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

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Chapter 7

Discussion

The interplay between cancer and the immune system is an intricate balance, and several immune evasion strategies are used by tumors when they progress. Ideally, a type I immune response is generated, leading to effective eradication of tumor cells by effector T cells. As described in **Chapter 1**, cancer impairs type I T cell responses by impeding the priming, trafficking, infiltration and/or function of T cells. Currently approved immune therapies aim to reinvigorate effector responses, by targeting mechanisms that inhibit T cell function in the tumor. These therapies have shown great potential in cancers with pre-existing T cell infiltrates. However, the failure in a large fraction of patients also stresses the need for therapies targeting other mechanisms of immune evasion. Our incomplete understanding of immune evasion mechanisms utilized by different cancer types impairs the development of novel therapies for all patients. Especially mechanisms that diminish T cell priming and infiltration require attention, as tackling these hurdles would potentiate the presence of a more robust T cell pool in tumors. Cancer vaccines are a promising treatment to overcome suboptimal T cell priming. Vaccines utilize the administration of tumor-specific antigens in combination with immune stimulatory agents, named adjuvants[1], to generate new effector T cells and to boost existing memory T cell populations. For optimal effector T cell function, priming requires antigen-presentation by fully matured, professional antigen-presenting cells (APCs), such as dendritic cells (DCs), as well as CD4 T cell help. With proper CD4 help, mature APCs are able to generate signals 1 to 3 required for appropriate T cell activation: T cell receptor (TCR) engagement, co-stimulation and cytokine signaling[2]. Vaccine adjuvants thus have to support tumor-specific antigen presentation (signal 1) by inducing both signals 2 and 3. We have a good understanding of the important factors required for full DC maturation and T cell help through studies on viral infections **(Chapter 2**). Vaccine strategies and adjuvants to protect against viral infections have thus been well established. It is unclear which of the currently available adjuvants are the most capable to support vaccine-induced responses in the context of cancer; and thus, which strategy can generate the most full-blown and durable systemic type 1 T cell response. In this thesis, we have studied the effects of different adjuvants on local and systemic immune responses, in patients with metastatic melanoma (**Chapter 3 and 4**). Once tumor-specific T cells are primed, tumors can still escape from this immune response by preventing T cells to enter the tumor and find their target. Barriers installed by tumors, preventing proper T cell infiltration and functional engagement with tumor cells, remain poorly understood. Although, these barriers likely involve manipulation of extracellular matrix (ECM) proteins in the tumor microenvironment and adhesion receptors on T cells, including integrins. In **Chapters 5 and 6** we aimed to unravel the dynamics and regulation of integrin expression on T cells, as well as their individual role in T cell adherence to ECM and tumor cells.

A need for a better mechanistic understanding of vaccine adjuvants.

In recent years, a large number of clinical trials evaluated peptide-based vaccine strategies as a treatment for solid tumors, including melanoma and breast cancer[3].

Results for these trials are primarily documented by evaluating either: IFNγ production by circulating T cells, upon stimulation with antigen through enzyme-linked immunospot assays (ELIspot); level of CD4 and CD8 T cell infiltrates in the tumor; or antigen-specific antibodies in serum[3]. These assays can provide very valuable information on the overall systemic effects of different vaccination strategies, but they fail to provide mechanistic understanding of the beneficial or detrimental effects of vaccine adjuvants. As described in **Chapter 1**, inflammation at the vaccine site, and the resulting level of DC maturation, are crucial for adjuvants to properly induce antigen-specific T cells. By contrast, in mice, certain adjuvants, or lack thereof, can reportedly support a suppressive vaccine site, in which T cells get sequestered and deleted[4,5]. With the current assays to evaluate T cell responses in patients, we fail to address how the composition of the response at the vaccine site affects the systemic response. If a systemic response is lacking: is that due to failure in DC maturation, CD4 help, induction of tolerance or suppressive mechanisms at the vaccine site? In order to address this question, we aimed to go beyond these "standard" peptide vaccine evaluations and compared systemic T cell responses to local inflammation and immune cell accumulation at the vaccine site (**Chapter 4**)[6,7].

So, what did we learn from our trials? **Chapter 4** showed that the depot-forming adjuvant IFA induces more robust and durable local immune cell accumulation at the vaccine site than TLR agonist cocktail AS15. Additionally, **Chapter 3** revealed that IFA administered with TLR agonists generates a better systemic immune response than TLR agonists alone. The robust local immune response supported by IFA likely further augments the systemic tumor-specific T cell response. The increase in early accumulation of innate immune cells, especially DCs **(Chapter 4)**, and constant supply of antigen to these innate immune cells[4], may contribute to robust and lasting inflammation, driving APC maturation and ultimately longer-lasting and effective T cell activation. The combination of TLR agonists and IFA leads to a systemic, type I immune response in melanoma patients, with circulating tumor-specific effector T cells (**Chapter 3**). This suggests that the addition of TLR agonists to IFA induced even more robust DC maturation at the vaccine site, followed by improved T cell activation in the draining lymph nodes.

In mice, peptide vaccination in large amounts of IFA was shown to result in excessive accumulation of activated T cells at the vaccine site[4,5]. Is a robust response at the vaccine site, therefore, a good or a bad thing? In the murine studies, T cells were properly activated, but then sequestered away from circulation and tumor into the vaccine site, leading to dysfunction and eventual deletion of tumor-specific T cells. However, when instead of short – exact MHC class I fitting - peptides, mice were vaccinated with longer peptides in IFA, the systemic T cell response was more durable[4]. Long peptides, in contrast to short peptides, have to be ingested and processed by APCs in order to be presented in the context of MHC class I molecules, whereas short peptides can be presented by any cell displaying MHC class I, including cells that can induce tolerance. It is speculated that by combining short peptides and IFA, surrounding, non-immunogenic cells at the

vaccine site continuously present the peptides to T cells, ultimately sequestering and silencing them. The deletion and suppression appears to be mostly mediated by FAS-induced apoptosis and myeloid-derived suppressor cells (MDSC)-induced suppression[5]. Alternatively, because antigen processing is not required with short peptides, T cell activation in the lymph node can be driven by APCs other than mature DCs[8]. These APCs are more likely to induce a tolerogenic response.

These opposing results between our trial and the murine studies can be explained in several ways:

1. Humans and mice may simply respond differently to the use of depot-forming IFA in combination with short peptides. In mice vaccinated with short peptide in IFA, MDSCs infiltrated and suppressed sequestered T cells at the vaccine site[5]. Our analysis in **Chapter 4** did not show increased expression of MDSC gene signatures at vaccine sites of melanoma patients vaccinated with short peptides in IFA[7]. It is thus possible that the presentation of short peptides by surrounding cells, induces less tolerance or suppression in humans, compared to mice.
2. In the clinical trial showing systemic benefit of IFA as adjuvant, it was always given together with either TLR agonist LPS or polyICLC. Furthermore, these vaccine regimens include a tetanus peptide to induce a CD4 helper response. Despite being non-specific to tumor antigen, this CD4 activation is envisioned to provide CD40 co-stimulation and cytokines to optimize CD8 T cell activation. CD4 help is crucial to induce a cytotoxic CD8 T cell response with neo-epitope vaccination strategies[9]. Importantly, in mice, TLR stimulants and/or CD4 help abolished the negative local effects of IFA[4]. The absence of CD4 help or TLR stimulation in the Overwijk study[5] could thus have caused the lack of a systemic CD8 response. By adding these components to our vaccine adjuvant regimen, the antigen depot effect of IFA may be optimally utilized, without the induction of suppressive mechanisms.
3. Our analysis in **Chapter 3** compared the amount of IFA used to administer normalized to average weight of mice and human. This showed that the murine studies used significantly larger amounts than our patient trial. This suggests there is likely a tipping point in the use of IFA: enough to create a proper depot for continuous antigen release and resulting circulating tumor-specific T cells; but not so much it leads to complete sequestration of these tumor-specific T cells at the activation site, through peptide presentation on suppressive surrounding cells.
4. In **Chapter 4,** we found that IFA induced robust expression of tertiary lymphoid structure (TLS) related genes. In humans, local accumulation of adaptive immune cells may therefore be caused by recruitment into newly formed lymphoid structures, which can lead to *in situ* activation of naïve T cells[10,11]. By creating TLS, IFA in humans could contribute to the systemic antigen-specific T cell pool from a local perspective, instead of sequestering T cells away from the circulation.

5. If the correct volume is given to patients, IFA addition to short peptides and TLR agonists LPS or polyICLC, induced responses of higher magnitude and greater durability than TLR agonists alone **(Chapter 3)** and we would recommend it as a vaccine adjuvant for melanoma patients. However, whether the beneficial systemic effects of IFA are due to or in spite of the accumulation of adaptive immune cells at the vaccine site needs to be elucidated in future experiments. For example, it will be important to link accumulation of the different immune cell subsets at the vaccine site directly to the magnitude of the systemic response, as well as patient survival. Additionally, the specific composition and balance of inflammatory versus suppressive immune cell subsets should be evaluated in relation to systemic response and patient survival. In doing so, the beneficial or detrimental effects of the local immune response, in the resulting magnitude and quality of the vaccine-induced immune response, can be determined.

How can vaccination strategies affect the quality of the response?

Irrespective of how many circulating effector T cells a vaccine generates, reaching the desired anti-tumor effect depends upon the ability of these cells to infiltrate tumors and find their target. The route of vaccine administration and strength of activation signals 1-3 play an important role in the homing and chemokine receptor repertoire expressed by a T cell[12–16]. In melanoma patients, vaccination with IFA, but not TLR agonist combination AS15, led to the expression of homing receptor on T cells (**Chapter 4**)[6], suggesting the use of IFA as a vaccine adjuvant does support T cell homing and infiltration. In addition to homing and chemokines receptors, **Chapter 5 and 6** showed that expression of collagen-binding integrins CD49a and CD49b and E-cadherin binding integrin CD103, also depends on activation signals and environmental cues. Increased CD49a expression was observed in vaccine sites of patients vaccinated with melanoma peptides and IFA, compared to control skin (**Chapter 4**)[6]. This suggests that collagen-binding CD49a is induced by currently explored vaccination strategies. The integrins CD49b and CD103 were not induced at the vaccine site by either IFA or AS15 adjuvants over control skin. However, a different study did observe IFA-driven CD103 expression on T cells at the vaccine site at a later time point[6]. Combined, our data indicate that expression of homing, collagen-binding and E-cadherin binding receptors depend on T cell activation signals and/or environmental cues; and thus, vaccination strategy and choice of adjuvant.

Current peptide-based vaccine trials largely evaluate either the magnitude of antigen-specific T cells in the blood or T cell accumulation in tumors to determine the effectiveness of the vaccine. These assessments do not allow for studying the homing and adhesion receptor repertoire, and thus their capacity to enter tumors and eradicate tumor cells. We propose that a comprehensive phenotypic analysis of T cells at the vaccine site, the circulation and in tumors after different vaccine adjuvant strategies, is required to further understand their full impact on T cell activation. This can be achieved by comparing the phenotype and functional

capacity of tetramer-positive T cells from each location, by flow cytometry. Furthermore, single cell RNA sequencing could provide a more unbiased analysis of T cell status. These assays would measure both the magnitude of the immune response at different sites, as well as the expression of homing and adhesion receptors after different vaccine approaches. The differential induction of homing and adhesion receptors can subsequently be analyzed in relation to T cell localization and engagement with tumor cells, through immunofluorescent staining of vaccine-induced T cells in tumors.

What is the individual role of CD49a, CD49b and CD103 in T cell function?

Prior studies have speculated that integrins CD49a, CD49b and CD103 are adhesion molecules, important for retaining T cells in peripheral tissues with abundant collagen or E-cadherin expression[17]. Blocking CD49a or CD49b *in vivo* diminished overall T cell numbers in models for rheumatoid arthritis, influenza or tumors[17–19]. Similarly, CD103 binding to E-cadherin is important for the presence of tissue resident memory-like populations in peripheral tissues or tumors[20,21]. More generally, however, integrins have been described either to provide strong adhesion or to drive cell motility and migration[22]. Integrin-mediated "retention" is thus likely obtained through one of two mechanisms; it can encompass durable adhesion to molecules or cells and thereby establish long-term residence in a tissue. Alternatively, ligand binding drives motility, leading to confined cell migration within the specific tissue or area that contains abundant ligand. The combination of integrin repertoire, ligand availability/organization and resulting integrin signaling pathways may then ultimately determine whether and how a T cell remains in a tissue.

The impact of CD103 signaling on T cell motility and adhesion has been studied *in vitro* and during *in vivo* influenza infection of murine lungs[23]. In this work, CD103 binding to E-cadherin *in vitro* specifically caused T cell arrest. Knocking out or blocking CD103 *in vivo* directly increased velocity of influenza-specific effector CD8 T cells in the lung. In addition to providing adhesion to E-cadherin^{+} cells, CD103/E-cadherin interactions cause active F-actin remodeling and polarization of cytolytic granules, thereby supporting degranulation and TCR-mediated target cell killing[24,25]. CD103 plays an important and very direct role in both T cell adhesion to target cells and subsequent killing.

CD49b signaling in the context of T cell motility is less well-studied. CD49b can mediate migration of other immune cell subsets[26], but in primary T cells CD49b expression has only been correlated with accumulation in collagen-rich matrices or tissues[27]. In line with these findings, we observed that T cell motility was not affected by blocking CD49b-collagen interactions in collagen-rich tumor slices **(Chapter 6)**, but T cell localization was altered and allowed them to reside closer to tumor cells. CD49b binding to collagen may thus provide adhesion, not active migration, similar to CD103. Although, in contrast to CD103, this interaction does not lead to long-term arrest, because blocking CD49b did not increase

overall migration. Other reports have shown a direct role for CD49b signaling in the protection of T cells against FAS- or drug-induced apoptosis[28,29]. Together, this evidence points to a role for CD49b-collagen binding in short-term T cell adhesion and survival, not migration.

On the other hand, collagen-binding integrin CD49a signaling has been directly linked to increased T cell motility of primary T cells both *in vitro* and in epithelial tissues *in vivo*, including lungs and tumors[23] (**Chapter 6**). *In vitro* CD49a binding to collagen increased T cell migration, suggesting that T cells utilize collagen-CD49a interactions to move within a collagen-rich tissue. It can be envisioned that CD49a provides a mechanism for T cells to scan tissues for cognate antigen.

How do the opposing functions of CD49a, CD49b and CD103 differentially guide T cell subset movement in peripheral tissues?

Elegant imaging work in a subcutaneous lung tumor model has shown that antigen-specific T cells either engage with tumor cells in long-term firm contact or establish sequential short-term interactions[30]. During optimal tumor eradication in regressing tumors, long-lasting interactions dominate. Multiple tumor and infection models confirm the importance of these durable interactions to induce target cell killing[30–32]. Additionally, antigen-specific effector T cells displayed higher velocity than non-specific cells, suggesting antigen-specific T cells move rapidly between target cells. For optimal "serial killing" of tumor cells, a complementary role for CD49a, CD49b and CD103 in migration and arrest can thus be envisioned to mediate both effective and rapid scanning for new target cells, as well as efficient adhesion and eradication once the target cell is spotted.
The complementary roles of integrins CD49a, CD49b and CD103 fit with the envisioned movement of different T cell subsets that express them. Effector cells can express CD49a and CD49b, but generally not CD103 (**Chapter 5**). In acute infections, scanning tissues for antigen is mediated by CD49a, and the addition of inflammatory signals from innate immune cells, such monocytes and macrophages, then likely direct the T cells to target cells[33,34]. At the same time, CD49b provides survival signals to effector cells[28,29], by generating short-term contacts with collagen while the cells are moving through the tissue. Contrastingly, tissue resident memory (TRM) T cells generally express both CD49a and CD103, but not CD49b. TRM T cells remain in tissues long after acute infections have dissipated, therefore inflammation and infection are normally absent[35]. In this case, CD49a can allow for rapid movement and scanning through collagen-structured tissues. While scanning, CD103 can bind to E-cadherin expressing cells, cause T cell arrest and create the opportunity to sample the cell for its antigen[23,25]. Together this would provide rapid migration, with intermittent stops to efficiently sample all cells in the tissue for potential re-infection.

In tumors, ligand availability and organization are not as straightforward as other peripheral tissues: collagen matrix is disorganized and E-cadherin is often downregulated on tumor cells, as a part of epithelial-mesenchymal transition

(EMT)[36]. In this context, tumor infiltrating CD8 T cells often express collagen- and E-cadherin-binding integrins CD49a, CD49b and/or CD103 (**Chapter 5 and 6**). Thus, we can envision a few possible scenarios. 1) If collagen is expressed as a mesh among tumors cells, and these tumor cells express E-cadherin, T cells utilize CD49a and CD103 to scan for and adhere to tumor cells, similar to how TRM may utilize these mechanisms. In this situation, because collagen is dispersed in the tumor microenvironment, CD49b may also allow for short-term adhesion and arrest among tumors cells to either complement or replace CD103. 2) When collagen is localized only outside of tumor cell nests, T cells are confined within the stromal regions mediated by CD49a and/or CD49b, unable to find and engage tumor cells. Here, it is irrelevant whether E-cadherin and CD103 are expressed. Interaction of CD49b, CD49a and/or other collagen-binding integrins with collagen needs to be blocked for T cells to reach and engage with tumor cells. 3) Lastly, if tumor cells don't express sufficient levels of E-cadherin, T cells may find and recognize tumors cells, but the cue for long-term arrest and adherence through CD103 is absent. In this situation, subsequent cytotoxic target cell killing will be impaired. When collagen is dispersed among tumor cells, CD49b may be able to replace the CD103-induced long-term arrest to a certain degree. However, whether short-term CD49b adherence to collagen is able to support a durable engagement with the tumor cell, as required for killing[30,31], is unclear and should be further investigated.

In any of these three scenarios, CD49b-mediated survival signals are of course beneficial for ultimate T cell numbers and the overall capacity to eradicate tumor cells. However, even then, when cells are only protected from apoptosis in dense stromal regions, as described in scenario 2, they continue to migrate within this region alone. T cells remain unable to find their target and T cell-mediated tumor cell killing will be suboptimal. Despite this, T cells confined in stromal regions could contribute by producing inflammatory cytokines, if in contact with APCs. This is supported by the notion that, patients who only have T cell infiltration outside of tumor cell regions still have a better prognosis than patients with no T cells at all[37]. An important remaining question is whether CD49b-mediated adhesion in tumors with dense stromal regions (and their lacking ability to interact with tumor cells), outweighs the positive effects that CD49b signaling has on T cell survival; especially if the T cells in stromal regions contribute with inflammatory cytokines. In summary, we propose that CD49a, CD49b and CD103 expression affect tumor control either positively or negatively, depending on the exact tumor-specific context. Under "normal" inflammatory conditions, with nicely organized ECM, blocking β1 integrins (which include CD49a and CD49b) impairs T cell motility and target cell killing[38,39]. This suggest that indeed, in normal ECM structures, integrin signaling benefits effector function. Therefore, understanding the specifics of ECM disorganization and E-cadherin expression in tumors, is crucial in regulating T cell motility and adhesion through integrins. We hypothesize that reorganization of the ECM to resemble normal epithelial tissues will optimize integrin signaling and subsequent T cell migration and function. Later in this chapter we will delve deeper into the different types of collagen organization that

can be found in tumors and how we could aim to normalize the distribution and nature of the ECM to facility better T cell motility.

How does collagen organization in tumors affect T cell motility and function?

A diffuse, mesh-like ECM distribution will have a vastly different effect on T cell motility and function than dense localized ECM. In general it is known that overall presence, collagen fiber thickness, rigidity and organization are all crucial for direction and speed of T cell motility[40]. Most normal epithelial tissues are in a tensional homeostatic state, which leads to a relaxed meshwork of collagens. However, as was described in **Chapter 1**, tumors often display increased collagen deposition, cross-linking and distorted organization[41,42]. Additionally, high levels of matrix metalloproteinases (MMPs) cause increased remodeling of collagen fibers in tumors[43]. Thus overall, collagen alignment, length, width, density and straightness is altered in tumors compared to adjacent normal tissue[44] and the level of disorganization is highly variable between tumors. Due to the high variability, the exact specifics of the collagen organization and structure within a tumor, likely determine the ultimate effect the ECM has on T cell motility and function. Few comprehensive analyses have been done to understand the role of differential collagen organization on T cell function in tumors. One major problem is that no standardized visualization method exists to characterize collagen organization[44]. Currently used methods range from conventional to sophisticated microscopic techniques visualizing collagen structures with ranging sensitivity, making it very difficult to compare between studies. Regardless, anecdotal studies, combined with the findings in this thesis, can teach us about different collagen structures and how they may affect T cell localization, motility and function.

A common collagen organization in stromal-rich tumors involves dense, aligned collagen fibers outside of tumor cell clusters. In these tumors, T cells are often confined within these collagen-dense regions and are unable to interact with tumor cells[45]. T cells can be liberated by collagenase treatment but not integrin blockade, suggesting that in this type of ECM organization, the collagen fibers form a physical barrier. Other collagen-rich tumors, including the breast carcinoma model described in **Chapter 6,** have collagen deposited more evenly throughout the tumor. Although, even when among tumor cells, these fibers are often still dense, linear and highly aligned. T cells, similar to many other cell types, have been shown to use these aligned collagen fibers as highways to migrate along[41,46]. **Chapter 6** and other research showed that CD49a may be involved in promoting this motility in epithelial tissues and tumors[23]. To successfully kill a tumor cell, a T cell has to arrest and engage for an extended time period[34]. When trafficking along collagen fibers at high velocity, it can be envisioned that the T cell is unable to engage as efficient and durable. Thus, tumors can have distinct forms of collagen deposition and create either structural barriers, preventing T cell from leaving stromal regions, or collagen-highways within tumor regions that distract T cells from engaging with target cells. Both forms of collagen deposition

can be mechanisms to hijack the ECM in the tumor microenvironment to prevent optimal T cell recognition, albeit in contrasting ways. Interestingly, treatment with recombinant Hyaluronan And Proteoglycan Link Protein 1 (HAPLN1) in a melanoma model, promoted a more "basket-weave" ECM structure, closer resembling normal epithelial tissue[47]. This correlated with improved T cell infiltration; however, whether it ultimately led to more frequent interactions with tumor cells is unknown.

Since we hypothesize that CD49a, CD49b and possibly other ECM-binding integrins play a role in the movement or adhesion of T cells along collagen fibers **(Chapter 6)**, the availability of the peptide sequence for integrin binding itself could also play a role. Rigid, dense collagen fibers may have structurally blocked the binding sequences, whereas smaller, loose collagen structure could allow for better T cell interactions via integrins. Importantly, MMP-mediated degradation of collagen generates small fragments that have chemotactic properties[48,49]. Binding sites on these fragments are likely more available than on complete collagen fibers. In this scenario, there is no collagen meshwork, as there would be in normal epithelial tissues. However, the small collagen fragments may still drive T cell motility in tumors by signaling through CD49a, but likely without directionality. MMP inhibitors have been shown to increase T cell function in a tumor model, suggesting that presence of collagen fragments can indeed be detrimental[48]. However, whether this is due to CD49a or CD49b signaling remains to be elucidated. Interestingly though, collagen fragments have been shown to antagonize CD49b function *in vitro* in sarcoma cells. The fragments bind to CD49b, but in contrast to full collagen fibers fail to elicit a signaling response[50]. An important question arising from this observation: do collagen fragments antagonize integrin signaling *in vivo*, and is this beneficial or detrimental for T cell function in tumors. We hypothesize that the answer of these questions, depends on the collagen organization of the tumor and changes thereof after MMP inhibition.

Taken together, comprehensive analyses should be conducted to characterize ECM matrix organization in relation to T cell function both in infections of epithelial tissues and different cancer types. Subsequently, novel and existing treatment strategies, altering either collagen deposition and/or organization or the integrin phenotype of T cells, can be deployed to successfully target this barrier to T cell function in tumors.

Regulation of integrin expression on human and murine T cells

Optimal T cell migration in tumors likely is obtained when ECM is organized in a relaxed, meshwork, resembling epithelial tissues. In addition to ECM organization, optimal T cell migration will require an integrin repertoire, that can mediate the desired migration pattern of the T cell. Therefore, it is important to fully understand how integrin expression is regulated on T cells. **Chapter 5 and 6** showed that CD49b is upregulated on a fraction of both human and murine

CD8 T cells after TCR-mediated activation *in vivo* and *in vitro*. However, only a fraction of the antigen-specific T cells upregulated CD49b, in any of these circumstances. This suggests that only a specific lineage of naïve CD8 T cells is capable of upregulating CD49b. With that in mind, we hypothesize that the correct epigenetic and transcriptional state of a naïve T cell is likely essential to support CD49b upregulation. Alternatively, components of the signals required for proper T cell activation, such as CD4 help, may be crucial for CD49b upregulation and are missing for a fraction of the cells. This latter explanation is deemed unlikely due to the existence of a relatively large CD49b^{neg} fraction after *in vitro* activation with CD3/CD28 activating antibodies **(Chapter 5)**. CD3/CD28 stimulation should provide signals for T cell activation equally to the all cultured T cells. In melanoma patients, vaccination either with peptides in IFA or protein in AS15 (adjuvant containing TLR agonists) alone did not increase CD49b expression on accumulated cells at the vaccine site, suggesting the lineage capable of inducing CD49b may not be targeted by these vaccine strategies **(Chapter 4)**. However, antigen-specific T cells in the blood and tumor will have to be evaluated to establish whether CD49b upregulation was absent completely with this vaccination strategy or whether CD49b^{+} T cells selectively do not accumulate at the vaccine site. Alternatively, CD49b may also be rapidly downregulated at the vaccine site. Future analyses of the epigenetic state and gene expression patterns, in relation to CD49b expression, both after *in vitro* activation and in the circulation and vaccine site of vaccinated patients, can illuminate which signaling pathways are crucial for CD49b expression. Furthermore, these analyses can establish the functional capacity of CD49b^{+} and CD49b^{neg} cells under different conditions.

In contrast to CD49b, upregulation of CD103 on human CD8 T cells requires an additional signal in the form of immune suppressive cytokine TGFβ[51] (**Chapter 5**). This finding has been corroborated by *in vivo* murine models for TRM formation[20,52] and CD103 is specifically upregulated within the TGFβ-rich tissue[53]. The availability of TGFβ during different stages of an immune response and in the target tissue itself thus likely determine whether TRM and tumor-infiltrating CD8 T cells ultimately express CD103[54]. It is not surprising that the inflammatory environment of vaccine sites after vaccination with IFA or TLR agonist AS15 did not increase CD103 expression, as TGFβ levels are likely low **(Chapter 4)**. CD103 is induced at the vaccine site, 7 weeks post vaccination with peptide in IFA[6], suggesting that TGFβ is eventually expressed at the vaccine site driven by IFA, likely to dampen the immune response. Future studies can determine whether these CD103^{+} T cells are specifically retained at the vaccine site and whether blocking TGFβ locally could improve T cell dissemination from vaccine site to circulation. In tumors, TGFβ determines what fraction of the T cells express CD103 and can utilize this to improve adhesion and effector function. However, T cells expressing CD103 have also been subject to TGFβ-driven suppression of effector function[51,55]. Therefore, even though CD103 promotes T cell adhesion and killing of tumor cells, the expression of the molecule itself suggests suppressed effector capacity by TGFβ. Future experiments can determine whether blocking TGFβ signaling in tumors relieves the suppression, while retaining the beneficial effects

of CD103 mediated adhesion. Potentially a regulated balance in TGFβ levels is required: enough to upregulate CD103, but not enough to induce high levels of suppression.

Lastly, CD49a regulation is the most complicated of these three integrins and is distinctly regulated between different subsets of human and murine lymphocytes, including T cells. Vaccination in IFA, increased expression of CD49a, but not CD49b and CD103 at the vaccine sites, when compared to normal skin **(Chapter 4)**. *In vitro* cultured human T cells upregulated CD49a on a fraction of cells after TCR-mediated activation. Further upregulation of CD49a, both in fraction and intensity, required additional signals from TGFβ or TNFα in these human CD8 T cells (**Chapter 5)**. IL-2 can induce CD49a expression independent of TCR signaling. Contrasting these findings in human T cells, *in vivo* activation of murine T cells in vaccination or tumor models showed that TCR-mediated activation alone is not sufficient. In this murine tumor model, TGFβ is also not responsible for the upregulation of CD49a on tumor infiltrating T cells **(Chapter 6)**. Other soluble factor(s) in the tumor microenvironment, and likely the vaccine site, induced CD49a in these mouse models. TGFβ does seem to play a role in CD49a expression on murine intestinal TRM[56] and TGFβ, in combination with IL-15, is also responsible for CD49a expression on murine innate lymphocyte cells (ILCs)[57,58]. The distinct regulation of CD49a between different subsets, suggests that CD49a induction by environmental and TCR signals is highly influenced by epigenetic state or cytokine receptor repertoire of the individual cell. Furthermore, our findings and current literature point to a regulation of CD49a expression through multiple pathways and thus suggests importance for CD49a in lymphocyte function under many different circumstances. More detailed studies are to be conducted to understand the relationship between epigenetic status and the signaling pathways required for the upregulation of CD49a. This creates an opportunity for therapeutic modulation of CD49a expression on various T cell subsets and thus regulating their motility in different situations.

The different ways of upregulating these integrins is linked to the functional CD8 T cell subsets expressing them. Under normal circumstances, CD103 is expressed on TRM, whose development is driven in part by TGFβ. Tumors also often contain high levels of TGFβ in the environment, explaining why TIL express CD103, even though they are not classical TRM. CD49b is predominantly expressed on a fraction of effector CD8 T cells both in infections and tumors, but generally not on memory subsets (**Chapter 6)**. This corresponds with the dependence on TCR activation for its expression, and the likely downregulation of CD49b when TCR signal is lost. Then lastly, CD49a induction appears to be driven by several different factors, depending on both the environment and the differentiation state of the cell. The discrepancy observed between CD49a-expressing human and murine CD8 TIL, and their phenotype and function in tumors (**Chapter 5 and 6**), can therefore be explained by the difference in environment and timing of the tumor-specific immune response. CD49a may simply be a sensor of the environment, similar to cytokine-driven expression of CD69 and subsequent downregulation of

T cell trafficking molecule S1P1[59]. By being a sensor of the environment, CD49a could function as a tool for the T cell to upregulate under conditions that require high motility, independent of what the phenotype or functional capacity of that particular cell is.

What other beneficial effects could targeting ECM or integrins have in tumors?

Collagen-binding integrins CD49a and CD49b are expressed on many other cell types, in addition to T cells. These include immune cell populations such as myeloid cells and neutrophils, but also endothelial cells and even tumor cells in some cases[60]. In **Chapter 6** we have shown that CD49a specifically drives T cell motility. In tumor cells and myeloid cells, CD49a also supports migration upon binding with collagens[61,62]. Targeting CD49a in tumors may not only revert T cell dysfunction by increasing arrest and engagement with tumor cells, but also directly address tumor cell migration and metastasis via collagen. It may decrease motility of suppressive myeloid populations and their capacity to inhibit T cell function throughout the tumor. In contrast, utilization of MMP inhibitors or other strategies to normalize the ECM structure could lead to increased and better guided migration of tumor cells and tumor-promoting myeloid cells. It would be important to evaluate the metastatic capacity of tumor cells in the context of these potential therapies to ensure safety in this regard.

Alternatively, there are ECM-binding integrins on T cells in addition to those described in **Chapter 5 and 6** of this thesis. Similar to CD49a and CD49b, these integrins may alter their motility/adhesion in reference to their ligands and affect engagement with tumor cells and ultimate T cell function. For example in a murine model for skin inflammation, integrins αVβ1 and αVβ3 mediated CD4 T cell migration along collagen fibers, visualized with second harmonics generation[38]. T cells can express also ECM-binding integrins α3β1, α4β1, α5β1, α6β1 and αXβ2[63], although much less is known about their regulation during an immune response or their role in T cell migration in different tissues. Given the important individual roles for CD49a, CD49b and CD103 **(Chapter 6)**[23,38,39]**,** we propose that reorganizing the ECM could allow for optimal utilization of these other ECM-binding integrins as well. Therefore, the comprehensive analysis of ECM organization and structure in relation to T cell function should include the specific ligands for each ECM-binding integrin expressed by T cells.

Overall, which potential therapeutic targets have been highlighted to improve T cell function in tumors?

Activation and environmental factors determine integrin expression pattern on CD8 T cells **(Chapter 4-6)**. These integrins can affect T cell infiltration, motility and engagement with tumor cells. A deeper understanding of the exact contributors to induction of individual integrins is necessary, and would create opportunities to adjust vaccination and adoptive transfer strategies to activate T cells with a

favorable integrin and homing receptor repertoire. In doing so, activated T cells are not simply capable of responding to their antigens, but able to arrive in target tissue and engage with target cells. Future clinical trials, in which different T cell activation conditions are evaluated, such as vaccination and adoptive T cell transfer, should assess the effect of activation conditions on T cell phenotype. Activation conditions should then be adjusted for those phenotypes that drive optimal homing to tumors and target recognition.

Separately, elements in the tumor microenvironment can be blocked or enhanced to shape the integrin repertoire for most optimal T cell motility and tumor cell engagement. **Chapter 5** stressed that cytokines TNFα, IL-2 and TGFβ may be involved in the upregulation of CD49a expression on human CD8 T cells. However, in our breast carcinoma model (**Chapter 6)**, TGFβ is not responsible for the expression of CD49a on CD8 TIL. A more comprehensive analysis of cytokines, chemokines, growth factors and other elements of the tumor microenvironment and vaccine site should be conducted, to establish the exact mechanism of CD49a expression. Understanding this mechanism would create the opportunity to block CD49a upregulation, by limiting the signaling of that particular factor. Because CD49a may be directly involved in T cell dysfunction in tumors (**Chapter 6),** blocking its upregulation could negate this mechanism of dysfunction directly. Furthermore, we observed that CD49a often coincides with expression of exhaustion markers, suggesting that blocking the upregulation of CD49a may also result in less inhibitory signals and thus possibly target another mechanism of T cell dysfunction. Inhibition of CD49a signaling through small molecule inhibitors or blocking antibodies could also tackle T cell dysfunction in tumors.

Finally, the ECM matrix in tumors itself can be targeted and reorganized to structurally resemble healthy tissue. This would allow T cells to optimally utilize their integrin repertoire and efficiently find and interact with tumor cells. **Chapter 6** lays the groundwork for understanding the role of activation and environment on integrin repertoire on T cells, as well as how this affects their ability to move and interact with tumor cells. However, comprehensive knowledge of the different ECM components and organization in tumors has to be established. Furthermore, strategies to target ECM organization are emerging, for example, as described above through MMP inhibitors and treatment with recombinant HAPLN1. Therefore, in addition to mapping the ECM in different tumors, the effect of these treatments in each ECM "phenotype" will have to be established to determine the most optimal strategy to normalize ECM structure and thus normalize T cell motility and localization within this structure.

Summary

A type I immune response is crucial for adequate tumor eradication by the immune system. However, tumors often gain evasion mechanisms that create barriers to the generation or effectiveness of a type I immune response. Among

these barriers is the suppression of effective T cell priming and the inhibition of proper T cell infiltration and function in tumors. At present, the only therapies to target these barriers are focused on direct inhibition of T cell function by the tumor, through checkpoint molecules. These therapies are thus dependent on an existing type I response, and are generally not successful when tumors have insufficient T cells primed or infiltrated. This thesis has revealed ways to improve T cell priming and the infiltration of T cells in tumors. The priming of new anti-tumor T cells with melanoma peptides can induce systemic CD8 T cells, capable of responding to antigen. As described in **Chapter 1**, the circumstances during which these T cells get activated ultimately determine their functional capacity in the tumor. The use of IFA as an adjuvant generates both local and systemic immune responses (**Chapter 3 and 4**). IFA induced higher accumulation of activated DCs and CD4 helper T cells at the vaccine site compared to a TLR agonist as adjuvant, suggesting IFA promotes CD8 T cell activation signals efficiently. Combination of IFA with TLR agonists led to an even higher systemic tumor-specific CD8 T cell response. Together, these data highlight important findings to optimize treatment for patients that have no pre-existing T cell response.

Additionally, this thesis focused on the adhesion and retention capabilities of tumor infiltrating CD8 T cells. These CD8 T cells displayed increased expression of integrins CD49a, CD49b and CD103 in human melanoma tumors, compared to circulating CD8 T cells from normal donors (**Chapter 5**). This suggests that T cells may need these integrins in order to stay in the tumor. However, human cancer studies do not provide the opportunity to interrogate T cell dynamics, hence it is impossible to determine whether T cells lacking expression of these integrins are not retained in the tumor or whether they never arose in the first place. We found that elements in the tumor microenvironment are responsible for the upregulation of CD49a and likely CD103. Therefore, the tumor itself can modulate the adhesion capabilities of T cells. The *in vivo* analyses also revealed a more complicated function of these integrins than simply "retention". CD49a drives T cell motility and thereby distracts T cells from engaging with tumor cells, essentially creating retention and T cell dysfunction simultaneously (**Chapter 6**). CD49b did not drive T cell motility or block engagement with tumor cells, suggesting a different role for CD49b ligation to collagen ligands. Other reports showed that CD103 mediated arrest and lasting engagement with E-cadherin expressing cells in lung infections[23], however, whether it serves a similar function in tumors remains to be determined. Nonetheless, these integrins serve different purposes that involve adhesion or adhesion-driven motility, with opposite results in terms of T cell function. Due to environmentally driven expression, tumors can alter the cues required for differential integrin expression, to favor integrin-driven T cell dysfunction. By therapeutically addressing these environmental cues or integrin function directly, overall T cell function in tumors and thus tumor control will be improved.

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