Overcoming barriers to T cell activation, infiltration and function in tumors

Melssen, M.M.

Chapter 1

General Introduction

1. THE IMMUNE SYSTEM

The immune system consists of two components: the innate and adaptive immune system. Innate immune cells are the first-line responders; they guard tissues from damage or invasion of foreign elements such as viruses and bacteria, in a non-specific manner. Among innate immune cells are the myeloid-derived cells, including monocytes, macrophages, dendritic cells (DCs) and granulocytes, as well as lymphoid-derived innate lymphoid cells (ILCs) including natural killer (NK) cells. Each cell type has its own mechanism of action, though in general innate cells recognize commonly shared receptors or secreted molecules by pathogens. Subsequently they respond by engulfing the pathogen or infected cells and/or secreting inflammatory signals in the form of cytokines and chemokines to recruit more immune cells. After engulfment, professional antigen-presenting cells (APCs), such as DCs, further process the pathogen or infected cells. This mechanism functions as a bridge between the innate and adaptive immune systems, where these APCs present processed, foreign antigens from the engulfed pathogens to adaptive immune cells in order to generate a very specialized, targeted response.

The adaptive immune compartment consists of B and T cells, which have somatically re-arranged B- or T-cell receptors, each recognizing a specific antigen sequence at low frequency. When activated by their cognate antigen, the adaptive immune cells rapidly divide to generate effector and memory cells meant to clear pathogens or infected cells and create long-lasting memory against the specific antigen that is encountered. Activated B cells engulf pathogens and produce antigen-specific antibodies, facilitating targeted engulfment by professional phagocytes and destruction through the complement system.

The T cell compartment is comprised of CD8 and CD4 T cell subsets. CD8 T cells are considered cytotoxic effector cells, designed to eradicate virus-infected or tumor cells. CD8 T cells recognize antigen sequences presented on MHC class I molecules. In lymph nodes, professional antigen-presenting cells (APCs) present antigen-MHC complexes to naïve CD8 T cells and upon recognition these naïve CD8 T cell become activated. Once activated, or "primed", the CD8 T cells rapidly proliferate and differentiate into cytotoxic effector CD8 T cells[1]. At the same time, CD8 T cells lose the S1P1 receptor, which they require to leave the lymphoid tissue. Effectively this traps the expanding T cells within the lymph node and allows them to fully utilize the activation signals for optimal expansion and differentiation. After expansion and differentiation, S1P1 is recovered and the T cell are able to leave the lymphoid tissue and travel to peripheral target tissues. In order to "find" the infected or tumor tissue, the T cell expresses a plethora of homing and chemokine receptors, which recognize ligands expressed by inflamed endothelial cells[2,3]. Once they have entered the tissue successfully, the T cell recognizes antigen-MHC class I complexes on target cells which triggers production and release of granzymes and cytotoxins. These molecules are specifically designed to force apoptosis of virus-infected cells or tumor cells. In this manner, one cytotoxic T cell is capable of efficiently killing multiple target cells in a row, a

concept described as T cell serial killing[1,4]. The frequency and efficiency of serial killing *in vivo* is not very well understood, but appears to be slower than *in vitro* and affected by external factors[5,6]. Then lastly, cytotoxic CD8 T cells produce effector cytokines to further increase inflammation and recruitment of immune cells. CD4 T cells recognize antigens presented on MHC class II molecules by APCs. A wide variety of distinct CD4 T cell lineages exists and each of them has a different role in guiding, inducing or dampening immune responses in different contexts, such as infections, allergy or autoimmunity. In viral infections and tumors specifically, the Th1 CD4 T cell lineage offers crucial support during naïve CD8 T cell activation and differentiation, through co-stimulation and expression of inflammatory cytokines[7]. Th1 CD4 cells can furthermore support effector function within the target tissue through co-stimulatory receptor interactions and generation of an inflammatory cytokine milieu[8–10]. In some instances, CD4 T cells can also directly induce apoptosis or senescence of target cells through FASL, TRAIL, granzyme B, perforin and IFNγ[11–13]. IFNγ from CD4 Th1 cells also helps to polarize monocytes/macrophages towards a pro-inflammatory and anti-tumorigenic M1 phenotype[14]. Through these mechanisms, effector CD8 and Th1 CD4 cells are the basis of a type I immune response, which is deemed crucial for the eradication of both virally-infected and tumor cells.

2. IMMUNE MEMORY FORMATION

An important aspect of adaptive immunity is the formation of immune memory. This is established by the generation of memory cells alongside terminal effector cells, during the acute phase of the immune response. Memory T cells are self-renewing and can often remain in the body for years after the initial antigen encounter. Three different memory T cell subsets have been described: the circulatory central memory and effector memory cells and a third, non-circulatory tissue-resident memory subset[15]. Central memory T cells remain in the circulation and secondary lymphoid organs only, with great self-renewal capacity and plasticity. Upon re-encounter with antigen they rapidly proliferate and generate new terminal effector T cells[16]. Effector memory T (TEM) cells can be found in the circulation and peripheral tissues, and though they are self-renewing, their main purpose is to rapidly localize to inflamed sites and provide immediate effector function upon antigen recognition[16,17]. Then lastly, non-circulatory tissue resident memory (TRM) T cells remain in peripheral tissues long-term, predominantly at barriers such as skin, intestines and lungs[15]. Though TRM T cells are antigen-specific and rapidly induce inflammation upon encounter with antigen, they are also capable of responding in an innate fashion by producing inflammatory cytokines in an antigen-independent way[18,19]. Immune memory formation is most optimally generated during acute infections, with long-lived memory cells staying behind after the infection is cleared and terminal effectors have dissipated[20]. Whether the fate decision of effector T cells to become memory precursor cells for the different lineages occurs early or late during the acute response is still controversial[15,21]. What is clear is that transcription factor repertoire and metabolic

regulation are important in the eventual differentiation path of memory cells[15], and it is envisioned that these can be skewed by TCR signal strength and environmental cues[22,23]. When antigen persists in chronic infections or cancer, memory cell formation is often distorted and the generation of the different memory subsets can be skewed or non-existent[20,24,25]. Chronic restimulation by their antigen renders T cells functionally exhausted, a phenomenon thoroughly described in both chronic infection and cancer. Instead of gaining memory cell properties, T cells obtain a specific epigenetic chromatin state which is accompanied by the upregulation of inhibitory receptors, loss of stem-like markers and suppression of effector function[26,27]. These cells are largely dysfunctional, even during re-encounter with their cognate antigen and often eventually undergo apoptosis.

3. CANCER-SPECIFIC IMMUNE RESPONSE AND IMMUNE EVASION

It has been long understood that the immune system plays an important role in the eradication of cancer. Because the "non-self" antigen is intrinsic to the tumor cell, a type I immune response comparable to an anti-viral response, is crucial for tumor rejection[28]. In a very comprehensive study across 33 different cancer types, the nature of immune responses against the tumor were mapped and indeed, those with a predominant type I signature were correlated with the best patient survival[29]. A type I immune response involves activation of both effector CD8 T cells and CD4 Th1 cells. These T cell subsets produce type I inflammatory cytokines and cytotoxins in response to antigen stimulation and these induce target cell apoptosis. Thus, in order to survive, tumors often utilize mechanisms to divert or silence the type I immune response.

The development of cancer is a multistep process driven by the acquisition of somatic mutations in critical genes for cell division, replicative immortality and protection against apoptosis, that together lead to uncontrolled division of cells[30]. Accumulation of "driver" mutation is generally accompanied by other "passenger" mutations that are not directly involved in driving cancer development, but still change the genome of the (pre)cancerous cell[31]. Due to the accumulation of both types of mutations, peptide sequences arise that are unknown to the immune system, thereby classifying them as "non-self" antigens or neo-antigens[32]. The multistep process of cancer development leads to overexpression or induction of proteins normally not expressed in the cell type that is transforming, which can lead to recognition of these proteins as antigens, named shared antigens[32]. When neo- or shared- antigens get processed and presented on MHC molecules, they can induce an immune response against the cancerous cells and initiate a process called immunoediting. The extent of the immune response and immunoediting thus varies between cancer types and likely depends on the mutational burden. Immunoediting consists of three phases: elimination, equilibrium and evasion. During the early phase of cancer development, the immune system can successfully recognize and eliminate the cancerous cells through immunosurveillance. It is

thought that the human body continuously eradicates precancerous cells through this process. However, some cells will survive, continue to evolve and gain new driver mutations, leading to the equilibrium phase of cancer immunoediting. During this phase the immune system is able to eradicate mutated tumor cells, while they continuously divide at a balanced rate. Subsequently the cancer cells further evolve, until immune evasion mechanisms arise, initiating the escape phase of cancer development. At this stage, the tumor successfully evades immune destruction in one way or another and is able to grow without supervision of the immune system. To evade immune destruction, cancers utilize a plethora of mechanisms at different steps of the cancer-specific immune response (Figure 1), as will be outlined in greater detail in this chapter. Targeting these immune evasion mechanisms through therapeutic strategies can successfully prolong survival of cancer patients. However, there are still a lot of gaps in our knowledge with regards to the exact mechanisms and how to most successfully target them in individual patients.

Antigen uptake and DC maturation

To start a successful adaptive immune response, professional APCs, such as DCs have to engulf foreign antigen and get properly activated. When activated the APC will traffic to the lymph node to present the antigens to the adaptive immune subsets. Immunogenic DC activation is most commonly driven through pattern recognition receptors (PRRs), which respond to pathogen-associated molecular patterns (PAMPs)[33]. DC activation can also be induced by inflammatory cytokine signals[34,35]. In the context of a tumor, PAMPs normally present during pathogenic infections are absent. In such an environment, whether a dying cell creates an immune response or instead induces tolerance depends on a variety of factors[36]. When a sufficient amount of damage-associated molecular patterns (DAMPs) and cytokines are released, they can replace PAMPs and induce proper DC activation[36] (Figure 1, Step 1). However, when these DAMPs are absent during engulfment of antigens, the DCs can become tolerogenic, activating regulatory T cell subsets to dampen further response against these antigens[37]. DAMPs capable of inducing immunogenic DC activation include HMGB1, uric acid, ATP, cytosolic DNA and heat shock proteins[38–40]. Each of these molecules can activate DCs through a different mechanism, for example, ATP or HGMB1 release leads to inflammasome or TLR4 mediated DCs activation. Contrastingly, uptake of cytosolic DNA by APCs leads to the activation through the STING pathway and expression of type I IFNs[40]. All of these mechanisms activate APCs to generate a tumor-specific T cell mediated immune response. If these signals are abundant enough to successfully induce mature DCs during engulfment they become immunogenic, though there is some evidence that mature DCs can be tolerogenic in some circumstances[37]. During maturation DCs upregulate CCR7, MHC class II and CD40[41,42]. CCR7 allows the cells to exit peripheral tissues and traffic to the lymph node for antigen presentation. CD40 is important for the interaction of CD4 helper T cells with DCs during antigen-presentation and the further upregulation of costimulatory, signal 2 receptors CD80, CD86 and CD70[7], which will be discussed in more detail below.

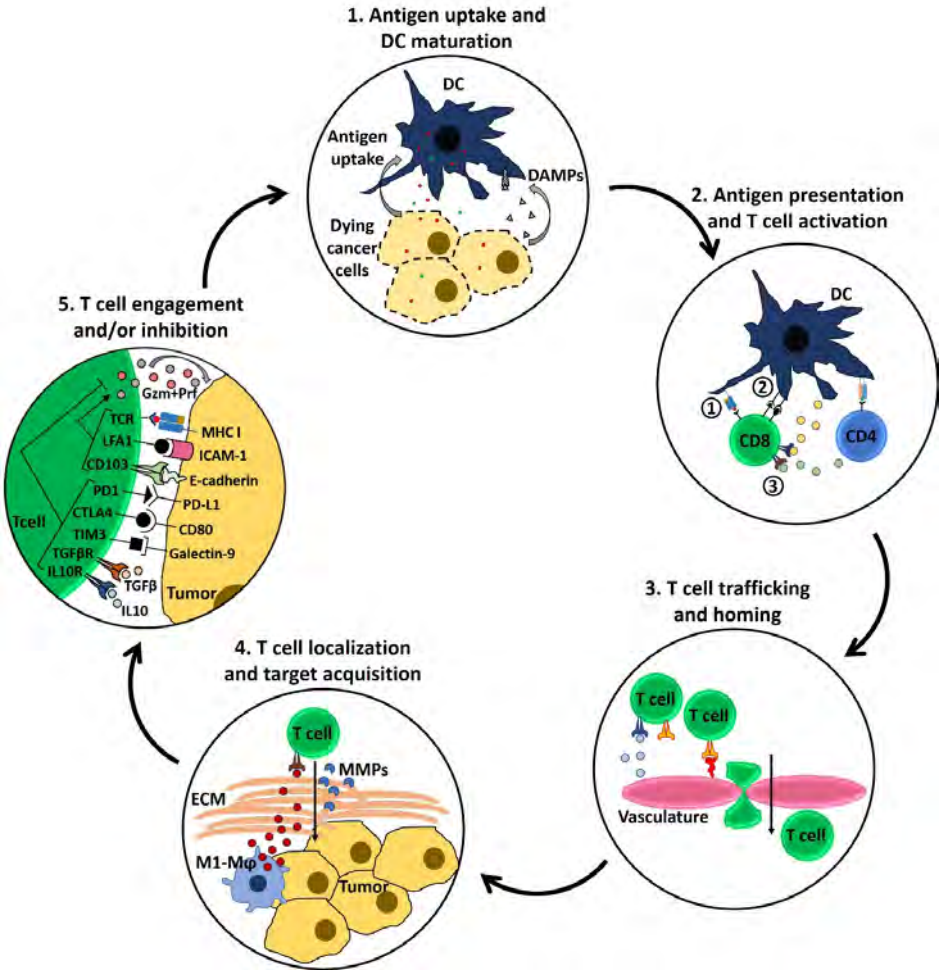

Figure 1. Cancer-specific Immune response. The cytotoxic, effector T cell driven anti-tumor immune response consists of several steps. Step 1. DCs take up antigen from dying cancer cells in the presence of DAMPs. These signals mature the DC and initiate migration to the lymph node. Step 2. The mature DC presents the antigen to CD4 and CD8 T cells (signal 1). CD4 helper T cells further mature the DC, leading to increased expression of MHC molecules (signal 1) and the expression of co-stimulatory molecules (signal 2). Furthermore, expression of cytokines by both mature DCs and helper CD4 T cells contribute to the activation and differentiation of CD8 T cells (signal 3). Step 3. Activated CD8 T cells traffic through the circulation to the tumor site. Inflammatory signals have upregulated chemokines and homing receptor ligands on the tumor vasculature, leading to a cascade or rolling, adherence and extravasation through the endothelial cells. Step 4. In order to localize among tumor cells and target them, T cells require chemokine gradients, often provided by macrophages and monocytes (M1-Mφ). Furthermore, expression of MMPs allows for migration through dense ECM molecules often surrounding tumor cells. Step 5. In addition to TCR-mediated MHC class I-antigen complex recognition, T

cells utilize integrins LFA1 and CD103 to generate long-term adhesion to tumor cells. This long-term adherence is required for the proper induction of the immunologic synapse. A mix of stimulatory and inhibitory receptors in the synapse will determine expression of granzymes (Gzm) and perforins (Prf) in the immunologic synapse; and thus, whether tumor cell apoptosis is induced. In case of apoptosis, tumor cells release more antigens in the tumor microenvironment, possibly eliciting T cell response.

Thus, to induce a proper immune response against tumor-antigen, cancer cells have to release DAMPs during apoptosis. Normal apoptotic processes are caspase-driven, which eventually causes pore formation and efficient removal of dead cell corpses in an immunologically silent manner[43–45]. Cancer cells often follow these same pathways, because they face many cell intrinsic stressors, and often undergo apoptosis without the release of DAMPs. However, in some tumors, caspase is actively blocked, while cell death still occurs. In such situations, cell death is accompanied by type I IFN response and NF-kb signaling[45]. Extrinsic stressors, such as FASL and TRAIL, can also induce NF-kb signaling in tumor cells[46]. Both these mechanisms can lead to a more immunogenic cell death (ICD) and the release of DC-activating factors in the microenvironment.

In addition to the potential release of DAMPs, tumor cells produce or induce the production of factors that have been shown to directly reduce the differentiation into DCs from progenitors. Progenitors are instead pushed to differentiate into suppressive monocyte populations, such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs)[47–49]. Involved factors include retinoic acid (RA), VEGF, TGFβ, IL1β, IL13, GM-CSF, CCL2 and prostaglandins. Interestingly, the increased activity of the PI3K-AKT oncogenic pathway has been linked to the enhanced expression of VEGF and CCL2 in a melanoma mouse model[50]. Additionally, decreased recruitment and activation of DCs has also been linked to the activation of oncogenic WNT-βcatenin pathway in melanoma models[51]. Through secretion of βcatenin, this pathway can inhibit DC recruitment through suppression of chemokines, such as CCL4. These findings suggest that specific oncogenic mutations in tumor cells can directly affect the tumor microenvironment and thereby actively prevent the recruitment and/or generation of functional DCs that can take up antigen. These tumors then lack functional, fully mature DCs and will fail to activate T cells in the draining lymph node.

Both suppression of ICD and DC recruitment/differentiation, can lead to a decreased population of mature DCs in tumors. Either mechanism results in decreased uptake of tumor antigen and subsequent presentation to tumor-specific CD4 and CD8 T cells in the tumor-draining lymph node. Even more so, when T cells get activated by immature or tolerogenic DCs they become anergic or suppressive, thereby not only decreasing the tumor-specific cytotoxic T cell pool, but also creating active immunosuppression[37].

Antigen presentation and T cell activation

Once DC's successfully capture antigen and gain a mature phenotype, CCR7 allows them to traffic to the draining lymph node where the DC presents antigen to T cells. At this point, the activation state of the DC is again important for the directionality and magnitude of the response. Fully matured DCs express high levels of MHC molecules and co-stimulatory receptors such as CD40, CD80 and CD86. Classically, the mature DC activates T cells through 3 independent signals (Figure 1, Step 2). Signal 1 is delivered by engaging the TCR of the specific naïve CD8 T cells through cross-presentation of antigen on MHC class I molecules, a specialized function of APCs[52]. At the same time, the DC presents antigen to helper CD4 T cells on MHC class II, which, mainly through additional CD40-CD40L interactions leads to the upregulation of co-stimulatory molecules CD80, CD86 and CD70 as well as MHC class I molecules on the surface of the DC[7]. The upregulation of MHC molecules strengthens signal 1 and the co-stimulatory receptors interact with receptors on CD8 T cells to induce signal 2. Thirdly, DCs and helper CD4 T cells secrete cytokines, including IL-12, IL-2, IL-15 and type I IFN, that promote further effector differentiation and proliferation[7]. The integration and strength of these three signals determine the eventual effector capacity and clonal expansion of the T cell. If part of the signal is missing, cells can become regulatory or anergic instead of full effectors, or the generation of memory cells can be suboptimal[33,53]. As described earlier, tumors often lack the proper immunogenic cues to mature DCs and fail to upregulate CD40. Loss of CD40 on APCs impairs the interaction with helper CD4 T cells, which is required for proper signal 2 support during CD8 activation[7]. Additionally, suppressive molecules, including TGFβ have been suggested to drain from the tumor microenvironment to the lymph node and directly impact the strength of the T cell activation signals[54–56]. Together, these mechanisms, resulting in diminished DC maturation, illuminate the role of tumor microenvironment composition in the initiation of the anti-tumor immune response. Additionally, they highlight the importance of finding alternative ways to generate tumor-specific effector T cells in tumors without adequate levels of mature DCs.

T cell trafficking and homing

After effector T cells are primed in the lymph node, they traffic through the circulation to the tumor. Subsequent entry of these T cells into specific peripheral tissues requires a multistep process, which involves sequential interactions between ligands or chemokines expressed by the vasculature and homing receptors on T cells (Figure 1, Step 3). Initial interactions lead to T cell rolling on the endothelial cells making up the vasculature wall[2]. Then subsequently chemokine receptors on the T cell are activated to bind to chemokine ligands expressed by the tissue. This results in T cell arrest and final transmigration through the endothelium[2]. The specific repertoire of homing receptors and homing receptor ligands required for T cell entry depends on anatomical location of the target tissue[2,3]. Each peripheral tissue expresses its own set of homing receptor ligands on the vasculature, which is further influenced by presence or absence of inflammation. In subcutaneous

tumors, ligand-receptor interactions VCAM1-α4β1, ICAM1-LFA1, ESL-E-selectin and HA-CD44, as well as chemokine signaling through CXCR3 are required for T cell infiltration[3]. In these tumors, VCAM1, ICAM1 and CXCL9 expression on endothelium are regulated by inflammatory cytokines and chemokines, TNFα, IFNγ and CCL5[57]. Tumors can thus influence the capacity of T cell homing by directly suppressing the production of these cytokines and chemokines, or indirectly by recruiting suppressive cell types.

Besides downregulation of homing receptor ligands, distorted organization of the vasculature can impair T cell infiltration in tumors. Because tumors often grow so rapidly, the vasculature is unable to generate new blood vessels fast enough to provide sufficient nutrients and oxygen, leading to massive hypoxia[58]. Hypoxia induces HIF-1α expression in both tumor cells and endothelial cells, which upregulates expression of pro-angiogenic genes including VEGF and other growth factors. The rapid neovascularization that occurs due to large amounts of these growth factors results in irregularly shaped, dilated and tortuous vessels, which are leaky and poorly covered by pericytes[59]. These structural defects in tumor vasculature alone are suggested to represent significant hurdles for T cell infiltration. In addition, the endothelial cells generated in rapid neovascularization often respond inefficiently to inflammatory signals, failing to upregulate sufficient levels of homing receptor ligands even when proper inflammatory cytokines and chemokines are present. VEGF furthermore has been shown to directly interfere with TNF-induced adhesion molecule expression on endothelial cells and other molecules expressed in the TME such as nitric oxide and epidermal growth factor like domain multiple 7 (egfl7) can also directly suppress endothelial cell activation[60–62].

T cell localization and target acquisition

To efficiently target tumor cells, infiltrating T cells have to be retained in the tissue overall, but also be able to traffic among and interact with tumor cells. For example, patients with diffuse immune cell infiltration amongst tumor cells have a better prognosis than patients who have limited immune cell infiltration in perivascular spaces[63]. Therefore, induction of barriers to diffuse immune cell infiltration and motility among tumor cell could provide immune evasion for tumors (Figure 1, Step 4).

When a cytotoxic T cell reaches its target organ after activation its main function is to eradicate infected or tumor cells. To optimize the serial killing capacity of T cells, they require rapid movement between cells to sample for antigen, then durable arrest once the target has been acquired[64,65]. In subcutaneous tumor rejection models, a large proportion of T cells indeed physically interacts with tumor cells leading to long-term arrest and display rapid motility between cells[64,66]. This efficient scanning and tumor cell engagement may be absent in non-rejected tumors, making serial killing more difficult. However, how tumors can evade T cell motility followed by long-term arrest is complex and can involve both direct cues

to change T cell motility and arrest as well as physical barriers in the environment. T cell movement is driven by both intrinsic motile capacity and extrinsic environmental organization and cues, such as matrix proteins and chemokines[67]. This makes T cell motility highly dependent on activation status, density of antigen, secretion of chemokines and target tissue type. In infection models and solid tumors it has been shown that myeloid-derived CXCL9 expression recruits effector T cells to target cells, through CXCR3[57,68,69]. Downregulating CXCL9 expression in myeloid cells thus provides an opportunity for immune evasion. Separately, in tissues with dense extracellular matrix (ECM), inflammatory signals such as TNFα, IFNγ and TGFβ can induce secretion of proteases to loosen the ECM matrix and allow integrin-mediated T cell motility within the tissue[70–72]. In viral infections, T cell motility along ECM molecules is driven by integrins α1β1 (or CD49a) and α2β1 (or CD49b)[73–75]. Thus, integrin expression on T cells and induction of proteases by inflammatory signals are both crucial to T cell motility and localization within tissues and provide targets for immune evasion for the tumor. The regulation and function of integrins on T cells, including CD49a and CD49b, under these circumstances is largely unexplored and gaining more understanding is crucial to find novel ways to tackle this barrier to T cell function in tumors.

Irrespective of whether T cells display proper integrins to traffic through ECM matrix, the organization of ECM itself determines the direction and efficiency of T cell movement. Most normal epithelial tissues are in a tensional homeostatic state, which leads to a relaxed meshwork of collagens and other ECM components, likely allowing for optimal lymphocyte motility. In tumors, however, activated cancer associated fibroblasts (CAFs), inflammation, high interstitial pressure and increased expression of collagen-processing lysyl oxidases can lead to increased collagen deposition, cross-linking and distorted organization[76,77]. Additionally, tumors often express higher levels of matrix metalloproteinases (MMPs) than normal epithelial tissue, leading to increased remodeling of ECM fibers[78]. In various solid tumor types, collagen alignment, length, width, density and straightness is altered compared to adjacent normal tissue[79]. As each of these components are dependent on all components highlighted above, collagen organization in tumors is highly variable, and the specifics affect T cell motility and function differently. For example, when tumors have dense stromal regions, T cells are confined within these regions, unable to move towards and engage with tumor cells[80–82]. Altering ECM organization could be an effective way for tumors to evade T cell motility and efficient “serial killing” of tumor cells. However, the exact effects of different tumor ECM structures on T cell motility have not been comprehensively studied in different tumor types beyond anecdotal observations.

3.5 T cell engagement and/or inhibition

Part of efficient T cell-mediated serial killing of tumor cells requires efficient motility through the interstitial spaces. Once a target cell has been acquired, the T cell needs to arrest motility and interact with the tumor cell long enough to create

the immunologic synapse[64,65] (Figure 1, Step 5). T cell arrest can be mediated by TCR signaling itself. Arrest and adhesion is further strengthened by integrins LFA-1 and CD103 on the T cell, interacting with ICAM-1 and E-cadherin, respectively, on the tumor cells[83–85]. In addition to adhesion, both of these integrins also support TCR-mediated killing itself[84,86,87]. One obvious way to evade T cell recognition and adhesion tumor often employ is the downregulation of MHC class I molecules on their surface[88]. In doing so, tumor cells actively make themselves "invisible" to the TCR on a T cell. Separately, tumor cells often lose E-cadherin expression as a part of epithelial-to-mesenchymal transition (EMT), which occurs as tumors progress and become metastatic. By driving the downregulation of E-cadherin, tumor cells not only become more invasive, they may also evade CD103 mediated engagement of T cells, possibly leading to decreased recognition and eradication[83,84]. However, direct evidence to support the relation between loss of E-cadherin and T cell recognition of tumor cells *in vivo* remains to be elucidated. Contrastingly, ICAM-1 is not often decreased as tumor progress, likely due to a possible role in metastatic capacity of tumor cells[85]. Instead, tumors secrete galectins, which bind to glycosylated receptors on tumor infiltrating T cells, including LFA1. This directly impairs the interaction between ICAM-1 and LFA-1 and synapse formation[89]. Through these mechanisms, tumors inhibit T cell adhesion to individual tumor cells and immunologic synapse formation.

As described above, appropriately activated antigen-specific T cells recognize peptides expressed on tumor cells through their TCR. Similar to the recognition of virus infected cells, TCR binding to antigen-MHC class I complexes on tumor cells leads to the formation of an immunologic synapse[90]. At the same time, T cell increase the expression of inflammatory cytokines, such as IFNγ and TNFα[91]. At the interface of the immunologic synapse, granzymes and perforins are expressed and directly targeted to kill the tumor cell[90,92]. During this process, tumor intrinsic and stromal aspects of the tumor can exert suppression mechanisms to dampen the direct eradication of tumor cells (Figure 1, Step 5). First of all, tumor cells express inhibitory ligands, such as PD-L1, which bind to inhibitory receptors on the T cell surface during the formation of the immunologic synapse. Because T cells have often become exhausted in the chronic antigen context of cancer, these inhibitory receptors are highly upregulated on tumor infiltrating T cells and engaging these receptors leads to suppressed release of cytokines and cytotoxins. The tumor microenvironment also contributes to the generation of exhausted cells, independent from chronic antigen stimulation, through high abundance of VEGF-A[93].

In addition to the evasion mechanisms tumor cells utilize to dampen T cell-mediated eradication directly, there are also several indirect mechanisms involving stromal cells or suppressive immune cell subsets. Among these are myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg) and cancer-associated fibroblasts (CAFs). MDSCs contain a collection of myeloid-derived cells that suppress the immune response in tumors. MDSCs are recruited through chemokines produced by tumor cells. Predominant chemokines include CCL2

and CCL5, though others have been shown capable of recruiting MDSCs and monocytes depending on the tumor type[94–96]. MDSCs suppress T cell homing, proliferation and function through secretion of Arginase-1, nitric oxide and ROS, as well as surface expression of IDO and PD-L1[61,96,97]. MDSCs also express suppressive cytokines IL-10 and TGFβ, induce Treg recruitment or differentiation and alter NK cell function[96,98]. Tregs are suppressive T cells, often found in tumors. They can be recruited from the circulation or differentiated from non-regulatory T cells in the TME. Tregs utilize a wide variety of suppression mechanisms to induce tolerance in mice and humans, though a few key mechanisms impact tumor immunity most predominantly. Among these are: sequestration of survival cytokine IL-2, limiting availability for other T cell subsets; the constitutive expression of inhibitory receptor CTLA-4; and expression of suppressive cytokines IL-10 and TGFβ[99]. Finally, tumors contain fibroblast-like cells, CAFs, representing the most abundant stromal cell population. In normal tissue, fibroblasts are quiescent stromal cells that only activate upon hypoxia, oxidative stress and growth factor signals, which are present during wound healing and inflammation[100]. Because tumors are essentially wounds that never heal, fibroblasts are constitutively activated and promote tumor growth by expressing growth factors, angiogenic factors and by inducing fibrosis. Fibroblasts are a multipotent cell type; thus, CAFs consist of a heterogeneous population of cells with different phenotypic markers and tumor-promoting mechanisms[100]. The phenotype and function of CAFs depend on fibroblast origin, recruitment and specific environmental signals[101]. For example, α-smooth muscle actin (αSMA)-expressing CAFs resemble activated myofibroblasts. These CAFs are involved in ECM remodeling, but also express chemokines and cytokines to recruit monocytes and drive their differentiation into tumor-promoting M2 macrophages[102]. CAFs subpopulations can also express TGFβ, IDO and PD-L1/2 to directly suppress effector function of lymphocytes and recruit MDSCs and Tregs[100,101]. Overall CAF function is dependent on tumor type and specific environment, though similarities exist, providing opportunities for CAFs targeted therapies.

4. THERAPIES TO TARGET IMMUNE EVASION

4.1 Current approved immune therapies

The majority of recently approved immune therapies to treat cancer are targeting immune checkpoint pathways, such as PD-1 and CTLA-4[103]. PD-1 and CTLA-4, among others, are inhibitory receptors expressed by T cells and engaging ligands effectively suppresses activation or effector function. Tumor often drive expression of ligands for these inhibitory receptors as an immune evasion strategy. Blocking the interaction has proven to be successful in boosting T cell mediated tumor eradication in patients and improve their survival[104,105]. It is clear that factors such as pre-existing T cell infiltration are strongly associated with successful checkpoint blockade treatment[106], and it has thus been most effective in melanoma and lung cancer, which generally have a high mutational burden. In patients without pre-existing T cell infiltrates, these therapies are often not

sufficient or effective at all. Other immune evasion mechanisms may be involved, especially those creating barriers for T cell priming and T cell infiltration, causing low to negligible T cell infiltrates. Therefore, it is important to study other potential strategies to overcome these barriers in particular, either alone or in combination with checkpoint inhibitor therapies. In this thesis we have therefore aimed to answer two broad research questions: 1). How can we overcome barriers to T cell priming?; and 2). What are the barriers to T cell infiltration and trafficking within the tumor microenvironment, and how can we overcome these?.

4.2 Overcoming barriers to T cell priming

The barriers to T cell priming are two-fold: First, the recruitment and differentiation of professional APCs in tumors can be impaired, leading to diminished opportunities of antigen-presentation to adaptive immune cells. Secondly, the availability of DAMPs and inflammatory cytokines to mature APCs can be low or negligible, thereby limiting the magnitude and quality of adaptive immune cell activation. Both can be targeted individually, for example, therapies aimed to drive monocyte differentiation into mature antigen-presenting DCs are being established[39]. Currently used chemotherapies and radiation can induce ICD and improve antigen-presentation and T cell priming. Optimization trials of these existing therapies and trials with new chemotherapies designed to induce ICD are underway[107]. Also, therapies designed to enhance DC maturation by inhibiting ATP degradation are investigated in *in vivo* tumor models[108].

An interesting strategy to simultaneously overcome both barriers to T cell priming involves cancer-targeted vaccination. The optimal vaccine would encompass all three activation signals required for proper induction of a type I immune response: antigen presentation, co-stimulation and stimulatory cytokine release. Current strategies to accomplish this range from injecting matured and antigen-pulsed DCs to injecting immunogenic peptides with immune-activating adjuvants[32]. Preclinical and clinical research has shown great potential of these strategies in generating a systemic immune response, especially in high-mutational cancers such as melanoma. Aspects that impact the capacity of vaccines to induce a systemic immune response include adjuvant choice, injection site and antigen delivery method, as each of these play a role in inducing immunogenic APCs. Current adjuvants that are being tested in clinical trials are, among others, the emulsion forming Incomplete Freund's Adjuvant (IFA), several toll-like receptor (TLR) agonists or DC activating cytokines such as GM-CSF. IFA is an oil-based emulsion, which in contrast to water-soluble compounds, protects antigen from dilution, degradation and elimination. In doing so it creates an antigen depot at the vaccine site, allowing for continued antigen exposure[109]. TLR agonists bind to PRR on DCs to mimic DAMP signals and induce maturation. In addition to adjuvant of choice, several antigen delivery methods are currently tested in clinical trials. Methods include injection of mature, peptide-pulsed DCs, tumor protein or (a cocktail of) tumor-specific peptides, whole tumor cells, tumor lysate or tumor RNA/DNA[32]. These methods are mostly being tested independently,

however one study in human melanoma elucidated that vaccines with peptides in IFA induced better systemic immunity than injection of cytokine-matured and peptide-pulsed APCs[110]. More comparative clinical and preclinical studies are required to understand how vaccine components influence the local immune response at the vaccine site as well as whether the local response supports a systemic anti-tumor immune response. Additionally, for cancer vaccines to induce fully functional T cell responses at the tumor site, barriers to T cell infiltration and function should be addressed in parallel, likely through combination therapies.

Adoptive transfer therapy is another approach to overcome T cell priming evasion. With adoptive transfer, the goal is to infuse patients with *ex vivo* expanded or generated effector T cells[111,112]. Either, tumor-specific T cells from tumor mass excisions are isolated and expanded, or, blood-derived T cells are manipulated to express a tumor-specific cloned TCR or a chimeric antigen receptor (CAR). Both methods aim to infuse a large army of activated, patient- and tumor-specific T cells into a cancer patient, thereby overriding the need for antigen presentation by mature DCs and *in vivo* activation of new T cells. However, the infused T cells have to be capable of infiltrating tumors and fully function in the tumor microenvironment. So far these strategies have therefore yielded limited success in solid cancers, with low actual infiltration of infused T cells into tumors[112]. Adoptive transfer approaches would greatly benefit from gaining more insight into how *ex vivo* activation methods affect homing and infiltration mechanisms.

4.3 Overcoming barriers to T cell infiltration

One major hurdle to T cell infiltration is the nature of tumor vasculature. The leaky structure and often lack of homing receptor ligands can severely diminish T cell extravasation from the circulation into the tumor tissue. Preclinical and clinical studies involving anti-angiogenic drugs, such as anti-VEGF antibodies, showed that they induce vessel pruning, maturation and improved perfusion[113]. By normalizing the vasculature, the expression of adhesion receptors and chemokines was also enhanced as well as the number of infiltrating T cells. Preclinical models have shown great potential in combining anti-angiogenic therapy with checkpoint blockade and current ongoing clinical trials are studying the efficacy of these combined immune therapies in human cancers[58,114].

Another hurdle is the localization of T cells in the tumor microenvironment. Physical and chemical barriers are able to prevent T cell engagement with T cells in some tumors. Breaking down ECM matrix barriers through matrix metalloprotease (MMP) regulation, could provide a therapeutic angle to improve T cell engagement and function in tumors. For example, blocking MMP9 and thereby ECM remodeling, in murine tumor models showed increased immune activation and reduced tumor outgrowth[115]. However, the exact mechanism of action with this kind of therapy remains to be elucidated, mainly because the interactions between ECM and T cells, as well as the resulting effects on T cell localization and function in different tumor types, is largely unclear at this point.

Interestingly, MMP9 also cleaves chemokines CXCL9 and CXCL10, suggesting MMP9 inhibitors may affect T cell infiltration and localization by breaking chemical barriers as well[115,116].

The function of infiltrating T cells can be directly inhibited by tumor cells, suppressive immune and stromal cells and/or cytokines in the microenvironment. In addition to the already approved checkpoint inhibitor blockade therapies, numerous therapeutic strategies tackling each of these issues are currently being investigated in preclinical and clinical settings. These include blocking the recruitment, generation and function of MDSCs and Tregs through various mechanisms, inhibiting TGFβ signaling and others[108,117].

5. BRIEF OUTLINE OF THE THESIS CHAPTERS

Despite advances in utilizing immunotherapies for cancer treatment, many gaps remain in our knowledge regarding immune evasion mechanism and how to overcome barriers to successful immune mediated cancer eradication in different cancer types. One hurdle is the lack of tumor-specific T cell activation, which can be tackled by cancer vaccines. In **Chapter 2** it is described where the field stands and why it is important to target CD4 helper T cells with cancer vaccines as a method to support CD8 T cell responses. Furthermore, to analyze which adjuvant combination allows for the most optimal systemic tumor-specific T cell activation, two different TLR agonists, LPS and polyICLC were tested as adjuvants, with or without emulsion-forming Incomplete Freund's adjuvant (IFA) in a peptide-based vaccine for metastatic melanoma patients (**Chapter 3**). In this study, the capacity of tumor-specific circulating T cells to produce IFNγ was assessed at different time points post vaccination, thereby unraveling the importance of each component (LPS, polyICLC and IFA) in the durability and magnitude of a vaccine-induced tumor-specific CD8 T cell response. Importantly, it revealed the role of the vaccine site in generating a systemic immune response. The vaccine sites from patients vaccinated with melanoma-specific antigens either in presence of IFA or TLR agonist cocktail AS15 were assessed for innate and adaptive immune cell subsets, as well as expression of tertiary lymphoid structure (TLS) genes and immune cell homing/retention genes **(Chapter 4)**.

A second hurdle is formed by barriers to T cell infiltration and motility in the tumor microenvironment. The role of integrins on T cells and the resulting effects of integrin-mediated interactions with ECM and E-cadherin on T cell localization and motility, are particularly poorly understood. From work in murine models of infection and autoimmunity, it is clear that collagen-binding integrins CD49a and CD49b are both upregulated during the course TCR-mediated activation of CD8 T cells. E-cadherin-binding integrin CD103 on the other hand, is only expressed on tissue resident memory cells and tumor infiltrating T cells. Beyond this, not much is known about the expression dynamics of these integrins on tumor infiltrating T cells, or how expression correlates with differentiation state and functional

capacity in tumors. Therefore, in **Chapter 5,** CD8 T cells from human metastatic melanoma lesions were categorized based on expression of these 3 integrins, and subsequently placed in a framework of memory and effector cell markers. Here we found that CD49a, CD49b and CD103 expression characterizes five phenotypically and functionally distinct intratumoral CD8 T cell subpopulations. Additionally, cytokines regulating the expression of CD49a and CD103 on human CD8 T cells in addition to TCR-mediated activation were illuminated. In **Chapter 6,** we delve deeper into the expression dynamics and specific cues driving collagen-binding integrins CD49a and CD49b in *in vivo* models for melanoma and breast carcinoma. Interestingly, we found that CD49b is induced on a fraction of antigen-specific cells fairly quickly after TCR-mediated activation. CD49a on the other hand, requires additional environmental cues specific to the tumor for its upregulation. CD49a signaling in these tumor-infiltrating T cells then drives rapid motility, which renders them unable to engage with and respond to tumor cells. Environment-driven expression of CD49a in the context of these tumors may thus be a novel Immune escape mechanism that can be therapeutically targeted.

Finally, in **Chapter 7,** the major findings on how to improve T cell activation, localization and function in tumors are summarized and implications for new therapeutic approaches to target barriers to T cell function in cancer are discussed. Furthermore, new and important scientific questions that arose from the work in this thesis are highlighted.

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