Dissecting the heterogeneity of circulating and tissue-resident memory T cells

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Discussion

The development of heterogeneous subsets of circulating as well as tissue-resident memory T (T_{RM}) cell populations upon vaccination and infection is incompletely understood. Memory $CD8^+$ T cells are heterogeneous in their phenotype and migratory properties as well as their functions (i.e. cytokine polyfunctionality, cytolytic capacity and proliferative potential). The circulating memory $CD8^+$ T cell pool can be largely classified in two major subsets, effector memory (T_{EM}) and central memory T (T_{CM}) cells. T_{RM} cells on the other hand have limited recirculation capacity and are characterized by the expression of tissue retention molecules, such as CD69, and are local specialists of immune defence. $CD8^+$ T_{RM} cells are found in a large variety of tissues and mediate potent local innate and adaptive immunity against pathogens and tumors. Within circulating and resident $CD8^+$ T cell subsets a variety of phenotypes and functions exists, with a continuum of memory T cell properties. Understanding the phenotypic heterogeneity of memory T cells is essential to enable specific targeting of these cells in order to improve vaccination strategies and immunotherapies of infectious and malignant disease.

Circulating and tissue-resident memory $CD8^+$ T cells

In this thesis we dissected the development and phenotypic heterogeneity of antigen-specific circulating and $CD8^+$ T_{RM} cells upon vaccination and infection. Upon vaccination or infection by subcutaneous, intraperitoneal or intravenous injection of vaccines or pathogens, antigen-specific $CD8^+$ T cells are induced in multiple organs, including the liver, lungs, salivary gland, bone marrow and spleen (**chapter 2 and 3**). We delineated the expression of various molecules on the surface of $CD8^+$ T cells by flow cytometry and high-dimensional single cell mass cytometry. We categorized three main memory T cell subsets (i.e., T_{CM}, T_{EM}, and T_{RM}) based on their CD69 and CD62L expression for further characterization. Historically, CD69 is known as an activation marker for $CD8^+$ T cells, as this molecule is transiently induced early activation (1). However, in contrast to the transient expression of CD69 on circulating T cell subsets, exclusively T_{RM} cells are able to constitutively maintain CD69 expression under steady state conditions. CD69 regulates peripheral T cell retention by inhibiting S1PR1 expression and function (1, 2). Parabiosis studies have demonstrated that the vast majority of T_{RM} cells in different tissues express CD69, however T_{RM} populations exist that lack CD69 expression (3, 4). In addition to the expression of cell surface markers, transcription factors

are used to identify T_{RM} cells. Homologue of Blimp-1 in T cells (Hobit) is a transcription factor specifically up-regulated in T_{RM} cells and contributes to tissue-residency together with the related transcription factor Blimp-1 (5). Using Hobit reporter mice we showed that upon vaccination as well as viral and bacterial infection, $CD8^+$ T cells that express CD69 are also expressing Hobit, and can thus be considered tissue-resident (**chapter 3**).

In this thesis, phenotypically heterogeneous subsets of circulating memory $CD8^+$ T cells and $CD8^+$ T_{RM} cells were characterized in different organs, upon dissimilar infections and vaccinations, to investigate factors that shape the development and heterogeneity of memory $CD8^+$ T cells. We aimed to detect the expression of numerous cell surface molecules that are linked to the different functions including migration, adhesion or exhaustion. We designed a mass cytometry panel consisting of 41 cell-surface markers including anti-PE and anti-APC labelled tetramers to detect and distinguish various antigen-specific $CD8^+$ T cell populations. In forthcoming experiments mass cytometry can also be used for the detection of intracellular molecules and additionally tissue mass cytometry can be performed to investigate the location of cells and interaction with other cell types within the tissue of interest. The function of T cell subsets is closely linked to the expression of specific cell-surface markers, however heterogeneity could also be examined by for example cytokine production or the expression of certain transcription factors. Sorting and/or adoptive transfer experiments of $CD8^+$ T cell subsets to identify differences in function however is challenging, because of the plethora of distinct memory $CD8^+$ T cell subsets (**chapter 2**) and survival of these cells *ex vivo*.

In addition to the phenotypic heterogeneity, we investigated the protective capacity of circulating and $CD8^+$ T_{RM} cells in malignant disease (**chapter 6**). We addressed the importance of T_{RM} cells in protection by capturing circulating cells in the lymph nodes by FTY720 treatment, or by specifically depleting T_{RM} cells via administration of depleting antibodies and subsequent comparison of settings in which T_{RM} cells were present and/or absent in tissues prior to (local) challenges. FTY720 inhibits lymphocyte egress from lymphoid tissues by downregulating S1P receptor, leading to sequestration of these cells in the lymph nodes which prevents them from contribution to immune responses (6). Moreover, we depleted either the circulating $CD8^+$ T cells by low dose anti-CD8 antibody treatment or $CD8^+$ T_{RM} cells by CXCR3 depleting antibodies (7). Upon administration of depleting antibodies, we

delineated the importance of circulating and resident CD8$^+$ T cells in protection against subsequent tumor challenges (**chapter 6**).

Factors influencing CD8$^+$ T cell heterogeneity

Factors that govern the complex formation of memory T cells are not completely understood. Antigen-specific naïve T cells have the ability to differentiate into multiple effector and memory T cell subsets upon antigen recognition and proper activation. Both clonal expansion and differentiation patterns are heterogeneous resulting in the generation of functionally diverse T cell subsets. Multiple factors, including tissue-environment, cellular interactions and induction of molecular pathways impact memory T cell differentiation (8-12). Transcriptional and epigenetic networks modulate the plasticity and differentiation of antigen-specific CD8$^+$ T cells (13). Upon re-challenge, T_{RM} cells expand locally and in draining lymph nodes, however T_{RM} cells can also be recruited in tissues (14-16). Park and colleagues found that T_{RM} cell reactivation triggers the recruitment of circulating memory CD8$^+$ T cells into the tissue, which undergo T_{RM} cell differentiation in situ, without displacing pre-existing memory pools (14).

Upon viral or bacterial infection, diverse antigen-specific CD8$^+$ T cells are generated that target various pathogen-specific epitopes. In our studies we used different pathogens, of which some were modified to express foreign epitopes, to investigate the phenotype of antigen-specific CD8$^+$ T cells directed towards the same epitope. In **chapter 2**, we showed that the phenotype of antigen-specific circulating and CD8$^+$ T_{RM} cells is prominently influenced by the pathogen-specific inflammatory milieu, and additionally shaped by the tissue micro-environment. Moreover, the chronicity of infection shapes the antigen-specific CD8$^+$ T cell phenotype. As we additionally showed in **chapter 2**, similar observations were made by analyzing the total CD8$^+$ T cell populations, comprising both GP33-specific CD8$^+$ T cells as well as other antigen-specific CD8$^+$ T cells, bystander-activated CD8$^+$ T cells, and naïve cells. Contrary to viral and bacterial infections, SLP vaccination allows the incorporation of a single MHC restricted epitope in the vaccine, and can thus be designed to induce exclusively antigen-specific CD8$^+$ T cells against the epitope provided (**chapter 3**). When comparing total liver CD8$^+$ T_{RM} cells upon SLP vaccination or different types of infection, these cells display phenotypic heterogeneity (**Figure 1**). Whereas the phenotype of total CD8$^+$ T_{RM} cells is

dissimilar upon prime, prime-boost or prime-boost-boost vaccination (**chapter 3**), compared to viral and bacterial infection the SLP-elicited cell populations resemble each other more as compared to cells provoked by infection. These data further illustrate that the inflammatory milieu in which the T cells are elicited has a profound impact on shaping the CD8$^+$ T_{RM} cell phenotypes. This is in line with the data displayed in **chapter 2** showing that tissue-specific signatures exist, however when analyzing one tissue, mainly chronicity of infection rather than antigen-triggering by SLP vaccination seems to shape total CD8$^+$ T_{RM} heterogeneity. Next, we will discuss the impact of chronicity of infection, antigen-triggering as well as the tissue-micro-environment on the development and heterogeneity of memory CD8$^+$ T cells.

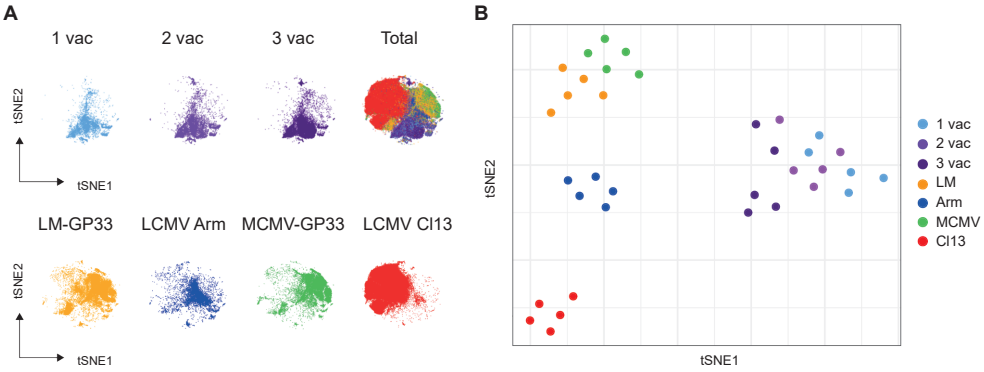

Figure 1. Heterogeneity of liver CD8$^+$ T_{RM} cells upon vaccination and infection. Mass cytometry analysis of total liver CD8$^+$ T_{RM} cells combined from 1, 2 and 3 GP34 SLP vaccinations, LM-GP33, LCMV Armstrong, MCMV-GP33, LCMV clone 13 infections day 50-70 after vaccination/infection. GP34 SLP vaccination (100ug + 20ug CpG) was administered s.c. in a prime-boost-boost setting with 2 week intervals. Mice were infected with LCMV Armstrong (2×10^5 PFU, intraperitoneally), LCMV Clone 13 (2×10^6 plaque-forming units (PFU), intravenously via retro-orbital injection), *Listeria monocytogenes* (LM) expressing GP33 (LM-GP33), 1×10^4 colony-forming units (CFU), intravenously via retro-orbital injection), MCMV-GP33 (2×10^5 PFU, intraperitoneally). Total CD8$^+$ T cells gated in FlowJo after which Cytosplore HSNE analysis was performed. Downsampled to 20.000 cells per sample and selected on CD69$^+$ cells for further analysis. Shown the fingerprints of the CD69$^+$ CD8$^+$ T cells of different vaccination/infection groups. B) tSNE analysis showing the distribution of the different samples color coded per vaccination/infection group.

Chronicity of infection

CD8$^+$ T_{RM} cells are present in naïve mice (**chapter 2**), however, infection and/or inflammation enhances T_{RM} cell formation (16). T_{RM} cells can be found in virtually every tissue but infection or vaccination enhances T_{RM} cell numbers. Upon vaccination or infection, we have seen lodgement of antigen-specific CD8$^+$ T_{RM} cells in multiple organs (**chapter 2, chapter 3**). In **chapter 2** we used various bacterial and viral infections to show that the chronicity of infection impacts the circulating as well as resident CD8$^+$ T cell compartment. Antigen-

specific $CD8^+$ T cells responding to pathogens provoking either acute, low-level persistent, or high-level chronic infection were phenotypically characterized. Importantly, viral persistence superseded memory $CD8^+$ T cell differentiation after acute infection.

In **chapter 4**, we investigated the impact of infectious dose on the $CD8^+$ T cell phenotype. The phenotypic antigen-specific $CD8^+$ T cell profiles and degree of accumulation over time was directly influenced by low or high-dose persistent CMV infection. Moreover, high-dose infection progressed the degree of differentiation and impaired the protective capacity of these $CD8^+$ T cells. This high-dose CMV infection hampered the control of heterologous LCMV infections. Thus, CMV infection shapes the memory $CD8^+$ T cell phenotype and function. CMV induces a persistent infection, where the virus is able to reactivate at certain times and thereby induce memory inflation. This characteristic of CMV infection is also seen upon adenoviral vector vaccination and typified by the maintenance of large populations of virus-specific $CD8^+$ T cells with an effector-memory-like phenotype (17, 18). These inflationary memory responses are induced by low-level intermittent persistence of antigen and importantly, the inflationary $CD8^+$ T cells remain functional over time (18). In **chapter 6**, we show that memory inflation enhances $CD8^+$ T_{RM} cell development. Vaccination with replication-deficient adenoviral vectors induced memory inflation and subsequent T_{RM} cell development, protective against cancer. Importantly, we showed that antigen-specific $CD8^+$ T_{RM} cells relatively increased in liver, not lung, compared to their circulating counterparts upon intravenous vaccination with adenoviral vectors. This is consistent with the liver being the site of infection on systemic administration of the adenoviral vaccine.

Low-level persistent infections such as CMV induce a different memory $CD8^+$ T cell phenotype compared to chronic infections such as LCMV clone 13. Contrary to low-level persistent CMV infection, $CD8^+$ T_{RM} cells express molecules as PD-1, CD223 and CD39 upon chronic LCMV infection. These molecules are described in exhaustion and their expression often reflects impaired cytotoxicity and cytokine production (19-21). This phenotype is seen in cancer as well, providing opportunities for immunotherapy (**chapter 5**).

Antigen-triggering

Prime-boost vaccination is commonly used to generate protective immune responses, however the impact of recurring antigen stimulation on the differentiation of heterogeneous memory

$CD8^+$ T cell populations is not fully understood. In **chapter 3**, we delineated the impact of high-level antigen triggering on circulating as well as resident memory T cell differentiation and protective capacity by SLP prime-boost vaccination. Using high-dimensional single-cell mass cytometry we identified that especially secondary immunizations sculp the heterogeneity of vaccine-induced $CD8^+$ T cells organ-wide. We showed that repeated antigen triggering has a dynamic and profound impact on the magnitude and diversification of antigen-specific circulating as well as $CD8^+$ T_{RM} cells. Interestingly, SLP vaccines can be designed to induce immune responses against numerous different infections and malignant diseases. Upon vaccination with SLP we included one MHC restricted epitope, and additionally included the TRL9 ligand CpG as adjuvant. It would be interesting to investigate T_{RM} cell development upon vaccination with different adjuvants.

As shown in **chapter 6**, memory $CD8^+$ T cells are not fully differentiated since both effector and central memory T cells can acquire a tissue-resident phenotype upon adoptive transfer. This is more pronounced when antigen is present locally, thus antigen-triggering plays a role in the formation of $CD8^+$ T_{RM} cells. Also, shown in **chapter 3**, upon antigen-triggering a small subset of $CD8^+$ T_{RM} cells can exit their tissue, enter the circulation again and become ex-T_{RM} cells. Using Hobit lineage tracer mice we found that $CD8^+$ ex-T_{RM} cells are induced upon antigen triggering and display an effector memory phenotype. However, ex-T_{RM} cells constitute a transcriptionally and functionally distinct effector memory subset (15). Thus, upon interaction with their cognate antigen, some T_{RM} cells can exit their tissue of origin and contribute to the circulating memory T cell pool.

As described in **chapter 6**, memory inflation is associated to increased $CD8^+$ T_{RM} cell formation. Here, adenoviral vectors were modified to induce memory inflation, generating an antigen-specific $CD8^+$ T cell response that is similar to responses provoked by CMV infection, characterized by a gradual rise in the magnitude of the antigen-specific memory T cell pool. One important difference between adenoviral vector vaccination and CMV infection is the fact that adenoviral vaccines do not replicate, although antigen remains present (22). Consistent with the liver being the site of infection on systemic administration of the adenoviral vaccine, antigen-specific $CD8^+$ T_{RM} cells were stably maintained in the liver for months after vaccination. Here, a high level of memory inflation correlated with an increase in $CD8^+$ T_{RM} cells. This is in line with the findings **in chapter 3**, were we show that antigen-

triggering leads to an increase in antigen-specific CD8$^+$ T cells and especially of CD8$^+$ T_{RM} cells. Also, strong antigen-triggering enhanced the formation of CD8$^+$ ex-T_{RM} cells upon SLP boosting. This formation of ex-T_{RM} cells was not evident upon MCMV infection, suggesting that low-level antigen-triggering is insufficient for the formation of ex-T_{RM} cells. Further research is needed to characterize the function of these ex-T_{RM} cells and to investigate the contribution of these circulating cells to the T_{RM} cell pool.

Tissue microenvironment

In **chapter 2** we showed that the phenotype of antigen-specific memory CD8$^+$ T cells is shaped by the tissue micro-environment, even though to a lesser extent than pathogen-specific cues. The difference in phenotypes was mainly evident between hematopoietic and non-hematopoietic tissues, spleen samples associated more with bone-marrow and blood samples, whereas lungs and liver samples clustered together. This difference is impacted of course by the presence of CD8$^+$ T_{RM} cells. However, phenotypic diversity was also obvious within the major subsets of circulating as well as resident CD8$^+$ T cell compartments upon infection. Various cytokines and cellular interactions in the tissue micro-environment could affect the phenotype of these antigen-specific CD8$^+$ T_{RM} cells since environmental cues are known to play a role in the fate of an individual naïve CD8$^+$ T cell responding to infection (23). Moreover, the development and persistence of CD8$^+$ T_{RM} cells is shaped by the cytokine milieu, with the cytokines IL-12, type I IFN, and IL-15 playing important roles in the differentiation of these memory T cells (8-10).

The pathogens we used generated systemic infections, leading to broad antigen-specific CD8$^+$ T_{EM} and CD8$^+$ T_{RM} development in many different organs. Many CD8$^+$ T_{RM} cells were found in the liver, which could relate to the fact that the liver is a site of infection and plays a major role in the clearance of pathogens entering the bloodstream. For example, upon LM infection, bacteria are rapidly cleared from the bloodstream by resident macrophages in the spleen and liver (24). Importantly, T_{RM} cells can be reactivated by numerous antigen-presenting cells, but the identity of the antigen-presenting cells that reactivate T_{RM} cells impacts the functional properties of the T_{RM} cells. Recently, Low et al. showed that activation of T_{RM} cells by hematopoietic or nonhematopoietic cells induced differential T_{RM} activation signatures and functional output (25).

Transcriptional profiling has revealed a unique transcriptional profile of T_{RM} cells, shared between T_{RM} cells at different locations, however, T_{RM} cells at different organs are characterized by tissue-specific gene expression profiles (11). Next to stimulation by TCR triggering, $CD8^+$ T cells can become activated in a process called bystander activation, i.e. by proinflammatory cytokines such as type I IFNs, IL-12, and IL-15 (26). Noncognate, bystander activation can trigger the sensing and alarming function of pulmonary $CD8^+$ T_{RM} cells (27). Recently, it was found that competition for TGFβ allows for selective retention of antigen-specific T_{RM} cells, not bystander T_{RM} cells, in the epidermal niche (28). These data suggest that preferentially antigen-specific T_{RM} cells are maintained. Bystander $CD8^+$ T cells have diverse phenotypes that overlap with antigen-specific cells. In this thesis, we focused on total $CD8^+$ T cells or antigen-specific $CD8^+$ T cells, consequently further studies are needed to define the contribution of bystander T cell activation to protective immunity in infectious and malignant disease upon vaccination.

$CD8^+$ T_{RM} cell targets

In this thesis we dissected the phenotypic heterogeneity of circulating and $CD8^+$ T_{RM} cells in different tissues. The expression of various molecules by these cells in different vaccination, infection or cancer settings, is linked to the function of these cells. In **chapter 2,** we identified heterogeneous subsets of antigen-specific $CD8^+$ T cells that could enhance the rational design of immunotherapies. Importantly, the phenotypic heterogeneity provides an excellent opportunity to target these cells. In chronic infections and cancer, T cells (transiently) express inhibitory receptors, such as PD-1, CTLA-4, and LAG-3, associated with $CD8^+$ T cell exhaustion. Consequently, many different immunotherapeutics target inhibitory immune checkpoints to enhance the functionality and protective capacity of these cells to improve immunotherapy. Especially, clinical trials with antibodies that block the interaction between the inhibitory receptor PD-1 with its ligand PD-L1, resulted in unprecedented clinical response rates for patients with advanced cancer (29). In **chapter 5**, we have seen that PD-L1 blockade therapy induced the expansion of tumor-infiltrating T cell subsets, co-expressing both activating (ICOS) and inhibitory (LAG-3, PD-1) molecules. By additional co-targeting of these molecules, T cell numbers further increased in the tumor micro-environment and improved tumor protection. Thus, the identification of various molecules expressed by T cells enables the

rational design of immunotherapy. In addition, the identification of phenotypically diverse T cells upon treatment could lead to the identification of biomarkers for clinical activity (30). High-dimensional single-cell technologies, including mass cytometry but also RNA and TCR sequencing, will increasingly be crucial to the analysis of immunotherapy and guidance of clinical decision-making (31, 32).

Antigen-specific memory $CD8^+$ T cell numbers or functionality can be targeted by both antibody-based therapy and vaccines. In **chapter 6**, we showed that targeting of costimulatory interactions during vaccination with adenoviral vectors enhances the frequency of antigen-specific $CD8^+$ T_{RM} cells. Costimulation is essential for the development of functional $CD8^+$ T cell responses and in line with an early impact of CD80/CD86 costimulatory interactions, we found that initial targeting of the inhibitory molecule CTLA-4 after immunization increased $CD8^+$ T_{RM} cells and protective tumor immunity. CTLA-4 antibody treatment however is not specifically targeting T_{RM} cells but also has a broader effect. It is of interest to further explore whether targeting other inhibitory molecules or costimulatory receptors could also lead to enhanced formation of $CD8^+$ T_{RM} cells and how the differentiation of other memory cells is impacted. Additionally, we showed that the formation of $CD8^+$ T_{RM} cells can be enhanced by booster immunization or by vaccination with vectors that induce high levels of memory inflation (**chapter 3, chapter 6**). More research on local treatment or delivery of vaccines (i.e. intra-tumoral injections) is needed to specifically target $CD8^+$ T_{RM} cells.

In this thesis we used different mouse tumor models to study the phenotype and protective capacity of memory $CD8^+$ T cells. We studied the immune composition in various subcutaneous tumor models, including TC-1, C3, MC38, as well as in a model of metastasis by intrasplenic injection of tumor cells. Using these mouse models we were able to delineate multiple factors underlying memory $CD8^+$ T cell heterogeneity and protective capacity. It would be interesting to perform similar studies on the phenotype of human $CD8^+$ T cells isolated from different tumors or metastasis, and in addition study the effect of combined immunotherapies on the immune composition.

As described in **chapter 7**, there are many possibilities to target $CD8^+$ T cells and enhance their numbers or functionality but specific targeting of $CD8^+$ T_{RM} cells remains challenging. Thus far, memory $CD8^+$ T cell subsets are mostly non-specifically targeted, while targeting

of specific CD8$^+$ T_{RM} cell subsets may be more beneficial. T_{RM} cell infiltration correlates with enhanced response to current immunotherapy and is often associated with improved clinical outcome in patients with cancer (33). The phenotypic heterogeneity of CD8$^+$ T_{RM} cells allows for targeting of these cells with agonistic antibodies against inhibitory or costimulatory molecules. In addition, targeting the specific metabolic requirements or transcription factors of these cells might also constitute a potential therapeutic approach. T_{RM} cells can be induced by local treatment or administration of vaccines and T_{RM} cell differentiation and survival can be targeted in various manners to enhance therapies. More focus is needed on the development of T_{RM} cells upon vaccination and their response to immunotherapy. Specific targeting of T_{RM} cells may provide a promising approach to improve vaccination and immunotherapies against cancer and infectious diseases but a better understanding of CD8$^+$ T_{RM} cell heterogeneity and development is essential to exploit T_{RM} cells for therapeutic purposes.

Concluding remarks

Diverse subsets of antigen-specific memory CD8$^+$ T cells are present in multiple tissues upon vaccination and infection. In this thesis we identified phenotypically heterogeneous subsets of circulating as well as resident CD8$^+$ T cells. Multiple factors including pathogen-specific cues, the tissue micro-environment and antigen-triggering were identified that shape the phenotypic heterogeneity of these memory CD8$^+$ T cells. CD8$^+$ T_{RM} cells are located at the frontlines of the immune defense and have a crucial role in immune protection against infectious and malignant disease. The unique properties of these cells make T_{RM} cells attractive therapeutic targets. Understanding the phenotypic diversity of memory CD8$^+$ T cells is essential to exploit their unique properties for the rational design of vaccines and immunotherapy approaches.

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