The power of help: mechanistic insights into CD4⁺ T cell differentiation in vaccination and cancer

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Chapter 1

General Introduction

Generation of T-cell responses

The immune system consists of a variety of cell subsets with different functions to combat pathogenic infections and cancer formation, while maintaining tolerance to host cells. It can be split up in the innate and adaptive system; the innate immune system responds fast but relatively non-specific by sensing pathogens via conserved motifs such as pathogen associated molecular patterns (PAMPs)[1], while the adaptive immune system, consisting of T cells and B cells, takes days to mount a response. This response is more durable, more specific and results in immune memory, protecting the host from subsequent reinfections[2]. T cells recognize small peptide sequences, called antigens by means of their T cell receptor (TCR). These antigens can be from the host, referred to as 'self', or of a pathogen or other foreign, 'non-self' entity. Successful immunity relies on maintaining balance between combatting pathogens without damaging the host. Typically, T cells are subdivided in $CD8^+$ and $CD4^+$ lineages, although other lineages such as natural killer (NK)-T cells and γδ T cells also exist. $CD8^+$ T cells recognize antigens as presented by major histocompatibility complex (MHC) class I (MHC-I) molecules, while $CD4^+$ T cells require presentation in MHC class II (MHC-II) molecules. While MHC-I molecules are widely expressed by all cell types, with some exceptions such as erythrocytes, MHC-II expression is largely restricted to antigen-presenting cells (APCs), specifically dendritic cells (DCs), other myeloid cells, as well as T- and B cells[3]. Due to this limited expression of MHC-II, $CD4^+$ T cells excel at modulating the functionality of other immune cells.

DCs are divided in three lineages, plasmacytoid (p)DCs, conventional (c)DC1s and cDC2s[4] that are further discerned into sublineages cDC2A and cDC2B[5]. Additionally, distinct differentiation states are recognized now, such as inflammatory cDC2s found in viral infection[6] and ISG^+ cDC2 observed in tumor tissue[7]. cDCs are critical for T-cell priming, but research of especially cDC2s is challenging due to lack of unique markers, and may be confounded by other subsets such as monocyte-derived cells[8]. cDC1s excel at capturing cell-associated antigens via for example CLEC9a[9], and have superior cross-presentation of exogenous antigens in MHC-I and are therefore the main drivers of $CD8^+$ T-cell differentiation[10,11]. This is further emphasized by genetic models lacking cDC1s[12,13]. By contrast, cDC2s preferentially present antigens in MHC-II and are important for $CD4^+$ T-cell priming[14,15], which was also evidenced by a lack of anti-helminth $CD4^+$ T-cell activation in mice lacking cDC2s[16]. A recent study directly compared the role of cDC1s and cDC2s in (cross)-presentation of different types of antigen in genetic mouse models lacking cDC1s (IRF8Δ32) or cDC2s (ZEB2 Δ1Δ2Δ3)[17]. This critical study highlighted that cDC1s are required and sufficient for (cross-)presentation of tumor cell-associated antigen to both $CD4^+$ and $CD8^+$ T cells, although inflammatory cDC2s were also capable of inducing $CD8^+$ T-cell responses. By contrast, both cDC1s and cDC2s could

cross-present soluble antigen to CD8⁺ T cells. Additionally, cDC2s could cross-present immune complexes (i.e. antibody-coated particulate antigen), but not antibody-coated cellular antigen to CD4⁺ and CD8⁺ T cells, while cDC1s could cross-present immune complexes to CD8⁺ T cells, but not to CD4⁺ T cells[17]. Furthermore, the antigen load may also affect which cDC type is capable of antigen uptake, as cDC1s and cDC2s are not homogeneously distributed in the lymph nodes[18].

Typically, T-cell priming takes place in secondary lymphoid organs (SLO); the lymph nodes (LNs) and spleen. For successful T-cell priming three signals are required: 1) antigen presentation in MHC, 2) costimulation by APCs 3) cytokine delivery by APCs[19–21]. Typically, antigen is transported from the site of infection by migratory cDCs, which move to the T-cell zone of the local draining (d)LNs, to initiate T-cell activation. However, whether migratory cDCs are required depends on antigen load and the location of infection[22]. In the dLN, antigen can be handed over to local resident cDCs[23]. Under specific inflammatory conditions, monocytes can differentiate to monocyte-derived DCs (MoDCs) that can aid in T-cell differentiation[24,25]. It is critical to gain deeper understanding on how DCs instruct T-cell differentiation, as more optimal vaccination or treatment strategies against infection and cancer rely on understanding which signals DCs require to optimize the desired T-cell responses.

CD4⁺ T cells as orchestrators of the immune response

CD4⁺ T cells have an unique position in the adaptive immune system, as they can attain a wide variety of functionally distinct differentiation states, depending on the type of pathogen that is encountered. Inflammation is often classified into three different types depending on the nature of the encountered pathogen[26]. Intracellular pathogens, such as viruses and intracellular bacteria, as well as cancer result in type 1 inflammation, depending on IFNγ-dominated responses. Parasitic infections result in type 2 inflammation characterized by among others IL-4 responses, while extracellular bacteria and fungi result in type 3 inflammation, which depends on IL-17. CD4⁺ T cells are activated in all these types of immune responses and differentiate into distinct functional cell states that aid in clearing the pathogen. The most well studied CD4⁺ T-cell differentiation trajectories are T-helper 1 (Th1), Th2 and Th17 cells, T follicular helper (Tfh) cells and regulatory T (Treg) cells[27,28]. These CD4⁺ T cell subsets have unique gene expression programs that orchestrate amongst others distinct cytokine producing profiles and chemokine receptor repertoires, and depend on master transcription factors that induce and maintain their status. These transcription factors are T-bet (Th1), GATA3 (Th2), RORγt (Th17), BCL6 (Tfh) and FOXP3 (Treg)[28,29] (**Figure 1A**). To clear pathogens, the immune system relies on a cellular response mediated by T cells,

NK cells and myeloid cells and a humoral response mediated by B cells via antibody production. Th1, Th2 and Th17 cells provide help for the cellular response, while Tfh cells support the humoral response. This subdivision of CD4$^+$ T-helper cell subsets is explained in a bifurcation model, where Th1, Th2 and Th17 cells are generated at the same time as Tfh cells[30] (**Figure 1B**). In the different immune challenges, Tfh cells also adapt their cytokine profile[31]. However, how bifurcation is regulated is incompletely understood. It has been suggested that higher TCR affinity may favor Tfh differentiation over Th1 differentiation[32,33], but other studies have shown TCR-independent decision making[34,35]. Furthermore, bifurcation is typically studied by making use of TCR-transgenic T cells, which does not formally demonstrate that Th1 and Tfh cells can be formed from a single naïve cell. However, limiting dilution studies have suggested that offspring from a single OTII TCR transgenic CD4$^+$ T cell can adopt multiple fates[34]. Studying CD4$^+$ T-cell biology has been more challenging compared to CD8$^+$ T cells, due to lower affinity MHC-II tetramers for detection, as well as the fact that CD4$^+$ T cells have much lower proliferative output than CD8$^+$ T cells[29,30]. Understanding how CD4$^+$ T cells decide to become either cellular or humoral helpers is of great importance to steer vaccination or anti-cancer T-cell responses.

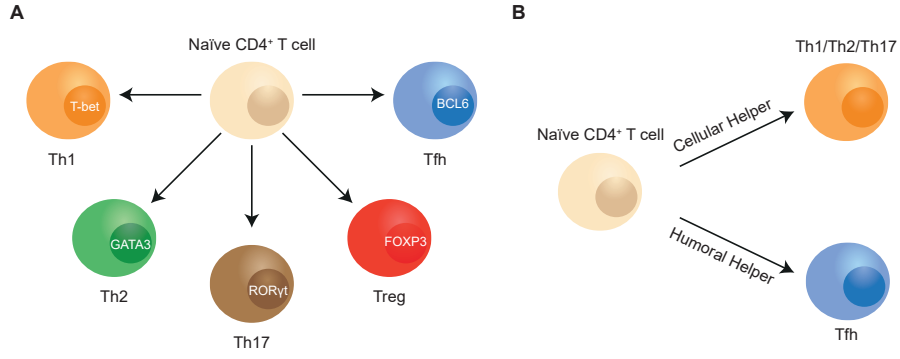

Figure 1. CD4$^+$ T cell differentiation trajectories and bifurcation.
A) Schematic overview of different CD4$^+$ T cell differentiation trajectories from naïve T cells. For each differentiation trajectory, the master transcription factor is highlighted. **B)** CD4$^+$ T cell bifurcation into cellular and humoral helpers. Differentiation to specific cellular helpers is dictated by the type of immune response.

On the other hand, Treg cells restrict immune responses to prevent pathologic damage as well as autoimmunity. T cells are selected in the thymus, where auto-reactive T cells are deleted from the nascent T-cell pool. However, certain T cells with affinity for 'self' antigens are rescued from this negative selection and become thymus-derived (t)Treg cells[36]. These tTreg cells are critical in restraining autoimmunity that could be induced

by presentation of self-antigens by migratory DCs that continuously travel to dLNs under steady state conditions[37–39]. Self-antigen presentation may induce priming of self-reactive T cells that survived negative selection in the thymus. Indeed, autoreactive CD4$^+$ T cells can spontaneously get activated and differentiate to Tfh-like cells in mice lacking Treg cells[40]. Additionally, lack of MHC-II on cDC1s that prevents their cognate interaction with Treg cells causes fast-onset, lethal autoimmunity in mice in a self-reactive CD8$^+$ T-cell dependent manner[41]. Furthermore, conventional CD4$^+$ T cells can also become peripherally induced (p)Tregs in the course of a response to foreign antigen. At mucosal sites, specifically in the gut, pTregs are crucial for tolerance to dietary and environmental antigens[42,43]. Similarly to effector T helper cells, Treg cells present in peripheral tissue can adapt to local cytokine milieus and gain expression of specific T-helper transcription factors, such as T-bet or BCL6. These differentiation states allow for Treg-specialized dampening of for example Th1 and Tfh responses[44]. This seems at least to be in part dependent on upregulation of chemokine receptors important for homing to the T-helper effector site such as CXCR3 and CXCR5[45,46]. Recent data show that TNFR2 costimulation can drive a specific gene program of non-lymphoid tissue-resident tTregs including improved migration, adhesion and suppressive function[47]. However, TNFR2 costimulation does not induce Th-like polarization, suggesting that this is mediated by other factors such as cytokine milieus[47]. Gaining insights in how Tregs respond and dampen immune responses is of great importance, as reducing Treg function can aid anti-cancer therapy and boosting it can combat autoimmunity.

Generation and roles of Th1 and Tfh cells in type 1 immune responses

In type 1 inflammation, CD8$^+$ T-cell differentiation into effector Cytotoxic T lymphocytes (CTLs) is required to clear infected or cancerous cells, while B-cell differentiation into high-affinity antibody producing plasma cells is needed to ensure that pathogens can no longer infect new cells[48]. Type 1 inflammation also induces Th1 and Tfh responses of CD4$^+$ T cells (**Figure 1**). Th1 cells are typically classified as T-bet dependent cells that secrete IFNγ and TNFα. Th1 cells can exit the SLOs and move into infected tissues, where they can help CD8$^+$ T cells, cDCs and macrophages[11,29,49,50]. Generation of Th1 cells is dependent on the presence of IL-12 and IL-2[51–53].

Intravital microscopy studies in mice have demonstrated that in infection, CD8$^+$ T cells and CD4$^+$ T cells are initially primed separately, both in time and space[54,55]. However, during a second-step of priming, activated CD8$^+$ T cells and CD4$^+$ Th1 cells come together on a cDC1, as guided by chemokine cues and dependent on specific antigen presentation by the cDC1. Here, the CD4$^+$ T cell provides 'help' signals to the cDC1,

which in turn relays this help to CD8⁺ T cells[13,56,57] (**Figure 2A**). Recently, it has been confirmed that human cDC1s, like their mouse counterparts, are also the recipients of CD4⁺ T-cell help[11]. Upon specific antigen recognition, the CD4⁺ T cell helps to install - via the cDC1 - an optimal CTL effector state in the CD8⁺ T cell, as most overtly evidenced by upregulation of cytotoxic molecules, downregulation of co-inhibitory molecules, improved migratory capacity, as well as effector-memory differentiation[58–60]. It has recently been shown in both human and murine studies that cDC1s are the unique recipient of CD4⁺ T cell help[11,13]. Our group demonstrated that CD4⁺ T cell help instills a specific program only in cDC1s, which optimizes antigen-presentation and the costimulatory capacity of cDC1s[11]. Mechanistically, CD4⁺ Th1 cell-dependent costimulation via CD40L to CD40 expressing cDC1s is a critical signal (**Figure 2A**). CD40 signaling into cDC1s induces a gene expression program that amongst others results in upregulation of the costimulatory molecule CD70[56,61,62], as well as upregulation of the cytokine IL-12[63,64]. These molecules play vital roles in CD8⁺ and CD4⁺ T-cell differentiation[58,59,65–67]. Furthermore, it was recently shown that CD40 signaling also induces other programs in cDC1s, such as improved survival[61]. Additionally, CD4⁺ T cells also produce IFNβ to optimize MHC-I antigen presentation by human cDC1s[62], and IFNγ production by T cells results in IL-12 upregulation[68].

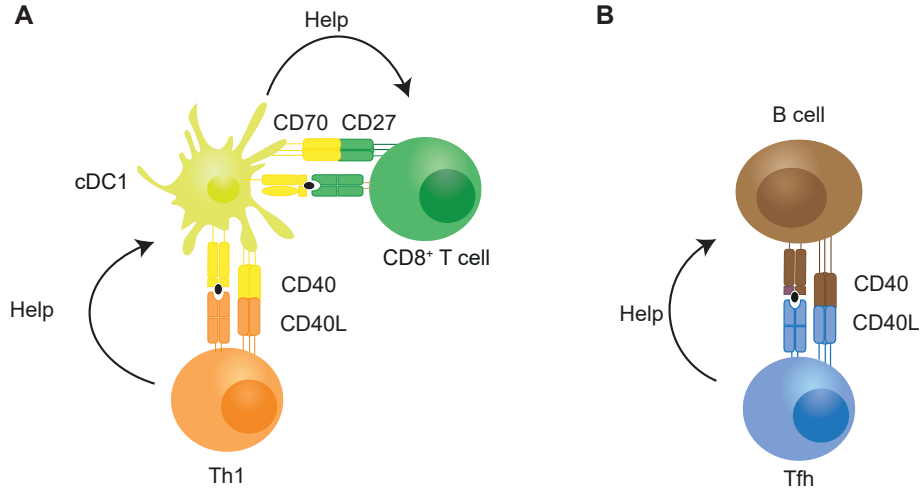

Figure 2. CD4⁺ Th1 and Tfh helper functions.
A) CD4⁺ T cells provide help for CD8⁺ T cell differentiation via cDC1. Upon antigen presentation by the cDC1, CD4⁺ Th1 cells provide costimulatory support, particularly CD40L, and cytokine support to the cDC1. As a result, cDC1s provide costimulatory and cytokine support to CD8⁺ T cells upon antigen presentation. **B)** CD4⁺ T cells are critical for selection of high affinity BCR B cells in the germinal center. Upon antigen capture by B cells, they present it to CD4⁺ Tfh cells in the light zone. As a result, this leads to provision of costimulation, in particular CD40L, and cytokine support for B cells. In turn, B cells also provide costimulation to Tfh cells.

Thus, this tri-cell interaction of Th1 $CD4^+$ T cells, cDC1 and $CD8^+$ T cells is required for inducing optimal cellular immunity to viruses and cancer. Typically, Th1 differentiation already takes place in SLO as evidenced by phenotype (e.g. T-bet^{hi}) or cytokine production (e.g. IFNγ). This has been observed for multiple pathogens, such as LCMV, influenza and malaria infection[69,70], but also in vaccination[24,71]. However, in some contexts further Th1 differentiation, as well as memory formation takes place in tissues; intestinal *Cryptosporidium* Th1 responses were dependent on cDC1 after $CD4^+$ T cells have left the LNs[72], while monocyte-derived cells are required to reactivate Th1 cells in response to vaginal HSV2 infection in mice[73]. In conclusion, these data suggest that although $CD4^+$ T cells can complete their differentiation trajectory in SLOs, they require additional input in the inflamed tissue depending on their priming. This may depend on pathogen sensing, as a vaccination strategy with LCMV-epitopes and adjuvant resulted in non-committed $CD4^+$ T-cell memory formation, which rapidly formed Th1 cells only upon rechallenge with LCMV[74]. Similarly, it was shown that vaccination with ovalbumin (OVA) combined with lipopolysaccharide (LPS) and incomplete Freund's adjuvant (IFA) dramatically promoted Th1 differentiation in the dLN compared to OVA with either LPS or IFA alone[71]. Altogether, these data imply that in certain immunizations, $CD4^+$ T-cell lineage commitment is not completed in the SLO, but can still occur in peripheral tissue.

Tfh cells help B cells in their affinity maturation and differentiation trajectory[75] (**Figure 2B**). Tfh cells are dependent on the transcription factor BCL6, and produce the hallmark cytokine IL-21[76,77]. Generation of Tfh cells is a multistage differentiation process. It is believed that cDCs are important for the induction of Tfh cells, as mice lacking MHC-II expression by cDCs do not form pre-Tfh cells[78]. This induction seems to be mainly dependent on migratory cDC2s as determined by comparison of genetic mouse models lacking either cDC1s or cDC2s[79]. After initial activation, Tfh cells upregulate CXCR5 and move to the border of the T and B cell zone of the SLOs, where they interact with antigen-specific B cells[75,80]. However, under certain conditions, Tfh cell priming can occur by B cells without a requirement for DCs[81]. Mechanistically, absence of IL-2 signaling and the presence of IL-6 is required, while ICOS-ICOSL and OX40-OX40L interactions optimize Tfh cell differentiation[49,51,82,83].

After initial antigen-mediated activation of B cells via membrane immunoglobulin (Ig), alias the B-cell receptor (BCR), and help from Tfh cells, B cells clonally expand and form germinal centers (GC), wherein they undergo Ig affinity maturation via somatic hypermutation and class switching. The GC is divided in two zones: 1) the dark zone, where somatic hypermutation and clonal expansion take place; 2) the light zone, where GC B cells are positively selected based on antigen recognition[84]. Tfh cells are located in the light zone, and provide costimulatory and cytokine signals to B cells upon specific

antigen recognition in MHC-II[85]. B cells with high affinity membrane Ig obtain higher amounts of antigen from follicular dendritic cells (FDCs), and are thus more likely to present antigen to T cells[86]. High affinity B cells interacting with Tfh cells have improved survival and increased proliferative capacity, resulting in the selection of B cells with the highest affinity antibodies[84]. Mechanistically, Tfh cells help high affinity GC B cells by providing IL-21, IL-4 and CD40L[77,87,88] (**Figure 2B**). It is currently appreciated that during a type 1 immune response, antigen-specific $CD4^+$ T cells differentiate into both Th1 and Tfh cells (**Figure 1B**), but how this is regulated, and how individual $CD4^+$ T cells are steered into either lineage is not well established.

$CD4^+$ T-cell responses to cancer

In the last decade, immune checkpoint blockade (ICB) as a therapy for cancer has become more and more prominent[89]. This therapy, mainly revolving around the co-inhibitory molecules PD-1 and CTLA-4, aims to release a brake on the T-cell response[90]. However, despite clinical success, ICB does not help all patients, nor does it prove curative in all settings[89]. This may be the result of several factors, including tumor-mediated suppression of T cells in the tumor microenvironment[91]. Typically, ICB has the best prognosis in cancers with a high mutational burden such as melanoma, as a high mutation rate results in a multitude of possible neoantigens, which can then be recognized by T cells[92].

However, the immune system constellation within the tumor is also of great importance; T cells may be excluded from the tumor (immune desert), T cells may be restricted from the tumor core (excluded) or T cells might have infiltrated the entire tumor[93]. As can be envisioned, increased tumor infiltration by immune cells is associated with improved patient survival. Indeed, tumors lacking T-cell infiltration likely have poor T-cell activation, or lack chemokines to attract T cells[94]. To achieve durable anti-cancer T cell responses, it is vital that cDCs get activated and traffic to the dLN to activate T cells[94]. Given their superior capacity to cross-present cellular antigen, cDC1s are critical in priming of T cells towards cancer. Indeed, when tumors were classified in 10 different cancer ecotypes, ecotypes containing cDC1s, $CD4^+$ and $CD8^+$ T cells were associated with better overall patient survival compared to other ecotypes, such as tumors with high myeloid infiltration[95]. The presence of cDC1s, $CD4^+$ and $CD8^+$ T cells may allow for a platform to relay $CD4^+$ help to $CD8^+$ T cells, also in the tumor. Indeed, the gene signature of human *in vitro* helped cDC1s correlates with improved survival, a favorable tumor-infiltrating DC phenotype, increased $CD8^+$ T-cell activation and Th1 differentiation[11,96]. Under certain conditions, cDC2 targeting may also be of interest. In case of immune-complex vaccination, murine tumor cells were

cleared in a CD8$^+$ T cell dependent, but cDC1-independent fashion[17], while ISG$^+$ cDC2s could induce cDC1-independent anti-tumor responses[7]. However, it is clear that cDC1s are the main responders to CD4$^+$ T-cell help, and that helped cDC1s provide superior anti-tumor responses[11]. Additionally, recent analysis confirmed that CD4$^+$ and CD8$^+$ T cells have to directly interact with the same co-presenting DC, and that the antigens for both T cell types should be expressed within the same cancer cells. This study did not specify the precise DC, but it is likely that that these are cDC1s[97].

Like CD8$^+$ T cells, CD4$^+$ T cells can upregulate cytotoxic molecules (referred to as ThCTL)[98], and these ThCTLs can directly kill MHC-II$^+$ cancer cells in mice[99]. Also in human bladder cancer, ThCTLs can kill cancer cells, and their gene signature is correlated to a response to anti-PD-1 therapy[100]. However, given the fact that most cancer cells do not express MHC-II, CD4$^+$ T-cell responses to cancer are mostly indirect. Depletion of CD4$^+$ T cells in several mouse models enhanced tumor growth, even if this also ablated Tregs[101]. As outlined above, CD4$^+$ Th1 cells are critical for proper priming and activation of CD8$^+$ T cells via cDC1s[13,59]. Additionally, IFNγ produced by Th1 cells can activate macrophages which can phagocytose cancer cells and destroy them[102]. Tfh cells can contribute to the formation of tertiary lymphoid structures, which are associated with improved survival and responsiveness to immune checkpoint blockade[103,104]. Additionally, Tfh derived IL-21 can promote CD8$^+$ T cell responses to cancer and viral infections[105], which may be dependent on coexpression of CD4$^+$ T-cell and B-cell epitopes[105].

As outlined above, in several immunization and vaccination settings, CD4$^+$ T cells already form Th1 cells in the SLOs, however, this differentiation process is impaired in suboptimal priming settings. Therefore, local, intratumoral reinforcement of T-cell fate is particularly important in the tumor context[11]. Boosting desired Th1 type CD4$^+$ T-cell differentiation for delivery of CD8$^+$ T cell and cDC1 help may be an attractive strategy in cancer. Additionally, anti-cancer vaccinations directed at CD4$^+$ T cells should result in T-helper differentiation rather than Treg responses. This is important as vaccination can result in Treg expansion[106], and Tregs can be tumor-specific[107]. Understanding how to properly induce CD4$^+$ T-helper differentiation while not activating or inducing Treg responses is a critical goal. Therefore, we need to define which signals CD4$^+$ T cells require to complete their correct differentiation strategy.

Focus in cancer immunotherapy has been primarily on CD8$^+$ T cells that can directly kill cancerous cells and seem to be the main responders to anti-PD-1 therapy[108,109]. However, in cancer and chronic infections, CD8$^+$ T cells often have an incomplete differentiation trajectory and they can become ‘exhausted’[110]. CD8$^+$ T-cell exhaustion is the result of incomplete priming combined with continuous antigen stimulation and is

associated with loss of proliferative capacity, high expression of co-inhibitory receptor expression and epigenetically closed effector loci, effectively rendering them incapable of having an anti-tumor effect[111,112]. It has been demonstrated that exhaustion and productive CTL differentiation is a bifurcation, with a common 'stem-like' $CD8^+$ T cell state as branchpoint[113]. These stem-like $CD8^+$ T cells have expression of PD-1 and TCF-1 and are the main responders to anti-PD-1 therapy[109,114].

Congruent with incomplete effector differentiation of $CD8^+$ T cells in cancer, scRNAseq data from a multitude of cancer types showed that bona fide $CD4^+$ Th1 cells seem mostly absent in human cancer[115]. These data highlighted a Th1/Tfh subpopulation, that had expression of both Th1 and Tfh gene expression, but also had expression of co-inhibitory molecules[115]. Thus, it could be that specific targeting of this population with for example cytokine or costimulatory receptor targeting therapy would allow them to differentiate into bona fide, functional Th1 cells. These Th1 cells could then help intratumoral cDC1s, which has been shown as a critical factor in overall survival and ICB response[11]. To specifically guide $CD4^+$ T cells to become Th1 rather than Tfh cells, it is critical to understand this differentiation trajectory, as well as the costimulatory and cytokine signals required to achieve Th1 differentiation. Furthermore, murine models show that tumor specific $CD4^+$ T cells are activated but seem paralyzed in the LNs[101]. Similarly to $CD8^+$ T cells, $CD4^+$ T cells might get exhausted in the tumor context. This has been more generally established in chronic infections, where 'exhausted' $CD4^+$ T cells are characterized by reduced cytokine production and increased expression of coinhibitory receptors such as PD-1[116]. In the tumor context, similarly 'exhausted' $CD4^+$ T cells are found[112]. However, in contrast to the vast information for the trajectory as well as the genetic program regulating $CD8^+$ T cell exhaustion, $CD4^+$ exhaustion is less well established at the moment[117]. Whether, like $CD8^+$ T cells, this 'exhaustion' is a fixed and irreversible fate remains to be investigated. Furthermore, $CD4^+$ T cell exhaustion may be confounded by a lack of proper definition, which results in terming cells 'exhausted' merely by seeing expression of e.g. PD-1 after RNA-sequencing, while PD-1 is also upregulated rapidly by T cells after activation[118].

Despite these unclarities, $CD4^+$ T cells are evidently of great importance in the response to ICB-based cancer therapy. In mouse melanoma models and human lung cancer, $PD\text{-}1^+$ $CXCL13^+$ $CD4^+$ T cells were found to be in direct communication with antigen presenting DCs, and that these cells were of great importance in the response to anti-PD-1 treatment[119]. In head and neck squamous cell carcinoma, combination therapy of anti-PD-L1 and anti-CTLA-4 increased the proportion of $CD4^+$ T cells with a Th1 phenotype[120], and successful anti-PD-1 treatment in breast cancer expands Th1 and Tfh populations[121]. However, the $CD4^+$ T cells raised in both these studies remained

PD-1 high, indicating that they did not complete effector differentiation. Finally, the importance of CD4$^+$ T-cell engagement is shown by tumor regression of murine pancreatic and colon carcinoma models after vaccination with tumor intrinsic CD4$^+$ T cell epitopes in combination with CD8$^+$ T cell epitopes[122,123]. Thus, understanding how CD4$^+$ T-cell responses to cancer can be unleashed by vaccination or ICB is of great promise.

Scope of the thesis

In this thesis, I investigated the nature and biology of CD4$^+$ T-cell differentiation in *in vivo* mouse models, both after vaccination as well as in tumor models. First, I studied how CD4$^+$ T cells decide to become Th1 and Tfh cells and how these lineage decisions are affected by different external stimuli. Next, I investigated the consequences of this CD4$^+$ T cell effector differentiation for the function of other immune cell types. Specifically, I wanted to understand how CD4$^+$ T cells reshape the myeloid composition in the dLN, as well as how CD4$^+$ T cells optimize the CTL response. Lastly, we studied how ICB affects conventional as well as Treg responses in cancer.

Understanding the differentiation process of CD4$^+$ T cells in vaccination, infection and cancer can provide insight in how to engage CD4$^+$ T cells in anti-cancer responses or vaccination. Additionally, it can provide information on which signals to deliver in combination with ICB to instruct CD4$^+$ T-cell effector responses while dampening Treg responses. Lastly, investigating how CD4$^+$ T cells can shape the fate of both myeloid cells and CD8$^+$ T cells can be informative for rational immunotherapy designs.

In Chapter 2, I used the DNA tattoo vaccination model that our group previously used for in depth study of CD8$^+$ T-cell differentiation to study CD4$^+$ T-cell differentiation. We identified that bifurcation of functional Th1 and Tfh differentiation pathways is preceded by the formation of a bipotent CD4$^+$ T-cell precursor that shares expression of Th1 and Tfh associated molecules, such as T-bet and BCL6. We demonstrated that this precursor is clonally related to mature Th1 and Tfh cells and formed independently of TCR signaling strength. By making use of antibody-dependent depletion of B cells and a genetic mouse model lacking cDC1s, we demonstrate that B cells are required for the formation of Tfh cells, while cDC1s instruct Th1 differentiation. However, neither impacted the generation of precursor cells. Similarly, blockade of the costimulatory ligands ICOSL and CD40L demonstrated their role in differentiation of Tfh and Th1 cells, respectively. By contrast, the generation of precursor cells was dependent on CD28 signaling.

This chapter gives insight in the molecular mechanisms of decision making by the immune system to stimulate the generation of Th1 and Tfh cells, while maintaining flexibility to steer the differentiation towards either path. These insights could be of interest in anti-cancer therapy, where generation of Th1 $CD4^+$ T cells might be favored. On the other hand, increasing the Tfh differentiation trajectory could be a therapeutic target in enhancing vaccine efficacy.

In Chapter 3, we investigated how the engagement of $CD4^+$ T cells affects myeloid APCs. We identified that the DNA vaccine used induces the attraction of monocytes to the dLN, where they locally differentiate to MoDCs. This process was amplified by the activation of $CD4^+$ T cells. Antibody-mediated depletion of monocytes and MoDCs did not impair $CD4^+$ and $CD8^+$ T cell priming, but hampered their differentiation to Th1 cells and CTLs. MoDCs activated in the presence of activated $CD4^+$ T cells displayed higher expression of costimulatory molecules CD40 and CD80 as well as the co-inhibitory molecule PD-L1, both in frequency as well as on a per cell basis. Mechanistically, we demonstrate that $CD4^+$ T cells enhance the MoDC response via CD40L and IFNγ.

These data are relevant given the scarcity of especially cDC1s in the dLN. By enhancing MoDC responses, the immune system provides a platform that can provide additional cytokine production or costimulation in need. Understanding how $CD4^+$ T cells affect myeloid cells, and thereby steer their own differentiation as well as that of $CD8^+$ T cells can aid in the generation of targeted strategies that steer monocytes into differentiation towards pro-inflammatory MoDCs in for example cancer.

$CD4^+$ T cells, and specifically Th1 cells provide 'help' for $CD8^+$ T cells via cDC1s. In Chapter 4, we map the differentiation of $CD8^+$ T cells in priming with or without $CD4^+$ T-cell activation after vaccination. We find that $CD8^+$ T cells primed in absence of $CD4^+$ T cells are 'stuck' in a stem-like state. This state is also found when $CD4^+$ T cells are engaged, however, these cells subsequently further differentiate into CTLs. By means of adoptive transfer experiments, we also show that stem-like cells can progress to CTL differentiation, but only when $CD4^+$ T cell help is provided. In MC38 tumors, despite the presence of endogenous $CD4^+$ T cell epitopes, help does not seem to be delivered, which impairs the differentiation of stem-like cells to effector, and results in T-cell exhaustion due to chronic antigen stimulation. These data argue that for effective tumor clearance by $CD8^+$ T cells, $CD4^+$ effector functions need to be optimized.

To counteract effector functions of conventional $CD4^+$ T cells, the immune system balances overt immune activation via inhibitory mechanisms, such as Treg responses. In Chapter 5, we studied the immune modulatory effects of radiotherapy (RT) as a

therapeutic agent in the lymphocyte-depleted tumor model TC1. We identified that RT acts an immunostimulatory agent that induces $CD8^+$ T-cell priming, but this effect is counteracted by simultaneous Treg priming. Clinically, RT is often combined with immune checkpoint blockade (ICB) against CTLA-4 or PD-1. However, we found that in this lymphocyte-depleted tumor model, ICB exacerbated Treg priming and thereby promoted inhibition of the CTL response. Treg priming depended on CD86 but not CD80 costimulation, and blockade of CD86 improved DC costimulatory status, $CD8^+$ T-cell priming and tumor control after RT. These data may be clinically relevant, since RT negatively impacts on lymphocyte-depleted human cancers and exacerbation of Treg responses after anti-PD-1 ICB has been observed.

Importantly, these findings highlight the double-edged sword of $CD4^+$ T-cell responses, which I further discuss in Chapter 6, considering the current literature.

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