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

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Chapter 8

General Discussion

Cancer immunotherapy is used as a strategy to boost the anti-tumor immune response, by raising new or improving existing tumor-specific T-cell responses. $CD8^+$ T-cell exhaustion, characterized by loss of cytotoxic function and high expression of inhibitory receptors, limits anti-tumor responses. Since the late 1990s, it was believed that exhausted cells derive from eroded effector CTLs[1,2], but this has been disproven in the last 10 years. It was discovered that exhausted cells are derived from PD-1^+TCF-1^+ stem-like cells[3–6]. More recent data have further attenuated this model by showing that also functional effector cells derive from stem-like cells, and therefore stem-like $CD8^+$ T cells form the branchpoint between effector and exhausted trajectories[7–10]. An important outstanding question remains which signals during priming decide the differentiation at this branchpoint, and how to steer stem-like cells away from exhaustion and towards the effector state. To answer these questions we need to investigate the cellular and molecular mechanisms of $CD8^+$ T-cell differentiation upon priming.

Effects of $CD4^+$ T-cell help on linear CTL differentiation

It has long been known that during priming, $CD4^+$ T helper cells can deliver 'help' for the CTL response via DCs[11]. Our research group previously identified molecular mechanisms of $CD4^+$ T-cell help using a vaccination model in which mice are vaccinated with DNA constructs encoding an immunodominant tumor-associated MHC-I epitope with or without tumor unrelated MHC-II helper epitopes. In this reductionistic approach, vaccine antigen-specific $CD4^+$ T cells are engaged during the priming of tumor-specific $CD8^+$ T cells or not. This method showed that helped CTLs, primed in the presence of $CD4^+$ T-cell help, display superior effector and migratory functions, and are more effective in tumor killing compared to their 'helpless' counterparts[12,13].

In Chapter 2, we show in the same system the effects of $CD4^+$ T-cell help on $CD8^+$ T-cell memory differentiation. Memory T cells are cells that persist long-term after clearance of their cognate antigen, following an immunogenic challenge such as vaccination or acute infection. Based on functional capacities, localization and marker expression, circulating memory cells are classically divided into central memory (T_{CM}) and effector memory (T_{EM}) subsets. T_{CM} cells, usually identified by a $CD44^+CD62L^+$ phenotype, circulate via the blood through the T-cell zone of secondary lymphoid organs (lymph nodes and spleen). T_{CM} cells are characterized by high self-renewal capacities and robust clonal expansion upon antigen encounter. T_{EM} cells, usually identified by a $CD44^+CD62L^-$ phenotype, circulate through blood, the red pulp of the spleen and non-lymphoid organs, and can rapidly respond to antigen by expressing cytolytic and pro-inflammatory proteins[14]. We show that $CD4^+$ T-cell help improves mostly the formation, maintenance and gene expression profile of T_{EM} cells. T_{CM} cells can still be formed in absence of help, although their long-term maintenance is impaired. Within the T_{EM} population, helped cells display a more effector-like phenotype, which is maintained upon help-independent secondary activation.

Models of CD8$^+$ T-cell differentiation

Memory and effector differentiation in T cells is driven by the expression of transcription factors, wherein the balance between pairs of opposing transcription factors, including T-bet/Eomes, Blimp1/Bcl6 or ID2/ID3, determines if a T cell will differentiate towards an effector or memory cell fate. These transcription factors are strictly regulated by epigenetic mechanisms, such as DNA methylation and histone modification[15,16]. Since we observed that CD4$^+$ T-cell help delivered during priming promotes both effector and memory CD8$^+$ T-cell formation, we aimed to find an overarching explanation for the effects of CD4$^+$ T-cell help on CD8$^+$ T-cell differentiation as a whole. For this purpose, one needs to consider the different models of effector and memory T-cell differentiation that are used in the field. According to the effector-first model[2,17], antigen-specific naïve CD8$^+$ T cells all become effector cells upon activation, during the expansion phase of the response. Effector cells can be subdivided into a population of short-lived effector cells (SLECs) and memory precursor effector cells (MPECs)[18,19]. Following antigen clearance, SLECs die during the contraction phase, while MPECs persist and de-differentiate to form steady-state T_{EM} and T_{CM} memory cells. Upon re-encounter with antigen, memory cells can differentiate again into effector cells during the secondary response (Figure 1A). This model implies that a division between SLEC and MPEC differentiation occurs upon priming, and that MPEC cells can differentiate in a circular manner, or back-and-forth between the memory and the effector state.

In contrast, the linear or progressive differentiation model[20] proposes that CD8$^+$ T-cell differentiation is unidirectional. During priming, a spectrum of T-cell differentiation states is formed, ranging from stem-cell memory (T_{SCM}) cells, T_{CM} cells, T_{EM} cells, to short-lived effector cells[21]. Cells in the highest differentiation state, which as proposed received the most TCR input during priming, will become short-lived effector cells and die during the contraction phase of the response. Cells that have attained a lower differentiation state after priming persist after antigen clearance and form the memory populations (Figure 1B). Upon antigen re-encounter, the entire spectrum is formed again from the lowly differentiated memory cells. This model implies that upon priming, all CD8$^+$ T cells follow the same differentiation path, just to different extents.

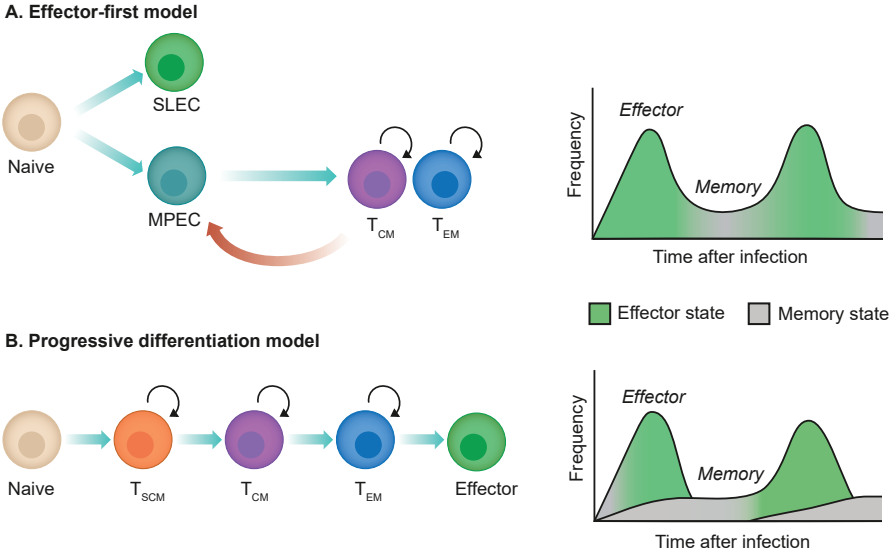

Figure 1. Effector-first vs progressive differentiation model.

While the effector-first model was originally thought to be true, more and more evidence from epigenetic, transcriptomic and TCR tracing studies argues that the progressive differentiation model is the correct one to describe T-cell differentiation[16,22–25]. Instead of the black-and-white subset division of effector and memory populations based on the expression of limited markers such as CD62L or CD44, a further refinement of T-cell differentiation states has been proposed based on proliferative state[26] and expression of additional differentiation markers such as CX3CR1 or KLRG1[27–29]. These studies also point towards a continuum of T-cell differentiation states. TCR input during priming, which is determined both by TCR affinity and antigen abundance at the time of priming, is a key determinant of the differentiation fate of a $CD8^+$ T cell[30–32].

Nowadays the progressive differentiation model is well-established in the $CD8^+$ T-cell differentiation field and supported by many studies. However, the question remains whether this model rules out the possibility of T cell de-differentiation, as suggested by the effector-first model[25]. Early studies describing de-differentiation of $CD8^+$ T cells mainly investigated this by comparing transcriptional and epigenetic properties of phenotypically defined total effector versus memory $CD8^+$ T-cell populations isolated at various stages of the response[33–37]. However, these type of analyses do not prove de-differentiation on a per-cell level, because changes in the total pool can also be explained by differences in outgrowth or maintenance of populations. A more convincing approach is by permanent fluorescent labeling of individual $CD8^+$ T cells that express effector molecules, in order to trace their differentiation fate over time. Using this system, it was shown that a subset of $KLRG1^+$ effector $CD8^+$ T cells could lose their KLRG1 expression and turn into long-lived “ex-KLRG1” memory cells[29]. While these

results can indeed be interpreted as de-differentiation of effector cells into memory cells, they may also reflect the formation of an intermediate population of $CD8^+$ T cells that transiently expresses effector marker molecules but are not stably committed to the effector fate after priming. These cells could still be part of a linear trajectory, highlighting the continuity of the differentiation spectrum. The same principle has been recently shown for the transient downregulation of memory-related genes, such as TCF-1, in a subset of $CD8^+$ T cells[38]. Earlier studies also demonstrated that a subset of $CD62L^-$ effector/T_{EM} $CD8^+$ T cells can re-express $CD62L^+$ upon adoptive transfer into a naïve host[28,39,40]. Taken together, there is evidence for some level of plasticity or "de-differentiation" of $CD8^+$ T cells via transient expression of effector (marker) genes or transient downregulation of memory genes. However, this is likely occurring in intermediate populations within the progressive differentiation spectrum, and does not imply an effector-first fate for all $CD8^+$ T cells.

$CD4^+$ T-cell help during priming shifts the CTL differentiation spectrum

Our results indicate that $CD4^+$ T-cell help during priming improves both effector and memory formation of $CD8^+$ T cells. How does this observation fit within the paradigm of progressive $CD8^+$ T-cell differentiation? We see that $CD4^+$ T-cell help delivered during priming promotes formation of more and better CTL effectors as revealed in the primary response, and formation of more T_{EM} with a more explicit effector-like phenotype, as revealed in the steady-state memory phase. These results are in line with the progressive differentiation model, wherein $CD4^+$ T-cell help shifts the differentiation spectrum towards more effector-like states (Figure 2). This hypothesis is laid out in Chapter 3. In Chapter 4, we provide the experimental evidence for this shift in differentiation as a result of $CD4^+$ T-cell help during priming. We demonstrate that help is required for $CD8^+$ T cells to differentiate past the PD-1^+TCF-1^+ stem-like state towards the KLRG1^+CX3CR1^+ cytotoxic effector state. If $CD4^+$ T-cell help is absent during priming, the so-called ‘helpless’ $CD8^+$ T-cell population displays lower levels of differentiation and cells accumulate mostly in the stem-like state. We also show that even after initial priming, stem-like $CD8^+$ T cells are still able to complete their CTL differentiation when they are provided with antigen and help signals. This indicates that stem-like cells lie within the progressive differentiation spectrum, but need $CD4^+$ T-cell help to progress further along this spectrum.

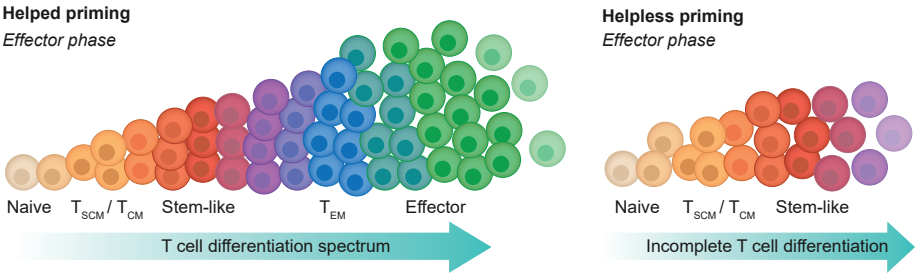

Figure 2. CD4⁺ T-cell help shifts the CTL differentiation spectrum.

Thus, not only TCR input but also costimulatory and cytokine input, including all signals resulting from CD4⁺ T-cell help determine how far CD8⁺ T cells progress along the differentiation spectrum upon priming. Potentially, the total input that determines how far a CD8⁺ T cell progresses on the spectrum, equals the cumulative TCR/costimulatory/cytokine signals from both the first and second priming steps (Figure 3). It is important to consider that even if CD4⁺ T cells are engaged in the response, it does not automatically mean that each CD8⁺ T cell will receive CD4⁺ T-cell help. This depends on the likelihood that a "help" platform on cDC1 is formed, engaging all three cell types. In the Help vaccination setting, we do not see all antigen-specific CD8⁺ T cells becoming terminally differentiated effectors, but rather formation of the entire differentiation spectrum, including low differentiation states as also seen in the No Help setting (Chapter 4). In fact, the effector cells are the only population of which we can safely conclude that have received CD4⁺ T-cell help, because these cells are lacking in the No Help setting. For the other states formed upon vaccination under the Help condition, such as the stem-like cells, we cannot determine whether these cells have actually received CD4⁺ T-cell help during priming or not. Since stem-like cells from Help and No Help are transcriptionally identical (Chapter 4), we propose that these cells have received similar input during priming. This means that stem-like cells generated in the Help vaccination condition are cells that have not (yet) received help. Alternatively, if the eventual differentiation state of a CD8⁺ T cell is determined by the sum of TCR/costimulatory/cytokine signals from both the first and the second priming steps, the relative contribution of these signals may be different between individual cells in the same differentiation state.

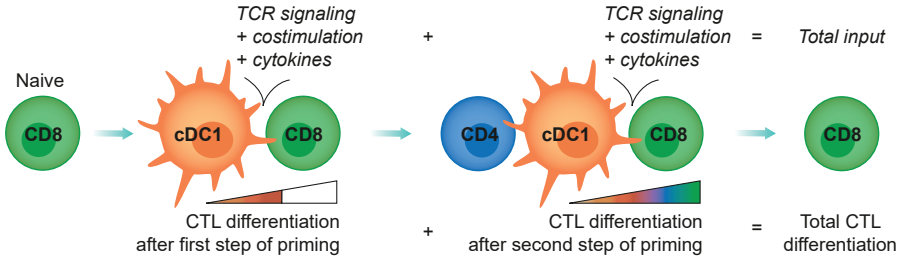

Figure 3. Total CTL differentiation state is determined by input during first + second priming step.

Requirement of CD4⁺ T-cell help for effector and memory CTL responses

Historically, it has been debated whether CD4⁺ T-cell help is always necessary for raising an effective CTL response to pathogens, with the suggestion that helpless priming may also be sufficient in some settings[41]. Early studies showed that primary infections such as by vaccinia virus or acute LCMV can be cleared independently of CD4⁺ T-cell help[42–44], but even in those circumstances there is a difference in helped versus helpless memory formation[43–47]. Of note, the effects of CD4⁺ T-cell help in infection models are typically studied by antibody-based or genetic depletion of the total CD4⁺ T-cell population, including Tregs. Co-depletion of Tregs removes a constraint on CD8⁺ T-cell priming, and thereby likely masks the effects of CD4⁺ T-cell help on the frequency and effector phenotype of primary CD8⁺ T cells, as recently shown in a vaccinia virus infection model[48].

It has been proposed that CD4⁺ T-cell help via CD40 signaling amplifies innate signals to DCs[49], suggesting that in settings with high innate signaling CD4⁺ T-cell help may not be necessary. However, the authors more recently attenuated this model, demonstrating that CD40 signaling can amplify innate signals to DCs, but can in addition induce specific transcriptional programs upon integration of innate signaling by IFN-I[50]. In light of the CTL differentiation trajectory, it can be envisaged that in settings with high levels of antigen and innate signals for DC activation (such as acute infection), the combined TCR/costimulatory/cytokine input CD8⁺ T cells receive during the first step of priming can drive their differentiation along the trajectory. As a consequence, the shift in differentiation between helped and helpless priming may be less pronounced in these circumstances (Figure 4). Thus, the model in which CD4⁺ T-cell help shifts the CD8⁺ T-cell differentiation spectrum further actually fits well with the differential dependence on CD4⁺ T-cell help for effector and/or memory responses in different infection, tumor or vaccination settings, in which the amount of antigen and inflammatory signals that initially drive the differentiation spectrum vary.

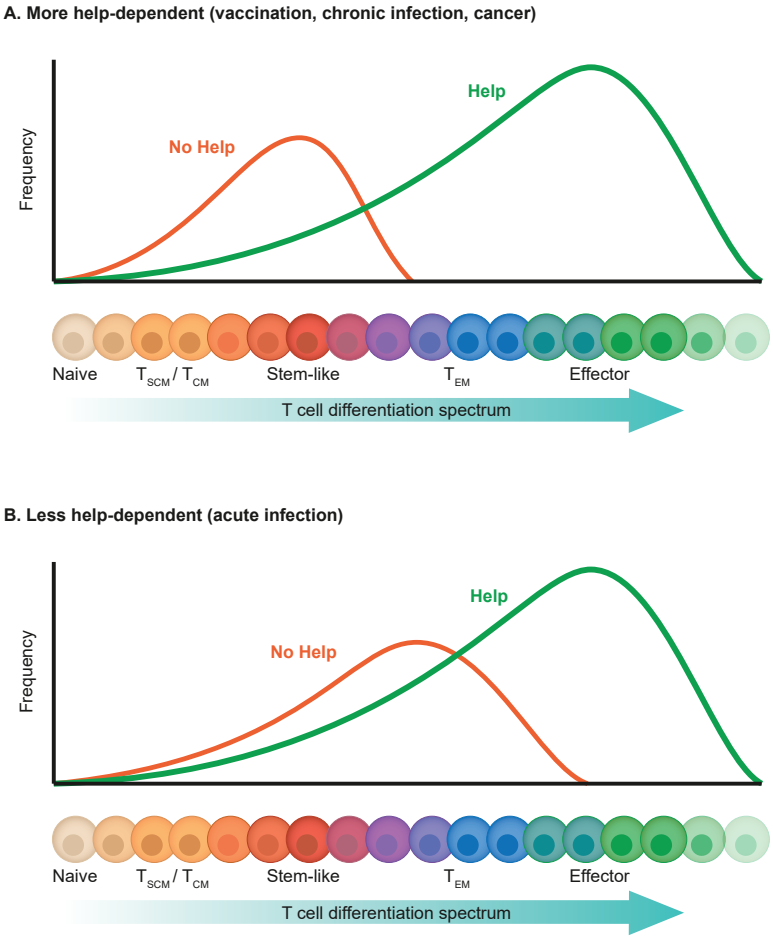

Figure 4. High or low dependence on $CD4^+$ T-cell help is determined by $CD8^+$ T-cell differentiation input during first priming step.

$CD4^+$ T-cell differentiation

In Chapter 5, we focus on $CD4^+$ T-cell differentiation. In type-1 immunity directed against intracellular antigens, such as virus infection or cancer, $CD4^+$ Th1 and Tfh responses are raised in parallel. Th1 cells can deliver help to $CD8^+$ T cells, while Tfh cells can help B cells by promoting isotype class switching and somatic hypermutation. Thus, $CD4^+$ T-cell differentiation coordinates both cellular and humoral immunity. Using our vaccination system, we show that Th1 and Tfh cells are clonally related and are generated from the same uncommitted Th1/Tfh population. Uncommitted Th1/Tfh cells are formed early in the response and express chemokine receptors and transcription factors characteristic for both Th1 and Tfh cells. Interactions with cDC1s or B cells are critical for differentiation of Th1 cells or Tfh cells respectively, but not for initial $CD4^+$ T-cell activation and the generation of the uncommitted Th1/Tfh population. We demonstrate that terminal Th1 differentiation also depends on CD40L-CD40

signaling, which is the main pathway in help delivery from CD4+ T cells to cDC1[51–53]. Thus, in line with earlier observations [54], our data indicate that cDC1-mediated help delivery during the second step of priming is not only crucial for stem-like CD8+ T cells to differentiate into effector CTLs, but also for uncommitted Th1/Tfh CD4+ T cells to differentiate into Th1 cells.

Direct versus indirect effects of CD4+ T-cell help on CTL differentiation

To understand the impact of CD4+ T-cell help on CD8+ T-cell differentiation, it is important to discriminate between direct and indirect effects of responder CD4+ T cells on the total antigen-specific CD8+ T-cell population. In Chapter 2, we demonstrated an increased frequency of T_{EM} cells after helped versus helpless priming. This seems to contrast with results from studies in mouse models of acute infection, where CD4+ T-cell depletion impaired T_{CM} formation and increased T_{EM} formation[46,47,55]. To reconcile these data, it is important to consider that antigen must be cleared to permit the formation of a stable T_{CM} cell population after priming. Accordingly, limiting exposure to cognate antigen improved generation of a CD8+ T_{CM} cell pool under helpless conditions in an allogeneic transplantation model[56]. This principle was also demonstrated in acute infection[47,57]. For example, it was shown that 7-9 weeks after acute LCMV infection under conditions of CD4+ T-cell depletion, the formation of a T_{CM} population was impaired, which was driven by the prolonged presence of antigen[47]. In the same study, the increased frequency of T_{EM} cells within the total memory pool after helpless priming was due to continued presence of antigen for at least 8 weeks. Thus, helpless priming likely resulted in lack of antigen clearance, which impacted T_{EM} formation and/or maintenance. In CD4+ T-cell depleted mice, CTLs are not helped and can therefore less efficiently clear antigen, but help for B cells is also lacking, which will add to antigen persistence driven by impaired antibody-mediated clearance. Due to these effects, CD4+ T-cell depletion in chronic or acute infection models does not allow for a distinction between the direct effects of CD4+ T-cell help during priming on CD8+ T-cell-intrinsic capabilities and the indirect effects of CD4+ T-cell help on CD8+ T-cell differentiation via antigen persistence[9,57]. In our vaccination model, the indirect effect of antigen persistence on memory CD8+ T-cell differentiation does not play a role, because antigen produced in transfected keratinocytes is rapidly lost regardless of the immune response[58,59].

Besides the effect of CD4+ T-cell help on cDC1 licensing, CD4+ T cells may additionally play a direct role in CD8+ T-cell memory maintenance. It has been shown that CD4+ T cells can support CD8+ T-cell responses via the production of IL-21 that acts directly on CD8+ T cells and sustains effector functions during chronic infection[60–62]. During acute infection, IL-21 signaling to CD8+ T cells was dispensable for effector and memory differentiation[63], indicating that the importance of this 'direct' IL-21-mediated help pathway is context-dependent. In our reductionistic Help / No Help vaccination system, vaccine-specific CD4+ T cells are not engaged during steady-state memory. Therefore, improved maintenance of memory CD8+ T cells after Help vaccination results from CD8+ T cell-intrinsic alterations resulting from help during priming.

Tissue-resident memory T cells

Tissue-resident memory T cells (T_{RM}) constitute another T-cell memory population that is not traditionally described as part of the differentiation spectrum, but has recently caught more attention in the cancer immunology field. After antigen clearance, T_{RM} cells differentiate from precursors upon entry in non-lymphoid tissue and exposure to local cues. T_{RM} cells are characterized by expression of the sphingosine-1 receptor inhibitor CD69, and the αEβ7 integrin CD103, which both play a role in tissue retention. They are long-lived, retain high proliferative capacity and exert rapid cytotoxicity upon antigen re-encounter[64]. Epigenetically, T_{RM} cells resemble T_{CM} cells, they have a high plasticity and can give rise to effector cells as well as circulating T_{CM} and T_{EM} cells after restimulation[65–67]. Within the linear $CD8^+$ T-cell differentiation spectrum, T_{RM} cells could therefore be considered as tissue-resident T_{CM} cells. In our vaccination system, we did not specifically investigate the role of $CD4^+$ T-cell help on the priming of T_{RM} $CD8^+$ T cells. However, $CD4^+$ T-cell depletion studies by others showed that $CD4^+$ T-cell help was required for the formation of a lung-resident T_{RM} $CD8^+$ T-cell population after influenza virus infection, and for brain-resident T_{RM} $CD8^+$ T-cell populations after VSV or mouse polyoma virus infections[68,69]. The rate of antigen clearance or quality of priming did not play a role in formation of the T_{RM} $CD8^+$ T-cell pool, but rather, tissue-resident antigen-specific $CD4^+$ T cells produced IL-21 to promote long-term maintenance of $CD8^+$ T_{RM} cells[70,71]. Thus, $CD4^+$ T-cell help for T_{RM} cells seems to be delivered during the steady-state memory phase rather than during priming.

Effects of $CD4^+$ T-cell help on dysfunctional $CD8^+$ T-cell differentiation in cancer

Helpless cells are characterized by impaired functionality and expression of inhibitory receptors such as PD-1, LAG3 and BTLA, but also by expression of the transcription factor TCF-1[12]. This phenotype resembles that of $CD8^+$ T cells found in mouse models of cancer and chronic infection, where they are termed TCF-1^+ stem-like cells, and identified as the precursors of exhausted cells[72]. In Chapter 3, we show the similarity in the transcriptomes of helpless $CD8^+$ T cells raised in our vaccination model and 'predysfunctional' or stem-like $CD8^+$ T cells identified in mouse models of cancer and chronic infection. We propose that stem-like, and eventually also exhausted $CD8^+$ T cells, as found in chronic infection and cancer result from helpless priming. This hypothesis is named the helpless dysfunction model (Figure 5). According to this model, $CD4^+$ T-cell help is needed for formation of the complete $CD8^+$ T-cell differentiation spectrum, including the optimal effector CTL state. These optimal effector CTLs are mainly responsible for antigen clearance, after which $CD8^+$ T cells with lower differentiation states establish memory pools that persist long-term. However, in the case of helpless priming, optimal effector CTLs are lacking, resulting in impaired antigen clearance. The resulting persistent antigen prevents the formation of proper memory pools, but instead drives the formation of exhausted cells from the stem-like population.

In Chapter 4 we provide experimental evidence for the helpless dysfunction model. We show, in line with two-step priming, that under conditions of CD4⁺ T-cell help initially stem-like CD8⁺ T cells are primed, but that these develop in time into effector cells that disseminate systemically. On the other hand, in absence of help, stem-like cells accumulate in the priming lymph node and do not reach the effector state. Moreover, we show that immunogenic MC38 tumors can prime neoantigen-specific stem-like CD8⁺ T cells, but fail to prime neoantigen-specific CD8⁺ T cells with a helped effector phenotype. Instead, stem-like cells in the TME increasingly adopt an exhausted phenotype over time during tumor progression.

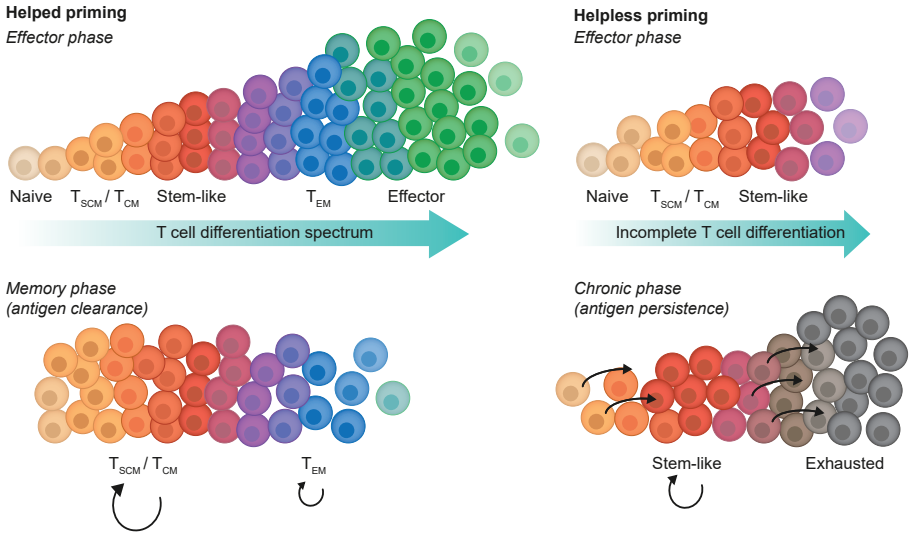

Figure 5. Helpless dysfunction model.

Chronic antigenic stimulation to drive exhaustion

Both aspects that are reportedly needed for CD8⁺ T-cell exhaustion, namely the formation of stem-like cells as well as driving their exhaustion by constant antigen exposure can be explained by helpless priming, according to the helpless dysfunction model. In mouse tumor models, CD8⁺ T cells that do not recognize tumor-specific antigen do not become exhausted, even if they are exposed to the same suppressive TME[73]. Also in human cancer, virus-specific bystander CD8⁺ T cells do not have an exhausted phenotype[74–76]. Thus, chronic antigen exposure is crucial for exhaustion of tumor-specific CD8⁺ T cells, rather than the effects of the suppressive TME. Exhaustion of tumor-specific CD8⁺ T cells can therefore be seen as a reflection of insufficient reduction of tumor antigen burden[77]. In line with this, we do not observe exhaustion of the helpless population in our vaccination model where antigen is quickly removed, but we do see exhaustion in a tumor model where antigen is constantly expressed (Chapter 4).

As discussed above, helpless priming can lead to chronic antigen persistence via lack of help for $CD8^+$ T cells, but probably also via lack of help for B cells. Although they are not as extensively studied as CTL responses, tumor-specific antibodies produced by B cells may contribute to tumor control via various mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or signaling interference on tumor cells[78]. In a mouse lung cancer model, CTL-mediated tumor control was decreased upon depletion of $CD4^+$ Tfh cells or B cells, indicating that these cell types can contribute to anti-tumor effector $CD8^+$ T-cell responses[79]. If tumors are unable to prime $CD4^+$ T helper cells, insufficient $CD4^+$ T-cell help to B cells may impair antibody-mediated antigen clearance, and thereby contribute to chronic antigen persistence that further drives exhaustion of helpless $CD8^+$ T cells. In our tumor model, we did not address this hypothesis, but it would be of interest for further investigation.

$CD8^+$ T-cell exhaustion as a spectrum

In chronic infection models, the $CD8^+$ T-cell differentiation trajectory from stem-like to terminally exhausted has also been described as a continuous spectrum (Figure 6). In early studies, the terms 'stem-like' and 'progenitor exhausted (T_{PEX})' were often used interchangeably, but more recent literature preferably uses stem-like for all TCF-1^+ $CD8^+$ T cells that have self-renewing capacity (including memory cells), while T_{PEX} refers specifically to TCF-1^+ $CD8^+$ T cells that are already on the trajectory towards exhaustion[80,81]. The differentiation spectrum towards terminal exhaustion also includes 'intermediate' or 'transitory' exhausted populations with an effector-like phenotype including expression of T-bet and CX3CR1[82–86]. In chronic LCMV infection, it was shown that transitory cells have cytolytic function and can contribute to viral control[82]. Transitory $CD8^+$ T cells have also been identified in human lung cancer and head and neck cancer patients, where they showed TCR sharing with stem-like and exhausted TIL populations[87,88]. Thus, while they express effector-related gene programs, transitory cells are distinct from bona-fide effector CTLs, and lie on the trajectory resulting in terminal exhaustion. Studies in chronic LCMV infection demonstrated that CX3CR1$^+$ transitory $CD8^+$ T cells raised under $CD4^+$ T-cell-depleted conditions became exhausted upon continuous antigen exposure[82], while CX3CR1$^+$ functional effector CTLs capable of clearing the virus followed a separate differentiation path and could only be raised in presence of $CD4^+$ T cells[9]. These results fit with our helpless dysfunction model (Chapter 3, 4), in which the presence of $CD4^+$ T-cell help during priming is required for stem-like cells to further differentiate into proper effector CTLs, but not for their differentiation towards exhausted cells (Figure 6).

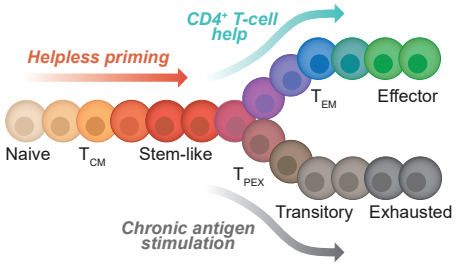

Figure 6. Stem-like CD8⁺ T cells at the branchpoint of CD8⁺ T-cell differentiation trajectories towards the functional effector (dependent on CD4⁺ T-cell help) or terminally exhausted state (driven by chronic antigen stimulation).

Localization of stem-like and exhausted populations

Stem-like CD8⁺ T cells in cancer were first identified as a small subset in the TME, but have later been discovered to mainly reside in TdLN, from where they travel to the tumor to sustain the short-lived exhausted population[89–91]. In accordance with this, we found that in our vaccination setting stem-like/helpless cells preferentially localized to the dLN (Chapter 4). While helped effector CD8⁺ T cells were able to leave the dLN into the circulation, stem-like cells were found to be mainly retained at the priming site. This is in line with a recent study showing that Notch signaling as part of the CD4⁺ T-cell help program is required for LN egress of CD8⁺ T cells after priming (Laurent *et al.*, manuscript in preparation). In mouse tumor models, stem-like and T_{PEX} cells present in the TdLN displayed a less exhausted phenotype than T_{PEX} cells in the TME[92,93], which agrees with the proposed spectrum of exhausted T-cell states. Stem-like cells in the TdLN, but not in the TME, have been suggested to be bona-fide memory cells, which maintained a memory phenotype when shielded from antigen exposure[92]. The same has been suggested for stem-like cells in chronic LCMV infection[10]. These views are in line with our data from the vaccination setting, where stem-like cells are mapped within the linear differentiation spectrum (Chapter 4).

In our tumor experiments using MC38 implantation in mice, we also observed a higher expression of PD-1 and TOX in stem-like cells from the TME compared to stem-like cells from the TdLN. In agreement with literature data, exhausted CD8⁺ T cells were found only in the TME, and not in the TdLN (Chapter 4). We also observed a higher frequency of stem-like cells in TME at an early timepoint and more exhausted cells at a later timepoint, in line with stem-like cells becoming progressively exhausted over time.

Impaired CD4⁺ T-cell help in cancer

In mouse tumor models, CD4⁺ T-cell help is often assumed to be in place unless CD4⁺ T cells are depleted. Recently, it was shown that CD4⁺ T-cell depletion in models of pancreatic cancer did not affect the frequency or phenotype of tumor-specific CD8⁺ T cells[94]. While the authors concluded that the anti-tumor CTL response occurred independently of CD4⁺ T-cell help, this data also supports the alternative hypothesis that tumor-specific CD8⁺ T cells raised in these models were

the result of helpless priming, regardless of whether CD4$^+$ T cells were depleted. In a squamous cell carcinoma model, providing help by adoptive transfer of tumor-specific CD4$^+$ T cells improved anti-tumor CTL responses[95], supporting that spontaneously raised tumor-specific CD8$^+$ T cells in this model lacked sufficient help. Taken together, these findings suggest that raising anti-tumor CD8$^+$ T-cell responses in mice with an intact CD4$^+$ T-cell population does not automatically result in helped CTLs. Our experimental results confirm this hypothesis and show that even in mice that are not depleted of CD4$^+$ T cells, immunogenic MC38 tumors fail to induce priming of helped effector CTLs (Chapter 4). We propose that the occurrence of stem-like, and eventually exhausted CD8$^+$ T cells in cancer can be explained by these tumors inducing helpless priming. With this model, the most important question that comes up is: Why would help be lacking in cancer settings?

There are probably two main reasons for this. Firstly, tumors may lack the expression of immunogenic MHC-II restricted epitopes necessary to raise tumor-specific CD4$^+$ T-cell responses. Potential neoantigens presented by a tumor can be predicted *in silico*, based on genetic profiling of the tumor and the calculated ability of mutated peptides to bind to MHC-I or MHC-II. Since most tumors types do not express MHC-II, testing the presence or immunogenicity of predicted neoantigens is often focused on MHC-I epitopes. As a result, less is known about the immunogenicity of MHC-II-restricted neoantigens, and the contribution of spontaneously raised CD4$^+$ T helper cells in anti-tumor immunity[96,97].

Secondly, the immunotolerant or suppressive TME may lead to impaired DC activation for the priming of helper CD4$^+$ T-cell responses, or impaired cDC1-mediated delivery of CD4$^+$ T-cell help to tumor-specific CD8$^+$ T cells[98]. In Chapter 4 we show that expression of strong helper epitopes by MC38 cells raised a tumor-specific CD4$^+$ T-cell response, but could not rescue the formation of tumor-specific CD8$^+$ T cells with a helped effector phenotype. Our results suggest that help for CD8$^+$ T cells is impaired in the MC38 model regardless of the formation of a tumor-specific CD4$^+$ T cell pool, which is in line with a previous study where MC38 tumors raised an antigen-specific, but dysfunctional CD4$^+$ T-cell response[99]. The absence of inflammatory signals and insufficient DC activation at early stages of tumorigenesis can induce priming of thymus-derived (t)Tregs, or steer differentiation of neoantigen-specific CD4$^+$ T cells towards peripherally induced (p)Tregs, which in turn contribute to the suppressive environment as tumors progress[100]. In this way, CD4$^+$ T-cell responses against a tumor can be impaired, even if MHC-II-restricted antigens are present. For example, it was recently demonstrated that tumor-specific CD4$^+$ T cells in a mouse prostate cancer model displayed a TCF-1$^+$ stem-like phenotype, and their differentiation into Th1 cells was impaired by Tregs. When Tregs were depleted, Th1 differentiation and CTL-based tumor control was improved[101]. In another recent study, proximity-dependent labeling in a mouse melanoma model showed that interactions between tumor-specific CD4$^+$ T cells and DCs in the TdLN decreased over the course of tumor progression[102]. In the same tumor model, the activation of cDC2s in the TME was inhibited by Tregs, leading to suboptimal priming of CD4$^+$ T cells in

the TdLN[103]. Also in a breast tumor model, CTLA4-mediated suppression by Tregs impaired proliferation and differentiation of tumor-specific CD4$^+$ T helper cells in the TdLN. Over the course of tumor progression, tumor-specific CD4$^+$ T cells acquired FOXP3 expression, indicative of Treg induction[99]. These findings suggest that tumors prime insufficient or dysfunctional CD4$^+$ T-cell responses, thereby leading to impaired CD4$^+$ T-cell help to CTLs.

In Chapter 6, we describe another possible underlying mechanism for impaired help in cancer, namely a lack of IFN-I signals in the TME. IFN-I is a crucial signal for initial cDC1 activation but also for delivery of CD4$^+$ T-cell help. Supported by current literature, we present an integrated model how IFN-I signals optimize CD4$^+$ T-cell help delivery by increasing cDC1 survival, antigen presentation on MHC-I and MHC-II, and expression of costimulatory molecules and cytokines. IFN-I also plays an important role in orchestrating the spatial localization of primed CD8$^+$ and CD4$^+$ T cells and cDC1s within the LN via the induction of chemokines, and formation of the help delivery platform. However, tumors often represent a IFN-I-poor environment, via suppression of IFN-I production by tumor cells or dysfunction of tumor-infiltrating immune cells. We propose that the lack of IFN-I signals in the TME could be an important underlying mechanism for the lack of CD4$^+$ T-cell help to tumor-specific CD8$^+$ T cells.

Help delivery in cancer

There are many different cancer types with different immune cell compositions and in certain types help delivery may take place in the TdLN and/or in the TME. For example, neoantigen-specific CD4$^+$ T cells were identified in human melanoma and correlated with improved CD8$^+$ T-cell activation and clinical responses[104–106]. In human kidney cancer, presence of CD4$^+$ Th1 cells in the TME or TdLN correlated with higher frequencies of effector CD8$^+$ T cells and response to immune therapy[101]. Intratumoral interactions between DCs, CD4$^+$ T cells and CD8$^+$ T cells, so-called 'triads', were found to be enriched in hepatocellular carcinoma and malignant plural mesothelioma patients that respond to ICB therapy[107,108]. Also tertiary lymphoid structures (TLS), which are more complex immune structures in close localization to the tumor where DCs and T cells colocalize, have been associated with better prognosis and response to ICB therapy in various human cancers[64,109]. These studies suggest that tumor-specific T cells can be primed or recurrently activated by APCs within intratumoral triads or TLS upon ICB therapy. They also suggests that the CD4$^+$ T-cell help scenario may play out within or close to the TME. However, to which extent this process happens spontaneously in treatment-naïve tumors remains to be investigated.

In recent years, efforts have intensified to characterize TIL populations or overall immune cell composition by transcriptome analysis across multiple types of human cancer. The aim in these studies is to identify overarching T-cell states, tumor immune archetypes or carcinoma ecotypes[8,110–112]. These pan-cancer analyses provide a wealth of information and may also be used to assess the presence or absence of spontaneous CD4$^+$ T-cell help in different TME types.

For example, a meta-analysis of about 6000 tumors representing 16 solid cancer types identified a specific carcinoma ecosystem (CE9) with co-localization of $CD8^+$ T cells, $CD4^+$ T cells and cDC1s, which were associated with the highest overall survival and response to PD-1 blockade therapy[110]. Among different ecosystems, CE9 contained the gene expression signature of helped human cDC1s[113], suggesting that in this type of TME help delivery takes place.

Is helped priming sufficient to eliminate a tumor?

Whether helped priming is necessary or sufficient to control tumor growth likely depends on the immunogenicity of the tumor. The total effect of the anti-tumor CTL response is determined by both the absolute numbers of raised $CD8^+$ T cells and their cytotoxic efficacy. In a highly immunogenic tumor setting which spontaneously raises a large and varied population of tumor-specific $CD8^+$ T cells, helpless priming of these cells may already be sufficient for tumor control, particularly at an early stage of tumorigenesis. For example, it was shown that poorly immunogenic PDAC-OVA tumors injected into the pancreas, but not highly immunogenic PDAC-OVA tumors injected subcutaneously, were dependent on $CD4^+$ T-cell help for tumor rejection[114]. In less immunogenic tumor settings, even helped CTL priming may not be sufficient to control tumor outgrowth, despite $CD4^+$ T-cell help improving the number and efficacy of the CTLs raised. For example, in a tumor model where helped $CD8^+$ T-cell responses were not sufficient for primary tumor control, they could protect against a secondary tumor after resection of the first[54]. Similar to infection, the amount of TCR/costimulatory/cytokine input tumor-specific $CD8^+$ T cells receive during first step of priming probably dictates to which extent the primary CTL response to the tumor relies on $CD4^+$ T-cell help.

It is important to realize that helped priming does not solve all problems regarding the required efficacy of tumor-specific CTL responses, since suppressive mechanisms in the TME may still impair helped CTL responses. Suppressive mechanisms include anti-inflammatory cytokines such as TGF-β or IL-10, inhibitory metabolites, limited nutrient availability and the presence of suppressive cells such as Tregs[115]. Tumor cell-intrinsic escape mechanisms may also occur, such as loss of MHC-I epitopes, downregulation of MHC-I or dysregulation of the antigen processing and presentation (APP) pathway[116]. Tumor cells that lose expression of an MHC-I epitope could in theory still induce help for priming of CTLs specific for other MHC-I restricted epitopes, as long as tumors retain expression of immunogenic MHC-II epitopes. Since MHC-II epitopes are presented by APCs rather than (generally) by the tumor cells themselves, MHC-II epitope loss from the tumor may be limited[96]. However, loss of MHC-II epitopes by tumor cells may indirectly lead to less efficient killing via the priming of helpless CTLs.

Clinical implications of the helpless dysfunction model

In the past decade, advances in immunotherapy have revolutionized cancer treatment, but clinical responses are still highly variable between patients. In order to improve immunotherapy strategies or to select the best immunotherapy regimen for different groups of patients, it is crucial to gain a better understanding of the various types of immune states or TMEs that can surround a tumor. In recent years, several large meta-analyses have been performed to profile different TME compositions across cancer types, in order to identify overlapping immune 'ecotypes' or 'archetypes' that can be used to guide immunotherapy strategies[8,110–112], but the presence or absence of $CD4^+$ T-cell help within these types of TMEs is not generally considered. Understanding $CD4^+$ T-cell help in cancer is important to make rational decisions on immune therapy, whether it is immune checkpoint blockade (ICB), adoptive cell therapy (ACT), or therapeutic vaccination.

$CD4^+$ T-cell help for ICB therapy

In Chapter 7, we link our findings to the mechanism of PD-(L)1 blockade therapy. Studies in mouse models of chronic LCMV infection[4–6] and cancer [117–119] have demonstrated that PD-(L)1 blockade acts on stem-like $CD8^+$ T cells by inducing their proliferation. We clarify the role of PD-1 signaling on the T-cell-cDC interface during priming in the LN, where it regulates TCR signaling and CD28 costimulation. We also outline several studies proving that the therapeutic effect of PD-(L)1 blockade is due to alleviating PD-1 mediated inhibition at the T cell-cDC1 interface and promoting CTL priming, rather than at the CTL/tumor cell interface and promoting CTL-mediated killing. In $CD4^+$ T-cell depleted mouse models of chronic LCMV infection, PD-(L)1 blockade drives expansion but also exhaustion of stem-like $CD8^+$ T cells, and is not sufficient to induce the differentiation of $CX3CR1^+$ effector CTLs[6,9]. In light of the helpless dysfunction model (Chapter 3, 4), we hypothesize in Chapter 7 that PD-(L)1 blockade transiently activates helpless/stem-like $CD8^+$ T cells. We suggest that PD-(L)1 blockade alone is not sufficient to completely 'mimic' or replace $CD4^+$ T-cell help signals that are needed for optimal effector differentiation.

However, recent data have led us to a somewhat different view. In melanoma patients, an increased frequency of $TCF\text{-}1^+$ stem-like $CD8^+$ TILs positively predicts clinical response to PD-1 targeted therapy[117,120]. More recent studies in various types of human cancer show that response to PD-(L)1 blockade therapy is associated with so-called 'triads' or 'immunity hubs' in the TME where T cells and DCs interact[107,108,121,122]. Upon PD-1 blockade therapy, breast cancer lesions showed an increase of $PD\text{-}1^+CXCL13^+$ $CD4^+$ T cells, which displayed a gene signature similar to tumor-specific $CD4^+$ T cells interacting with DCs in the TME or TdLN in mouse tumor models[121,123]. $CXCL13^+$ CD4 T cells in the TME showed expression of both Th1 and Tfh-related genes[107,123], which may suggest an uncommitted phenotype such as described in Chapter 5. In the TME of hepatocellular cancer patients that responded to PD-1 blockade, interactions were observed between stem-

like PD-1$^+$TCF-1$^+$ CD8$^+$ T cells, CXCL13$^+$ CD4$^+$ T cells and DCs with an activated phenotype that also express PD-L1[107]. Importantly, these so-called mature regulatory DCs (mregDCs)[124] shared their transcriptional signature with helped cDC1s as recently shown by our research group[113]. The presence of the helped cDC1 signature in melanoma patients positively correlated with response to PD-1 blockade therapy[113]. Taken together, these studies indicate that the hypothesis that PD-(L)1 blockade therapy acts on stem-like CD8$^+$ T cells by partially substituting for help signals, as described in Chapter 7, is probably incomplete. In addition, successful PD-(L)1 blockade therapy likely works via activating helper CD4$^+$ T cells in the TdLN and/or TME, allowing the CD4$^+$ T-cell help scenario to take place. This model is supported by clinical data from lung cancer, where CD8$^+$ T-cell proliferation and clinical response upon PD-(L)1 blockade was only observed in patients harboring functional systemic CD4$^+$ T-cell responses before treatment[125].

Fewer studies have focused on the role of CD4$^+$ T-cell help in CTLA-4 blockade therapy. CTLA-4-mediated suppression via downregulation of CD80/CD86 on APCs is a key suppressive mechanism of Tregs[100]. Some studies suggest that clinically effective tumor control by CTLA-4 blockade therapy may be achieved via depletion of Tregs rather than blocking CTLA-4-CD80/CD86 interactions[126–128]. Removing CTLA-4-mediated suppression during CD8$^+$ T-cell activation in the LN allows for CD28 costimulation and thereby to improved CTL priming. Recent studies in mouse tumor models also suggest a role for CD4$^+$ T-cell help during this scenario. For example, CTLA-4 expression on Tregs but also on tumor-specific CD4$^+$ T cells themselves was shown to mediate dysfunction of tumor-specific CD4$^+$ T cells, which resulted in impaired CD4$^+$ T-cell help delivery and tumor control. Treg depletion or CTLA-4 blockade resulted in increased numbers of cDC2s in the TdLN and higher expression of CD80 and CD86 on these cells, indicating that Tregs and CTLA-4 induce dysfunction of tumor-specific CD4$^+$ T cells by limiting CD28 signals for their activation[99]. These results are in line with an earlier study, showing that Treg-mediated suppression of cDC2s leads to suboptimal CD4$^+$ T-cell priming[103]. Using proximity-dependent labeling, it was recently shown that CTLA-4 blockade induced higher frequencies of cDC2s and more interactions between DCs and CD4$^+$ T cells in the TdLN and the TME[102]. Thus, CTLA-4 blockade may promote CD4$^+$ T-cell activation and subsequent help delivery by limiting Treg-mediated suppression of cDC2s.

CD4$^+$ T-cell help for ACT

ACT is a personalized cancer therapy that relies on the administration of tumor-specific CD8$^+$ T cells to a patient. These cells can be *ex vivo* expanded TILs, or T cells from blood that are genetically modified to express a tumor-specific TCR or chimeric antigen receptor (CAR). The persistence and anti-tumor activity of CD8$^+$ T cells used for ACT is highly dependent on their differentiation status. Clinical studies have shown that ACT using less differentiated CD8$^+$ T cells is associated with prolonged T-cell persistence and improved therapeutic control, while more differentiated effector-like CD8$^+$ T cells are more prone to become dysfunctional[129]. The role of CD4$^+$ T-cell help for the differentiation of tumor-specific CD8$^+$ T cells is not widely considered in

the ACT field, although there are clinical data showing that adoptive transfer of tumor-specific $CD4^+$ T cells along with $CD8^+$ T cells increases treatment efficacy[130,131].

In CAR-T cells, variable regions of the antibody heavy and light chains are linked to the intracellular signaling chain of CD3ζ and additional costimulatory domains such as CD28 or 4-1BB, which results in MHC-I-independent activation of CAR-T cells upon binding to surface antigens[132]. Thus, CAR-T cells are not activated by cDC1s in the way endogenous $CD8^+$ T cells are, and likely do not receive cDC1-mediated $CD4^+$ T-cell help. In solid tumors, CAR-T cell therapy has demonstrated low clinical efficacy, which is mediated by various factors such as low tumor infiltration, poor T-cell persistence, and CAR-T cells acquiring an exhausted phenotype[133]. These characteristics indeed match those of helpless $CD8^+$ T cells. Thus, understanding the molecular mechanisms underlying $CD4^+$ T-cell help delivery, and incorporating relevant costimulatory domains in CAR design to 'mimic' help signals, may improve CAR-T cell efficacy. For example, adding CD27 to CD28/4-1BB costimulation domains in CAR-T cells has recently been shown to enhance CAR-T cell persistence and tumor control in a xenograft mouse model, while reducing expression of exhaustion markers[134].

$CD4^+$ T-cell help for therapeutic vaccination

A possible strategy to overcome helpless priming in cancer is to specifically raise or activate $CD4^+$ T-cell responses along with $CD8^+$ T-cell responses, for example via vaccination. Several studies have demonstrated that the inclusion of helper epitopes improves vaccine efficacy against tumors[135–139]. Our research group previously showed that in a TC-1 tumor model, which expresses HPV-E7, vaccination with E7-Help but not with E7-No Help resulted in tumor clearance[13]. In these vaccines, which are also used in Chapter 2 and Chapter 4, MHC-II restricted T helper cell epitopes P30 and PADRE are E7-unrelated. Help can be delivered because upon vaccination, MHC-II restricted helper epitopes and MHC-I restricted E7-derived CTL epitope are expressed on the same transfected keratinocyte[59] and can therefore be presented on the same DC. In peptide vaccination studies, vaccination with coupled MHC-I and MHC-II epitopes raised more potent anti-tumor $CD8^+$ T-cell responses than combined vaccination with separate epitopes[140,141]. Also tumor cells expressing both MHC-I and MHC-II restricted epitopes induced stronger anti-tumor $CD8^+$ T-cell responses and better protection than tumors consisting of mixed cells expressing MHC-I or MHC-II epitopes only[108,142]. Taken together, these findings indicate that for therapeutic vaccination, both helper and CTL epitopes should be presented on the same DC in order to facilitate $CD4^+$ T-cell help delivery.

Most therapeutic vaccination strategies aim to raise both $CD4^+$ and $CD8^+$ T-cell responses by vaccination. Another strategy is to raise $CD4^+$ T cells by vaccination that can deliver help to endogenous $CD8^+$ T cells primed by the tumor. For example, neoantigen-specific $CD4^+$ T cells raised by vaccination could help $CD8^+$ T cells against tumor antigens not included in the vaccine in a mouse model of multiple myeloma[143]. Our own experiments so far suggest that vaccinating

tumor-bearing mice with tumor-unrelated helper epitopes does raise a CD4+ T-cell response, but does not effectively deliver help to tumor-primed CD8+ T cells. This is probably because the MHC-I epitopes from the tumor and the MHC-II epitopes from the vaccine are not presented by the same DC for help delivery. This is in line with studies reporting improved protection against tumor outgrowth by tumor-specific rather than tumor-unrelated memory CD4+ T cells, effects that depended on CD4+ T-cell help delivery to CTLs[95,137,144]. When tumor-specific helper epitopes are used for vaccination, it is more likely that DCs presenting tumor-derived antigens can benefit from the help of tumor-specific CD4+ T cells raised by the vaccination. However, tumor-specific helper epitopes may be weakly immunogenic and fail to induce CD8+ T-cell effector differentiation (Chapter 4). In this thesis, we focus on the effect of CD4+ T-cell help on differentiation of CTLs, however direct cytotoxic effects of CD4+ T cells could also contribute to tumor control in case of MHC-II expression by the tumor[145,146]. Alternatively, cytokines released by tumor-specific CD4+ T cells can act on tumor cells or immune cells in the TME, which may also contribute to anti-tumor immune responses[97,147].

A downside of using tumor-specific help for therapeutic vaccination is the challenge to select the right MHC-II neoepitopes to include in the vaccine. In several clinical trials using vaccination with neoantigen peptides, predominant CD4+ T-cell responses were observed without clinical benefit[148–150]. In contrast to mouse studies, where immunogenicity of neoepitopes can be tested, most clinical trials use only prediction algorithms, which may result in selection of suboptimal epitopes, especially for MHC-II[138]. Another possibility is that vaccination with tumor-specific MHC-II epitopes raises Tregs rather than helper CD4+ T cells, when vaccine-induced CD4+ T cells are converted into Tregs under tolerogenic or immune-suppressive conditions in the TME. Tumor-specific Treg induction as a result of therapeutic vaccination has been reported in preclinical and clinical studies[151–153] and was also observed in our experiments. These results underline the importance of selecting optimal administration routes and immune-stimulating adjuvants to induce proper DC activation and T-cell priming upon therapeutic vaccination[154].

Overall, the helpless dysfunction model provides a new perspective on the mechanism of T-cell priming in cancer, and understanding this mechanism is crucial to make rational decisions on novel or combination immune therapies. Gene expression signatures of helped or helpless CD8+ T cells could be used for immune profiling of tumors, in order to stratify patients for immune therapy treatment.

Concluding remarks

In summary, this thesis provides experimental evidence for a new conceptual model on $CD8^+$ T-cell differentiation, by showing that $CD4^+$ T-cell help shifts the CTL differentiation spectrum towards a more effector-like state. More importantly, it identifies helpless priming as underlying mechanism for the occurrence of stem-like and eventually exhausted $CD8^+$ T cells in cancer. These findings offer a new perspective to re-interpret studies on tumor-specific $CD8^+$ T-cell differentiation, and highlight the potential of stem-like/helpless $CD8^+$ T cells to still complete their CTL differentiation when provided with help signals. Presence or absence of $CD4^+$ T-cell help is an important aspect to consider as part of immune profiling of tumors, to tailor immune therapy for cancer patients.

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