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

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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

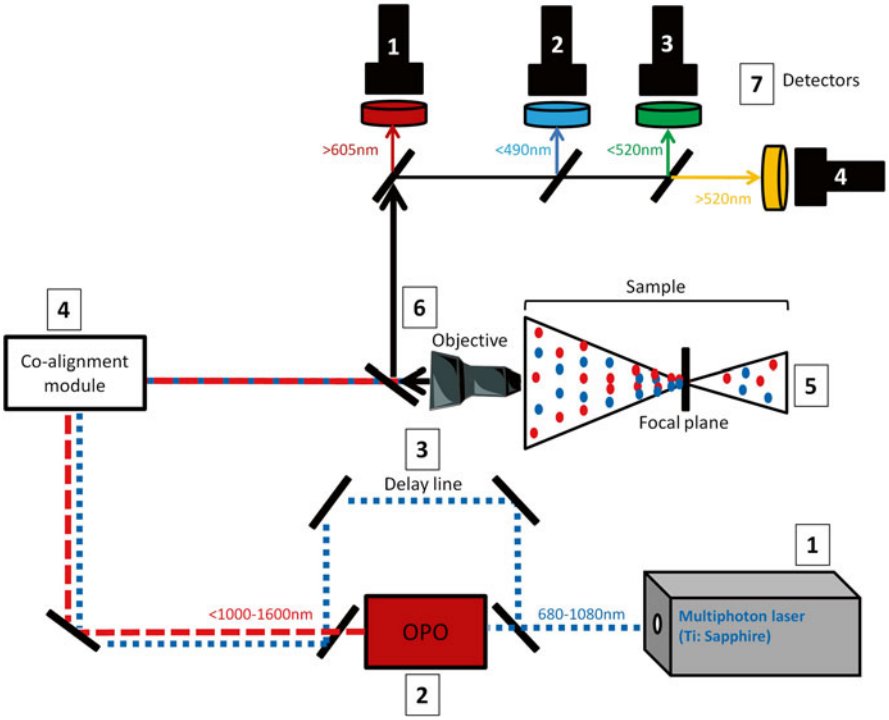

Fig. 2.1 Example of Two-photon instrument design. (1) Multiphoton excitation laser (TI:Saphire) generates femtosecond near-infrared (680–1080 nm) pulses. (2) Optical parametric oscillator (OPO) generates additional beam between (1000–1600 nm). Ti:saphire and OPO beams are temporally synchronized using 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

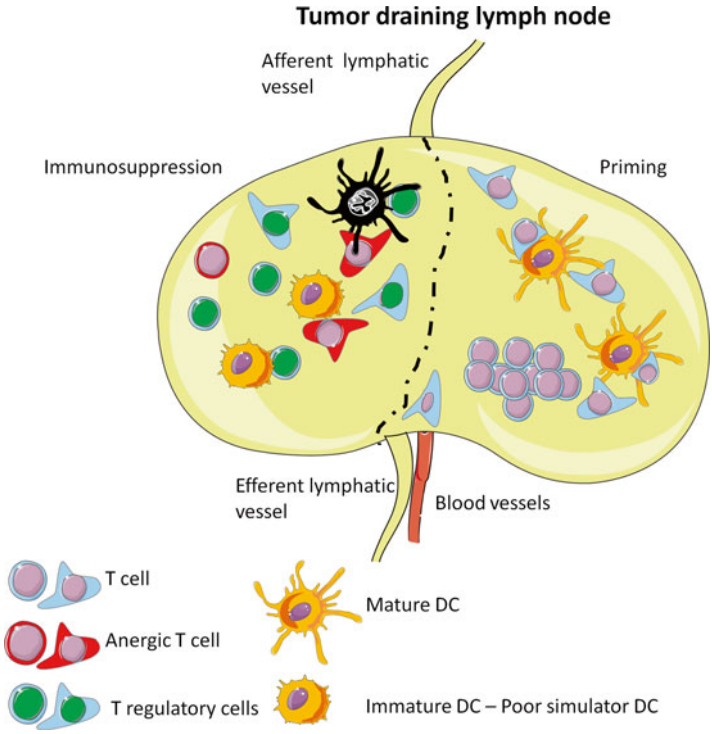

Fig. 2.2 Dynamic cellular interactions during antitumor T cell priming. IVM of the tumor draining lymph nodes allows to visualize the cellular interactions during T cell priming and activation but also the processes of immunosuppression. Efficient priming is characterized by the stable interaction of 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

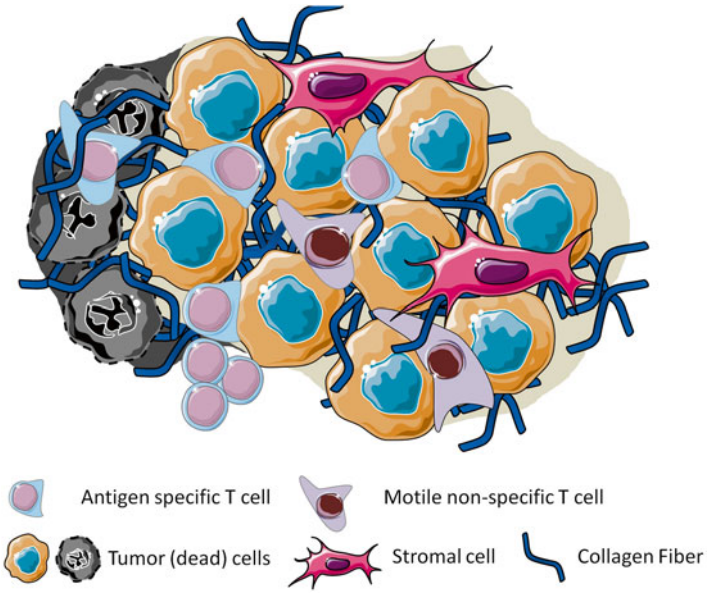

Fig. 2.3 Regulation of CTLs retention by antigen specificity. Both antigen specific and non-specific CTLs may infiltrate the tumors but only antigen specific ones make long lasting interactions with tumor cells. These interactions are thought to extend the time of residency of the antigen-specific CTLs 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

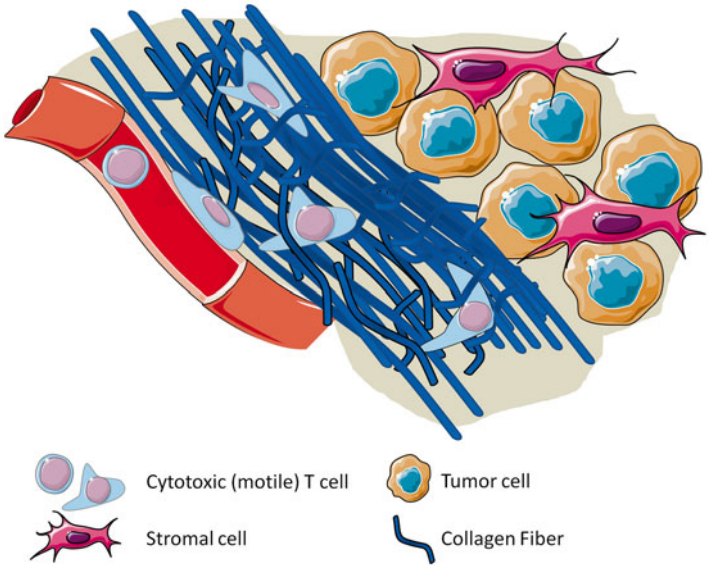

Fig. 2.4 Control of T cell migration in the interstitial space of tumors. IVM of T cell behavior in tumor has allowed to determine the components of TME that control T cell migration. After extravasation, T cells were shown to migrate along peripheral tumor vessels that can serve as a route in the interstitial 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-

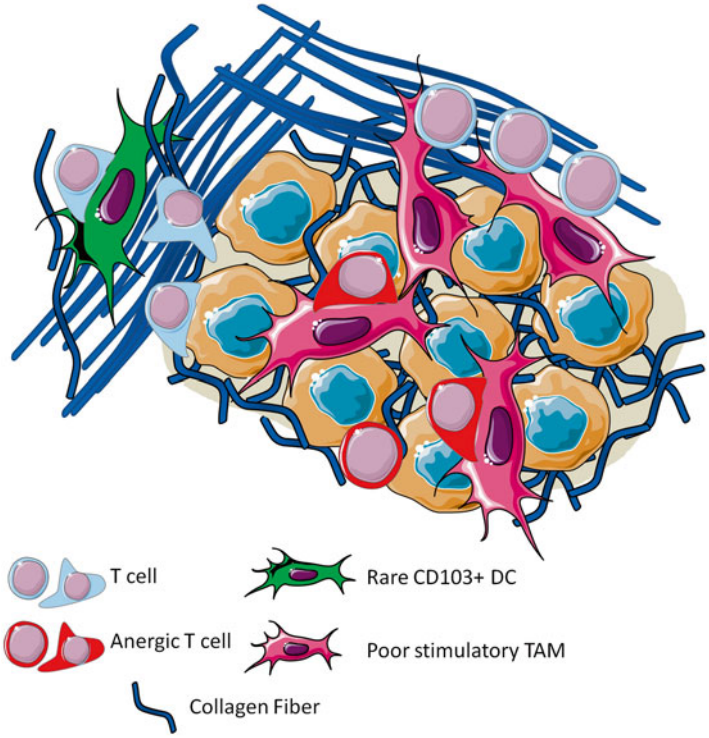

Fig. 2.5 Regulation of T cell activation and infiltration by tumor-associated antigen presenting cells. In most tumors, mature populations of macrophages compose more than 50 % of the CD45+ stroma. These have mainly been associated with dampening the T cell response and promoting tumor growth via various 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.

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