How CD4+ T-cell help shapes functional and dysfunctional CD8+ T-cell differentiation

Busselaar, J.L.C.

Chapter 1

General Introduction & Scope of Thesis

T cells play a crucial role in the immunological protection of the host against infections and cancer. They can recognize pathogen-derived antigens expressed by infected cells, tumor-associated antigens or neoantigens expressed by cancer cells as a result of mutations. However, in cancer there are often barriers for an effective T-cell mediated immune response, which include a lack of non-self antigens, insufficient immune-activating signals, and an immune-suppressive tumor microenvironment (TME). Immune therapies aim to overcome these barriers and thereby to stimulate the host's immune system to fight the cancer. Clinical responses to immune therapies are variable, and a better fundamental understanding of the underlying immunological mechanisms is needed.

$CD8^+$ cytotoxic T lymphocytes (CTL) are particularly effective for anti-tumor immune responses, since they kill target cells upon recognition of intracellular antigens in the context of MHC class I (MHC-I) molecules, which are expressed by all nucleated cells including tumor cells. To undergo clonal expansion and gain effector functions, naïve tumor-specific $CD8^+$ T cells expressing a T cell receptor (TCR) that recognizes a specific (tumor) antigen, have to be activated (primed) by dendritic cells (DCs) in secondary lymphoid organs, generally the lymph node (LN). After priming, antigen recognition by a properly activated and differentiated antigen-specific $CD8^+$ T cell can lead to direct killing of the (tumor) target cell. However, $CD8^+$ T cells in cancer are often found in a dysfunctional or "exhausted" state, which is characterized by loss of effector function, loss of proliferation, and high expression of inhibitory receptors such as PD-1[1]. Upon interaction with their ligand, inhibitory receptors relay a negative signal that counteracts T cell activation, and thereby act as a brake or so-called "checkpoint" for anti-tumor immune responses. Immune checkpoint blockade (ICB) therapies, such as PD-1 blockade, aim to release this brake and thereby reactivate $CD8^+$ T cells within the tumor context[2].

$CD4^+$ T cells recognize antigens in the context of MHC class II (MHC-II), which is expressed by professional antigen-presenting cells (APCs), and typically not by solid tumor cells. $CD4^+$ T cells have a dual role in cancer: conventional helper T cells can help $CD8^+$ T cells or B cells, while regulatory T cells (Tregs) suppress the CTL response[3]. Tregs express high levels of the inhibitory receptor CTLA-4, which downregulates CD80/86 on DCs and thereby inhibits CD28 costimulation needed for CTL priming. CTLA-4 blockade is another checkpoint inhibition therapy, which aims to block this interaction and thereby allow for better activation of $CD8^+$ T cells. In comparison to CTLs, conventional $CD4^+$ T cells have generally received less attention within the ICB field, because most tumors are MHC-II negative and therefore not directly recognized by $CD4^+$ T cells. However, for an optimal anti-tumor $CD8^+$ T cell response, $CD4^+$ T-cell help via the DC interface is essential[4].

Priming of tumor-specific $CD8^+$ and $CD4^+$ T cells relies on antigen uptake by conventional (c) DCs in the tumor, DC activation and migration to the tumor-draining lymph node (TdLN) where tumor antigens are presented to T cells. In viral infection and vaccination settings, naïve $CD8^+$ and $CD4^+$ T cells are initially activated independently by migratory cDCs in distinct regions of the LN[5–7]. Type 1 cDCs (cDC1s), specialized in exogenous cell-derived antigen crosspresentation

on MHC-I, provide the first activation step for CD8⁺ T cells, while generally type 2 cDCs (cDC2s) initially activate CD4⁺ T cells via MHC-II[8,9]. The cDC1s subset can effectively present exogenous cell-derived antigens on both MHC-I and MCH-II, and thereby forms the platform for CD4⁺ T-cell help delivery to CD8⁺ T cells, as shown in mouse and human settings[5,6,10–12].

According to the two-step priming model[4], CD4⁺ T-cell help delivery likely takes place during the second step of T cell priming (Figure 1). This is supported by studies in infection and vaccination models, demonstrating that the initial activation of CD4⁺ and CD8⁺ T cells by migratory DCs takes place prior to CD4⁺ T-cell help delivery by resident cDC1s in distinct regions of the LN[5,6,13–15]. In cancer, initial priming of CD8⁺ T cells and delivery of CD4⁺ T-cell help also depend on crosspresenting cDC1s[10,12,16], but whether these events happen in two consecutive priming steps is less clear. A CD8⁺ T cell may receive its initial activation by one (migratory) cDC1, and the CD4⁺ T-cell help signal by another (resident) cDC1. However, these steps may also occur on the same 'helped', also called 'licensed' cDC1, that is interacting or has interacted with an activated CD4⁺ T cell. It has been proposed that cDC1s in cancer may also provide early activation signals for CD4⁺ T cells[10], although the possibility of their initial activation by cDC2s, as previously shown by others[17] was not excluded in this study.

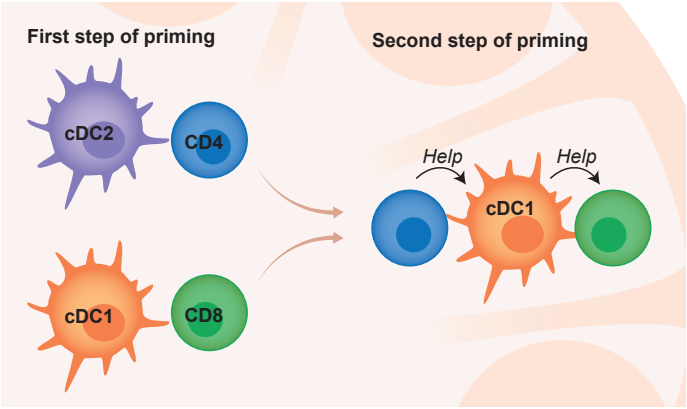

Figure 1. Two-step priming model of CD8⁺ and CD4⁺ T-cell activation in the LN.

Help signals (Figure 2) are relayed to the cDC1 via CD40 ligand (L) and type I interferon (IFN-I) produced by the activated CD4⁺ T cell[18–21]. These signals lead to increased survival, crosspresentation and upregulation of costimulatory molecules such as CD80/86 and CD70, as well as the production of cytokines such as IL-12 and IL-15 by the helped cDC1[4,12,22]. The delivery of these costimulatory and cytokine-mediated signals, in addition to the TCR-mediated activation signal via MHC-I, eventually results in the priming of 'helped' CD8⁺ T cells with optimal effector and memory functions [4,11]. Recent studies have suggested that in addition to cDC1 licensing, CD4⁺ T-cell help during priming may also be delivered to CD8⁺ T cells via cDC1-independent mechanisms, for example via other APCs such as monocyte-derived (Mo)DCs, or perhaps directly via CD4⁺ T-cell–derived IL-2[15,23].

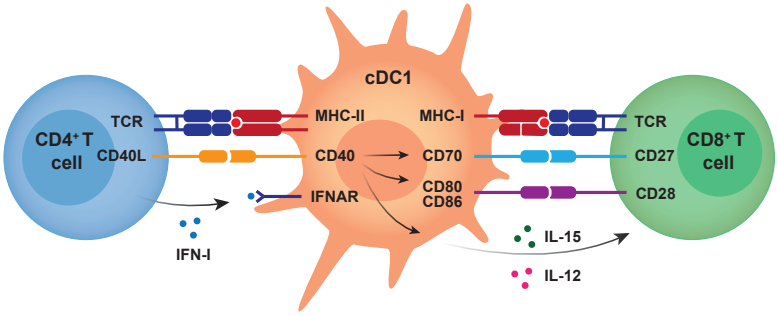

Figure 2. Signals involved in CD4$^+$ T-cell help delivery to CD8$^+$ T cells, mediated by cDC1.

Cancer immune therapies aim to improve tumor-specific CTL responses, by sustaining and activating previously primed tumor-specific CD8$^+$ T cells, or by new priming of such CD8$^+$ T cells. The importance of CD4$^+$ T-cell help for the anti-tumor CTL response is recognized mostly in the therapeutic vaccination field, where it has long been known that inclusion of MHC-II epitopes in the vaccine formulation improves CTL-mediated tumor control[24–26]. However, in the checkpoint inhibition field, the importance of CD4$^+$ T-cell help is not so generally considered. In recent years, accumulating evidence supports the idea that clinical efficiency of PD-1 blockade therapy does not solely depend on PD-1 expression on CD8$^+$ T cells, but also on PD-1 expression on conventional CD4$^+$ T helper cells[27,28]. Whether the spontaneous or ICB-induced priming of tumor-specific CD8$^+$ T cells happens in presence or absence of CD4$^+$ T-cell help, remains unclear.

In preclinical studies that investigate the role of CD4$^+$ T cells on CTL priming and differentiation, researchers often make use of antibody-based CD4$^+$ T-cell depletion in mouse models of chronic or acute infection[29–35]. These CD4$^+$ T-cell depletion experiments provide strong evidence for the importance of CD4$^+$ T-cell help for CTL effector and memory differentiation and function in the context of infection. However, the CD8$^+$ T-cell states in these type of experiments may additionally be affected by depletion of Tregs and/or the absence of CD4$^+$ T-cell-derived cytokines for CTL maintenance[15,36]. CD8$^+$ T-cell states may also be indirectly affected by the impact of CD4$^+$ T-cell depletion on antigen load or antigen persistence[37–39].

In order to specifically investigate CD8$^+$ T cells that are primed with or without CD4$^+$ T-cell help, outside the context of infection or chronic antigen stimulation, we made use of a DNA vaccination model wherein CD4$^+$ T cells are not depleted, but are simply engaged in the response or not via the addition of MHC-II restricted epitopes in the vaccine construct[40]. Using this model, our group has previously identified molecular mechanisms of CD4$^+$ T-cell help for the CD8$^+$ T-cell response during the effector phase. “Helped” CTLs undergo more clonal expansion, express higher levels of effector molecules such as Granzyme-B, IFNγ and TNF and are superior on a per-cell basis in antigen-specific killing of target cells[41]. They also show improved migratory and invasive capacities, which allows them to infiltrate tumors. On the other hand, “helpless” CD8$^+$ T

cells, primed in absence of CD4$^+$ T-cell help, expand less, show impaired cytotoxic and migratory function and have a higher expression of various inhibitory receptors including PD-1[41].

Besides shaping size and quality of the effector CTL pool, CD4$^+$ T-cell help also impacts CD8$^+$ T-cell memory. After antigen clearance and contraction of the effector CTL population, memory CD8$^+$ T cells persist long-term and respond efficiently upon secondary antigen encounter. Effector memory (T_{EM}) cells retain a phenotype resembling effector cells, and can rapidly respond to antigen by expressing cytolytic molecules. Central memory (T_{CM}) cells are characterized by their self-renewal capacities and robust clonal expansion upon antigen encounter, and are phenotypically more similar to naïve CD8$^+$ T cells[42]. In Chapter 2, we demonstrate that CD4$^+$ T-cell help during priming impacts CD8$^+$ T-cell memory by promoting the long-term maintenance, but not so much the gene expression profile of T_{CM} cells and by increasing and qualitatively altering the T_{EM} pool that transcriptionally and epigenetically resembles helped effector CTLs. Importantly, memory CD8$^+$ T cells that received help during priming, no longer depend on CD4$^+$ T-cell help during the secondary response, indicating that CD4$^+$ T-cell help improves CD8$^+$ T cell-intrinsic memory capacities.

After identifying the molecular mechanisms underlying CD4$^+$ T-cell help for effector and memory CD8$^+$ T-cell responses, we aimed to conceptualize an overarching model on how CD4$^+$ T-cell help shapes mature CD8$^+$ T-cell differentiation after antigen recognition in general. For therapeutic interventions, it is important to understand this differentiation process both in the context of an acute immune response such as after vaccination or transient infection, but also upon chronic immune stimulation as in the case of cancer or chronic infection.

When exhausted CD8$^+$ T cells were first discovered in the late 1990s[30], it was thought for years that these cells were derived from 'eroded' effector CTLs as a result of constant antigen stimulation[32]. However, in 2016 it was discovered that exhausted CD8$^+$ T cells do not derive from previously functional effector CTLs, but instead are generated from a population of PD-1$^+$TCF-1$^+$ 'stem-like' cells[33,43–45]. Many different names have been given to this population, such as 'memory-like', 'predysfunctional', 'precursor exhausted', 'progenitor exhausted (T_{PEX})', or simply TCF-1$^+$ CD8$^+$ T cells. Importantly, stem-like cells are the CD8$^+$ T cells responding to PD-(L)1 blockade therapy in preclinical tumor models[46,47]. In human cancer, effective PD-(L)1 blockade therapy induces proliferation and further differentiation of PD-1$^+$TCF-1$^+$ stem-like cells in the TdLN[48] or in intratumoral niches where they interact with DCs and CD4$^+$ T cells[49]. Thus, stem-like CD8$^+$ T cells are of particular interest for cancer immunotherapy.

In Chapter 3, we show that helpless CD8$^+$ T cells transcriptionally resemble stem-like cells. We compare the transcriptomic signature of helpless cells from our vaccination model to published signatures of predysfunctional stem-like cells from chronic infection and cancer. Based on the transcriptional overlap and supported by literature, we pose the hypothesis that stem-like CD8$^+$ T

cells equal helpless cells, and are generated as a result of priming in absence of $CD4^+$ T-cell help. Next, we provide experimental evidence for this hypothesis in Chapter 4. We show that upon priming, naïve $CD8^+$ T cells adopt an entire spectrum of differentiation states, ranging from lowly differentiated T_{CM} cells, to stem-like cells, T_{EM} cells and eventually terminally differentiated effector CTLs. The generation of this spectrum of $CD8^+$ T-cell states after priming, also known as the progressive differentiation model, has been proposed before and is supported by a large body of evidence epigenetic, transcriptomic and TCR tracing studies[50–54]. Until now, $CD8^+$ T-cell fate was proposed to be mainly dependent on TCR signaling strength. Our experiments argue that it is also dependent on $CD4^+$ T-cell help and all its associated signals, which shifts the CTL differentiation spectrum in the direction of a more effector-like state. We demonstrate that $CD4^+$ T-cell help is required for $CD8^+$ T cells to differentiate past the PD-1^+TCF-1^+ stem-like state towards the CTL effector state. We show that in an immunogenic tumor setting, tumor neoantigen-specific $CD8^+$ T cells that are spontaneously primed by the tumor have a stem-like, but not helped effector state, suggesting that $CD4^+$ T-cell help delivery is impaired. Our results support that stem-like $CD8^+$ T cells as found in cancer are in fact helpless cells that lie at an early stage on the 'normal' differentiation spectrum. Importantly, we show that helpless/stem-like cells are still able to complete their CTL differentiation when they are provided with help signals after initial priming.

That the second step of priming is not only important for $CD8^+$ T-cell differentiation but also for $CD4^+$ T-cell differentiation, is shown in Chapter 5. In type-1 immune responses raised against intracellular antigens, $CD4^+$ T cells differentiate into T helper 1 (Th1) cells that can deliver help to $CD8^+$ T cells, and T follicular helper (Tfh) cells that help the humoral response by promoting isotype class switching, somatic hypermutation and memory formation of B cells[55]. We show that upon initial activation, conventional $CD4^+$ T cells adopt a uncommitted Th1/Tfh phenotype. Interactions between $CD4^+$ T cells and cDC1s, which take place during the second step of priming, are dispensable for initial $CD4^+$ T-cell activation and formation of the uncommitted Th1/Tfh phenotype but essential for uncommitted $CD4^+$ T cells to differentiate into Th1 cells. The formation of Tfh cells on the other hand, depends on a second priming step on B cells.

One of the underlying mechanisms for impaired $CD4^+$ T-cell help in cancer may be a lack of inflammatory signals, such as type I interferon (IFN-I) in the TME. How this works is described in Chapter 6. We provide an overview of literature showing that IFN-I optimizes the help delivery capacity of cDC1, by promoting antigen presentation on MHC-I and MHC-II, costimulation and cytokine production. IFN-I also induces the expression of chemokines that are essential for the spatial organization of the help delivery platform within the LN. However, in a tumor setting the IFN-I pathway is often suppressed, resulting in suboptimal cDC1 activation and likely impaired delivery of $CD4^+$ T-cell help.

In recent years, evidence has accumulated showing that the target cells of PD-1 blockade therapy in cancer are not terminally exhausted CD8$^+$ T cells, as previously thought, but tumor-specific stem-like CD8$^+$ T cells[46]. Previous chapters have elaborated on stem-like cells being helpless CD8$^+$ T cells, indicating that it is the helpless cells are the likely target for PD-1 blockade therapy. Moreover, the PD-1/PD-L1 axis that is important for the response to ICB lies at the interface between tumor-specific T cells and cDC1, not at the interface between CTLs and their tumor target cells. The data that support this are reviewed in Chapter 7. We propose on basis of these data that PD-1 blockade leads to delivery of CD4$^+$ T-cell help for the CTL response, and discuss the clinical implications of this view.

Finally, Chapter 8 places the concepts and experimental findings as described in this thesis in the context of current literature.

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