally two of these receptors, as they are good examples of how the physiological expression patterns of the receptor influences its expression in tumor:

The receptor for the homeostatic chemokine CXCL12, CXCR4, has a ubiquitous expression range in physiological conditions [10]. Mirroring this fact, it has been found in most tumors (ovarian, prostate, melanoma, lung cancer, bladder, oesophageal, colorectal, neuroblastoma, pancreatic, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, stomach cancer, renal cancer, as well as in cancer stem cells) [1, 2, 11]. Melanoma and breast cancer also express CXCR7/ACKR3 [11], which is an atypical receptor for CXCL12, with different downstream signaling effects compared to CXCR4 [12]. Targeting CXCR4 with a competitive inhibitor, AMD3100, was able to improve outcomes in a preclinical model of hepatocellular carcinoma, suggesting a role for the receptor in aiding tumor growth [13].

The receptors CCR7 and CXCR5 are essential for establishing the distribution of lymphocytes within secondary lymphoid organs. Reflecting this, both are expressed in tumors of lymphoid tissue: CCR7 is expressed in T cell leukemia and Non-Hodgkin's lymphoma [2], whilst CXCR5 is highly expressed in chronic lymphocytic leukemia [14].

7.3 Accessory Roles of Chemokines in Cancer

Intriguingly, as hinted from the above, chemokines may actively aid the growth and survival of tumor cells [2]. CCL20 plays a role in the growth of colorectal cancer, CXCL1, CXCL2 and CXCL3 are involved in the growth of pancreatic cancer,

melanoma, lung cancer, gastric cancer and adenocarcinoma, whilst CCL27 is involved in melanoma growth [14]. Further, the chemokines CCL2, CCL11, CCL16, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL12 have pro-angiogenic effects at the tumor site [2, 14, 15].

Metastasis involves the migration of tumor cells away from the primary tumor mass. As tumor cells express chemokine receptors, the metastatic process is thought to be driven by chemokine gradients. In most tumors, this involves CXCR4 and its ligand, CXCL12. CXCR4 correlates with metastasis formation in breast, ovarian, pancreatic and prostate cancer [2, 15, 16] as well as in melanoma and glioblastoma [1, 8, 17]. It is noteworthy that CXCR4 is the only chemokine expressed robustly and consistently on cancer stem cells, which are believed to be important for the metastatic properties of many tumors (e.g., breast, colorectal carcinoma, melanoma) [1, 15, 18]. As mentioned above, the orchestration of the lymph node architecture involves CCR7 and its ligands, CCL19 and CCL21. It is thus unsurprising that secondary metastasis to lymph nodes in melanoma and many other tumors involves CCR7 [1, 2, 15, 17]. Several other chemokine receptors have been shown to be involved in the metastasis of different tumors: CXCR3 in experimental colon cancer metastasis, CCR9 in melanoma metastasis [14], CXCR5 in colorectal carcinoma metastasis [1], to name but a few. The wide range of chemokines and chemokine receptors associated with several cancer-promoting processes highlights the complexity of chemokine functions in the tumor and the tumor stroma. This complexity, however, may even create opportunities for therapeutic strategies, as we will discuss below.

7.4 Chemokines Mediate Recruitment of Pro- and Anti-tumoral Cells to the Tumor

The chemokine function of most interest to us in this chapter is the recruitment of immune cell subpopulations to the tumor site. Tumor cells secrete chemokines that recruit both tumor-promoting cells but also cells with anti-tumor activity. Inflammatory cytokines, such as CCL2 in breast cancer [19] and prostate cancer [20] or CCL5 [19] enable the recruitment of malignancy-promoting tumor-associated macrophages (TAMs) [1, 21]. TAMs display an alternative ("M2-like") polarization state which favors tumor growth by promoting vascularization, angiogenesis, tumor cell proliferation and suppression of anti-tumoral immunity [2, 15]. Indeed, CCL2 and CCL5 expression in human breast cancer, melanoma, colon, prostate and pancreatic cancer is correlated with TAM presence. The source of the chemokines attracting TAMs is not limited to the tumor as they may also be produced by stromal cells [1]. The fibroblasts that form part of the tumor stroma itself may also be recruited to the site via the action of chemokines [2]. Finally, chemokines also recruit the heterogeneous population of Myeloid-Derived Suppressor Cells (MDSC), that favor tumor growth [15, 22].

The immune cells with tumor-promoting activity are not limited to the cells of the innate immune system, such as TAM and MDSC. Regulatory T cells (Treg), the main immunosuppressive T lymphocyte subpopulation [23] have the ability to block $CD8^+$ cytotoxic T cell responses [24], which could combat tumor growth; Treg presence in tumors has been correlated with CCL2 expression by the tumor [25], though more often it is associated with CCL17/CCL22 expression, as Treg are thought to express the cognate receptor, CCR4 [26]. CCR4, along with CCR3, may also mediate the recruitment of Th2-polarized T cells, which can promote M2-polarization among macrophages and oppose type-1 polarized cytotoxic responses, contributing to the formation of an environment that fosters tumor growth [1, 2].

Luckily, pro-inflammatory chemokines such as CCL5 in ovarian cancer [8] or CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10 in metastatic melanoma patients [19, 27] can recruit $CD8^+$ cytotoxic T cells with anti-tumor activity, whose presence in the tumor as Tumor Infiltrating Lymphocytes (TIL) is associated with improved survival for the patient [15]. CXCL9 and CXCL10, which are signature chemokines of M1-polarized, "classical" inflammation, are also thought to be responsible for the recruitment of Natural Killer (NK) cells inside the tumor mass. In colorectal and gastric carcinomas these two chemokines are mostly produced by stromal macrophages [1].

In summary, whilst chemokines are involved in a variety of functions in the microenvironment of the tumor and the tumor stroma that promote tumor growth, they also recruit and/or retain both pro- and anti-tumoral cell populations to the tumor site.

7.5 Barriers to Entry: Functional and Physical Obstacles to T Cell Infiltration into Tumors

And yet, in many tumors, such as colorectal cancer, pancreatic cancer and ovarian cancer, the anti-tumoral cytotoxic T cells appear to be excluded from the tumor mass. This is an issue of major importance, as the inability of the T cells to infiltrate into the tumor correlates with poor patient survival [28]. Therapy protocols aiming to combat cancer using anti-tumoral T cells, such Adoptive Cell Therapy (ACT) with cytotoxic T cells [29] will have to resolve this issue in order to maximize their efficacy. As a first step in addressing this problem, it is imperative to understand what keeps the T cells from accessing the tumor.

The ancient Chinese strategist Sun Tzu, in his text "The Art of War", states that "*the worst policy of all is to besiege walled cities*". As recent pioneering studies have uncovered, this aphorism appears to be a suitable metaphor for the means through which tumors manage to protect themselves from T cell attack. Tumors form barriers that impair T cell infiltration. The barriers can spatially inhibit T cell entry, or they can be functional barriers, where the tumor microenvironment undergoes changes that render the T cells unable to infiltrate, even in the absence of physical obstacles.

7.6 Functional Barriers to T Cell Entry

In several human and mouse tumors, the tumor-associated vasculature, unlike healthy vasculature, expresses high levels of the "death receptor" ligand FasL. FasL expression is driven by vascular endothelial growth factor (VEGF), interleukin-10 and prostaglandin E_2. As a result, upon arriving in tumor-associated vessels, anti-tumoral $CD8^+$ cytotoxic T cells are eliminated via FasL-induced apoptosis. At the same time, tumor-promoting Treg are not affected by this mechanism. The result is a functional barrier that inhibits T cell entry into the tumor [30]. A similar blockade of T cell entry into tumors has been reported to result from other defects of tumor vessels, such as increased endothelin B receptor expression [28, 31].

In mouse and human tumors, reactive nitrogen species produced by the tumor have been shown to lead to nitration of the chemokine CCL2. The nitration of the chemokine modifies its binding properties. As a result, the tumor is unable to recruit anti-tumoral $CD8^+$ T cells, whilst pro-tumoral myeloid cells are still recruited; this was explained by the substantially higher levels of expression of the cognate receptor, CCR2, on myeloid cells compared to $CD8^+$ T cells. Experimental dampening of the reactive nitrogen species combined with adoptive T cell therapy in mouse models enabled higher cytotoxic T cell infiltration and a subsequent protection from the tumor [32]. Skin tumors have been shown to evade anti-tumoral T cell responses by reducing the expression of keratinocyte-specific chemokine CCL27, which T cells utilize in order to home to the skin [33].

Finally, targeting of the chemokine CXCL12 by inhibition of its cognate receptor CXCR4, or indeed elimination of Fibroblast Activation Protein-α (FAP)-expressing stromal cells was able to reverse the exclusion of infiltrating T cells in a mouse model of pancreatic ductal carcinoma, via a yet-to-be determined mechanism of functional inhibition of T cell entry [34].

7.7 Physical Barriers to T Cell Entry

Recent work has shown that, in lung and pancreatic tumors, a densely-packed extra cellular matrix (ECM) structure surrounds the tumor. This fibrotic capsule may be the product of Cancer-Associated Fibroblasts (CAFs) that express FAP [28, 35] and it inhibits the infiltration of anti-tumoral T cells into the tumor. Donnadieu and co-workers showed in pioneering ex vivo imaging experiments that T cells were unable to infiltrate human lung tumor due to the dense, parallel collagen fibers that were surrounding the tumor mass [36–38]. Thus, effectively, the collagen fibers act as "walls", through which the T cells cannot traverse easily. Examples of this barrier can be seen in Fig. 7.1, where two sections of the periphery of a mouse solid tumor are presented. A pancreatic carcinoma cell line, PANC02, was injected subcutaneously, leading to the formation of solid tumors, which where subsequently harvested from recipient mice 3 weeks after inoculation. In Fig. 7.1a Masson's trichrome

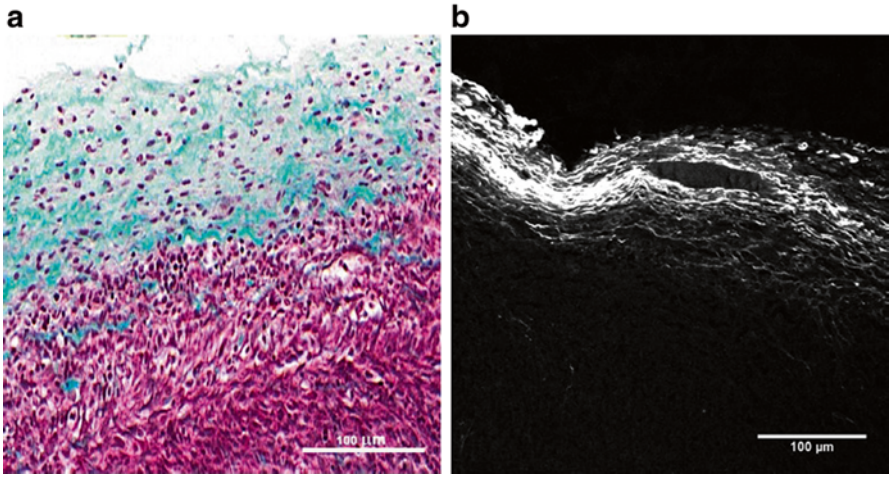

Fig. 7.1 Example of a peri-tumoral fibrotic capsule section. (**a**): Representative image of a tumor section from C57BL/6 J mice with a solid subcutaneous tumor derived from PANC02 murine pancreas ductal adenocarcinoma cells. Harvested tumors were fixed in 4 % formalin, processed for paraffin embedding and sectioned at 3 µm. Tissue collagen content was visualized by staining sections in Masson's trichrome. Images were acquired with an Olympus BX53 microscope with a digital camera. The collagen fibers are *stained cyan*, the nuclei are *stained purple/black* and the cytoplasm is *stained red/pink*. The collagen fibers (*cyan*) form a capsule around the solid tumor (*red/pink*, lower part of the image). (**b**): Representative image of a similar tumor section as in (**a**), acquired using LaVision TrimScope II upright 2-photon microscope. The image shows the collagen capsule visualized via Second Harmonic Generation (SHG) and Multiphoton Microscopy (excitation wavelength 840 nm). Collagen signal is shown in white. The tumor is on the lower part of the image. SHG is an intrinsic signal that does not require any staining and does not suffer from photobleaching. It mostly marks collagen I and to a lower extent other types of collagen. Fibrotic content can thus be quantified as a density of the SHG signal over the tissue area, while other acquisition and analysis techniques can be used to assess the fibrillar order and directionality. As such, it is a powerful technique for the study of fibrosis with subcellular resolution on a wide range of samples from tissue section slides to in vivo models

staining, observed in light microscopy, identifies the collagen fibers (in cyan coloration) in the capsule. Taking advantage of the spontaneous second harmonic generation by collagen under 2-photon microscopy, which enables the detection of a signal from fibrillar collagen without the use of any additional dyes or antibodies [39], the structure of the collagen matrix can be also be clearly visualized, as shown in Fig. 7.1b (collagen fibers shown in white). The role of the stroma in dictating T cell movement is not unique to the tumor environment. Indeed, in lymph nodes, fibroblastic reticular cells, which can also express FAP, form a conduit-like structure that regulates T cell movement and localization [40, 41]. Yet in the case of solid tumors, the stroma, instead of channelling T cell infiltration, appears to keep the cytotoxic T cells out.

Intriguingly, the physical barrier formed by the ECM may not only restrict T cell access to the tumor but also the delivery of chemotherapeutic agents [37, 42–44].

Further, the fibrotic capsule may enhance the aggressive nature of the tumor itself through direct activation of pro-metastatic, growth-enhancing signals in the tumor cells. Work on mouse breast cancer models suggests that the tumor can become more aggressive, aided by signals from the dense collagen matrix that surrounds it. This, for example, can lead to activation of the PI3K pathway, driving tumor progression towards more invasive forms [42, 45].

Whilst this activation is occurring, the tumor takes advantage of the protection offered by the matrix barrier against immune attack or "immunosurveillance" [46], as the process is usually termed. Perhaps ironically, in cases where the tumor is accessible, complete surgical removal of the tumor at this encapsulated stage—when it is constrained by the matrix—would presumably be the only beneficial option created by the existence of the fibrotic capsule. Yet, once tumors reach more advanced and invasive states, they are associated with a peritumoral fibrotic structure that is more radial in orientation, enabling the escape of metastasis-forming cells [42]. Unfortunately, in many cases the tumor or metastasis is not treatable by surgery, and indeed these are many of the clinical contexts where adoptive cell therapy is considered as a potential therapeutic approach. In these cases, the physical barrier described above remains an important obstacle.

7.8 Breaking Through the Barrier

Experimentally, the barrier can be overcome. Indeed there have been many experimental attempts to target the fibrotic structure or the CAF cells that are required for its formation, leading to reduced tumor growth [43, 47]. Examples include enzymatic depletion of the hyaluronan component of the ECM in a model pancreatic tumor, which in combination with chemotherapy led to improved survival [48]. Genetic ablation of Caveolin-1, which is also expressed in CAFs, decreased tumor growth and invasiveness [49]. In a landmark paper, Kraman and colleagues demonstrated that genetic depletion of FAP-expressing CAFs enables the T cell-mediated control of the growth of injected Lewis lung carcinomas or pancreatic tumors [35].

In more recent work in lung cancer ex vivo cultures, T cell infiltration into the tumor was made possible when the peritumoral fibrotic capsule was degraded via the administration of collagenase. As collagen is one of the main components of the capsule, this result confirms that the capsule stands as a physical barrier to T cell entry. Enhanced T cell recruitment into the tumor was also made possible when the tumor cells were manipulated ex vivo so as to over-express the chemokine CCL5, before being injected as a xenograft into immunodeficient mice [36, 38]. The enhanced migration is compatible with the increased expression of the chemokine receptor CCR5 in activated T cells [50]; thus the increased chemokine expression by the tumor must have been sufficient to force T cell migration through the barrier. This matches previous findings that transduction of chemokines in tumors may lead to a reduction in tumor growth, attributable to the influx of T cells [51, 52].

A highly compatible and complementary set of data has been produced by Ozdemir and colleagues. They studied a mouse model of pancreatic ductal carcinoma, where the tumor mass is surrounded by a desmoplastic stroma, mostly composed of myofibroblasts. By genetically depleting the myofibroblasts, the collagen-I-rich fibrosis surrounding the tumor was substantially reduced. This, surprisingly, led to a boost in tumor growth; this was attributable to the increased infiltration of pro-tumoral Treg cells [44]. Nonetheless, these results show that the fibrotic capsule in the pancreatic tumor inhibits T cell access into the tumor; in a pathological context, these T cells may well be pro-tumoral Treg; however during cell therapy, the same barrier would inhibit the entry of anti-tumoral $CD8^+$ cytotoxic T cells.

Whilst the experiments outlined above have been essential in deciphering the mechanisms involved in inhibition of T cell entry, many of them cannot be easily translated to the clinic, as they involve genetic manipulation of the tumor, which is not feasible in a patient. On the other hand, pharmacological treatment of the barrier, whether functional or physical, does have a substantial translational value and extensive work is currently aimed at bringing it closer to the clinic. In parallel, however, there also exist solutions with a focus on the therapeutic T cells that are required to penetrate the tumor. We will discuss these in the next section. Used in combination with approaches targeting the stroma itself, these strategies may assist in providing better future therapeutic options for fighting tumors.

7.9 Modifying T Cells in Order to Improve T Cell Homing to the Tumor

Genetic manipulation of chemokine expression in the tumor so as to attract antitumoral T cells expressing the cognate receptor is not a solution that can be easily translated to the clinic. Some chemotherapeutic drugs [53] as well as the TNF superfamily 14 protein LIGHT are able to induce intratumoral chemokine expression, favoring T cell migration [54]. Nonetheless, the tumors relevant to the application of adoptive T cell therapy are likely to be inaccessible, as otherwise they would have been surgically removed prior to the consideration of immunotherapy. Additionally, if the tumor is metastatic, the site of metastasis may well be diffuse. Thus modifying the chemoattractant features of the tumor itself may not be a simple task. On the other hand, the opposite strategy could be a viable alternative: the cytotoxic T cells used in adoptive T cell therapy can be made to express chemokine receptors that match the chemokines released by the tumor. The current consensus practice in adoptive T cell therapy, irrespective of whether the T cells used are peripheral blood T cells or purified tumor-infiltrating T cells, often involves expanding the T cells ex vivo prior to administration to the patient [29]. During this phase, the T cells are not only accessible, but can be readily transduced with viral constructs. Indeed the antigen-specificity of the T cells, via the transduction of cloned T cell antigen receptors (TCRs) or chimeric molecules that enable antigen

recognition (Chimeric Antigen Receptors—CARs), is usually modified during an expansion via ex vivo culture [29]. If a chemokine receptor matching the chemokine expressed by the tumor were to be transduced into the T cells at the same time, the resulting cytotoxic T cells would have not only improved antigen specificity for the tumor, but also the "homing instructions" necessary to infiltrate into the tumor mass. Much like the increased tumor chemokine production, this would hopefully be sufficient to counteract the barriers behind which the tumor lies, and enable tumor infiltration. This strategy of "enhanced migration" has been shown to function experimentally by our work in a different pathological context [50]. The application of this concept in tumors was outlined by Kershaw and colleagues, who demonstrated that tumor cells from melanoma patients secreted CXCL1. They thus cloned the cognate receptor, CXCR2, and transduced it in T cells, showing that the resulting cells had higher in vitro migration capacity towards the chemokine [55]. Subsequent studies by the same group showed that in vivo, CXCR2-tranduced T cells could migrate with higher efficiency towards implanted CXCL1-expressing tumor cells, leading to better anti-tumor responses [56].

As Treg and Th2 cells, both tumor-promoting populations, express the chemokine receptor CCR4, reports have shown that transducing $CD8^+$ T cells with this receptor improves their homing and anti-tumor efficiency. The strategy was demonstrated using human T cells, double-transduced with the chemokine receptor and a CAR, in a mouse xenograft model of Hodgkin's lymphoma [57]. A more recent study has confirmed similar findings in a mouse model of injected pancreatic tumor [58].

Craddock and colleagues argued that many tumors express CCL2, and thus they transduced human T cells with the cognate receptor, CCR2b. They proceeded to show enhanced migration to tumors and improved anti-tumor activity in immunodeficient mice injected with the modified T cells and a CCL2-producing neuroblastoma cell line [59]. Moon and co-workers found that mesothelioma cells produce CCL2, and demonstrated a similar effect using immunodeficient mice injected with the modified T cells and a mesothelioma cell line [60].

The above studies clearly demonstrate that "enhanced" migration, engineered by the transduction of a suitable chemokine receptor, can lead to improved infiltration of the cytotoxic T cells into the tumor, with measurable beneficial effects for therapy. Whilst not necessarily all the tumor models used were characterized by a peritumoral fibrotic capsule, it is likely that the ectopic chemokine receptor expression may aid in penetrating this physical barrier. In a bid to further extend the translational potential of this strategy, we have recently addressed one of the few remaining limitations of the prior studies, namely, that in all cases an intentionally inoculated tumor was targeted. We thus applied the above chemokine receptor modification strategy albeit in a spontaneous model of mouse prostate tumor, the TRAMP model [61], which—importantly—forms spontaneous metastases in lymph nodes starting from week 24 of age. We thus identified the spontaneous metastases, characterized their chemokine expression profiles, identified the most promising candidate chemokine and cloned its matching receptor. We then co-transduced cytotoxic T cells with both the chemokine receptor as well as a TCR specific for an antigen expressed by the tumor. As with the studies discussed above, we obtained improved homing of

the T cells, in this case towards spontaneous lymph node metastases [62]. The main benefit of this approach is that it highlights the flexibility of the chemokine modification strategy: T cell immunotherapy is likely to be a realistic clinical alternative only in cases where more traditional therapeutic strategies have failed. In such cases, which may well be characterized by diffuse metastases, characterizing the chemokine profile of an accessible metastatic focus may provide a gateway to obtaining the necessary access information required to "push" T cells to sites of metastasis that are inaccessible or even unknown. Admittedly, this would be applicable to those metastases that originate from the same clone, which is not always the case [63]. Yet, in this manner, a biopsy of an accessible metastasis could be used to "calibrate and instruct" the therapeutic T cells to home to (and infiltrate into) similar but inaccessible metastases.

Interestingly, in situations where metastatic foci are visible and accessible, a novel and innovatively alternative approach has been recently proposed. A CXCR4 gene was fused with an optogenetic-control construct, enabling T cells transduced with the engineered receptor to switch on chemokine receptor signalling (and thus migration) in response to light. The result was that light shone on a melanoma site could activate T cell infiltration into the tumor, enabling enhanced anti-tumor activity [64]. Conversely to the above, this approach would require clear prior identification of all tumor or metastatic sites.

7.10 Conclusion

Taken together, the chemokine receptor modification strategies highlighted here offer a range of tools that could be easily integrated into adoptive T cell therapy protocols. As an additional benefit, the risks associated with the viral transduction process, such as insertional mutagenesis, are the same as those associated with protocols of TCR or CAR transduction. Thus one can be hopeful that solutions found to these issues, such as more advanced gene delivery technologies [65] or added safety features [66] should automatically be applicable to chemokine receptor delivery [67]. Modifying chemokine receptor expression is likely to improve adoptively transferred T cell migration to the tumor and infiltration into the tumor mass. It may not completely overcome the barriers of a different nature, as described above; further experimentation will be required to assess this in greater detail. Yet, if combined with therapeutic strategies targeting the barriers, chemokine receptor modification of T cells has the potential to become a useful strategy to enhance T cell therapy, hopefully leading to better clinical outcomes in cancer.

Acknowledgments This work was supported by AIRC (MFAG10752), the Italian Ministry of Health (GR-2009-1558698) and FIRB RBFR10HP97. We would like to thank Dr R Bonecchi for helpful comments.

Conflict of interest statement: The authors declare that they have no potential conflicts of interest.

References

1. Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R. The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev. 2010;21:27–39.
2. Balkwill FR. The chemokine system and cancer. J Pathol. 2012;226:148–57.
3. Savino B, Caronni N, Anselmo A, Pasqualini F, Borroni EM, Basso G, Celesti G, Laghi L, Tourlaki A, Boneschi V, Brambilla L, Nebuloni M, Vago G, Mantovani A, Locati M, Bonecchi R. ERK-dependent downregulation of the atypical chemokine receptor D6 drives tumor aggressiveness in Kaposi sarcoma. Cancer Immunol Res. 2014;2:679–89.
4. Sarris M, Masson JB, Maurin D, Van der Aa LM, Boudinot P, Lortat-Jacob H, Herbomel P. Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. Curr Biol. 2012;22:2375–82.
5. Ellem SJ, Taylor RA, Furic L, Larsson O, Frydenberg M, Pook D, Pedersen J, Cawsey B, Trotta A, Need E, Buchanan G, Risbridger GP. A pro-tumourigenic loop at the human prostate tumour interface orchestrated by oestrogen, CXCL12 and mast cell recruitment. J Pathol. 2014;234:86–98.
6. Conley-LaComb MK, Saliganan A, Kandagatla P, Chen YQ, Cher ML, Chinni SR. PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling. Mol Cancer. 2013;12:85.
7. Lanca T, Costa MF, Goncalves-Sousa N, Rei M, Grosso AR, Penido C, Silva-Santos B. Protective role of the inflammatory CCR2/CCL2 chemokine pathway through recruitment of type 1 cytotoxic gammadelta T lymphocytes to tumor beds. J Immunol. 2013;190:6673–80.
8. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer. 2004;4:540–50.
9. Tsaur I, Noack A, Makarevic J, Oppermann E, Waaga-Gasser AM, Gasser M, Borgmann H, Huesch T, Gust KM, Reiter M, Schilling D, Bartsch G, Haferkamp A, Blaheta RA. CCL2 chemokine as a potential biomarker for prostate cancer: a pilot study. Cancer Res Treat. 2014;47(2):306–12.
10. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A. 1998;95:9448–53.
11. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410:50–6.
12. Naumann U, Cameroni E, Pruenster M, Mahabaleshwar H, Raz E, Zerwes HG, Rot A, Thelen M. CXCR7 functions as a scavenger for CXCL12 and CXCL11. PLoS One. 2010;5, e9175.
13. Chen Y, Ramjiawan RR, Reiberger T, Ng MR, Hato T, Huang Y, Ochiai H, Kitahara S, Unan EC, Reddy TP, Fan C, Huang P, Bardeesy N, Zhu AX, Jain RK, Duda DG. CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenib-treated hepatocellular carcinoma in mice. Hepatology. 2015;61:1591–602.
14. Sarvaiya PJ, Guo D, Ulasov I, Gabikian P, Lesniak MS. Chemokines in tumor progression and metastasis. Oncotarget. 2013;4:2171–85.
15. Allavena P, Germano G, Marchesi F, Mantovani A. Chemokines in cancer related inflammation. Exp Cell Res. 2011;317:664–73.
16. Boimel PJ, Smirnova T, Zhou ZN, Wyckoff J, Park H, Coniglio SJ, Qian BZ, Stanley ER, Cox D, Pollard JW, Muller WJ, Condeelis J, Segall JE. Contribution of CXCL12 secretion to invasion of breast cancer cells. Breast Cancer Res. 2012;14:R23.
17. Murakami T, Cardones AR, Hwang ST. Chemokine receptors and melanoma metastasis. J Dermatol Sci. 2004;36:71–8.
18. Jachetti E, Caputo S, Mazzoleni S, Brambillasca CS, Parigi SM, Grioni M, Piras IS, Restuccia U, Calcinotto A, Freschi M, Bachi A, Galli R, Bellone M. Tenascin-C protects cancer stem-like cells from immune surveillance by arresting T-cell activation. Cancer Res. 2015;75:2095–108.
19. Ben-Baruch A. The multifaceted roles of chemokines in malignancy. Cancer Metastasis Rev. 2006;25:357–71.

20. Izhak L, Wildbaum G, Uri W, Shaked Y, Alami J, Dumont D, Stein A, Karin N. Predominant expression of CCL2 at the tumor site of prostate cancer patients directs a selective loss of immunological tolerance to CCL2 that could be amplified in a beneficial manner. J Immunol. 2010;184:1092–101.
21. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29:313–26.
22. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity and subset definition. Curr Opin Immunol. 2010;22:238–44.
23. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol. 2010;11:7–13.
24. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. J Exp Med. 2003;198:889–901.
25. Fridlender ZG, Buchlis G, Kapoor V, Cheng G, Sun J, Singhal S, Crisanti C, Wang LC, Heitjan D, Snyder LA, Albelda SM. CCL2 blockade augments cancer immunotherapy. Cancer Res. 2010;70:109–18.
26. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, Foster AE, Heslop HE, Brenner MK, Dotti G, Savoldo B. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. Blood. 2009;113:6392–402.
27. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, McKee M, Gajewski TF. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res. 2009;69:3077–85.
28. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348:74–80.
29. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12:269–81.
30. Motz GT, Santoro SP, Wang LP, Garrabrant T, Lastra RR, Hagemann IS, Lal P, Feldman MD, Benencia F, Coukos G. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. Nat Med. 2014;20:607–15.
31. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med. 2008;14:28–36.
32. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208(10):1949–62.
33. Pivarcsi A, Müller A, Hippe A, Rieker J, van Lierop A, Steinhoff M, Seeliger S, Kubitza R, Pippirs U, Meller S, Gerber PA, Liersch R, Buenemann E, Sonkoly E, Wiesner U, Hoffmann TK, Schneider L, Piekorz R, Enderlein E, Reifenberger J, Rohr UP, Haas R, Boukamp P, Haase I, Nürnberg B, Ruzicka T, Zlotnik A, Homey B. Tumor immune escape by the loss of homeostatic chemokine expression. Proc Natl Acad Sci U S A. 2007;104:19055–60.
34. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL, Teichmann SA, Janowitz T, Jodrell DI, Tuveson DA, Fearon DT. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013;110:20212–7.
35. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, Gopinathan A, Tuveson DA, Fearon DT. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science. 2010;330:827–30.
36. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, Mami-Chouaib F, Donnadieu E. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 2012;122:899–910.

37. Salmon H, Donnadieu E. Within tumors, interactions between T cells and tumor cells are impeded by the extracellular matrix. Oncoimmunology. 2012;1:992–4.
38. Peranzoni E, Rivas-Caicedo A, Bougherara H, Salmon H, Donnadieu E. Positive and negative influence of the matrix architecture on antitumor immune surveillance. Cell Mol Life Sci. 2013;70(23):4431–48.
39. Martin TP, Norris G, McConnell G, Currie S. A novel approach for assessing cardiac fibrosis using label-free second harmonic generation. Int J Cardiovasc Imaging. 2013;29(8):1733–40.
40. Bajenoff M, Egen JG, Koo LY, Laugier JP, Brau F, Glaichenhaus N, Germain RN. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity. 2006;25:989–1001.
41. Denton AE, Roberts EW, Linterman MA, Fearon DT. Fibroblastic reticular cells of the lymph node are required for retention of resting but not activated CD8+ T cells. Proc Natl Acad Sci U S A. 2014;111:12139–44.
42. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ. Collagen density promotes mammary tumor initiation and progression. BMC Med. 2008;6:11.
43. Erkan M, Hausmann S, Michalski CW, Fingerle AA, Dobritz M, Kleeff J, Friess H. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. Nat Rev Gastroenterol Hepatol. 2012;9:454–67.
44. Ozdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, De Jesus-Acosta A, Sharma P, Heidari P, Mahmood U, Chin L, Moses HL, Weaver VM, Maitra A, Allison JP, LeBleu VS, Kalluri R. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell. 2014;25:719–34.
45. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139:891–906.
46. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410:1107–11.
47. Spector I, Zilberstein Y, Lavy A, Nagler A, Genin O, Pines M. Involvement of host stroma cells and tissue fibrosis in pancreatic tumor development in transgenic mice. PLoS One. 2012;7, e41833.
48. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. Cancer Cell. 2012;21:418–29.
49. Goetz JG, Minguet S, Navarro-Lerida I, Lazcano JJ, Samaniego R, Calvo E, Tello M, Osteso-Ibanez T, Pellinen T, Echarri A, Cerezo A, Klein-Szanto AJ, Garcia R, Keely PJ, Sanchez-Mateos P, Cukierman E, Del Pozo MA. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. Cell. 2011;146:148–63.
50. Kallikourdis M, Andersen KG, Welch KA, Betz AG. Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus. Proc Natl Acad Sci U S A. 2007; 104:594–9.
51. Mulé JJ, Custer M, Averbook B, Yang JC, Weber JS, Goeddel DV, Rosenberg SA, Schall TJ. RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity in vivo: role of immune cell subpopulations. Hum Gene Ther. 1996;7:1545–53.
52. Ruffini PA, Morandi P, Cabioglu N, Altundag K, Cristofanilli M. Manipulating the chemokine-chemokine receptor network to treat cancer. Cancer. 2007;109:2392–404.
53. Tan KW, Evrard M, Tham M, Hong M, Huang C, Kato M, Prevost-Blondel A, Donnadieu E, Ng LG, Abastado JP. Tumor stroma and chemokines control T-cell migration into melanoma following Temozolomide treatment. Oncoimmunology. 2015;4, e978709.
54. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol. 2013;14:1014–22.

55. Kershaw MH, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, Wang E, Young HA, Murphy PM, Hwu P. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum Gene Ther. 2002;13:1971–80.
56. Peng W, Ye Y, Rabinovich BA, Liu C, Lou Y, Zhang M, Whittington M, Yang Y, Overwijk WW, Lizee G, Hwu P. Transduction of tumor-specific T cells with CXCR2 chemokine receptor improves migration to tumor and antitumor immune responses. Clin Cancer Res. 2010;16:5458–68.
57. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, Foster AE, Heslop HE, Brenner MK, Dotti G, Savoldo B. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. Blood. 2009;113:6392-402. doi: 10.1182/blood-2009-03-209650.
58. Rapp M, Grassmann S, Endres S, Anz D, Kobold S. ITOC2–025. Transduction with C-C-chemokine receptor type 4 (CCR4) enhances tumour-specific migration of adoptively transferred T cells in a model of pancreatic cancer. Eur J Cancer. 2015;51 Suppl 1:S9.
59. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM, Foster AE. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother. 2010;33:780–8.
60. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, Powell DJJ, Riley JL, June CH, Albelda SM. Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human t cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res. 2011;17:4719–30.
61. Hurwitz AA, Foster BA, Allison JP, Greenberg NM, Kwon ED. The TRAMP mouse as a model for prostate cancer. Curr Protoc Immunol. 2001;Chapter 20:Unit 20.5.
62. Garetto S, Sardi C, Martini E, Roselli G, Morone D, Angioni R, Cianciotti BC, Trovato AE, Franchina DG, Castino GF, Vignali D, Erreni M, Marchesi F, Rumio C, Kallikourdis M. Tailored chemokine receptor modification improves homing of adoptive therapy T cells in a spontaneous tumor model. Oncotarget. 2016; [Epub ahead of print]. doi: 10.18632/oncotarget.9280.
63. Hafner C, Knuechel R, Stoehr R, Hartmann A. Clonality of multifocal urothelial carcinomas: 10 years of molecular genetic studies. Int J Cancer. 2002;101:1–6.
64. Xu Y, Hyun YM, Lim K, Lee H, Cummings RJ, Gerber SA, Bae S, Cho TY, Lord EM, Kim M. Optogenetic control of chemokine receptor signal and T-cell migration. Proc Natl Acad Sci U S A. 2014;111:6371–6.
65. Beane JD, Lee G, Zheng Z, Mendel M, Abate-Daga D, Bharathan M, Black M, Gandhi N, Yu Z, Chandran S, Giedlin M, Ando D, Miller J, Paschon D, Guschin D, Rebar EJ, Reik A, Holmes MC, Gregory PD, Restifo NP, Rosenberg SA, Morgan RA, Feldman SA. Clinical scale zinc finger nuclease-mediated gene editing of PD-1 in tumor infiltrating lymphocytes for the treatment of metastatic melanoma. Mol Ther. 2015;23(8):1380–90.
66. June CH. Adoptive T, cell therapy for cancer in the clinic. J Clin Invest. 2007;117:1466–76.
67. Engels B, Uckert W. Redirecting T lymphocyte specificity by T cell receptor gene transfer—a new era for immunotherapy. Mol Aspects Med. 2007;28:115–42.

Chapter 8
Strategies to Enhance Migration and Persistence of Chimeric Antigen Receptor (CAR)-T Cells into Tumors

Yuhui Chen, Gianpietro Dotti, and Barbara Savoldo

Abstract Since the past few decades, immunotherapy based on the adoptive transfer of tumor-specific T-cells is providing a promising form of cancer treatment. This result has been obtained through the improvement of the methodologies used to expand ex vivo antigen-specific T cells, and through technology advancements in T-cell engineering aimed at transferring into these cells chimeric antigen receptors (CAR) or αβTCRs chains. High enthusiasm is especially directed to the CAR technology, as this confers MHC-independent antigen-specificity to T cells, thus allowing broad clinical applications. The greatest advances have been achieved in lymphoid malignancies, while the clinical benefits of CAR-T cells to solid tumors still remain uncertain. In this review we will explore two of the problems that are currently perceived as limiting the success of the clinical translation of CAR-T cells in solid tumors: namely, the recruitment and survival of tumor-specific T cells in the microenvironment.

Keywords Chimeric antigen receptor • Immunotherapy • Chemokines • Migration and Infiltration • Improving Persistence • Overcoming immune-evasion

---

Y. Chen
Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

G. Dotti
Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA

B. Savoldo (✉)
Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Department of Pediatrics, University of North Carolina, Chapel Hill, NC, USA
e-mail: bsavoldo@med.unc.edu

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7_8

Abbreviations

Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
CAR	Chimeric antigen receptor
CD	Cluster of Differentiation
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DPP4	Dipeptidyl peptidase 4
ECM	Extra cellular matrix
ERK	Extracellular signal-regulated kinases
GD2	Ganglioside Diasialo 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gro-α	Growth-regulated oncogene-α
HVEM	Herpes Virus Entry Mediator
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
IL	Interleukin
LAG3	Lymphocyte-activation gene 3
M2	Macrophages type 2
MDC	Macrophage-derived chemokine
MHC	major histocompatibility complex
MDSC	Myeloid derived suppressor cells
NF-kB	Nuclear factor-kB
NKGD2	Natural Killer Group 2D
PD1	Programmed cell death protein 1
PD-L1/PD-L2	Programmed death-ligand 1/2
PGs	Proteoglycans
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
ROS	Reactive oxygen species
siRNA	Small interfering RNA
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
Tregs	Regulatory T cells

8.1 Introduction

Since the past few decades, immunotherapy based on the adoptive transfer of tumor-specific T-cells is providing a promising form of cancer treatment. This result has been obtained through the improvement of the methodologies used to expand ex vivo antigen-specific T cells, and through technology advancements in T-cell engineering aimed at transferring into these cells chimeric antigen receptors (CAR) or αβTCRs chains. High enthusiasm is especially directed to the CAR technology,

as this confers MHC-independent antigen-specificity to T cells, thus allowing broad clinical application. The greatest advances have been achieved in lymphoid malignancies, while the clinical benefits of CAR-T cells to solid tumors still remain uncertain. In this review we will explore two of the problems that are currently perceived as limiting the success of the clinical translation of CAR-T cells in solid tumors: namely, the recruitment and survival of tumor-specific T cells in the microenvironment.

8.2 CAR-T Cells and Solid Tumors

The typical basic structure of CARs consists of a single chain variable fragment (scFv) derived from a monoclonal antibody, to confer antigen binding specificity, joined to one component of the T cell receptor (TCR) complex, usually the ζ chain, that grants activation of the T-cell killing machinery (1st generation CAR) [1]. It is now well recognized that the engagement of CAR molecules with the tumor antigen is often inadequate for optimal activation of T cells, as the majority of tumor cells lack the expression of co-stimulatory molecules, or express inhibitory ligands that concur in impairing T cell functions [1]. To address the former issue efforts have been made to incorporate relevant co-stimulatory endodomains within CARs, including CD28, 4-1BB, OX40 or a combination of them to provide full activation of the T cells (2nd and 3rd generations CAR).

The adoptive transfer of CAR-T cells specific for the CD19 antigen has resulted in complete tumor regressions in almost 80 % of patients with relapsed acute lymphoblastic leukemia [2]. Promising clinical responses occur also in patients with chronic lymphocytic leukemia and lymphomas [3, 4]. Complete and sustained responses appear to correlate with the expansion of CAR-T cells post infusion in vivo in the peripheral blood and their long-term persistence (>6 months) [4–15]. CAR-T cells in responding patients have been detected not only in the peripheral blood, but also in the bone marrow and in lymph nodes that are the usual sites of tumor localization [4].

Provided that an appropriate antigen is identified for targeting solid tumors and that a CAR targeting this antigen can be successfully constructed, several challenges remain in the solid tumor setting for CAR-T cells to promote clinically relevant antitumor effects. First of all, leukemic cells or normal B lymphocytes expressing the CD19 antigen are immediately available for attack by CD19-specific CAR-T cells infused intravenously. In addition, secondary lymphoid organs, such as lymph nodes and bone marrow where B cell-derived tumor cells are located, are usually accessible to T cells. By contrast, the migration and infiltration of CAR-T cells to solid tumors has proved more demanding. Second, when CAR-T cells reach the tumor environment, they must maintain high proliferative capacity in order to capsize tumor cells. In solid tumors CAR-T cells are facing a more complex and immunosuppressive scenarios as compared to "liquid tumors". The tumor microenvironment in solid tumors is indeed represented by a multitude of stromal cell types, including

endothelial cells, pericytes, fibroblasts, mesenchymal stem cells, macrophages, myeloid suppressor cells and regulatory T cells (Tregs) [16]. Finally, tumor and tumor-associated cells produce a plethora of cytokines and molecules capable of directly inhibiting T cells or altering the biological properties of surrounding cells.

8.3 CAR-T Cells and Migration to Tumors

A critical step for successful tumor destruction by CAR-T cells is their robust recruitment to the tumor site (Fig. 8.1). This process consists of at least two critical steps: T-cell trafficking to the antigen site and active T-cell infiltration within the extra cellular matrix (ECM) that sustains the tridimensional structure of the tumor. The migration of antigen-specific T cells from the blood to peripheral tissues includes T-cell tethering, rolling, adhesion and transmigration through the endothelial cells that ultimately relocate T cells through post-capillary venules into the antigenic site [17]. In the majority of the cases, adoptively transferred CAR-T cells are delivered by intravenous infusion, implicating that they need to actively traffic from the blood vessels to peripheral tissues. Soluble and tissue-bonded chemokines direct T cells in a specific location of the vascular wall, where T cells initiate their extravasation and, therefore, a match between the chemokines produced by the tumors and the chemokine receptors expressed by T cells is critical for their recruitment [18]. However, antigen-specific T cells also actively secrete enzymes, such as elastase, metalloproteinases, hyaluronidase, and heparanase, which disrupt the ECM and facilitate leukocyte tissue-infiltration [19].

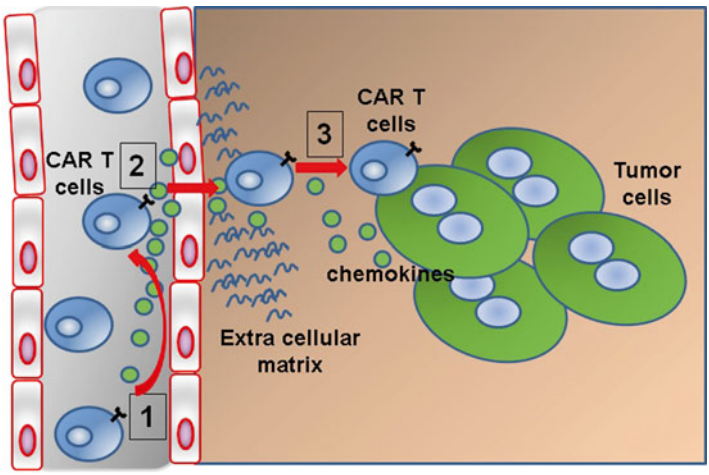

Fig. 8.1 Trafficking of T cells to tumors. This figure illustrates the critical steps required by antigen-specific T cells to effectively accumulate within the tumor microenvironment. *1*. Migration; *2*. Extravasation; *3*. Modification of the extra cellular matrix

8.3.1 T-Cell Trafficking

Despite tumor cells or surrounding stroma cells often secrete abundant and numerous chemokines, adoptively transferred CAR-T cells frequently lack the expression of the appropriate chemokine receptor. To overcome this deficiency, chemokine receptors can be expressed in association with CAR molecules in T cells. For example, to improve the antitumor effects of CAR-T cells in Hodgkin's lymphoma, the CD30-specific CAR has been coupled with the chemokine receptor CCR4 [20]. Indeed, CCR4 is not expressed by effector T cells, while Reed-Stemberg cells are well documented to produce the chemokines thymus and activation-regulated chemokine/CC chemokine ligand 17 (TARC/CCL17) and macrophage-derived chemokine (MDC)/CCL22 which play a role in recruiting Th2 and Tregs within the lymphoma environment [21]. Therefore, by co-expressing the CAR targeting the CD30 molecule, which is found on Hodgkin's tumor cells, and the chemokine receptor CCR4, CAR-T cells can be induced to migrate preferentially to the lymphoma and accomplish an improved antitumor activity [20]. Another well studied chemokine receptor is CCR2 that is also not very well expressed by effector T cells. The corresponding chemokine CCL2 is secreted by a variety of malignancies, including neuroblastoma, glioma, melanoma and breast cancer [22]. The co-expression of a subunit of the CCR2 receptor (CCR2b) and of a CAR targeting the neuroblastoma-associated antigen GD2 has been explored preclinically, demonstrating an enhanced migration to neuroblastoma tumors and consequent increased antitumor activity [23]. Another successful example is the expression of the CXCR2 receptor, whose ligand growth-regulated oncogene-α (Gro-α; CXCL1) is produced by a wide range of tumors [24].

An alternative strategy to the ectopic expression of a chemokine receptor to guide T-cell trafficking to the tumor site is the modification of the tumor microenvironment to produce chemokines for which adoptively transferred CAR-T cells constitutively express the specific receptor. A good example is represented by the chemokine RANTES since its receptors, CCR1, CCR3 and CCR5, are usually retained by ex vivo expanded CAR-T cells [25]. To promote the release of a specific chemokine such as RANTES by tumor cells, armed oncolytic viruses that have a specific "tropism" for tumor cells have been used. Tumors infected by a RANTES/IL-15-armed oncolytic virus locally release these factors, and when the administration of the oncolytic virus is combined with CAR-T cells a robust migration and survival of CAR-T cells within the tumor is achieved with consequent significantly enhanced antitumor activity [26]. New and improved biological agents (vaccinia virus) or inert vehicles (nanoparticles) can also be engineered to locally deliver attractive chemokines, among other molecules, and will certainly fuel the field of CAR-T cells in solid tumors. Moreover, inhibition of in vivo post-translational processing of chemokines may also prove a useful approach to improve migration to tumor sites. For instance, the inhibition of Dipeptidyl peptidase 4 (DPP4), a member of the large family of proteases that can cleave several chemokines, by sitagliptin enhances antitumor responses to melanoma by enhanced CXCL10 and CXCR3-dependent tumor immunity when combined with adjuvant therapies [27].

8.3.2 CAR-T Cell and Extra Cellular Matrix (ECM)

While the chemokine gradient drives CAR-T cells to the tumor site, this process needs to be complemented by an active infiltration of T cells into the antigen-rich tissues. The infiltration of effector T cells within the tumor often positively correlates with clinical responses and prognosis [28–30]. The same concept applies to CAR-T cells, with infiltration into bone marrow and lymph nodes frequently being associated with prolonged and sustained responses in patients infused with CD19-specific CAR-T cells [4–15]. However, unlike lymphoid tissues, solid tumors are frequently richer in stroma, which includes a complex milieu of non-malignant cells, such as fibroblasts and mesenchymal cells, and abundant ECM. The interaction between tumor cells and the surrounding stroma facilities the initiation, progression, metastasis and chemoresistance of solid tumors [31]. CAR-T cells must actively degrade the main components of the sub-endothelial basement membrane and of the ECM to reach the tumor cells. While this property is physiologically present in circulating T cells, ex vivo cultured T lymphocytes, such as CAR-T cells, are defective in degrading heparan sulfate proteoglycans because they lack the enzyme heparanase [32]. Re-expressing the enzyme in CAR-T cells clearly restores the capability of CAR-T cells to infiltrate the tumor microenvironment and promote better control of the tumor growth [32]. There are more than 300 proteins in the ECM in mammals, which included core matrisome, collagen, proteoglycans (PGs) and glycoprotein [33]. The endopeptidases that degrade and cleave ECM components include matrix metalloproteinases, adamalysins, meprins and other remodeling enzymes such as Serine proteases, cathepsins and sulphatases [19]. These enzymes and molecules are likely participating in the degradation and/or formation of the ECM and may contribute differently to the matrix formation of different tumors. Therefore, specific tuning may be required for successful tumor infiltration by CAR-T cells.

8.4 Persistence of CAR-T Cells at the Tumor Site

Even if tumor-specific T cells succeed in migrating to and infiltrating the tumor site, multiple mechanisms exploited by tumor cells themselves or by surrounding stromal cells can block an effective immune response (Fig. 8.2). Therefore, it is pivotal that CAR-T cells are well equipped to overcome inhibitory mechanisms and to persist once in the tumor.

Human tumor cells have evolved to express many different molecules that contribute to hamper T-cell responses. The great majority of the inhibitory molecules, cells and cell-surface ligands found in the tumor microenvironment act by limiting T cell signaling, cytokine production and cell cycle progression. Many evidences clearly point at the need for lymphodepleting conditioning regimens, to allow CAR-T cell expansion upon adoptive transfer [34, 35]. Lymphodepletion creates

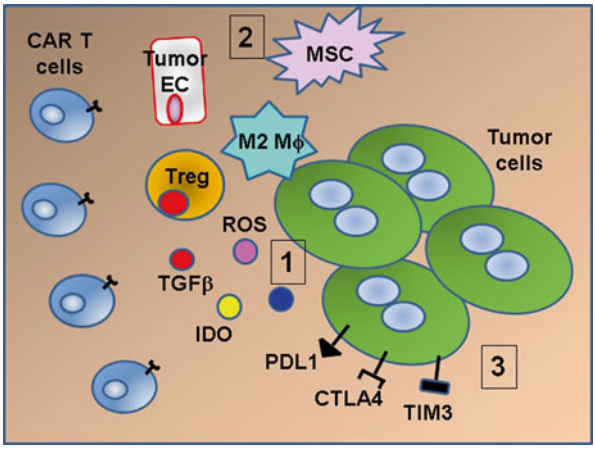

Fig. 8.2 Tumor suppressive microenvironment. This figure illustrates complex network of inhibitory mechanisms that tumor-specific T cells can encounter within the tumor microenvironment. *1*. Inhibitory molecules; *2*. Inhibitory cells; *3*. Inhibitory ligands. *EC* endothelial cells, *MSC* myeloid suppressor cells, *M2 Mf* M2-macrophages, *Treg* regulatory T cells, *ROS* reactive oxygen species

less competition for the availability of homeostatic cytokines, such as IL-7 and IL-15 that sustain T cell expansion [36]. When considering solid tumors, lymphodepleting chemotherapy may also act in disrupting the tumor microenvironment, thus improving the prospects for CAR-T cell persistence, expansion and antitumor activity. However, experiences in other adoptive immunotherapy settings suggest that further strategies will likely need to be included [37].

8.4.1 CAR-T Cells and Inhibitory Molecules

One of the first molecules implicated in tumor escape is TGF-β, which is produced by many tumor cells and stroma cells [38]. Tumor-derived TGF-β hinders antigen presentation by antigen-presenting cells and T-cell proliferation [39, 40]. TGF-β also renders the tumor microenvironment unfavorable for T cells, by transforming macrophages into a more tumor-supportive phenotype (M2 cells) [39, 41], converting $CD4^+$ $CD25^-$ T cells into Tregs [42] and attenuating the acquisition and expression of effector function of tumor specific T cells [43]. Blocking TGF-β signaling in T cells enhances their effectiveness [44]. One example takes advantage of the expression of a dominant negative TGFβ receptor in tumor-specific T cells that disrupts the phosphorylation of Smad2 in the TGFβ signaling pathway. As a result, TGFβ-resistant T cells can eliminate tumor cells in the presence of TGFβ without being affected in their function, phenotype, or growth characteristics [45, 46].

Metabolic inhibition of T cells by tryptophan catabolism allows tumors to resist immune destruction. Local degradation of tryptophan results in T cell inhibition

caused by cell cycle arrest, apoptosis mediated by the catabolites kynurenine and its derivatives and T cell differentiation into induced Tregs [47, 48]. Indoleamine 2, 3-dioxygenase (IDO) is a tryptophan-degrading enzyme, ubiquitously produced in mammalian tissues and found often upregulated in tumor cells [49]. IDO impairs T cells since tryptophan metabolites inhibit the secretion of IL-2, IL-7, and IL-15 and increase T cell apoptosis [50]. Co-stimulation provided into CAR-T cells may only partially counteract the inhibitory effects of TGFβ and metabolic inhibition, and thus CAR-T cells may benefit from the combination with molecules that antagonize TGFβ, such a dominant negative receptor, and the effects of IDO. Specifically, in a xenograft lymphoma model, inhibition of CAR-T cells IDO-mediated could be reverted by using an IDO inhibitor (1-methyl-tryptophan) [50].

8.4.2 CAR-T Cells and Inhibitory/Suppressive Cells

Tumors often recruit and sustain the proliferation and differentiation of immune-suppressive immune cells such as Tregs, myeloid-derived suppressor cells (MDSC), type 2 macrophages and mesenchymal stromal cells to create an inhibitory environment. The incorporation of co-stimulatory endodomains within CARs can per se confer T cells with resilience. For example, the incorporation of the CD28 signaling domain into CAR not only augments NF-kB expression and IL-2-dependent proliferation of CAR-T cells after antigen engagement, but also reduces their inhibition from Tregs and/or inhibitory cytokines like IL-10 and partially TGFβ [51]. More recently it has been reported that the deletion of the *lck* binding site of the CD28 endodomain abrogates IL-2 release upon CAR engagement, which in turn is no longer available to sustain Treg proliferation and inhibitory function. This effect is obtained without impairing IFN-γ secretion, proliferation, and cytolysis of CAR-T cells warranting antitumor activity in the presence of Tregs [52].

Provision of cytokines either systemically or endogenously from T cells or during the ex vivo culture process may also prove advantageous to overcome inhibition from Tregs. Recombinant IL-2 has been administered with the purpose to sustain expansion and prolong T cell persistence of adoptively transferred tumor-specific T cells in patients with melanoma [53, 54]. However, IL-2 also benefits the proliferation and function of Tregs [55]. As alternative, the systemic administration of IL-15 may be explored as this γ-chain cytokine seems to preferentially sustain the proliferation of T cells rather than Tregs, while reducing activation-induced cell death [56]. Interestingly, preclinical data suggest that the IL-15-mediated advantages may be imprinted to T cells during the ex vivo culture [56]. Alternatively, the provision of cytokines with more contained potential for toxicities can be achieved by genetic modification of T cells. For example, IL-15 has been incorporated into CAR to confer superior persistence and improved antitumor activity in preclinical models [57]. Finally oncolytic viruses, due to their great cargo capacity, have been used in mouse models to deliver IL-15 at the tumor site, so that in combination with CAR-T cells their local survival is highly promoted, leading to improved overall survival

[26]. Another cytokine of interest is IL-12, which can induce Th1-type responses and tumor infiltration by both natural killer cells and macrophages [58]. CAR-T cells have therefore been explored as a mean to induce local release of IL-12 [59]. In addition to locally attract macrophages, IL-12 also activates T cells, contributing to the changes of the immunosuppressive environment with resulting increased amounts of Th1 cytokines and reduced IL-4 and IL-5 levels. Finally, CARs can be coupled with the expression of specific cytokine receptors to favor their use of homeostatic cytokines and restore their proliferation even in the presence of immunosuppressive cells [60].

Myeloid derived suppressor cells (MDSC) blunt lymphocyte responses through the superoxide pathway [61] and production of IDO [62]. Strategies to counter MDSC have been explored in combination with CAR-T cells. Interestingly, the lymphodepleting regimen based on fludarabine and cyclophosphamide, which is frequently used before CAR-T cell administration, has also the advantage of down regulating IDO expression in lymphoma cells and, thus, contributes in improving antitumor activity [50].

Pro-tumor-associated macrophages inhibit T cell responses through the release of IL-10, prostaglandins and reactive oxygen species (ROS) [63]. Strategies have been explored to overturn these cells, for example using NKG2D-CAR-T cells that in addition to eliminate tumor cells also cause the activation of anti-tumor-associated macrophages through the secretion of GM-CSF and IFN-γ [64]. Finally, the combinations with molecules that inhibit the CSF-1R pathway, such as PLX3397, have shown to decrease tumor-infiltrating macrophages with consequent increase in the expansion, distribution, and functional activation of intratumoral T lymphocytes [65].

8.4.3 CAR-T Cells and Inhibitory Ligand

T cells, especially upon cytokine activation and antigen engagement, upregulate a wide array of inhibitory receptors (PD-1, LAG-3, TIM-3, CTLA4, CD160 and CD57) that cause contraction of T cell responses. Ligands for these receptors (PD-L1/PD-L2, MHC class II, galectin 9, CD80/CD86, HVEM, L/P selectins) are often hijacked by tumor cells to drive immune cell exhaustion and anergy [37, 66–69].

Clinical studies have shown that the expression of PD-L1 by tumors correlates with poor prognosis [28]. However, the expression of inhibitory molecules by tumor cells is a dynamic process since recent evidences suggest that PD-L1 may be upregulated after activation by cytokines or antigen engagement [70], making its role in suppressing immune responses more critical than initially thought. For example, tumor infiltrating T lymphocytes (TIL) express higher PD-1 and have limited effector cytokine production when compared to T cells present in normal tissues and peripheral blood in the same patients [71]. Blocking antibodies against PD-1 and PD-L1 can effectively reverse T cell exhaustion and restore antitumor immunity [72].

Preclinical studies suggest that these inhibitory pathways remain critical roadblocks also for CAR-T cells. Therefore, increasing interests are directed in combining CAR-T cells and checkpoints inhibitors [37, 73]. Preclinical studies have shown that the incorporation of the co-stimulatory endodomain 4-1BB is associated with a less pronounced expression of surface inhibitory receptors by CAR-T cells, enhancing T-cell proliferation and cytokine production [74]. The incorporation of the IL-15 gene in the CAR has also been associated with downregulated expression of PD-1 by CAR-T cells [57].

Using an alternative approach to the combination with check-point inhibitors, investigators have also explored the engineering of PD-1 to revert its inhibitory pathway into a co-stimulatory pathway. For instance, PD-1 has been coupled with the CD28 intracytoplasmic signaling. With this strategy T cells expressing the chimera engage the PD-L1 molecule expressed by tumors, but the signaling is switched into a positive pathway that increases ERK phosphorylation, augments cytokine secretion, increases proliferation, and enhances expression of granzyme B [75].

The tumor endothelial barrier may also contribute to the failure of the CAR-T cell therapy. The tumor-associated endothelium often provides a selective barrier allowing only certain T-cell subsets, notably Tregs, to traffic effectively into the tissues [76]. In addition, tumor endothelial cells induce apoptosis of effector T cells through activation of the FasL/Fas pathway [77]. Approaches that may prevent T cell apoptosis by down-modulation of Fas on T cell using small interfering RNA (siRNA) [78] or over-expression of Bcl-2 or Bcl-xL have also been tested to prevent the IL-2 withdrawal-induced apoptosis in lymphocytes [79].

8.5 Concluding Remarks

While compelling activity has been documented in patients with lymphoid malignancies treated with CD19-specific CAR-T cells, barriers remain to be defeated before equal activity is accomplished in solid tumors. CAR-T cells must actively traffic to the sites of tumor burden, which is rarely a physiological destination for T lymphocytes, survive and function in a highly immunosuppressive environment. To accomplish these tasks CAR-T cells can confide on two distinct important strategies: combination therapies and further genetic engineering. The former includes consolidation with chemotherapy and radiotherapy, for example to deplete suppressive T cell populations at the tumor sites, or combination with checkpoint inhibitors, including PD-1/PD-L1 to keep CAR-T cells functional when they reach the tumor. The latter is being actively explored to improve T cell trafficking, infiltration and endorse improved T cell survival. Clinical trials with these next generations CAR-T cells are warranted.

Acknowledgements This work was supported in part by a SCOR grant from the Leukemia and Lymphoma Society, RO1 1145564 from NHLBI, RO1CA193130 from NCI and Tier 2: Stimulus Award, Lineberger Comprehensive Cancer Center, University of North Carolina. The authors

Disclosure conflict-of-interest disclosure: No disclosure.

References

1. Savoldo B, Dotti G. Chimeric antigen receptors (CARs) from bench-to-bedside. Immunol Lett. 2013;155:40–2.
2. Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, Liu H, Creighton CJ, Gee AP, Heslop HE, Rooney CM, Savoldo B, Dotti G. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. Blood. 2014;123:3750–9.
3. Ramos CA, Savoldo B, Dotti G. CD19-CAR trials. Cancer J. 2014;20:112–8.
4. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011;365:725–33.
5. Cruz CR, Micklethwaite KP, Savoldo B, Ramos CA, Lam S, Ku S, Diouf O, Liu E, Barrett AJ, Ito S, Shpall EJ, Krance RA, Kamble RT, Carrum G, Hosing CM, Gee AP, Mei Z, Grilley BJ, Heslop HE, Rooney CM, Brenner MK, Bollard CM, Dotti G. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. Blood. 2013;122:2965–73.
6. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, Maric I, Raffeld M, Nathan DA, Lanier BJ, Morgan RA, Rosenberg SA. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood. 2010;116:4099–102.
7. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, Yang JC, Kammula US, Devillier L, Carpenter R, Nathan DA, Morgan RA, Laurencot C, Rosenberg SA. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood. 2012;119:2709–20.
8. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med. 2011;3:95ra73.
9. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, Lindgren CG, Lin Y, Pagel JM, Budde LE, Raubitschek A, Forman SJ, Greenberg PD, Riddell SR, Press OW. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood. 2012;119:3940–50.
10. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, Fry TJ, Orentas R, Sabatino M, Shah NN, Steinberg SM, Stroncek D, Tschernia N, Yuan C, Zhang H, Zhang L, Rosenberg SA, Wayne AS, Mackall CL. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet. 2015;385:517–28.
11. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, Yang JC, Phan GQ, Hughes MS, Sherry RM, Raffeld M, Feldman S, Lu L, Li YF, Ngo LT, Goy A, Feldman T, Spaner DE, Wang ML, Chen CC, Kranick SM, Nath A, Nathan DA, Morton KE, Toomey MA, Rosenberg SA. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J Clin Oncol. 2015;33:540–9.
12. Kochenderfer JN, Dudley ME, Carpenter RO, Kassim SH, Rose JJ, Telford WG, Hakim FT, Halverson DC, Fowler DH, Hardy NM, Mato AR, Hickstein DD, Gea-Banacloche JC, Pavletic SZ, Sportes C, Maric I, Feldman SA, Hansen BG, Wilder JS, Blacklock-Schuver B, Jena B, Bishop MR, Gress RE, Rosenberg SA. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. Blood. 2013;122:4129–39.
13. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med. 2013;5:177.

14. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, Chung SS, Stefanski J, Borquez-Ojeda O, Olszewska M, Qu J, Wasielewska T, He Q, Fink M, Shinglot H, Youssif M, Satter M, Wang Y, Hosey J, Quintanilla H, Halton E, Bernal Y, Bouhassira DC, Arcila ME, Gonen M, Roboz GJ, Maslak P, Douer D, Frattini MG, Giralt S, Sadelain M, Brentjens R. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci Transl Med. 2014;6:224ra225.
15. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, Kamble RT, Bollard CM, Gee AP, Mei Z, Liu H, Grilley B, Rooney CM, Heslop HE, Brenner MK, Dotti G. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. J Clin Invest. 2011;121:1822–6.
16. Huye L, Savoldo B. Cancer battlefield: six characters in search of an author. Immunotherapy. 2012;4:753–5.
17. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343:1020–34.
18. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002;298:850–4.
19. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15:786–801.
20. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, Foster AE, Heslop HE, Brenner MK, Dotti G, Savoldo B. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. Blood. 2009;113:6392–402.
21. van den Berg A, Visser L, Poppema S. High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltratein Hodgkin's lymphoma. Am J Pathol. 1999;154:1685–91.
22. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer. 2004;4:540–50.
23. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM, Foster AE. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother. 2010;33:780–8.
24. Kershaw MH, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, Wang E, Young HA, Murphy PM, Hwu P. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum Gene Ther. 2002;13:1971–80.
25. Li J, O'Malley M, Urban J, Sampath P, Guo ZS, Kalinski P, Thorne SH, Bartlett DL. Chemokine expression from oncolytic vaccinia virus enhances vaccine therapies of cancer. Mol Ther. 2011;19:650–7.
26. Nishio N, Diaconu I, Liu H, Cerullo V, Caruana I, Hoyos V, Bouchier-Hayes L, Savoldo B, Dotti G. Armed oncolytic virus enhances immune functions of chimeric antigen receptor-modified T cells in solid tumors. Cancer Res. 2014;74:5195–205.
27. Barreira da Silva R, Laird ME, Yatim N, Fiette L, Ingersoll MA, Albert ML. Dipeptidylpeptidase 4 inhibition enhances lymphocyte trafficking, improving both naturally occurring tumor immunity and immunotherapy. Nat Immunol. 2015;16:850–8.
28. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, Higuchi T, Yagi H, Takakura K, Minato N, Honjo T, Fujii S. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci U S A. 2007;104:3360–5.
29. Schatton T, Scolyer RA, Thompson JF, Mihm Jr MC. Tumor-infiltrating lymphocytes and their significance in melanoma prognosis. Methods Mol Biol. 2014;1102:287–324.
30. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JM, Mahmoud SM, Rakha E, Ellis IO, Liu S, Gao D, Nielsen TO, Pharoah PD, Caldas C. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Ann Oncol. 2014;25:1536–43.
31. Turley SJ, Cremasco V, Astarita JL. Immunological hallmarks of stromal cells in the tumour microenvironment. Nat Rev Immunol. 2015;15:669–82.

32. Caruana I, Savoldo B, Hoyos V, Weber G, Liu H, Kim ES, Ittmann MM, Marchetti D, Dotti G. Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. Nat Med. 2015;21:524–9.
33. Hynes RO, Naba A. Overview of the matrisome—an inventory of extracellular matrix constituents and functions. Cold Spring Harb Perspect Biol. 2012;4:a004903.
34. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A, Rosenberg SA. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol. 2005;23:2346–57.
35. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science. 2006;314:126–9.
36. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, Restifo NP. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. J Exp Med. 2005;202:907–12.
37. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12:252–64.
38. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nat Rev Immunol. 2010;10:554–67.
39. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol. 2006;24:99–146.
40. Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, Whitesell LJ, Ramanathapuram LV, Arteaga CL, Akporiaye ET. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res. 2003;63:1860–4.
41. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol. 2000;164:6166–73.
42. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med. 2005;201:1061–7.
43. Ahmadzadeh M, Rosenberg SA. TGF-beta 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. J Immunol. 2005;174:5215–23.
44. Wallace A, Kapoor V, Sun J, Mrass P, Weninger W, Heitjan DF, June C, Kaiser LR, Ling LE, Albelda SM. Transforming growth factor-beta receptor blockade augments the effectiveness of adoptive T-cell therapy of established solid cancers. Clin Cancer Res. 2008;14:3966–74.
45. Bollard CM, Rossig C, Calonge MJ, Huls MH, Wagner HJ, Massague J, Brenner MK, Heslop HE, Rooney CM. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. Blood. 2002;99:3179–87.
46. Foster AE, Dotti G, Lu A, Khalil M, Brenner MK, Heslop HE, Rooney CM, Bollard CM. Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-beta receptor. J Immunother. 2008;31:500–5.
47. van Baren N, Van den Eynde BJ. Tumoral immune resistance mediated by enzymes that degrade tryptophan. Cancer Immunol Res. 2015;3:978–85.
48. Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med. 2002;196:459–68.
49. Godin-Ethier J, Hanafi LA, Piccirillo CA, Lapointe R. Indoleamine 2,3-dioxygenase expression in human cancers: clinical and immunologic perspectives. Clin Cancer Res. 2011;17:6985–91.
50. Ninomiya S, Narala N, Huye L, Yagyu S, Savoldo B, Dotti G, Heslop HE, Brenner MK, Rooney CM, Ramos CA. Tumor indoleamine 2,3-dioxygenase (IDO) inhibits CD19-CAR T cells and is downregulated by lymphodepleting drugs. Blood. 2015;125:3905–16.

51. Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK. Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia. 2006;20:1819–28.
52. Kofler DM, Chmielewski M, Rappl G, Hombach A, Riet T, Schmidt A, Hombach AA, Wendtner CM, Abken H. CD28 costimulation Impairs the efficacy of a redirected t-cell antitumor attack in the presence of regulatory t cells which can be overcome by preventing Lck activation. Mol Ther. 2011;19:760–7.
53. Lo AS, Ma Q, Liu DL, Junghans RP. Anti-GD3 chimeric sFv-CD28/T-cell receptor zeta designer T cells for treatment of metastatic melanoma and other neuroectodermal tumors. Clin Cancer Res. 2010;16:2769–80.
54. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, Simpson C, Carter C, Bock S, Schwartzentruber D, Wei JP, White DE. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med. 1988;319:1676–80.
55. Malek TR. The main function of IL-2 is to promote the development of T regulatory cells. J Leukoc Biol. 2003;74:961–5.
56. Perna SK, De Angelis B, Pagliara D, Hasan ST, Zhang L, Mahendravada A, Heslop HE, Brenner MK, Rooney CM, Dotti G, Savoldo B. Interleukin 15 provides relief to CTLs from regulatory T cell-mediated inhibition: implications for adoptive T cell-based therapies for lymphoma. Clin Cancer Res. 2013;19:106–17.
57. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, Heslop HE, Rooney CM, Brenner MK, Dotti G. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia. 2010;24:1160–70.
58. van Herpen CM, Looman M, Zonneveld M, Scharenborg N, de Wilde PC, van de Locht L, Merkx MA, Adema GJ, de Mulder PH. Intratumoral administration of recombinant human interleukin 12 in head and neck squamous cell carcinoma patients elicits a T-helper 1 profile in the locoregional lymph nodes. Clin Cancer Res. 2004;10:2626–35.
59. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. Expert Opin Biol Ther. 2015;15:1145–54. doi:10.1517/14712598.2015.1046430.
60. Perna SK, Pagliara D, Mahendravada A, Liu H, Brenner MK, Savoldo B, Dotti G. Interleukin-7 mediates selective expansion of tumor-redirected cytotoxic T lymphocytes (CTLs) without enhancement of regulatory T-cell inhibition. Clin Cancer Res. 2014;20:131–9.
61. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64:5839–49.
62. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest. 2007;117:1147–54.
63. Mantovani G, Maccio A, Madeddu C, Mura L, Massa E, Gramignano G, Lusso MR, Murgia V, Camboni P, Ferreli L. Reactive oxygen species, antioxidant mechanisms, and serum cytokine levels in cancer patients: impact of an antioxidant treatment. J Environ Pathol Toxicol Oncol. 2003;22:17–28.
64. Spear P, Barber A, Rynda-Apple A, Sentman CL. Chimeric antigen receptor T cells shape myeloid cell function within the tumor microenvironment through IFN-gamma and GM-CSF. J Immunol. 2012;188:6389–98.
65. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L, Graeber TG, West BL, Bollag G, Ribas A. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. Cancer Res. 2014;74:153–61.
66. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12:492–9.
67. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, Chapuy B, Takeyama K, Neuberg D, Golub TR, Kutok JL, Shipp MA. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. Blood. 2010;16:3268–77.

68. Steidl C, Shah SP, Woolcock BW, Rui L, Kawahara M, Farinha P, Johnson NA, Zhao Y, Telenius A, Neriah SB, McPherson A, Meissner B, Okoye UC, Diepstra A, van den Berg A, Sun M, Leung G, Jones SJ, Connors JM, Huntsman DG, Savage KJ, Rimsza LM, Horsman DE, Staudt LM, Steidl U, Marra MA, Gascoyne RD. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. Nature. 2011;471:377–81.
69. Dondero A, Pastorino F, Della CM, Corrias MV, Morandi F, Pistoia V, Olive D, Bellora F, Locatelli F, Castellano A, Moretta L, Moretta A, Bottino C, Castriconi R. PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance. Oncoimmunology. 2016;5, e1064578.
70. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother. 2005;54:307–14.
71. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood. 2009;114:1537–44.
72. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, Schuster SJ, Millenson MM, Cattry D, Freeman GJ, Rodig SJ, Chapuy B, Ligon AH, Zhu L, Grosso JF, Kim SY, Timmerman JM, Shipp MA, Armand P. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N Engl J Med. 2015;372:311–9.
73. Beavis PA, Slaney CY, Kershaw MH, Neeson PJ, Darcy PK. Enhancing the efficacy of adoptive cellular therapy by targeting tumor-induced immunosuppression. Immunotherapy. 2015;7:499–512.
74. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, Smith JP, Walker AJ, Kohler ME, Venkateshwara VR, Kaplan RN, Patterson GH, Fry TJ, Orentas RJ, Mackall CL. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med. 2015;21:581–90.
75. Prosser ME, Brown CE, Shami AF, Forman SJ, Jensen MC. Tumor PD-L1 co-stimulates primary human CD8(+) cytotoxic T cells modified to express a PD1:CD28 chimeric receptor. Mol Immunol. 2012;51:263–72.
76. Shrimali RK, Yu Z, Theoret MR, Chinnasamy D, Restifo NP, Rosenberg SA. Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. Cancer Res. 2010;70:6171–80.
77. Motz GT, Santoro SP, Wang LP, Garrabrant T, Lastra RR, Hagemann IS, Lal P, Feldman MD, Benencia F, Coukos G. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. Nat Med. 2014;20:607–15.
78. Dotti G, Savoldo B, Pule M, Straathof KC, Biagi E, Yvon E, Vigouroux S, Brenner MK, Rooney CM. Human cytotoxic T lymphocytes with reduced sensitivity to Fas-induced apoptosis. Blood. 2005;105:4677–84.
79. Kalbasi A, Shrimali RK, Chinnasamy D, Rosenberg SA. Prevention of interleukin-2 withdrawal-induced apoptosis in lymphocytes retrovirally cotransduced with genes encoding an antitumor T-cell receptor and an antiapoptotic protein. J Immunother. 2010;33:672–83.

Index

E. Donnadieu (ed.), *Defects in T Cell Trafficking and Resistance to Cancer Immunotherapy*, Resistance to Targeted Anti-Cancer Therapeutics 9,
DOI 10.1007/978-3-319-42223-7