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

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Chapter 6

General Discussion

General discussion

The importance of immune protection against pathogens has been known for centuries. Written reports on variolation (i.e. knowingly infecting people with smallpox to induce milder disease) date back to 16th century China[1], and in 450 BC Thucydides noted that during the Peloponnesian war, the plague in Athens 'never attacked the same man twice'[2], both indicating a sense of long-lasting immune memory towards pathogens. Edward Jenner is considered the inventor of vaccination, by performing variolation with cowpox rather than smallpox which increased the safety with the same effectiveness[3]. Since then, major advancements have been made in understanding how the immune system recognizes non-self, while maintaining tolerance to itself. Recently, focus has been on rapid development of vaccines during pandemics, such as the COVID-19 pandemic, and activating the immune system in cancer, as exemplified by the success of immune checkpoint blockade (ICB). In this thesis, I have focused on the role of $CD4^+$ T cells in response to vaccination as well as cancer in mice, as a model system for human immunity. $CD4^+$ T cells can differentiate into a plethora of different cell states with unique helper properties, making them of great importance in battling wide varieties of pathogens. Understanding which factors influence $CD4^+$ T cell differentiation trajectories can therefore help to design and optimize future therapeutics and preventive and therapeutic vaccination.

Summary

In Chapter 2, we identified that $CD4^+$ T cell bifurcation is a temporal process in which $CD4^+$ T cells acquire an activated non-committed Th1/Tfh precursor state prior to their commitment to Th1 or Tfh lineages. This state is dependent on CD28 signaling and is characterized by coexpression of Th1 and Tfh markers, such as CXCR3 and CXCR5. To further commit to Th1 differentiation, these cells are dependent on cDC1s as well as on CD40-CD40L interactions. By contrast, Tfh cells depend on B cells, as well as on ICOS-ICOSL interactions. The results from this Chapter allow for a better understanding in the decision-making process of $CD4^+$ T cells to differentiate to terminal effector cells and can help us develop therapeutic strategies to steer $CD4^+$ T cell responses in desired directions.

In Chapter 3, we further investigated how myeloid cells respond to $CD4^+$ T cells by using a vaccination setting in which $CD4^+$ T cells are either engaged or not. After vaccination, monocytes were recruited to the draining lymph node (dLN) where they differentiated locally into monocyte-derived DCs (MoDC), which was amplified by activation of $CD4^+$ T cells. In turn, depletion of MoDCs hampered $CD4^+$ Th1 and $CD8^+$ T-cell effector differentiation. Mechanistically, IFNγ and CD40L were required for amplification of the

MoDC response by CD4+ T cells. This Chapter highlights a feed-forward loop by CD4+ T cells and MoDCs that is essential for optimal T-cell effector differentiation.

It has been well established that CD4+ T-cell help is required for optimal CD8+ T-cell effector differentiation. In Chapter 4, we demonstrate that stem-like CD8+ T cells, which lie at the branchpoint of either effector- or exhaustion trajectories, are formed in absence of CD4+ T-cell activation, but that subsequent effector differentiation is dependent on CD4+ T-cell engagement. These findings argue that for optimal CD8+ T cell-targeted immunotherapy, CD4+ T-cell help should also be provided. Nevertheless, we also demonstrate in the context of an inflammatory tumor that the mere presence of CD4+ T-cell epitopes and CD4+ T-cell activation does not ensure improved CD8+ T -cell effector differentiation.

Some tumors do not elicit a T-cell response, despite carrying immunogenic antigens. For a long time, it was believed that radiotherapy (RT) could invite a T-cell response by inducing immunogenic cancer cell death. In Chapter 5, we demonstrate that in a 'cold', immune-depleted but antigenic tumor, RT indeed can elicit a CD8+ T-cell response. However, RT simultaneously induces a thymus derived-regulatory T cell (tTreg) response, which counteracts the CD8+ T-cell response. This tTreg response is enhanced when mice are treated with anti-PD-1 or anti-CTLA-4 blocking antibodies and depends on CD28 costimulation via CD86 but not CD80. So, in this context, ICB is unfavorable rather than favorable for tumor control. These findings underscore that fully understanding the tumor microenvironment (TME) and the immune modulating effect of conventional therapies is of great importance to advance ICB, and to rationally design treatment options for 'cold' tumors.

CD4+ T-cell priming as a multistep program

Intravital imaging has demonstrated that CD8+ T cells undergo 'two-step' priming[4–6]. During the first step, they get activated separately from CD4+ T cells, before they come together on the same (lymph-node resident) cDC1[5,6]. In this second step, CD4+ T cell help is delivered, which is mediated via direct contact with the cDC1, as well as cytokine production that activates the DC and IL-2 production for CD8+ T cells[4,7–10]. This data argues that the stem-like, helpless cells we describe in Chapter 4 have likely only undergone the first step of priming. Importantly, in this study adoptive transfer experiments demonstrated that these 'first-step primed' or stem-like cells can still receive and respond to help.

For CD4+ T cells, their activation and differentiation fate are often separated in time. The diversity of CD4+ T-cell differentiation trajectories has likely complicated rather than simplified our view of CD4+ T-cell priming. Key questions in the field revolve around which signals CD4+ T cells are receiving and how these optimize T-helper differentiation. In this light, several recent reviews highlight that it is mainly the interacting APC that decides T-cell differentiation fate[11,12]. For example, it has been postulated that cDC2s induce Th2 responses, and B cells induce Tfh responses. Alternatively, models exist where one or two dedicated APCs induce multiple CD4+ T-cell responses, depending on the pathogen[13]. However, this view focuses on a binary 'yes or no' fate decision, leading to for example either Th1 versus Th2 differentiation, or Th1 versus Tfh differentiation, whereas multiple immune responses are raised simultaneously, including Th1 and Tfh cells[14]. These thoughts become even more challenged when considering that humans, rather than specific pathogen-free (SPF) mice are continuously challenged by diverse (opportunistic) pathogens that require different immunological defenses. Moreover, in contrast to SPF mice, the vast majority of the human population is challenged by chronic viral infections, such as Herpes Simplex Virus (HSV) 1 or -2 and Epstein-Barr virus (EBV) that have an estimated prevalence of 67% and >90% in adults worldwide, respectively[15,16]. In line with this, it is of no surprise that compared to farm-housed or pet-shop mice, the immune system of SPF mice is more comparable to newborn, rather than adult humans[17]. Thus, it seems likely that several APCs are working together to optimize the right outcome for antigen-specific T cells.

Our findings in Chapter 2 and Chapter 3 highlight that in contrast to the 'one-APC-does-all' strategy, CD4+ T-cell differentiation depends on multiple interaction partners and costimulatory molecules. By combining temporal spectral flow cytometry analysis in combination with scRNA-seq and TCR-seq, we demonstrated that endogenous, polyclonal CD4+ T cells differentiate into an activated, 'uncommitted' Th1/Tfh precursor state prior to bifurcation into Th1 and Tfh cells. Generation of the uncommitted Th1/Tfh precursor pool depended on CD28 signaling delivered by either CD80 or CD86, and it was formed independently of cDC1s and B cells, as demonstrated with genetic KO models and antibody-based interventions. These activated CD4+ T cells shared expression of the Th1 and Tfh master transcription factors T-bet and BCL6, as well as Th1- and Tfh-associated chemokine receptors CXCR3 and CXCR5. It is likely that this initial Th1/Tfh precursor state is dependent on cDC2s and combined TCR-CD28 input. However, formal testing in a model that lacks cDC2, like ZEB2 Δ1Δ2Δ3 mice will be required to confirm this[18]. Another possibility could be that multiple APC subsets can initiate the Th1/Tfh precursor state, and that depletion of only one subset such as cDC1s does not capture this. Lastly, it seems likely that amount and nature of the antigen shape the dependency on specific APC types. For Tfh differentiation, B

cells may also initiate differentiation if antigen levels are high enough, and often the antigen doses used in vaccination or infection studies in mice are supra-natural[19]. The trafficking of the antigen affects the dependency on APCs too; higher load of antigen will increase its passive drainage to the dLN, overruling dependence on migratory cDCs. Within the LN, resident cDC1s and cDC2s occupy different sites, with cDC2s having more access to draining antigen, thereby lowering the dose needed for $CD4^+$ T-cell activation[20,21]. For our vaccine however, fluorescent protein-antigen tracing demonstrated dependency on migratory DCs for antigen trafficking and initial activation, as resident DCs and B cells were antigen-negative[22].

The CXCR3 ligands CXCL9 and CXCL10 are expressed in the interfollicular zone (IFR) of the dLN, while the ligand for CXCR5, CXCL13, is expressed in B cell follicles[23,24]. CXCR3 expression is critical for formation of Th1 cells, while CXCR5 is required to form Tfh cells[23,25], which seems to be associated with reduced CCR7 expression[25,26]. Mechanistically, dynamic changes in expression of these chemokine receptors likely allow activated $CD4^+$ T cells to move from the CCR7-ligand dominated T-cell zone to the T-B cell border or IFR, where they can interact with a secondary interaction partners such as B cells or cDC1s. It is tempting to speculate that coexpression of CXCR3 and CXCR5 by Th1/Tfh precursors allows for stochastic movements to either cDC1 or B cells and thus stochastic decisions between Th1 and Tfh fate. This would explain why Th1 and Tfh cells have such a high degree of TCR sharing as described in Chapter 2. Such stochasticity could optimize pathogen-specific decision making, depending on for example cytokine expression and timing[27]. However, to formally conclude this, intravital imaging strategies following $CD4^+$ T-cell activation and migration will be required. This is technically challenging, as for intravital imaging often B cells are deleted to visualize the IFR, and multiple fluorescent tags are required.

Monocyte-derived cells such as MoDCs described in Chapter 3, further reinforce Th1 fate. Monocyte-derived cells can produce CXCR3 chemokines CXCL9 and 10 and can be present in the IFRs, as well as in the T cell zones[28,29]. As can be observed when comparing MoDC deletion by anti-CCR2 treatment (Chapter 3) with genetic deletion of cDC1s in *Batf3*$^{-/-}$ mice (Chapter 2), lack of cDC1s had a more drastic effect on Th1 differentiation. Since MoDC amplification was dependent on Th1 factors CD40L and IFNγ, at least some Th1 differentiation must take place prior to MoDC-dependent enforcement of Th1 fate. Given the scarcity of cDC1s, MoDCs may primarily function to expand the pool of interaction partners for T cells or increase the amount of cytokine producing cells after initial lineage commitment. Indeed, MoDC function is dependent on IL-12 production, and although MoDCs were poor in producing IL-12 compared to cDC1s frequency-wise, the amount of IL-12-producing MoDCs vastly outnumbered

cDC1s[29–31]. Furthermore, microscopic analyses demonstrated that MoDCs were present in T-cell zones where CD4⁺ and CD8⁺ T cells were in an activated T-bet⁺TCF-1⁻ state, arguing that they are enforcing differentiation of already activated T cells[29].

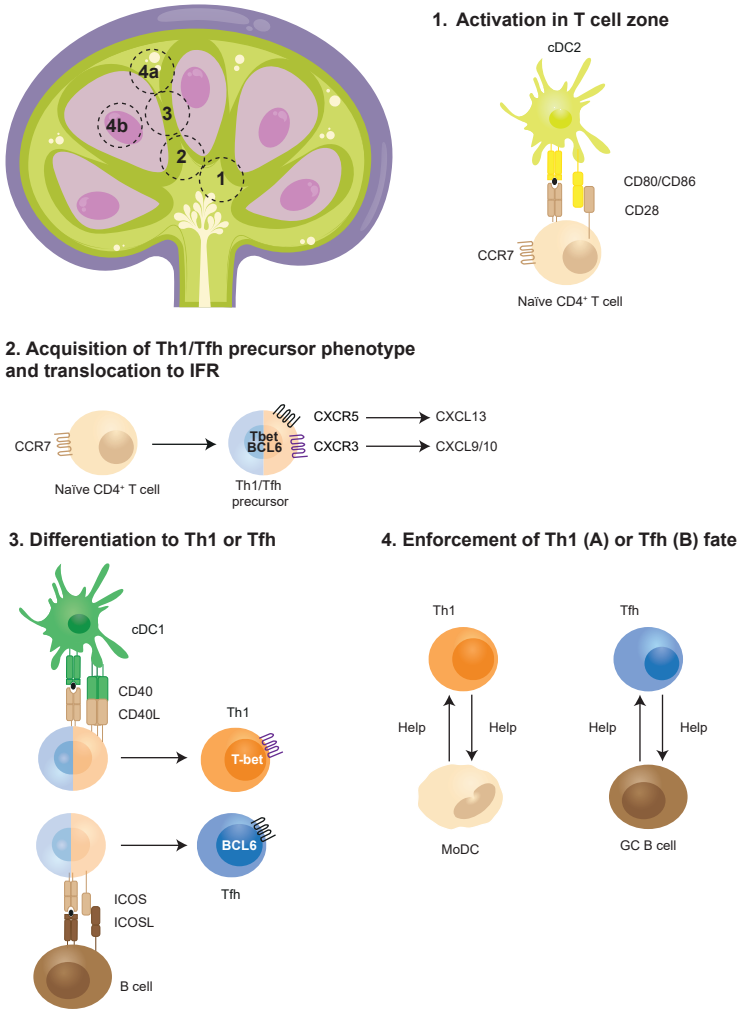

Figure 1. CD4⁺ T cell differentiation is a multistep program.
Model depicting the different steps and locations of CD4⁺ T cell activation and differentiation in the dLN (top left). **1)** Naïve CD4⁺ T cells are first activated by cDC2s, in a TCR- and CD28 signaling-dependent manner**. 2)** As a result of CD4⁺ T cells start acquiring the Th1/Tfh phenotype. Due to CXCR3 and CXCR5 expression and CCR7 downregulation, Th1/Tfh precursor cells move to the IFR. **3)** In the IFR, CD4⁺ T cells encounter B cells and cDC1, and as a result of CD40/CD40L and ICOS/ICOSL input, they differentiate to Th1 or Tfh respectively. **4)** Within the IFR (for Th1 cells) or the GC (for Tfh cells), Th1 and Tfh fate is further enforced by MoDCs and GC B cells, which form a feedforward loop with CD4⁺ T cells.
Lymph node vector was obtained from Adobe Stock.

Tfh cells also undergo stepwise differentiation, in which intermediate states are formed prior to full GC-Tfh differentiation[32,33]. Tfh cells primarily depend on B cells for their fate reinforcement and require continuous input from B cells to enforce their GC-Tfh program[24]. It has been described that after activation, $CD4^+$ T cells adopt a 'pre-Tfh' phenotype, and move to the IFR and B-cell border where they must interact with B cells to form Tfh cells[34]. Interestingly, not all $CD4^+$ T cells maintain this 'pre-Tfh' phenotype, as they lose CXCR5 and PD-1 expression, while others form $CXCR5^+$ $PD\text{-}1^+$ Tfh cells[24,34]. Recently, TIGIT expression has been proposed as a marker of GC-Tfh precursors, while $TIGIT^-$ cells were memory precursors[32]. An IL-21-fate-mapping mouse model highlighted a Tfh-progenitor of the GC-Tfh differentiation program, which depended on both intrinsic transcription factor expression and extrinsic regulation by T-follicular regulatory (Tfr) cells[33]. Thus, both Th1 and Tfh differentiation depend on multiple steps, which are regulated by interaction with APCs, the nature of the pathogen, costimulatory status, the cytokine milieu, and other external regulation.

Overall, our results in Chapters 2 and 3 argue that antigen activated $CD4^+$ T cells, like $CD8^+$ T cells[4], undergo a multistep differentiation path (**Figure 1)**; 1) activation by an APC, likely a cDC2; 2) acquisition of a Th1/Tfh precursor phenotype and migration to the T-B border or IFR; 3) interactions with B cells or cDC1 to differentiate to Tfh or Th1 lineages; 4) continued interactions with B cells to enforce GC-Tfh program or interactions with MoDCs to enforce the Th1 program.

The formation and function of precursor $CD4^+$ T cells in infection and disease

Precursor $CD4^+$ T cells that share expression of Th1 and Tfh genes have been described previously. Here, I will place the findings of our Th1/Tfh precursors in context of other findings and speculate on their role in infection and disease. After *in vitro* activation of murine $CD4^+$ T cells, transient co-expression of T-bet and BCL6 was noted. In this setting, IL-12 surprisingly resulted in upregulation of BCL6 to a greater extent than IL-6, and at the same time also induced IL-21 production by $CD4^+$ T cells[35]. T-bet expression repressed this 'Tfh-like' transition and resulted in physical interaction between T-bet and BCL6. Interaction with T-bet inhibits BCL6-mediated repression of BLIMP-1, which downregulates the Tfh cell program. It has been challenging to generate Tfh cells *in vitro*, but recently such a model has been generated, in which Tfh generation depended on TGF-β[36]. In this system, Tfh and Th17 shared similar cytokine requirements, with the difference that IL-21 was added to the Tfh cultures. Also, in these Tfh cultures, a hybrid, $IL17^+CXCR5^+$ population was observed, and scRNA-seq demonstrated a bifurcation of *in vitro* generated cells in Tfh-conditions, splitting from a common starting point to either a

Tfh or a Th17-like branch. Both *in vitro* and *in vivo*, the transition of precursors to Tfh cells may depend on c-Maf[32,36]. Future research could be aimed at studying precursor cells in either Th1- or Tfh steering conditions, but both cited studies already indicate that bipotent precursors can be formed in *in vitro* cultures.

Perhaps more interesting is the formation of T-helper – Tfh intermediates or precursors in *in vivo* settings. A study focusing on IL-17-expressing CD4$^+$ T cells in steady-state as well as in experimental autoimmune encephalomyelitis (EAE), modelling multiple sclerosis in mice, identified a SLAMF6$^+$ population in spleen that had TCR sharing with effector Th17 cells in colon. TCR sharing identified high overlap of SLAMF6$^+$ precursors, Th17 effector cells and Tfh-like Th17 cells in colon. Additionally, upon adoptive transfer these SLAMF6$^+$ precursors gave rise to effector cells in EAE, where Th17 effector cells also expressed key Th1 associated genes, such as *Tbx21* (encoding T-bet), *IFNγ* and *Bhlhe40*[37]. This study does not formally prove that the precursor subset differentiated to both Tfh and Th17 cells, but TCR sharing between the populations, as well as the fact that the precursors differentiated to Th17 effector cells implies that they may be bipotent for Th17 and Tfh cells. The first description of a bipotent precursor for both Th1 and Tfh cells came from experiments with adoptive transfer of *Plasmodium chabaudi*-specific PbTII CD4$^+$ T cells, combined with scRNA-seq after different days post immunization (day 1, 2, 4 and 7)[38]. Computational analyses revealed that prior to day 7, Th1 or Tfh fate could not be discerned. Additionally, combined endogenous TCR-seq of PbTII cells revealed clonal relationship between Th1 and Tfh cells, arguing that they shared common precursors. It must be said that the power of this finding was limited, since it was based on very few clones and on TCR-transgenic CD4$^+$ T cells. Computationally, it was predicted that prior to bifurcation, set at a pseudotime where Th1 and Tfh fate could be discerned, precursor CD4$^+$ T cells coexpressed CXCR3 and CXCR5. In another study, a 'memory-like' cell precursor was formed during lymphocytic choriomeningitis virus (LCMV) infection, that was clonally related to Th1 and Tfh cells as determined by TCR-sequencing[39]. The precursor was marked by expression of TCF-1, PD-1 and SLAMF6 and depended on BCL6 expression. In contrast to the study by Lonnberg *et al.*[38], total tetramer positive cells were assessed, arguing that this differentiation stage is part of a common trajectory. However, adoptive transfer experiments showed only limited capability of these precursors to differentiate into Th1 and Tfh cells and in fact, the frequency Th1 cells was higher upon adoptive transfer of Tfh cells[39]. This may be explained by a loss of BCL6 expression in Tfh cells, which allows transdifferentiation to Th1 cells[40]. This differentiation fate was determined 21 days after chronic LCMV infection, which may have had this specific impact. We noted significant overlap between the gene signature of our Th1/Tfh precursor with that of the early stage LCMV-specific CD4$^+$ T cell population that shared TCR-expression with both Th1 and Tfh cells in both acute

LCMV-Armstrong and chronic LCMV-Clone 13 infection[39]. An IL-21 fate mapping system revealed a Tfh precursor cell state expressing CXCR5 but not IL-21, which had a greater expansion capacity than Tfh cells and further differentiated into Tfh cells[33]. However, its potential to differentiate to other T-helper subsets was not assessed.

Stem-like $CD8^+$ T cells found in cancer as well as chronic and acute infection have gained much interest as they seem to be the main responders to anti-PD-1 therapy in cancers[41]. Similarly, 'stem-like' $CD4^+$ T cells, precursors for Th1 cells, have been identified in alloreactive transplantation rejection as well as cancer[42,43]. In line with our findings on Th1/Tfh precursors, tumor-derived stem-like $CD4^+$ T cells could differentiate to $BCL6^+$ cells *in vitro* under specific culture conditions and in the context of implanted tumors, deletion of Tregs was sufficient to induce 'stem-like' to Th1 differentiation[42]. This result argues that 'stem-like' $CD4^+$ T cells isolated in these conditions might be multipotent and can differentiate into both Th1 and Tfh cells, if the conditions *in vivo* are appropriate[44]. In specific tumor models where $CD4^+$ T cells contribute to tumor control, such as MC38, tumor-specific $CD4^+$ T cells are paralyzed in proliferation and differentiation, and adopt a 'Tfh'- like state, that however does lack key molecules like CXCR5[45]. Combined CTLA-4 blockade with Treg depletion allowed the generation of $IL\text{-}2^+IFN\gamma^+$ $CD4^+$ T cells in these settings. Thus, it is possible that this 'Tfh'-like state, actually represents a precursor for both Th1 and Tfh cells.

In line with our studies on the role of Tregs in restraining $CD8^+$ T-cell responses after RT (Chapter 5), it was identified that tumor-specific $CD4^+$ T-cell differentiation and proliferation are simultaneously inhibited by Tregs, as well as CTLA-4[42,45]. One potential mechanism could revolve around IL-2 availability. Tregs have high expression of the IL-2Rα chain CD25[46], and IL-2 has been described as a central cytokine to steer $CD4^+$ T-cell differentiation to the Th1[47]. However, the findings on IL-2 are not corroborated in all models, as CD25 upregulation is noted in LCMV but not influenza infection[48]. Similarly, in our model we did not find any CD25 upregulation on antigen-specific $CD4^+$ T cells after vaccination (data not shown).

Consistent with a memory and stem-like phenotype found in murine studies, circulating $CD62L^+$ $CCR7^+$ $CXCR3^+$ $CXCR5^+$ $PD\text{-}1^{lo}$ cells have been identified in humans[49]. These cells were associated with improved neutralizing antibody responses in HIV controllers, and with increased anti-influenza antibody responses[50–52]. Based on these findings, they were suggested to be 'circulatory memory Tfh' cells, but it seems likely that these cells are a circulating memory counterpart of precursors found in our studies and in those described above. Interestingly, apart from $CXCR3^+T\text{-}bet^+$ Th1-like circulatory Tfh cells, also circulatory Tfh cells with GATA3 or RORγt expression were found, suggesting

that precursor cells are formed in multiple types of immune responses[49]. However, whether these human 'circulatory Tfh' cells can differentiate into mature Th1, Th2 or Th17 cells remains to be determined.

In mouse models of pancreatic autoimmunity, continuous influx of TCF-1^{hi}CD62L^{+} CD4^{+} T cells was required to maintain immune responses to pancreatic beta cells[53]. Although not specifically studied, within the pancreas both T-bet^{hi}TCF-1^{lo} and TOX^{hi}TCF-1^{hi} effector cells are formed. These TOX^{hi} cells were described as exhausted, but it seems more likely that these cells have a Tfh phenotype, as they also displayed open chromatin at the IL-21 locus. In a pan-cancer analysis of 21 tumor types, a prominent potentially tumor specific Tfh/Th1 population was found, with a variety of Tfh and Th1 genes[54]. It is tempting to speculate that these cells are similar to the Th1/Tfh precursor cells found in our studies, and that they are lacking specific signals to differentiate towards full effector Th1 cells. Similarly to CD8^{+} T cells not forming proper effector cells (Chapter 4) in cancer, it may be that critical activation signals are lacking in the TME or in dLNs. Indeed, we show that merely the addition of tumor-specific CD4^{+} T cell epitopes and priming of CD4^{+} T cells does not induce helped effector CD8^{+} T cells. These data indicate that activation of CD4^{+} T cells is not sufficient to relay CD4^{+} T cell help. It is likely that both the CD4^{+} and the CD8^{+} T cells primed in these settings have not received the input to differentiate to effector Th1 or cytotoxic T lymphocyte (CTL). A specific target to improve this is the cDC1, which is the platform for Th1 differentiation and for help-delivery[7]. However, for the cDC1 to provide help to CD8^{+} T cells and to enforce Th1 differentiation, initial CD4^{+} T cell activation must already occur. As cDC2s are relatively poor at presenting cell-associated antigen, it may be that the CD4^{+} T cells are not fully activated to begin with. Alternatively, it could be that activated CD4^{+} T cells do not properly interact with cDC1s, thereby preventing help delivery and Th1 differentiation, as we noted in MC38-HELP (Chapter 4), thereby keeping part of the cells in a less differentiated state. Thus, in various settings of infection, autoimmunity and cancer, cells with similar characteristics as the Th1/Tfh precursors identified in Chapter 2 are found, and it will be critical to understand how to optimize or inhibit their effector differentiation trajectories to rationally optimize treatment design.

Analogies between CD8^{+} T cell and CD4^{+} T cell differentiation trajectories

It has been well established that CD8^{+} T cells during type 1 inflammation can differentiate into two directions: a productive CTL response, or a dysfunctional exhausted response. For this exhausted response to take place however, chronic antigen stimulation in form of cancer or chronic viral infection or autoimmunity

must be present[55]. Both CTL and exhaustion trajectories pass through a cellular differentiation state that is referred to as stem-like, progenitor exhausted or pre-dysfunctional[56,57]. Significant focus has been on this population, as stem-like $CD8^+$ T cells are the main responders to anti-PD-1 therapy[41]. Additionally, as they are at a branchpoint of effector or exhaustion differentiation trajectories, it is of great importance to understand the factors deciding their differentiation fate. During stem-like to exhausted differentiation, $CD8^+$ T cells become impaired in cytokine production, cytotoxicity, and proliferative capacity, which is accompanied by epigenetic closing of effector genes, and upregulation of co-inhibitory molecules[55].

In Chapter 4, we demonstrate that for stem-like cells to progress to the CTL effector state, they require $CD4^+$ T-cell help. Initially, $CD4^+$ and $CD8^+$ T cells are activated separately, after which they come together on the same cDC1, where $CD4^+$ T cell help is delivered[4-6] (**Figure 2)**. Molecularly, it has been shown that $CD4^+$ T cell help is mediated via CD40-CD40L interactions with cDC1 and IFNβ production by $CD4^+$ T cells, which together among other factors results in upregulation of costimulatory molecules, MHC-I-associated molecules, and improved survival[8,10]. Furthermore, $CD4^+$ T-cell produced IL-2 is critical for effector differentiation in viral infections[9]. We show that Th1/Tfh precursors with a stem-like phenotype similarly depend on cDC1 and CD40 signaling (Chapter 2), and that like Th1/Tfh precursors stem-like $CD8^+$ T cells express CXCR3 (Chapter 4) and CXCR5[56]. Thus, $CD4^+$ stem-like to Th1 differentiation fate and $CD8^+$ stem-like to CTL differentiation seems to be linked. Additionally, like for Th1 differentiation, MoDCs were important in further enforcing CTL differentiation (Chapter 3), which likely occurs in the IFR (**Figure 2**)[28,29].

Beyond their role in anti-PD-1 therapy, stem-like $CD8^+$ T cells are also critical for maintaining continuous immune responses. In cancer it has been observed that tumor-specific $CD8^+$ T cells first form stem-like $CD8^+$ T cells in the dLN that upon migration to the tumor site acquire effector functions[58]. As T-cell effectors are short lived, it is logical to reason that under conditions of chronic antigen stimulation, stem-like cells form the reservoir from which effector responses are replenished. Indeed, adoptive transfer of as few as 20 stem-like autoimmune $CD8^+$ T cells was sufficient to induce type 1 diabetes in mouse models, while 100,000 effector cells did not[59]. This is similar to adoptive transfer of stem-like $CD4^+$ T cells versus effector Th1 cells in alloreactive transplant studies[60]. The local costimulatory status of APCs is crucial to allow for stem-like to effector differentiation[58], where specifically cDC1s are crucial in inducing durable effector responses by relaying $CD4^+$ T-cell help[7], which results in the hallmarks of successful CTL differentiation[61]. This enforcement of effector fate can occur in the dLN as our data (Chapter 4) clearly demonstrates, but also in peripheral tissues. For $CD4^+$ T

cells, similar observations have been made; in our acute vaccination model, but also in other models[23], Th1 cells can be formed in the dLN already, while in others, activated CD4⁺ T cells must travel to the site of infection for cDC1 dependent Th1 differentiation in *Cryptosporidium* infection, or for CD4⁺ T cell dependent tumor rejection[62,63].

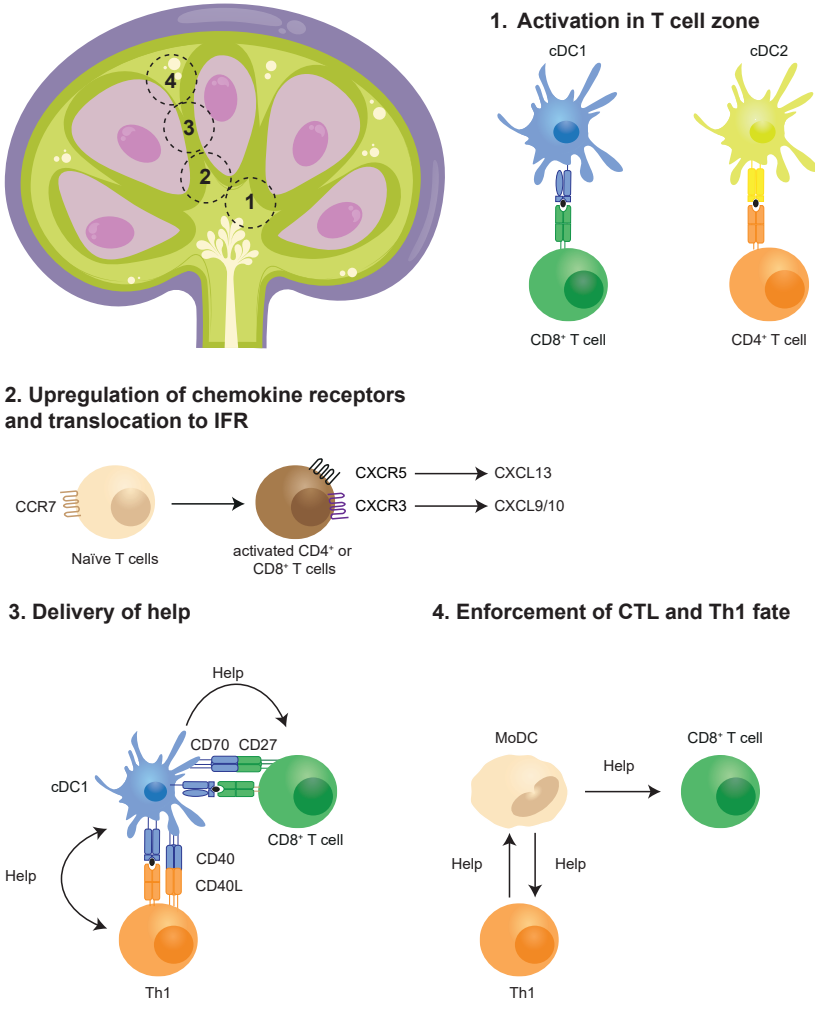

Figure 2. Analogies between CD8⁺ and CD4⁺ T cell differentiation and the delivery of help.
Model depicting the different steps and locations of CD4⁺ and CD8⁺ T cell activation and differentiation in the dLN (top left). **1)** Naïve CD4⁺ and CD8⁺ T cells are activated separately. CD8⁺ T cells are primed by cDC1s, while CD4⁺ T cells are activated by cDC2s. **2)** As a result T cells start upregulating CXCR3 and CXCR5. Due to CXCR3 and CXCR5 expression and CCR7 downregulation, activated CD4⁺ and CD8⁺ T cells move to the IFR or T-B border. **3)** In the IFR, CD4⁺ T cells provide help to cDC1, in part via CD40/CD40L. This help is relayed and results in differentiation of CD8⁺ T cells to CTL effector and effector memory cells, while also ensuring Th1 differentiation. **4)** Within the IFR, MoDCs and CD4⁺ T cells engage in a feedforward loop that enhances MoDC function and enforces Th1 and CTL fate.

Similar to stem-like Th1/Tfh precursors, stem-like CD8$^+$ T cells retain expression of TCF-1, upregulate SLAMF6 and express PD-1. We and others have noted that similar to Tfh cells, stem-like to exhaustion trajectories are characterized by similar transcriptional requirements and protein expression[64,65], such as increased expression of molecules like CXCR5, PD-1, SLAMF6, TCF-1, TOX and ID3[66,67], and a similar epigenetic transition post PD-1 blockade[65]. Expression of CXCR5 in combination with CXCR3 may result in CD8$^+$ T cell translocation from the T cell zone to the T-B border and IFR in the dLN. Indeed, during chronic LCMV infection and in HIV infected individuals stem-like CD8$^+$ T cells were found in B cell follicles[68,69]. Additionally, EBV specific-CXCR5$^+$ CD8$^+$ T cells were found in human tonsils, and stem-like CD8$^+$ T cells were capable of restriction of virus-infected Tfh cells and B cells in mice[69]. Thus, stem-like CD8$^+$ T cells can have a similar localization to Tfh cells in the B cell follicle. Additionally, both stem-like to exhausted CD8$^+$ T cells and Tfh cells critically depend on the expression of the transcription factors TOX and TOX2 for their function[70–72], as these transcription factors drive expression of key transcription factors such as BCL6[73] as well as the expression of PD-1[74]. TOX is induced by NFAT signaling and chronic TCR-stimulation[74,75], and ectopic expression of TOX was sufficient to drive a global T exhaustion program in CD8$^+$ T cells[74]. In parallel, chronic LCMV infection shifts CD4$^+$ T-cell differentiation to the Tfh direction, and adoptively transferred antigen-specific CXCR5$^-$ SMARTA cells differentiated to Tfh cells in chronic LCMV infection, which depended on continuous TCR signaling[76]. Although underexplored, it is plausible that continuous TCR triggering in CD4$^+$ T cells also induces high TOX and TOX2 expression, making this a likely mechanism that connects CD4$^+$ Tfh and CD8$^+$ exhaustion trajectories.

In addition to the variety in T-helper responses during infection, Tregs can also adopt an inflammation specific phenotype, e.g., by forming Tfr cells or by expressing T-bet, mirroring Th1 and CTL development[77,78]. Although it has been suggested that exhaustion trajectories are formed to prevent autoimmunity or excessive tissue damage in chronic infections[55], they may have a different origin. For example, chemokine receptor expression is dependent on specific transcription factors, such as CXCR3 on T-bet and CXCR5 on BCL6[79–81]. Thus, for correct homing of CD8$^+$ and CD4$^+$ Tconv cells and Treg cells towards specific chemokine cues, such as CXCL9, 10 and 11 in inflamed tissue, these cells must rely on similar transcription factor expression. Perhaps stem-like CD8$^+$ T-cell differentiation trajectories have developed in evolution due to co-existence and co-evolution with chronic viral infections that target Tfh cells or B cells, such as EBV and associated ancestors[82], which establishes life-long infection[16]. This would allow constraint of viral reactivation in B-cell follicles as was demonstrated before[69]. As high antigen load and chronic antigen stimulation also increases the proportion of Tfh cells and since TOX expression is linked to chronic

TCR triggering in $CD8^+$ T cells and CAR T cells[74–76], this could potentially explain why Tfh cells dominate in such settings. It was recently demonstrated that autoreactive $CD4^+$ T cells, which are continuously stimulated, adopt a Tfh-like phenotype and move to B-cell follicles[83]. Perhaps, high, continuous antigen loads require stronger B-cell responses, as a stronger $CD8^+$ T-cell response would cause too much cellular damage. However, such hypotheses are difficult to test as these are evolutionary traits that likely developed over millions of years.

Whether as for $CD8^+$ T cells, $CD4^+$ T-cell exhaustion truly exists remains understudied. $CD8^+$ T-cell exhaustion is observed upon chronic antigen stimulation and is typically considered to include the following hallmarks: poor proliferative capacity, altered transcriptomic, epigenetic and metabolic prolife, high co-inhibitory molecule expression and impaired effector function and cytokine production[55]. Although impaired cytokine production and proliferative potential of $CD4^+$ T cells in cancer have been documented, this may very well be linked to: 1) anergy due to insufficient APC activation; 2) Treg conversion; 3) Treg-mediated suppression. Indeed, in multiple mouse models paralyzed, improperly primed $CD4^+$ T cells became functional after Treg inhibition or blockade[42,45,84]. Provision of additional CpG in tumor models increased CD86 expression by both cDC1 and cDC2, but this did not improve $CD4^+$ T-cell responses[84]. This may be linked to a preferential expansion of tTregs by CD86, as we have described (Chapter 5). We also observed a poor expansion of PADRE-specific $CD4^+$ T cells in MC38 transduced with the Help cassette (Chapter 4), while the PADRE epitope elicited strong $CD4^+$ T-cell responses in vaccination (Chapter 2, 3). Additionally, $CD4^+$ T-cell exhaustion is sometimes confused with Tfh differentiation, as merely expression of TOX and PD-1 is seen as 'exhaustion' or 'functional exhausted cells' by direct extrapolation of $CD8^+$ T-cell phenotypes[53,85]. As Tfh cells are typically determined based on gating on e.g. CXCR5 and PD-1, potential loss of such markers due to specific cues may limit detection of Tfh-like cells. To define Tfh cells, it should be determined whether they are capable of B-cell help. For example, it was reported that in aged mice, anti-PD-1 and anti-PD-L1 therapy induced immune related adverse effects, which was dependent on B cells, $ICOS^+TOX^+MAF^+PD\text{-}1^+CXCR5^-$ $CD4^+$ T cells, ICOS signaling and IL-21. All these data point to Tfh-like differentiation, but this was excluded by the authors as '$CD4^+$ T cells did not upregulate the Tfh marker CXCR5 in bronchoalveolar lavage fluid upon anti-PD-1'[86]. Lastly, sometimes 'exhaustion' has been reported based on a reduced, but not absent cytokine production or proliferative capacity in a subset of $CD4^+$ T cells, e.g. after adoptive transfer of antigen-specific T cells in cancer[87]. Thus, for formal conclusions on exhaustion of $CD4^+$ T-cell responses in cancer or chronic infection, it is crucial to: 1) exclude Tfh fate; 2) assess whether and how potential precursors can be saved from anergy; 3) assess whether there is full and

maintained loss of function. Additionally, future studies should be aimed at uncovering a terminal fate such as with epigenetic remodeling and closing of effector loci[88], rather than altered cytokine production or upregulation of co-inhibitory molecules[76,89].

Optimizing CD4$^+$ and CD8$^+$ T-cell responses to cancer

Harnessing functional effector CD4$^+$ T cells in cancer is of great promise. CD4$^+$ T-cell help for CD8$^+$ T cells via cDC1 in immunogenic mouse models results in tumor clearance[90,91], and a helped cDC1 state correlates with prolonged overall survival, checkpoint inhibition response and effector tumor infiltrating T cells in human cancer[7]. IFNγ and TNFα can induce cancer cell senescence or death in MHC-dependent and independent mechanisms, and IFNγ results in upregulation of MHC-I and MHC-II on tumor cells as well as chemokines such as CXCL9[92–94]. Additionally, CD4$^+$ T cell derived IL-21 increases CD8$^+$ T cell functionality[95,96]. With the successes of ICB, it becomes crucial to understand how CD4$^+$ T-cell responses are impacted.

Immune checkpoint blockade signaling pathways

Most research on checkpoint inhibition has been on targeting PD-1, which is primarily linked to expansion of stem-like CD8$^+$ T cells[41]. However, despite clinical success, most patients do not show complete responses, and patients may experience pathogenic immune related adverse events[97]. Upon ligation with PD-L1 or PD-L2, PD-1 inhibits CD28 signaling via SHP-2[98–100], thereby dampening TCR signaling in T cells. Unlike PD-L2, which is only expressed by limited cell types, PD-L1 can be expressed by multiple cell subsets, including cDCs, other immune cells, endothelium, and tumor cells[97]. Functionally, PD-L1 can exists as monomers or form heterodimers with CD80 *in cis*[101,102]. The PD-L1:CD80 heterodimer can no longer interact with PD-1 and is protected from transendocytosis via CTLA-4[103], effectively converting it into a costimulatory molecule. Perhaps, this is one of the reasons that CD86 is favored by Tregs over CD80 (Chapter 5), as CD80 can be shielded from CTLA-4 interaction.

These findings also critically highlight that anti-PD-1 and anti-PD-L1 are not interchangeable therapies from different pharmaceutical companies, but that they target distinct pathways. The commonly used anti-PD-L1 antibodies atezolizumab and durvalumab, also inhibit the interaction of PD-L1 with CD80[103,104]. From an immunological standpoint, this is counterproductive as it does not increase CD28 signaling in T cells which is required for function of anti-PD-1[105], as this disruption of the dimer can allow CD80 downregulation by Tregs via CTLA-4. To restore CD28 signaling availability via CD80, it could be opted to also provide anti-CTLA-4. Indeed, this combination, compared to anti-PD-L1 alone, specifically induces activation and

expansion of CD4$^+$ T cells in head and neck squamous cell carcinoma[106]. However, combining anti-PD-L1 and anti-CTLA-4 increases high grade immune related adverse events in cancer patients[107].

Anti-PD-1 treatment is more complex than currently perceived with focus on CD8$^+$ T cells, since NK cells, CD4$^+$ T cells, B cells and ILCs can also express PD-1. Currently, the effects of ICB on CD4$^+$ T cells are only partially understood and are probably complicated by the different differentiation trajectories of CD4$^+$ T cells. As described in Chapter 2 and by others, PD-1 is upregulated by T cells rapidly after activation[102,108]. Upon completion of the differentiation trajectory to the Th1 or CTL state, PD-1 is downregulated, while it is present on stem-like and exhausted CD8$^+$ T cells, Th1/Tfh precursor and Tfh cells (Chapter 2, 4). Notably, GC-Tfh cells express even higher levels of PD-1 than other CD4$^+$ T cells[67], arguing that they are also targeted by anti-PD-1 therapy.

The effects of immune checkpoint blockade on CD4$^+$ Tfh responses

The precise roles of PD-1 in Tfh biology are not fully understood. Several lines of evidence suggest biological function, as Tfh cells highly express PD-1. A key study by Shi *et al.*[109] highlights how PD-1 affects Tfh localization. PD-1 signaling inhibits PI3K signaling downstream of CXCR5, which can be overcome by strong ICOS signaling. Additionally, PD-1-PD-L1 interactions between Tfh cells and B cells dampen CXCR3 expression which retains Tfh cells inside the GC, thereby optimizing B-cell affinity maturation[109]. Additionally, GC B cells express CD28 ligands CD86 and CD80[110,111]. As PD-1 increases the threshold for TCR/CD28 signaling, anti-PD-1 treatment increases CD4$^+$ T-cell proliferation[112,113]. Thus PD-1 may enforce stringent selection of the highest affinity B cells by limiting Tfh cell expansion. Increased Tfh numbers would increase the available T-cell help and thereby decrease the threshold for affinity selection of B cells. Indeed, in peanut-allergic mice anti-PD-1 treatment increased Tfh and GC B cell development and altered isotype switching from IgE to IgG. Additionally, anti-PD-1 treatment increased the level of antibodies in serum, which had low affinity[114]. Also in *Schistosoma mansoni* infection and keyhole limpet hemocyanin (KLH) vaccination, PD-L1 knockout mice or anti-PD-1 treatment increased the GC response, as well as the proportion of Tfh cells[115]. The exact mechanism by which PD-1 dampens helper function of Tfh cells is currently unknown, but reduced help to B cells via CD40L and IL-21 are likely involved.

IL-21 provided by Tfh cells can be beneficial for CD8$^+$ T cell function[96]. In colorectal cancer, PD-L1 expressing tumor cells dampened IL-21 production by Tfh-like cells, which became more apparent in later disease stages[116]. Tfh-like cells (IL-21$^+$ CXCL13$^+$) formed triads with activated DCs and CD8$^+$ T cells in the tumor, which correlated

with response to PD-1 blockade in hepatocellular carcinoma[117]. Currently a uniform nomenclature for intratumoral Tfh-like cells is lacking[118], which complicates formal conclusions on cellular states and their comparison to acute immune responses. In B16-OVA mouse melanoma, adoptive transfer of *in vitro* generated Th1 or Tfh cells induced similar reduction in tumor outgrowth. Tfh cell-induced therapeutic effects were abrogated upon $CD8^+$ T-cell depletion, IL-21 or IFNγ blockade, but not B-cell depletion[119]. Mechanistically, Tfh cell recruitment to the tumor in several mouse models of cancer was dependent on CXCL13 production by $CD8^+$ T cells, and Tfh cells were critical for anti-PD-L1 treatment efficacy[60,119]. By contrast, in other models, (antigen-specific) B cells were required for interactions with Tfh cells, which produced IL-21 to optimize $CD8^+$ T-cell functionality[120,121]. These findings likely differ based on the origin of Tfh-like cells; for example, in the study of Niogret *et al.*[119] Tfh-like cells were generated *in vitro*, thereby bypassing the role of B cells in Tfh cell differentiation (Chapter 2), while in Cui *et al.*[120], Tfh cell differentiation depended on tumor antigen-specific B cells. Thus, it is critical to understand the differences in models, prior to making conclusions. Optimizing $CD8^+$ T-cell differentiation via Tfh cells is challenging, as it requires antigen-specific B cells, $CD4^+$ T cells and $CD8^+$ T cells. Critically, these antigens also must be expressed by the same cancer cells for optimal treatment effect and antigen-presentation by DCs[91]. Altogether, these data point to a functional role of Tfh-like cells in anti-PD-1 therapy and in optimizing the $CD8^+$ T-cell response.

The effects of immune checkpoint blockade on $CD4^+$ Th1 responses

Th1 cells are critical for tumor control. Th1 cells were associated with better prognosis in several types of cancer, including colon, gastric, lung and leukemia[122]. IFNγ induces expression of MHC-I and MHC-II on cancer cells, can lead to cellular senescence and can optimize anti-tumor properties of other immune cells[92]. IFNγ-related gene signatures are associated with responses to anti-PD-1 in over 9 different cancer types[123]. Furthermore, CXCL9 and CXCL10 are IFNγ response genes[124], and the upregulation of these chemokines is critical for tumor invasion by $CD8^+$ T cells in response to anti-PD-1 and CTLA-4 treatment[125]. Several lines of evidence point to improved $CD4^+$ Th1 cell functionality after PD-1 therapy. In chronic LCMV infection, a combination of IL-10 and PD-L1 restricted the function of Th1 cells, impairing $CD8^+$ T-cell function, arguing that PD-1 blockade could restore Th1 responses[126]. *In vitro,* anti-PD-1 increased T-bet, IFNγ and IL-2 expression in head and neck squamous cell carcinoma derived tumor-infiltrating lymphocytes upon TCR and CD28 triggering and both $CD4^+$ and $CD8^+$ T cells from tumor tissue showed higher expression of SHP-2[127], which inhibits CD28 upon PD-1 ligation. IFNγ signaling in DCs, especially cDC1s can result in the production of IL-12, which in turn upregulates IFNγ production by T cells[128]. In murine models, it was shown that IFNγ production by $CD8^+$ T cells upon anti-PD-1

treatment was critical for IL-12 production by DCs[128], but IFNγ production by other cellular subsets such as Th1 cells or NK cells may have the same effect. Furthermore, early induction of Th1 differentiation after cryo-thermal therapy depended on IFNγ production, and this was associated with improved survival[129]. Additionally, Treg depletion with anti-CTLA-4 allowed for differentiation of cancer-specific stem-like $CD4^+$ T cells to Th1 cells, which increased effector $CD8^+$ T-cell responses and critically contributed to improved outcomes in murine cancer models and correlated with better overall survival after ICB in kidney cancer[42]. In human breast cancer, anti-PD-1 therapy increased proliferation of intratumoral 'experienced' (marked by e.g., PD-1, LAG3, IFNγ, GZMB) $CD8^+$ T cells as well as Th1 and Tfh cells. In this setting, Tfh and Th1 cells also had a high degree of TCR sharing, arguing that these cells come from a shared precursor cell. Indeed, trajectory analysis showed a common precursor cell state prior to branching[130]. In conclusion, these data point to a crucial role of Th1 cells in cancer therapy and show how ICB can enhance Th1 function.

The role of PD-1 in deciding early $CD4^+$ T-cell differentiation fates

We aimed to investigate the role of PD-1 during the early differentiation fates as described in Chapter 2. To this end, we employed two strategies; antibody-mediated intervention and genetic deletion using CRISPR/Cas9 in adoptively transferred OTII cells (results not shown). In antibody-based intervention studies, we noted a decrease in the frequency of antigen-specific $CD4^+$ T cells, both for OTII and PADRE-tetramer$^+$ cells, in contrast to our expectation. This decrease was independent of antibody responses to the anti-PD-1 antibody or Fc-receptor dependent effects such as antibody-dependent cellular cytotoxicity or complement activation, as verified using a LALA-PG mutated, murine derived modified antibody that is immunologically silent[131]. Additionally in line with previous findings, antibody intervention increased the proportion of Tfh cells[115]. By contrast, genetic deletion of PD-1 in adoptively transferred OTII cells did not alter the frequency of OTII cells post vaccination, although the genetic deletion was clear. Likely, antibody-mediated intervention targeted different PD-1 expressing subsets that influenced the overall $CD4^+$ T- cell response, while the genetic deletion was OTII T cell-specific. Tregs can also express PD-1 and in cancer PD-1^+ Tregs associate with poor ICB responses[132]. Potentially also after vaccination in combination with anti-PD-1 Tregs were targeted which then constrained the new anti-vaccine $CD4^+$ T-cell response. These results also highlight the importance of assessing research outcomes in different models to be able to generalize concepts. They also demonstrate that antibody interventions may cause experimental artefacts or have confounding effects by targeting other cell populations than desired and should be interpreted with care. This also applies to depletion studies. For example, targeting Ly6C to deplete monocytes[30], indeed depletes monocytes, but can also deplete effector Ly6C$^+$ $CD8^+$ T

cells and Ly6C$^+$ Th1 cells[133,134], potentially confounding results if the loss of these T-cell subsets is measured as the primary outcome. Therefore, it is crucial to assess target expression and depletion efficacy, as performed for CCR2-depletion in Chapter 3.

Provision of CD4$^+$ T-cell help for CD8$^+$ T-cell responses

In chronic LCMV infection, overcoming a blockade of CD4$^+$ Th1 differentiation via blocking of IL-10 and PD-L1 overcomes CD8$^+$ T-cell exhaustion[126], while CD4$^+$ T-cell depletion exacerbates chronic LCMV infection[135]. The role of CD4$^+$ T cells in acute infections may have been underestimated, as depletion is often done with anti-CD4 antibody[135]. While this approach effectively removes CD4$^+$ T cells, it also depletes Tregs. Indeed, a 'CD4$^+$ Tconv-specific' model of depletion that utilizes a FOXP3-induced Cre-Lox system to excise a CD4-driven diphtheria toxin receptor demonstrated that in absence of helper, but not total CD4$^+$ T cells, CD8$^+$ T-cell proliferation was dramatically reduced in viral infection, while differentiation to effector cells was hampered in both total and helper CD4$^+$ T cell depletion[9]. Thus, the effect of helper CD4$^+$ T-cell depletion on CD8$^+$ T cell expansion was obscured by the simultaneous depletion of Tregs. In the Help/No Help vaccination strategy used in this thesis, we avoid Treg depletion and directly interrogate how CD4$^+$ T-cell help impacts CD8$^+$ T-cell differentiation or optimizes myeloid cell functionality (Chapter 2-4). Using this strategy, we clarify how CD8$^+$ T-cell differentiation from naïve to effector via stem-like cells takes place (Chapter 4). Along this trajectory, naïve, central-memory and stem-like cells are formed in absence of CD4$^+$ T-cell help and can be observed in both vaccination settings. By contrast, effector and effector-memory cells can only be formed in the presence of CD4$^+$ T-cell help, which optimizes antigen-specific CD8$^+$ T cells with anti-viral and anti-cancer properties, such as improved cytotoxicity and migratory properties[61,136,137]. This result underscores the importance of engaging CD4$^+$ T cells in anti-tumor immunity. However, as we demonstrate with the MC38-HELP tumor cell line that expresses the strong CD4$^+$ T cell epitope PADRE and spontaneously induces a PADRE-specific CD4$^+$ T-cell response, CD4$^+$ T-cell activation does not automatically result in improved anti-tumor CD8$^+$ T-cell responses. This is in line with the fact that MC38-intrinsic CD4$^+$ T-cell epitopes do not prevent outgrowth, but can be targeted therapeutically to improve tumor control, especially when combined with CD8$^+$ T cell epitopes[138]. This highlights that antigenicity does not equal immunogenicity, and that to achieve improve tumor control, the help signals need to be delivered for optimization of costimulatory and cytokine signals. Finally, Tregs can also hamper anti-tumor responses by restraining T-cell activation and function via direct mechanisms or via DCs (Chapter 5), and deletion of Tregs allows differentiation of CD4$^+$ T cells and optimized CD8$^+$ T-cell responses[42,139]. Thus, future research should be aimed at understanding how CD4$^+$ T-cell priming and differentiation can be optimized in the tumor context, so that help can be properly delivered to CD8$^+$ T cells.

The role of dendritic cells in CD4$^+$ and CD8$^+$ T-cell responses to cancer

Meta-analysis of DC states using scRNAseq across several human cancers including breast, colorectal and lung cancer identified a common, 'DC3' state[140]. This DC3 was tumor-specific, and its gene signature also included that of other 'tumor-specific' favorable DC states, such as mregDCs[141]. It was predicted that this DC3 state could be derived from both cDC1 and cDC2, but recently our group demonstrated that only human cDC1s adopt this phenotype after CD4$^+$ T-cell help, which is associated with improved clinical outcome, intratumoral Th1 and activated CD8$^+$ T-cell responses and immune checkpoint blockade responsiveness[7]. While this does not formally exclude cDC2s from adopting such a state as murine studies using SITE-seq showed both XCR1$^+$ (cDC1-like) and CD11b$^+$ (cDC2-like) mregDCs[141], these findings strongly suggest that cDC1s are the primary recipients of CD4$^+$ T-cell help that induces this activated DC phenotype. Furthermore, the mechanism by which cDC2s would adopt the DC3 state is currently not known. As the DC3 state could be induced *ex vivo* by CD4$^+$ T-cell help, this argues that this is a conserved immune response between different species, at least for cDC1s. Indeed, a similar LAMP3$^+$ DC subset with similarity to DC3s was found in autoimmune Crohn's disease, highlighting that this is a common immune response in different types of immunological challenges[140,142].

It has been well established that cDC1s are critical for anti-tumor immunity. Mouse models that lack (migratory) cDC1s such as *Batf3*$^{-/-}$ or *Irf8+32*$^{-/-}$ mice fail to control immunogenic tumors[143,144]. This is the result of poor CD8$^+$ T-cell activation, as cDC1s excel at cross-presentation of cell-associated antigen, and relay of CD4$^+$ T-cell help[4,7,90,143]. It is important to discern the priming of CD8$^+$ T cells during different immune challenges, since during viral infection, cDC2s can also prime CD8$^+$ T cells[5,6]. Mechanistically, cDC1s express CLEC9a that recognizes exposed f-actin-myosin complexes and enables uptake of cellular debris. Subsequently, CLEC9a also facilitates phagosomal rupture, allowing translocation of endocytosed extracellular material to the cytosol[145]. An elegant study using intravenous injection of cytochrome C, which induces apoptosis only upon cytoplasmic entry, demonstrated specific cDC1 death, indicating that cDC1s take up extracellular contents and translocate those to their cytoplasm[146]. In tumor stroma of prostaglandin-negative mouse melanoma, MHC-II^{hi}CCR7$^-$ cDC1s attracted CD8$^+$ T cells in a CXCL9/CXCR3-dependent fashion and induced CD8$^+$ T-cell proliferation and effector functions. Moreover, cDC1 clusters also constituted niches for T-cell proliferation in head and neck squamous cell carcinoma[147]. Although CD4$^+$ T cells were not assessed in this study, it seems likely that CXCL9 can also attract CXCR3$^+$ CD4$^+$ Tconv or Treg. Indeed, the microscopy data from Meiser *et al*.[147] show CD8$^-$ CD3$^+$ cells in vicinity of CD103$^+$ cDC1s. It was previously shown that NK cells are required for attraction of cDC1s via CCL5 and XCL1[148] and their survival via

FLT3L[149], and NK cells may also optimize cDC1s via IFNγ[150]. These findings highlight that anti-tumor immunity relies on cooperation of multiple immune cell types suggesting that therapeutic strategies must target several cell subsets simultaneously. Although cDC2s are classically described as the main driver of $CD4^+$ T-cell differentiation[151], cDC1s are important for $CD4^+$ T-cell priming in tumor models, as MHC-II-deletion in cDC1s hampered $CD4^+$ T-cell function and tumor control[90]. The dependence on cDC1 or cDC2 may vary depending on tumor type, as well as the extent and mode of spontaneous or induced cancer cell death.

A role for cDC2 in antigen uptake and presentation comes from multiple lines of evidence. For example, in Chapter 5, we described how RT can induce a $CD8^+$ T-cell response combined with a Treg expansion in cold tumors. This coincided with upregulation of CD86 on cDC2s and blockade of CD86 hampered Treg expansion. This suggests that cDC2s may be the main drivers of Treg expansion post RT via CD86, and for this, they would have needed to take up antigen. Alternatively, as a means to acquire tumor-derived antigen, horizontal vesicle dependent antigen-transfer between different cell types could take place[152], but it seems unlikely that it is only dependent on cDC1s, as tumor-derived monocytes, macrophages, cDC1 and cDC2 were all capable of taking up tumor associated proteins in B16 melanoma models[152]. Furthermore, CD40-CD40L interaction-dependent labelling after $CD4^+$ T cell-DC interactions in B16 melanoma demonstrated that both cDC1s and cDC2s could present tumor antigens to $CD4^+$ T cells, with a most pronounced difference in gene signature in cDC2s[153]. Another line of evidence for cDC2 dependent $CD4^+$ T-cell activation in cancer is that Tregs can suppress tumor-derived cDC2s. Upon Treg depletion these cDC2s can activate Tconv $CD4^+$ T cells and induce a Th1-like $CD4^+$ T-cell program that was critical for tumor rejection in mice. The cDC2:Treg ratio correlated with $ICOS^{hi}PD\text{-}1^{lo}$ $CD4^+$ T cells and increased progression free survival in head and neck cancer and melanoma patients[139]. The gene expression imprint of CD40-signaling was mainly found back in cDC2s upon Treg cell depletion[153]. This suggests that Tregs either directly block CD40 signaling into cDC2s, or that Treg cells inhibit $CD4^+$ Tconv cells activation, which in turn disallows CD40 signaling cDC2s. Altogether, these data highlight that the intratumoral state of both cDC1s and cDC2s is critical for tumor immune responses by both $CD4^+$ and $CD8^+$ T cells.

Targeting CD40 to improve $CD8^+$ and $CD4^+$ T-cell responses

A potential strategy to enhance Th1 or CTL differentiation based on our data could be targeting cDC1s with CD40 agonistic antibodies. Several anti-CD40 antibodies have been tested in human cancer and have shown some promise but generally low response rates combined with toxicity[154]. However, as anti-PD-1 treatment does not equal proper priming for $CD4^+$ T cells or effective help delivery to $CD8^+$ T cells, targeting

CD40 may be of interest. This may be of interest especially in cold tumors that do not respond to anti-PD-1 therapy such as pancreatic ductal adenocarcinoma[155], in which CD40 agonism showed non-durable cancer regression by a T-cell independent mechanism[156]. CD40 targeting can also optimize MoDCs (Chapter 3), and in murine cancer results in more activated MoDCs that are associated with improved $CD4^+$ and $CD8^+$ T-cell effector functions[157].

In breast- and pancreatic cancer models, the combination of vaccination, PD-1 blockade and CD40 agonism improved survival and tumor control[158]. For durable responses, preferably tumor-specific B cells, $CD4^+$ T cells and $CD8^+$ T cells work together[120]. CD40 agonism evidently does not overcome lack of tumor antigen, but can compensate for otherwise weak $CD4^+$ T-cell activation. Furthermore, CD40 targeting on B cells potentially supports formation of tertiary lymphoid structures in tumors, though current data are limited[154]. Given the role of CD40 signaling in cDC1s as key component of $CD4^+$ T-cell help[8,10], bispecific antibodies targeting both CD40 and cDC1 hallmark proteins like XCR1 or CLEC9A may increase cDC1 functionality while avoiding potentially toxic effects on other cell types. In MC38 and MCA-205 mouse models, anti-CD40 monotherapy reduced tumor burden which depended on $BATF3^+$ cDC1s and a CD11c/CD40 bispecific antibody targeting DCs significantly improved tumor control in MC38 and B16 models compared to CD40 antibody alone[159]. Mechanistically, CD11c/CD40 bispecific antibody increased the proportion of cDC1s and cDC2s in the tumor and improved the frequency of naive and effector $CD8^+$ T cell subsets compared to Treg and dysfunctional $CD8^+$ T cells, while reducing liver toxicity. Lastly, bispecific antibody co-targeting CD40 and CLEC9a (cDC1) dramatically improved $CD8^+$ T-cell responses and tumor control compared to CD40 antibody alone, but the effect was not compared to that of bispecific antibody targeting CD40 and CD11c (all DCs)[159]. In an alternative approach, bispecific antibodies targeting both DCs and PD-1 have been tested, to bring activated or stem-like T cells together with DCs[160]. However, this did not overcome inefficient T-cell differentiation, likely because a preformed, but hampered $CD4^+$ T-cell response as described above was lacking. In lung adenocarcinoma models, combination of Flt3L with anti-CD40 increased cDC1 frequency in tumor dLNs and cDC1s were critical for maintaining stem-like $CD8^+$ T cells and aided in tumor control[161]. However, all these strategies ultimately rely on at least $CD8^+$ T-cell specific (neo)-antigen expression by tumor cells, as well as DC stimulatory IFN-I[4,8,162,163]. Additionally, as we show in chapter 2, CD40 signaling is important for inducing Th1 responses, and thus CD40-targeting antibodies may also enhance $CD4^+$ T-cell responses.

Overcoming lack of tumor-specific T cell priming by vaccination and adoptive DC therapy

Another option to improve anti-tumor $CD4^+$ and $CD8^+$ T-cell responses is vaccination or adoptive cell therapy. Adoptive cell therapy in form of CAR T cells have shown great promise in B-cell malignancies but is often hampered by limited tumor-specific targets and escape variants[164]. Crucially, antigen-specific $CD4^+$ T cells, $CD8^+$ T cells and B cells can cooperate in tumor rejection[120]. Ideally, tumor-specific T cells should be introduced or primed against multiple tumor-specific epitopes, but this has been especially challenging for $CD4^+$ T cells, as MHC-II-binding prediction algorithms remain poorly predictive[165] and therefore identification of tumor-specific $CD4^+$ T cells is limited.

For tumors that arise due to expression of viral oncogenes, such as EBV-, KSHV- and HPV-induced cancers, vaccination against viral proteins presents a unique opportunity, as viral proteins like the CD40-mimicking protein LMP1 in EBV are critical in oncogenesis[92,166], and viruses only express limited proteins to be tested in context of human MHCs[167]. Because most tumor cells do not express MHC-II, eluting peptides from cancer-cell derived MHC is not a feasible strategy for standardized epitope prediction. Forced expression of the MHC-II machinery by CIITA overexpression in murine cancer cell lines in combination with mass-spectrometry provided a platform for identifying $CD4^+$ T cell epitopes[138], but this approach is only feasible in immortalized cells that can be transduced. An alternative could be to vaccinate with tumor derived-$CD8^+$ T-cell epitopes in conjunction with general, non-tumor specific helper epitopes. However, the $CD4^+$ T cells raised will likely be limited in efficacy to secondary lymphoid organs, and will likely not result in triad formation with $CD8^+$ T cells and cDC1 in the tumor where the $CD4^+$ T-cell epitope will be lacking, which is critical for rejection[63]. A combined RNA-sequencing with mRNA-vaccination strategy demonstrated clear induction of $CD4^+$ and $CD8^+$ T-cell responses post vaccination in melanoma patients which led to reduced metastatic rate and prolonged progression-free survival[168]. RNA vaccines are easily altered, and the COVID-19 pandemic has shown the clear clinical success and safety of mRNA vaccines from Pfizer and Moderna, highlighting the potential of RNA based vaccination strategies. Similarly, phase I/II clinical trials demonstrated functional T-cell responses in multiple melanoma patients as well as $HPV16^+$ oropharyngeal squamous cell carcinoma patients after peptide vaccination for $CD4^+$ T cells with or without anti-PD-1 therapy[169–171]. For optimal vaccination, also the route of administration and adjuvants will be critical to obtain optimal and durable responses[172], as well as obtaining the desired T-helper differentiation trajectory[173,174]. Improper activation or tumor-derived factors may result in $CD4^+$ Tconv to Treg conversion or tTreg expansion upon vaccination[175]. Thus, it is critical to ensure optimal DC maturation in combination with providing highly specific antigens to induce correct $CD8^+$ and $CD4^+$ T-cell priming.

Alternatively, *ex vivo* DC loading with cancer cell debris or peptide vaccines could be a viable strategy, with clear benefits. First, the environment of DC maturation can be controlled, avoiding suppression by the TME or Tregs. Second, either whole tumor lysates as well as peptides or genetic vaccines could be used. Most strategies have focused on *ex vivo* differentiated MoDCs as they are easy to obtain and scale up. However, MoDCs have reduced T-cell stimulatory capacity compared to cDCs and may have limited migratory capacity to lymph nodes[176,177]. Additionally, only cDC1s were receptive of $CD4^+$ T-cell help for $CD8^+$ T-cell responses, which also argues that cDC1 transfer should be the main goal[7]. However, cDC1s are notoriously scarce, and are difficult to generate *ex vivo*, although some optimization in FLT3L culture settings has been achieved[176,178]. While these hurdles are still present, hopefully future research can overcome these, and combined with in depth knowledge of $CD4^+$ and $CD8^+$ T-cell differentiation trajectories could further rationalize vaccine design, DC-targeting strategies, and ICB-based or immunostimulatory therapies to optimally activate anti-cancer T-cell immunity.

Concluding Remarks

Understanding how $CD4^+$ T-cell differentiation is shaped is critically important to optimize immune responses to viral infections and cancer. Additionally, critical insights in $CD4^+$ T-cell biology will also be important for type 2 and type 3 immune responses as well as autoimmunity. In this thesis, I have provided new insights in the differentiation trajectory of $CD4^+$ T cells. By identifying and characterizing a common Th1/Tfh precursor that lies at the branchpoint of subsequent $CD8^+$ T cell and B cell helping $CD4^+$ T cells, we provide a new target for improving vaccination against pathogens or cancer, and immunotherapy of cancer and autoimmunity. A thorough understanding of how to engage or block the differentiation trajectory of $CD4^+$ T cells could be crucial in improving clinical outcomes. Furthermore, I characterized and investigated a MoDC population that optimizes Th1 differentiation and $CD8^+$ T cell differentiation, through a feed forward loop with Th1 cells. This feed-forward loop can be taken into consideration for future vaccination strategies, as they have been shown to be important for $IFN\gamma^+$ T cell responses. In addition to insights in $CD4^+$ T-cell differentiation, I also investigated how $CD4^+$ T-cell help optimizes $CD8^+$ T-cell responses. We demonstrate that the provision of $CD4^+$ T-cell helper signals is critical in the formation of effector $CD8^+$ T cells derived from stem-like $CD8^+$ T cells. Furthermore, by generating a novel MC38-HELP model, we show that presence of $CD4^+$ T-cell epitopes and $CD4^+$ T-cell priming per se does not equal help delivery, providing a framework for future research to optimize $CD4^+$ and $CD8^+$ T-cell responses in cancer immunity. Lastly, I aided in investigating the effects of overcoming immune

resistance in cold tumors. In these tumors, RT elicits a $CD8^+$ T cell response, which is counteracted by concomitant Treg induction by upregulation of CD86 on cDC2s which was promoted by anti-PD-1 or anti-CTLA-4 blockade. These data help highlight that when searching for new strategies to induce T-cell responses, we need to take antigenicity, Treg activation and DC states into account.

In summary, this thesis provides a framework to interrogate $CD4^+$ T-cell differentiation after vaccination, which can be compared to cancer, chronic viral infection, and autoimmunity. Furthermore, I described key APC-$CD4^+$ T cell interactions that optimize both $CD4^+$ and $CD8^+$ T-cell immunity. Altogether, this information can guide the design of rational strategies to induce specific $CD4^+$ T-cell differentiation in different pathological contexts.

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