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

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General introduction

The immune system

The immune system provides protection against pathogens, such as viruses, bacteria and parasites, and malignant cells. The immune system can be largely divided into innate and adaptive immune responses, and the interaction between these two systems provides long-lasting immunity, e.g. resistance to infectious disease. The innate immune system is responsible for the initial protection and is the rapid, first line of defence. This initial defence is relatively non-specific and includes physical and chemical barriers as well as innate immune cells that are essential for immediate response upon pathogen recognition. Innate immune cells such as dendritic cells and macrophages can act as antigen-presenting cells (APCs) that recognize danger, these cells take up and process antigen and present it to other cells. APCs are activated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that are recognized by their pattern-recognition receptors and subsequently release pro-inflammatory cytokines upon activation, to alarm and recruit other cells (1).

Contrary to this pre-existing innate immune system, the adaptive immune system is acquired, more specific, and essential for lifelong protection. The adaptive immune system includes B and T lymphocytes that are vital for humoral and cellular immunity respectively. B cells develop in the bone marrow and upon activation in secondary lymphoid organs differentiate into plasma cells or memory B cells (2). Plasma cells are responsible for the production of protective antigen-specific antibodies, which neutralize and tag microbes for elimination. T cells on the other hand develop in the thymus and are responsible for cellular immunity. T cells recognize peptide antigens presented in major histocompatibility complex (MHC) molecules expressed by APCs and activation of these T cells can lead to direct killing of infected or aberrant cells. Like B cells, also T cells can differentiate after activation into memory cells that are able to rapidly respond to pathogen re-encounter.

T cell precursors originate from hematopoietic stem cells in the bone marrow and mature in the thymus where they undergo clonal selection. Critical in this step is the expression of a functional T cell receptor (TCR) on the cell surface of T cells, which enables them to recognize and react to peptides (3). In the thymus, those cells that are able to recognize peptide presented by APCs with sufficient TCR binding are selected by positive selection. On

the contrary, a process called negative selection limits excessive TCR binding and excludes the development of T cells that are strongly autoreactive against self-peptides. The $CD4^+CD8^+$ thymocytes develop eventually into either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. $CD4^+$ T helper cells ensure optimal responses by B cells and $CD8^+$ T cells (4). These two major T cell subsets further differentiate in the periphery.

T cell activation and differentiation

Antigen-presenting cells take up antigen locally, which can be from dead or infected cells or debris, and transfer it to secondary lymphoid organs were they interact with T cells (5). T cells mediate immunity by their T cell receptor that specifically recognizes peptide presented by MHC molecules. $CD4^+$ T cells recognize antigen presented by MHC class II. These antigens are for example from extracellular pathogens that have been taken up by endocytosis and processed by lysosomal proteolysis. $CD8^+$ T cells on the other hand recognize antigen presented by MHC class I, and these antigens are derived from intracellular proteins obtained through proteasomal proteolysis, from e.g. intracellular pathogens. However, cross-presentation also occurs, where peptides derived from extracellular sources are presented by MHC class I molecules. In cross-presentation, specialized dendritic cells relay helper signal from $CD4^+$ T cells to $CD8^+$ T cells to optimize T cell responses (6, 7).

For proper T cell activation, three signals are required (8). The first signal is provided when T cells recognize peptide presented in MHC molecules leading to TCR triggering. The second signal consists of interaction with costimulatory ligands, such as CD80, CD86 or CD70, on APCs that interact with costimulatory receptors such as CD28 and CD27 expressed constitutively by T cells. T cells express also other costimulatory molecules on their cell surface that are upregulated after activation (9, 10). These markers include members of the immunoglobulin superfamily (e.g., ICOS) and the tumor necrosis factor receptor (TNFR) superfamily including 4-1BB, OX40 and HVEM. The third signal for proper T cell activation is mediated by cytokines, such as IL-12 and type 1 interferons (IFN), which are essential for optimal T cell proliferation and function.

Upon antigen recognition and proper activation, $CD4^+$ and $CD8^+$ T cells undergo proliferative

expansion and differentiation into effector and memory T cells, to achieve sufficient numbers to eradicate invading pathogens or malignant cells. Multiple mechanisms are thought to play a role in the selection and differentiation of heterogeneous effector and memory T cells, such as TCR strength, costimulation, tissue micro-environment and gene expression (11, 12). However, the precise mechanisms that control T cell heterogeneity upon expansion to generate multiple T cell fates remain incompletely understood.

Upon activation and expansion, T cells migrate from the lymph node into the circulation. T cells reduce the expression of adhesion molecules and migrate based on chemokine gradients in the lymph nodes, blood and tissues. Sphingosine-1-phosphate (S1P) gradients, that are sensed by the S1P receptor 1 (S1PR1) expressed on T cells, induce T cell egress into the lymph and blood (13). T cells constantly recirculate between the blood and peripheral lymphoid organs. Depending on the anatomic location of the lymph node, various trafficking molecules are expressed on the expanding activated T cell population. The migration of T cells is also controlled by chemokines (14). During infection, T cells subsequently migrate to non-lymphoid tissues and sites of infection based on their specific tissue-homing properties.

Upon antigen encounter in the periphery, $CD8^+$ T cells can eradicate infected or malignant cells by the release of cytotoxic molecules such as perforin and granzyme B or the activation of the Fas ligand pathway (15). Perforin induces pores in the cell membrane of target cells, which facilitates the entry of the protease granzyme that subsequently initiates apoptosis, induced cell death. Additionally, T cells expressing Fas ligand interact with Fas (CD95) leading to cleavage of caspases and subsequent cell death. Moreover, T cells secrete various cytokines including tumor necrosis factor (TNF) and interferon-γ (IFN-γ) that exert multiple actions that contribute to protection, including the induction of various signaling events leading to cell death as well as the antiviral effects that include activation and enhanced antigen-presentation of other immune cells that are important for elimination of target cells. Various types of T cells mediate cell-type specific responses upon inflammation or antigen encounter.

Circulating memory CD8$^+$ T cells

Circulating memory CD8$^+$ T cells are heterogeneous in their phenotype and migratory properties as well as their cytokine polyfunctionality, cytolytic capacity and proliferative potential (16). The circulating memory CD8$^+$ T cell pool can be largely classified in two major subsets, effector and central memory CD8$^+$ T cells. Effector memory T cells (T_{EM}, phenotype: CD62L$^-$CD69$^-$) are mainly present in non-lymphoid tissue and exert immediate effector functions. On the contrary, central memory T cells (T_{CM}, CD62L$^+$CD69$^-$) are localized mainly in lymphoid tissues, and rapidly expand upon antigen-encounter. Even within these subsets a variety of phenotypes and functions exists, with a continuum of memory T cell properties with respect to their location and recirculation capacity, metabolism, epigenetic regulation and longevity (17). Hence, in addition to the main circulating T_{EM} and T_{CM} subsets, many more subdivisions of circulating CD8$^+$ T cells can be defined, including but not limited to stem cell memory T (T_{SCM}) cells and exhausted T (T_{EX}) cells.

The formation and heterogeneity of the circulating memory CD8$^+$ T cell pool is shaped by antigen-triggering, inflammation and infection. For example, upon infection with cytomegalovirus (CMV) or modified adenoviral vaccines, a phenomenon called memory inflation occurs (8). CMV induces a persistent infection, in which the virus is not cleared but becomes latent and reactivates at certain times. Therefore, antigen remains present and certain antigen-specific CD8$^+$ T cells persist at high levels, called inflationary T cells. Memory inflation is typified by the maintenance of large populations of virus-specific CD8$^+$ T cells with an effector-memory-like phenotype. The low-level intermittent persistence of antigen is critical for the induction of inflationary memory responses, and importantly, the inflationary CD8$^+$ T cells remain functional over time. Here, the viral inoculum dose impacts the level of memory T cell inflation (18). In contrast to the development of sustained functional CD8$^+$ T cell responses upon low-level replicating CMV, chronic infections with high-level active replication elicit virus-specific exhausted CD8$^+$ T cells. Exhausted T cells typically express PD-1 and are characterized by impaired cytotoxicity and cytokine production, however also here heterogeneity exists (19).

Exposure to pathogens as well as aging shape the memory T cell composition and functionality. Immune senescence is the age-related decline in immunological competence.

The progressive decline in the ability to induce effective immune responses leads to an increased susceptibility to infectious diseases and cancer, and a reduced efficacy of vaccines (20). In immune senescence, mainly the adaptive immune system is affected and the inability to respond to new antigens is linked to a decreased number of peripheral naïve T cells. In addition, an accumulation of memory T cells and low-grade inflammation is characteristic of immune senescence. Therefore, immune senescence is largely affected by the history of infections, especially by chronic infections that induce sustained memory T cell responses.

Tissue-resident memory CD8$^+$ T cells

Contrary to the classical view that all lymphocytes circulate, tissue-resident memory T (T_{RM}) cells have been discovered as cells with a limited recirculation capacity. T_{RM} cells retain the tissue-homing molecules and acquire a molecular program that contributes to the maintenance of these cells in peripheral tissue. T_{RM} cells are characterized by the expression of tissue retention molecules, such as CD69, and downregulation of genes involved in tissue egress, including S1pr1 and Ccr7 (21). CD69 regulates peripheral T cell retention by inhibiting S1PR1 expression and function (22, 23). In addition, inflammatory cytokines, including TGF-β, interleukin-33 (IL-33), and TNF, also mediate suppression of S1PR1 expression via downregulation of the transcription factor Krüppel-like factor 2 (24). Integrins, such as CD69, CD49a, and CD103, mediate the tissue retention of CD8$^+$ T cells. In addition, T_{RM} cells express various transcription factors that induce tissue-residency, such as Hobit and Blimp (25). The limited recirculation capacity of T_{RM} cells was discovered by parabiosis studies where two animals share the same blood circulation (26). Also in humans, transplantation experiments have demonstrated the minimal recirculation capacity of these cells and persistence for long periods of time under steady-state conditions (27, 28).

Antigen-specific CD8$^+$ T_{RM} cells are induced upon vaccination or infection and localize to many different tissues, including barrier tissues, where they play a crucial role in protection against infectious and malignant disease. CD8$^+$ T_{RM} cells can be induced in various non-hematopoietic tissues such as the liver, lung and salivary gland but also the brain, skin and gastro-intestinal tract, where they mediate potent local immunity (29). Upon re-stimulation, by the recognition of cognate antigen or bystander activation, T_{RM} cells rapidly release effector molecules, such

as pro-inflammatory cytokines IFN-γ and TNF, as well as granzyme B. This initiates activation of multiple cells including local dendritic cells and natural killer cells, and induces recruitment of other immune cells, such as memory T cells and B cells, to the site of challenge (29). $CD8^+$ T_{RM} cells can rapidly trigger an antiviral state by amplifying receptor-derived signals from previously encountered pathogens, providing protection against numerous viral pathogens in multiple tissues (30, 31). Thus, $CD8^+$ T_{RM} cells are local specialists in immune defence.

The formation and heterogeneity of $CD8^+$ T_{RM} cells is differentially regulated by multiple factors, including local inflammatory microenvironments, cytokines as well as antigen-triggering and cellular interactions. Consequently, $CD8^+$ T_{RM} cells arising at different sites and responding to different infections are characterized by diverse phenotypes. This phenotypic heterogeneity is exemplified by the expression of various cell surface molecules such as costimulatory and inhibitory receptors as well as chemokine receptors. In this thesis, we study the heterogeneity of $CD8^+$ T_{RM} cells upon vaccination and infection. In addition, we address the importance of T_{RM} cells in protection by capturing circulating cells in the lymph nodes, or specifically depleting circulating $CD8^+$ T cells or T_{RM} cells by administration of depleting antibodies and subsequent tumor challenges.

Although $CD8^+$ T_{RM} cells have limited recirculation capacity, some of these cells are able to re-enter the circulation upon pathogen re-challenge (32). These cells mainly acquire an effector memory $CD8^+$ T cell phenotype. Even though these ex-T_{RM} cells share developmental plasticity with other circulating memory subsets, they remain epigenetically poised for tissue migration and T_{RM} cell redifferentiation (33).

Phenotypic heterogeneity of $CD8^+$ T cells

T cells subsets such as central, effector memory and tissue-resident T cells can be distinguished by the expression of molecules on their outer cell membrane such as CD62L and CD69. The expression of these molecules is linked to the function of these cells, for example their migration and location. Additionally, T cells express various markers such as costimulatory, inhibitory and chemokine receptors. The phenotype of these cells is dependent on their location, i.e. the tissue micro-environment and other influences such as

signals from pathogens and interactions with other cell types. Understanding the phenotypic heterogeneity of T cells is essential to enable specific targeting of these cells for vaccination or immunotherapy approaches.

Conventionally, immune cells are phenotypically characterized by flow cytometry. In flow cytometry, cells are labelled with specific antibodies conjugated to fluorochromes in order to characterize the properties of each cell. Labelled cells are passed through a laser beam in a single cell suspension, fluorochromes are excited by the laser light and subsequently detected by their fluorescence emission spectrum. Using flow cytometry, the spectral overlap in the emitted fluorescence limits the amount of markers that can be used to characterize the cells measured and thereby limits the identification of heterogeneous cell subsets.

To study in depth the phenotypic heterogeneity of T cells we used single-cell high dimensional mass cytometry. In mass cytometry cells are labelled with antibodies conjugated to heavy metals instead of fluorochromes, which can be distinguished based on their unique mass allowing the inclusion of over 40 antibodies simultaneously. To distinguish various immune cell subsets, we designed a panel consisting of 41 cell-surface markers including tetramers for the detection of antigen-specific $CD8^+$ T cells. Any spill-over in signal that could be due to impurity of the heavy metals was compensated using Catalyst (34). For flow cytometry data, various T cell subsets are identified by manual gating. Mass cytometry however generates more complex datasets and requires a tailored bioinformatics pipeline for proper data analysis. Here we used clustering algorithms such as FlowSOM and Cytosplore, to identify various cell subsets (35, 36). We performed Jensen-Shannon divergence analysis to study the similarity between different tSNE plots generated by Cytosplore. To visualize the subsets and quantify statistical differences we used Cytofast, which was developed in house (37). In addition, we performed principal component analysis (PCA) and dual tSNE analysis to visualize the sample and cluster distribution in various experimental settings.

Vaccination and infection

T cell mediated immune responses play an important role in vaccine-induced immunity against persistent infections and malignant disease. Prophylactic vaccines are used to

prevent disease and have eradicated infectious diseases such as smallpox (38). Contrary to preventive prophylactic vaccination, therapeutic vaccination is used direct and activate the immune system to target infected or aberrant cells after the onset of disease. Importantly, the generation of durable T cell responses of sufficient magnitude and quality is key in the process of developing vaccine-induced immunity (39).

In this thesis we investigate circulating and $CD8^+$ T_{RM} cell development upon various vaccination strategies and infections, and identify factors that impact antigen-specific $CD8^+$ T cell formation such as antigen triggering and chronicity of infection. We investigate memory $CD8^+$ T cell development and protective capacity upon vaccination with synthetic long peptides (SLP) and adenoviral vectors. These vaccine platforms allow for the incorporation of any MHC restricted epitope in the vaccine, corresponding to for example the sequence of viral or tumor antigens, and can thus be designed to induce immune responses against numerous different infections and malignant diseases. Prime-boost vaccination with SLPs, where the same vaccination is administered sequentially, provides a robust model of specific and temporal antigen triggering. Vaccination with adenoviral vectors on the other hand induces low-level persistence, as these vectors do not replicate but the antigen remains present. Replication-deficient adenoviral vectors are generated by deletion of early proteins (E1/E3) that are essential for viral replication. Recombinant replication-deficient HuAd5 vectors can be modified by engineering a mini-gene into the vector to express specific epitopes eliciting memory T cell inflation (40, 41).

In addition to vaccines, we use various viral and bacterial pathogens that induce acute, low-level persistent or chronic infections. In acute infection the pathogen is cleared a few days after infection, whereas in persistent or chronic infections the pathogen remains present and continues to induce and shape T cell responses. In our studies we used different pathogens, modified to express certain epitopes (virus or tumor derived, or from model antigens such as ovalbumin), to investigate $CD8^+$ T cells directed towards the same epitopes. We used the pathogens *Listeria Monocytogenes* (LM) and lymphocytic choriomeningitis virus (LCMV) Armstrong to induce acute infections. LM is a gram-positive bacterium that replicates within the host cell cytosol and thereby stimulates strong $CD8^+$ T cell responses (42). The RNA virus LCMV Armstrong generates a strong initial immune response after which the pathogen is cleared. Next to these acute infections, we studied the $CD8^+$ T cell response upon low-level

persistent and chronic infections with mouse cytomegalovirus (MCMV) and LCMV Clone 13, respectively. CMV is a common double-stranded DNA virus, and upon infection remains present and becomes latent but can reactivate at certain times, inducing memory inflation against specific epitopes. Chronic LCMV Clone 13 infection on the other hand, generates strong immune responses resulting in T cell exhaustion. The different levels of chronicity of induced infections shape the memory $CD8^+$ T cell response.

Cancer immunotherapies

Cancer is the uncontrolled growth of abnormal cells, that potentially invade other tissues, called metastasis. Hallmarks of cancer include sustaining proliferative signalling, evading growth suppressors, resisting cell death and enabling replicative immortality (43). Genome instability is an important underlying mechanisms for the sustained proliferation of malignant cells. Genome instability of aberrant cells can be induced by mistakes in the DNA repair (e.g. caused by smoking or sunlight) or by viral infections such as human papilloma virus (HPV) that induces cervical cancer. Evasion of the immune system is another important mechanism by which cancer cells keep proliferating, once cancer is established malignant cells are often not removed by the immune system.

Current therapies for cancer include chemotherapy or radiotherapy often in combination with immunotherapies (44-46). Immunotherapy has become an important treatment option for cancer patients and aims to induce or enhance immune responses that target malignant cells. Immunotherapy includes vaccination, adoptive cell therapy and antibody-based immune checkpoint blockade therapy. Prophylactic vaccination has been very effective in protection from HPV and thereby protects against HPV-associated malignant disease. In addition, many therapeutic vaccines are being developed, to generate functional $CD8^+$ T cell responses against tumor cells after onset of disease (47).

In chronic infections and cancer, both circulating and T_{RM} cells (transiently) express inhibitory receptors, such as PD-1, CTLA-4, and LAG-3, which serve as immune checkpoints by dampening further T cell activation. The expression of these inhibitory molecules is associated with $CD8^+$ T cell exhaustion, and consequently, many different immune-therapeutics target

inhibitory immune checkpoints to enhance the functionality and protective capacity of these cells to improve cancer 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 (48). In addition, combined therapy of PD-1 blockade with additional antibody therapies that target costimulatory interactions such as CD27 or CTLA-4 is used to enhance T cell priming and infiltration (49). Thus, targeting costimulatory and/or inhibitory molecules on $CD8^+$ T cells is a successful approach to enhance the frequency or functionality of $CD8^+$ T cells and improve clinical outcomes.

In many human cancers, large quantities of tumor-infiltrating T_{RM}-like cells correlate with an improved overall survival (50-52). In addition, T_{RM} cells play a crucial role in controlling tumor outgrowth in preclinical models (53). However, we are only starting to understand the impact of immunotherapy on the $CD8^+$ T_{RM} cell populations. The possibility to increase T_{RM} cell numbers and thereby enhance local protective immunity constitutes a promising immunotherapeutic approach, however specific targeting of $CD8^+$ T_{RM} cells remains challenging. In this thesis we study the presence and protective capacity of $CD8^+$ T_{RM} cells in different cancer models. In addition, we focus on the phenotypic heterogeneity of circulating and $CD8^+$ T_{RM} cells at different tissue sites and discuss the possibilities of targeting their unique cell surface molecules for therapeutic purposes.

Scope of this thesis

The development of heterogeneous subsets of circulating as well as tissue-resident memory $CD8^+$ T cell populations upon vaccination and infection is incompletely understood. In this thesis we investigate the development and heterogeneity of antigen-specific memory $CD8^+$ T cells upon vaccination and infection and investigate their protective capacity in infectious and malignant disease. We further discuss the possibilities of targeting $CD8^+$ T_{RM} cells to improve vaccination strategies.

In the first chapters, we investigate factors that shape the development and heterogeneity of circulating memory $CD8^+$ T cells and $CD8^+$ T_{RM} cells in multiple organs. In **chapter 2**, we

investigate the influence of the pathogen-specific inflammatory milieu as well as tissue-specific cues on the heterogeneity of antigen-specific $CD8^+$ T_{EM} and $CD8^+$ T_{RM} cells using single-cell high dimensional mass cytometry. Using various viral and bacterial infections, we show the impact of the chronicity of infection as well as the influence of tissue micro-environment on the development of heterogeneous phenotypes of antigen-specific $CD8^+$ T_{EM} and $CD8^+$ T_{RM} cells. In **chapter 3**, we delineate the impact of antigen triggering on memory $CD8^+$ T cell differentiation using sequential SLP vaccinations. We show that prime-boost vaccination provides increased protection against infectious and malignant disease and we examine the impact of vaccine boosting on the differentiation of antigen-specific memory $CD8^+$ T_{RM} cells and the development of circulating $CD8^+$ ex-T_{RM} cells.

In the next chapters, we focus on the protective capacity of $CD8^+$ T cells in infectious and malignant disease. In **chapter 4,** we investigate the impact of viral infection on immune senescence and the capacity to protect from subsequent viral infection. We study the effect of the infectious dose on the differentiation and protective capacity of memory $CD8^+$ T cells in long-lasting infections. In **chapter 5** we show that $CD8^+$ T cells can be exploited for immunotherapy of cancer. We characterize T cells in the tumor micro-environment and show that based on the phenotype of these cells immunotherapy can be designed that leads to improved tumor immunity. In **chapter 6** we focus on the development of $CD8^+$ T_{RM} cells upon vaccination and the protective capacity against cancer. We examine the connection between memory inflation and T_{RM} cell formation and investigate the protective capacity of $CD8^+$ T_{RM} cells induced upon vaccination with replication deficient adenoviral vectors. In addition, we show that the formation of $CD8^+$ T_{RM} cells is dependent on costimulatory interactions and can be enhanced by targeting these molecules. The phenotypic heterogeneity of $CD8^+$ T_{RM} cells and their unique functions can be exploited for vaccines and immunotherapies of infectious and malignant disease (**chapter 7**). Finally, all studies in this thesis are discussed in **chapter 8.**

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