In search of a master regulator of cytotoxicity: Transcriptional control of cytolytic effector function in human lymphocytes
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Citation for published version (APA):
Hertoghs, K. M. L. (2016). In search of a master regulator of cytotoxicity: Transcriptional control of cytolytic effector function in human lymphocytes

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The immune system

The immune system protects the host from invading pathogens such as bacteria, parasites and viruses. It comprises of two branches: the innate and the adaptive immune system. The innate immune system is the first line of defense against infection and immediately responds to pathogens in an antigen non-specific manner. Innate reactions do not confer long-lasting or protective immunity to the host. Natural Killer (NK) cells participate in the innate defense mechanisms. Rather than directly killing invading pathogens, they destroy compromised host cells. Tumors and intracellular microbial infections may induce downregulation of cell surface MHC (Major Histocompatibility Complex) molecules, a condition known as ‘missing self’. NK cells monitor all cell surfaces and directly display cytolytic activity when no MHC molecules are detected.

Adaptive immunity allows the vertebrate immune system to specifically recognize and remember microbes, preparing the host for future challenges. V(D)J recombination enables lymphocytes to generate a vast number of antigen receptors from a limited amount of antigen receptor gene segments that allows the lymphocytes to ‘adapt’ to specific pathogens.

CD8+ T-lymphocytes, which belong to the adaptive immune system, recognize infected cells or antigen presenting cells (APCs) that display pathogen-derived peptides by MHC class I molecules on their cell surface. The primed CD8+ T cells can halt the viral replication and/or destruct infected cells by directly lysing target cells and secretion of soluble anti-viral factors. The emergence of sufficient numbers of CD8+ T cells with effector functions at the start of a primary infection takes several days and therefore forms a second line of defense after the immediate response of the innate immune cells. These cytotoxic T lymphocytes offer a sophisticated manner to recognize and combat viral infection and are able to clear viruses that cannot be resolved by innate immune cells alone. After the pathogen is cleared, the CD8+ T cells provide immunological memory for the specific pathogen.

CD8+ and NK cells share many similarities in components of the system that allows killing of target cells. Furthermore, NK cells can signal to cells of the adaptive immune system such as CD8+ T cells, by the release of cytokines.

This chapter will describe the differentiation pathways of antigen-induced CD8+ T cells and summarizes how cell extrinsic factors regulate their development. In addition, we will focus on the transcription factors that play a major role in CD8+ T cell differentiation. TF play center stage in the process of CD8+ T cell maturation because they bind to specific DNA sequences and thereby control the transcription of specific genes that are necessary to become fully functional effector cells.

Stages of the CD8+ T cell response

When naïve cells differentiate upon antigen encounter, roughly four distinct stages can be distinguished. During phase 1 naïve T cells undergo activation by stably interacting with mature DCs that present antigen (Ag) in the context of the appropriate co-stimulatory signals. Extensive proliferation follows in phase 2 (‘the expansion phase’) in which the Ag-specific T cells may expand more than 10,000 fold. This phase lasts for several days,
in which the gene expression profile is reprogrammed. Changes in chromatin accessibility and induction of transcription factors lead to the formation of different subtypes of CD8\(^+\) T cells. The pool of cells that arises, the ‘effector cells’, can clear a typical viral infection within days. Subsequently, in stage 3 the ‘contraction phase’, the majority of the effector cells that have cleared the virus die and the cell pool contracts until usually >90% of the cells are eliminated by apoptosis in the following week, a process governed by Bcl-2 and Bim\(^6\). At the onset of contraction the expression of anti-apoptotic protein Bcl-2 is diminished, and restoring of these levels inhibits the death of effector cells\(^7\). Lowering of anti-apoptotic factors is followed by an increment of Bcl-2 family member Bim, a pro-apoptotic protein, to induce cell death. In agreement with a central role for Bim in the contraction of the effector T cell pool, Bim deficiency leads to accumulation of activated T cells after for example HSV infection\(^8\). In the final ‘memory’ stage cells are maintained that provide a faster and more vigorous response upon re-challenge with the same pathogen and can guard the body throughout life against infection\(^9\). For the maintenance of memory cells antigen persistence is not required\(^10\). After clearance of the pathogen during acute infection, memory cells can be maintained by the cytokines IL7 and IL15 that deliver survival and proliferation signals\(^11\).

### Markers that distinguish CD8\(^+\) subsets

In mice two receptors, IL7Ra and KLRG1, have proven to be useful markers to distinguish effector subsets known as short lived effector cells (SLECs) and memory precursor effector cells (MPECs) that will generate the memory pool. The alpha chain of the IL7 receptor is almost uniformly expressed on naïve cells\(^12\). However, during primary infection, at the peak of T cell expansion most cells have downregulated their IL7Rα to become SLECs while a small percentage of Ag-specific T cells retains their IL7Rα and will form the memory population\(^13-16\). The inhibitory NK cell receptor Killer-cell Lectin-like Receptor G1 (KLRG1) is inversely expressed, expression being high on SLECs and low on MPECs. However, it is important to note that this distinction is not absolute, since KLRG1\(^+\)IL7Rα\(^-\) cells can persist after infection, while not all KLRG1\(^-\)IL7Rα\(^+\) cells will survive and become memory cells\(^17\).

During resting conditions a subdivision of various CD8\(^+\) subsets is made based on the expression of the lymph node homing marker CD62L and CD44 to distinguish naïve (CD44 CD62L\(^+\)), effector-memory (T\(_{EM}\), CD44\(^+\)CD62L\(^-\)) and central memory (T\(_{CM}\), CD44\(^+\)CD62L\(^+\)) cells. Upon reactivation, T\(_{EM}\) cells directly produce effector cytokines like Interferon-γ (IFNγ) and cytotoxic molecules, while T\(_{CM}\) cells proliferate rapidly and can give rise to newly formed effector cells. Although T\(_{CM}\) show low cytotoxicity and secrete IL2 but no IFNγ, the cytolytic potential is not completely abolished. A discrimination can be made between T\(_{EM}\) and T\(_{CM}\) based on CCR7 expression, a receptor that allows lymphocyte homing to secondary lymphoid organs\(^18\). T\(_{CM}\) that are CCR7 positive, home to lymphoid structures such as lymph nodes, while the CCR7 negative T\(_{EM}\) mostly reside in non-lymphoid tissues and mucosal sites. While the distinction between these groups of memory cells is widely used, it is still a matter of debate if there is indeed a sharp demarcation in immediate effector function between these memory cells, both in mice and human T lymphocytes\(^19,20\).
Recently, a novel type of non-circulating memory T cells was found that provides superior local immunity, resident memory T cells (T\textsubscript{RM}). These pathogen-specific T cells guard the mucosal areas of the gut, skin or lungs and undergo migrational imprinting within the draining lymph nodes, which results in memory CD8\textsuperscript{+} T cells that provide local and systemic protection mediated by GzmB (reviewed in 21-23). The majority of the T\textsubscript{RM} express CD103 which sustains their residency at barrier surfaces. Upon resolution of infection these cells retain CD69 expression and maintain their cytolytic capacity\textsuperscript{24}. To fully understand the distinct differentiation pathways of memory cells and the range of phenotypes and functions that arise, additional markers may be helpful to further make distinctions within the T cell pool\textsuperscript{25}.

In humans, other phenotypic markers are often utilized to separate CD8\textsuperscript{+} T cell subsets. CD45RA and CD27 provide good discriminatory properties to distinguish naïve (CD45RA\textsuperscript{+}CD27\textsuperscript{+}) cells from resting effector-type and memory-type cells (CD45RA\textsuperscript{-}CD27\textsuperscript{+})\textsuperscript{26}. Resting effector-type cells are distinct from effector cells during primary infection. Acute effector cells are mostly CD45R0\textsuperscript{-}CD27\textsuperscript{+}, and have high levels of Ki-67 expression reflecting rapid proliferation\textsuperscript{20,27}. Activation markers like HLA-DR and CD38 are expressed on the cell surface of acute effector cells and high levels of IFN\gamma and cytotoxic molecules such as Granzyme B (GzmB) and Perforin are produced by these cells.

When compared to naïve cells, memory type cells are more sensitive to antigenic stimulation, are less dependent on co-stimulation and show higher expression of CD40L after activation, which provides a more effective feedback to the APC. These cells have little or no effector function, but upon T cell receptor (TCR) triggering these cells show rapid clonal expansion and give rise to effector cells that produce large quantities of IFN\gamma and cytotoxic molecules. During both acute and chronic infection, memory cells do not express activation markers (e.g. CD38, HLA-DR, Ki67).

Based on CD27 and CD28 expression, memory cells that arise after chronic infection can be subdivided in early, intermediate or late phenotypes, in which sequential downregulation of CCR7, CD28 and CD27 is accompanied by upregulation of cytotoxic factors. For example, most memory CD8\textsuperscript{+} T cells responding to Hepatitis C (HCV) or Epstein Barr virus express CCR7, CD27 and CD28 and have a strong ability to proliferate, but have low cytotoxic potential\textsuperscript{19}. In contrast, human cytomegalovirus (CMV) has a different impact on CD8\textsuperscript{+} differentiation, in that a large stable pool of resting effector cells arise that functionally resemble the effector cells found in acute infection\textsuperscript{28,30}. Unlike their memory counterparts these vigilant effector cells contain Perforin, Granzyme B (GzmB) and CD95L to directly execute cytolysis\textsuperscript{26}. An important difference between acute effector and resting effector cells is that while the acute effector cell pool contracts after viral clearance, large numbers of resting effector cells are maintained in a chronic setting in the absence of detectable viral load\textsuperscript{31}.

**Signals required for optimal CTL programming**

Antigen presented by APC stimulates naïve cells through the T cell receptor together with CD28 and other co-stimulatory receptors such as ICOS, CD27 and 4-1BB\textsuperscript{32}. These activation signals can induce several rounds of cell division, but are not sufficient to induce fully
functional CTLs. Although primed, these cells fail to undergo further clonal expansion, do not develop cytolytic effector functions, have a poor survival rate and persistent cells become tolerant. Upon Toll-like Receptor engagement or interaction with CD4+ helper T cells, DCs mature and subsequently produce ‘signal 3’ cytokines to provide the right context in which antigen is presented to CD8+ T cells. Interleukin 12 (IL12) and type I Interferons (IFNαβ) are well known signal 3 cytokines. Also T cell-derived type II interferon (IFNγ) can act as a signal 3 cytokine. More recently, further evidence has shown that next to these inflammatory signals, also common γ chain cytokines such as IL2, IL15 and IL21 provide stimuli that contribute to the differential development of effector versus memory CTLs (Reviewed by 33-35).

The pro-inflammatory cytokine IL12 induces the production of IFNγ, favors differentiation of T\textsubscript{H}1 cells and forms a link between the innate and adaptive branch of the immune response. Activated mature DCs and macrophages are the main source of IL12 early during infection, but over time their production declines. In CD8+ T cells IL12 has an adjuvant effect during the primary response which optimizes CD8+ expansion in vitro and in vivo, as shown by experiments where in vitro priming of CD8+ T cells in the presence of IL12 generates effectors that confer better in vivo protection against viral challenge. Thus, IL12 is necessary for full clonal expansion and has the ability to promote survival and differentiation of naïve cells into effector cells capable of cytolysis when present during primary infection.

IFNα has a direct effect on antigen (Ag) specific cells and improves their expansion in vivo. For example, adoptive transfer of IFN-I receptor-deficient cells into wt hosts after Lymphocytic Choriomeningitis Virus (LCMV) infection resulted in strongly impaired numbers of Ag-specific CD8+ T cells compared to adoptively transferred wt CD8+ T cells. The importance of Type I Interferons was corroborated by two other studies that showed that IFN-I signaling promotes survival and differentiation into effector cells during primary infection.

Another factor that regulates the size of the CD8+ T cell effector pool is the inflammatory cytokine IFNγ which is predominantly produced by NK cells, NKT cells and primed T cells. After LCMV or L. monocytogenes infection, CD8+ T cells deficient in IFNγ or its receptor show a markedly reduced expansion. Specifically, adoptive transfer of wt CD8+ T cells into IFNγ/− mice generate a CD8+ T cell response of only 20% when compared to wt mice after immunization. Interestingly, the contraction is also impaired during infection of IFNγ/− and IFNγR−/− mice with L. monocytogenes or LCMV infection, resulting in normal numbers of memory CD8+ T cells. The experiments with IFNγ deficient mice have shown that very early during infection, IFNγ affects T cell differentiation and induces effector formation at the cost of memory cells. However, the effects of IFNγ on T cell responses can vary depending on the nature of the virus. The kinetics of some viral infections in IFNγ deficient mice such as vesicular stomatitis were reported to be normal.
As described above, next to the inflammatory signal 3 cytokines, also growth factors belonging to the \( \gamma_c \) chain cytokines have essential roles in modulating proliferation and effector functions. IL2 has long been regarded as a T-cell growth factor because of its in vitro effect on T-cells, although it can have positive as well as negative effects on cell proliferation\(^{55,56}\). Administration of IL2 \textit{in vivo} during the early stages of the viral response hampers CD8\(^+\) T cell survival, while addition of IL2 during the contraction phase promotes T cell survival\(^{57}\). It is mainly produced by CD4\(^+\) cells, although primed CD8\(^+\) T cells also excrete IL2. Responsiveness is conferred by the IL2 receptor complex consisting of the high affinity IL2R\(\alpha\) chain (CD25), IL2R\(\beta\) chain (CD122) and the \( \gamma_c \) chain (CD132). The high affinity component of the IL2R is rapidly induced after infection, but expression starts to wane after 2 days after infection until it is uniformly down regulated on antigen specific CD8\(^+\) T cells after 6 days of infection\(^{58}\).

The \( \gamma_c \) cytokine IL21 is produced by NKT and most subpopulations of CD4\(^+\) cells, such as \( T_{H17} \) lineage cells\(^{59-61}\). IL21 exerts its effects on many lymphoid (CD8, NK, B-cells) and myeloid cell types as well as non-immune cells, although the common theme is the induction of differentiation\(^{60}\). Together with other \( \gamma_c \) cytokines such as IL7 and IL15, IL21 has a markedly synergistic effect on proliferation of CD8\(^+\) T cells \(^{58}\). IL21 signals via ligation to the IL21R which is constitutively expressed on B cells and NK cells, but on T cells only following activation. IL21R-deficient mice have equal numbers of T cells compared to wt mice under steady state conditions\(^{60-63}\). However, during chronic infection such as with LCMV Clone 13, lack of IL21R decreased the number of anti-viral CTLs generated during the chronic phase\(^{64}\). No effects were observed during the acute phase or during infection with the LCMV Armstrong strain, suggesting that IL21 sustains CD8\(^+\) T cells during chronic infections\(^{64-66}\). Indeed, adoptive transfer experiments showed that IL21R deficient cells became exhausted or were deleted.

Exhausted cells are dysfunctional antigen-dependent cells which are prone to undergo apoptosis, although this process can be reversed by blocking inhibitory signals such as PD-1 ligation. The degree of exhaustion is dependent on antigen levels, ranging from partial loss of cytokine production to total loss of cytotoxic capacity and cytokine secretion. \textit{In vitro} studies have provided a mechanism which might explain why exhaustion is prevented. Addition of IL15 to naive CD8\(^+\) T cells leads to removal of CD28 and CD62L from the cell surface, inducing an effector phenotype. During a chronic infection CD4\(^+\) cells are the principle producers of IL21. IL21 triggering retains CD28 on the membrane, which maintains its co-stimulatory function. Furthermore, stimulation of CD28 can override the dampening effect of PD-1 ligation on TCR signaling\(^{67}\), and the combination of IL15 and IL21 leads to an increased production of IFN\(\gamma\) compared to IL15 alone\(^{68}\). Thus, binding of IL21 to its receptor on CTLs seems to avert exhaustion and induce a differentiation program that enables CD8\(^+\) T cells to better clear persistent viruses.

In summary, many studies showed that there is a certain level of redundancy between all type 3 signals\(^{33,42}\). For example, when IL12 is highly expressed such as during \textit{Listeria} infection, the role of IFN-I in promoting CTL responses seems to be small\(^{42,69}\). Thus, the immune response can react to different pathogens with the production of other combinations of type 3 signals. Depending on the pathogen, differences in the relative quantities of IL2, IL12,
IFN-I, IFNγ or IL21 during the acute phase of infection guide the outcome of CD8+ T cell expansion and memory formation into different directions.

**Transcription factors**

The superior ability of memory T cells to battle invading pathogens is caused by an altered gene expression profile after T cell priming. The encounter of naïve CD8+ T cells with APCs and associated factors leads to epigenetic changes such as DNA methylation, histone modifications and reorganization of chromatin structure. This is brought about by changes in the quantity and location of transcription factors which leads to altered gene expression. In turn this can further modify other sets of transcription factors leading to cellular reprogramming. Three transcription factors, T-bet, Eomes and Blimp-1 have proven to be key players in the generation of fully functional memory CD8+ T cells.

**T-box family, T-bet and Eomes**

T-bet is a member of a family of more than 50 members that share a 200 amino acid DNA binding domain, the T-box. T-box proteins have been shown to be crucial transcription factors that regulate differentiation and organogenesis in many vertebrate species. T-bet shares a high sequence homology (72% identical in T-box regions) to Eomes, a family member with a related function in immune regulation. T-bet expression is detected in CD4+ T cells, B-cells and cytotoxic cells such as CD8+ T cells, NK cells, and NKT cells that are able to produce IFNγ. Upon IFNγ exposure in mice, T-bet is also induced in myeloid cells such as monocytes and DCs. Eomes is more restricted to cytotoxic cells with high expression levels in primed CD8+ T cells and resting as well as activated NK cells. In contrast, CD4+ T cells do not express Eomes.

T-bet was first identified as a transcription factor whose expression coincided with IFNγ production and was capable of redirecting polarized TH2 cells into IFNγ producing TH1 cells. T-bet is now recognized as a master regulator that drives TH1 differentiation that can directly bind to the Ifnγ promoter and induce its expression in vitro. In mice harboring a targeted disruption of the T-bet locus, CD4+ cells are severely impaired in their ability to produce IFNγ and the secretion of hallmark TH2 subset cytokines IL4 and IL5 is elevated. Although T-bet deficiency in CD4+ cells leads to a clear shift in balance from TH1 to TH2, in CD8+ T cells no change in IFNγ production or cytotoxicity was observed. However, in a different model system using antigen-driven stimulation, T-bet deficient CD8+ T cells from OT-I T cell receptor transgenic mice indeed secreted low levels of IFNγ, whereas IL4, IL2 and IL10 production was upregulated. CD8+ T cells lacking T-bet proliferated and expanded normally but failed to acquire effector cell surface markers, showed reduced cytotoxicity and poorly cleared infection with LCMV, a virus of which the eradication is mainly CD8+ T cell dependent.

Because T-bet deficient mice only exhibited minimal defects in the CD8+ compartment under steady state conditions, Pearce and co-workers used the conserved DNA binding sequence of T-bet to amplify cDNA from activated CD8+ T cells to measure expression of
other T-box factors. They detected that Eomes transcripts were expressed in activated CD8+ T cells. Enforced expression of Eomes in T\(_{\gamma\delta}\) cells resulted in expression of CTL effector molecules IFN\(\gamma\), Perforin and GzmB and inhibition of Eomes by retrovirally transfecting a dominant negative from led to diminished IFN\(\gamma\) production and reduced cytotoxicity\(^{72}\).

Analysis of haploinsufficient Eomes\(^{+/}\) mice revealed that IFN\(\gamma\) production and effector cell formation were relatively unaffected\(^{72,84}\). Many reports demonstrate that deletion of one or both alleles of the T-box factors T-bet or Eomes can be counteracted by the other\(^{72,77,83,85-87}\). For example, in T-bet deficient mice expression of Eomes can restore CD8+ development. This indicates that Eomes and T-bet have complementary roles in generating and maintaining effector functions of primed CD8+ T cells. Deletion of T-bet or Eomes in cells that possess transcripts of only one of the two genes would therefore have a more pronounced effect. Indeed, mature NKT cells that do not express Eomes are almost depleted in T-bet deficient mice\(^{88}\).

Eomes homozygote knockout mice die early in embryogenesis\(^{89}\). Therefore, mice with a deletion of a single allele on a T-bet null-background were generated to test if Eomes and T-bet have redundant functions in regulation of the genes encoding IFN\(\gamma\) and cytolitic molecules. Eomes\(^+/\) T-bet\(^{-/-}\) mice showed almost a complete loss of all cells that depend on IL15 for maintenance in the periphery such as NK cells, NKT cells and memory CD8+ T cells\(^{84}\). Basal expression of CD122, the shared beta-chain of IL2R and IL15R, is required for IL2 and 15 signaling and enhanced expression (CD122\(^{hi}\)) confers stronger IL15 responsiveness to cells\(^{90}\). Deletion of one or both alleles of T-bet on a wt Eomes background did not have a considerable effect on CD122 expression. However, T-bet\(^{+/}\) Eomes\(^{-/-}\) and especially T-bet\(^{-/-}\) Eomes\(^{+/}\) mice showed a marked reduction in CD122 positive CD8+ T cells and total NK cells. This reduction in CD122\(^{hi}\) cells coincided with a reduction of IFN\(\gamma\) protein and Perforin mRNA production. Furthermore, ectopic expression of T-bet or Eomes in T\(_{\gamma\delta}\) cells that normally do not express T-bet or Eomes, increased the amount of CD122 mRNA transcripts. This clearly shows that both Eomes and T-bet are critical for inducing a CD122 responsive state in naïve cells that differentiate into effector cells and in maintaining elevated CD122 expression in mature cells\(^{84}\).

The molecular mechanism by which CD122 expression is induced has been elucidated for Eomes. ChIP analysis showed that Eomes is able to directly transactivate the IL2R\(\beta\) locus that encodes CD122. This experiment also demonstrated direct binding to the promoters of GzmB and Perforin, indicating that next to IFN\(\gamma\) more effector molecules are induced by direct binding of Eomes to target promoters\(^{84}\). This can explain why the mice harboring deletions in T-bet and Eomes alleles show a reduction in Perforin and Granzyme B and why those NK cell lines that have low levels of T-bet and Eomes are less potent in cytotoxicity assays\(^{84}\). Thus, Eomes and T-bet cooperate in regulating IL15 responsiveness and effector cell function in cytotoxic cells.

Homoygous deletion of Eomes specifically in CD4+ and CD8+ T cells (Eomes KO) together with germline deletion of Tbx21 (T-bet KO) showed similar results as described above\(^{91}\). Although conventional T cell development was unaffected, Eomes KO or double KO mice were deficient in CD44\(^{hi}\) CD122\(^{hi}\) CD8+ memory cells\(^{92}\). Double KO mice were not able to clear
LCMV and demonstrated severe impairment of cytolytic activity, which is again evidence for a redundant role of the T-box factors in the differentiation towards functional CTL. Surprisingly, LCMV specific double KO CD8^+ T cells produced IL17, a cytokine that is able to induce many cytokines and chemokines leading to e.g. neutrophil infiltration.

Indeed, virus-induced wasting disease that is characterized by neutrophil infiltration was observed in double KO mice. Elevated expression of transcription factor RORγt, IL23R, IL21 and IL22 in double KO CD8^+ T cells further demonstrated that in wt cells Eomes and T-bet prevent differentiation into \( \text{T}_\text{H}17 \) cells. Differentiation of CD4^+ into \( \text{T}_\text{H}17 \) cells can be prevented by IL4, IL27 and IFNγ. In addition, forced expression of T-bet represses \( \text{T}_\text{H}17 \) differentiation whereas deletion of T-bet leads to an increase in \( \text{T}_\text{H}17 \) cells. Together with the notion that T-bet deficiency induces IL17-producing CD8^+ T cells, the results from CD4^+ T cells suggest that IL17 expression might arise in CTLs when the extent of inflammatory signals that increase Eomes or T-bet levels are insufficient or absent.

### Models describing CD8^+ memory T cell formation

The question how effector CD8^+ T cells differentiate into memory cells has been under investigation for more than a decade of extensive research. Although intricate methods have enabled detailed quantitative analysis of the subsets of cells that arise after primary infection, the exact pathway of differentiation of these subsets remains controversial. Three models were used to describe the emergence of effector and memory T cells.

The linear differentiation model states that during the expansion phase activated T cells arise with an effector phenotype (Fig. 1). After viral clearance and the subsequent contraction phase, these cells die due to apoptosis and only CD62L^lo \( \text{T}_{\text{EM}} \) cells remain. In the memory phase \( \text{T}_{\text{CM}} \) cells emerge, most likely derived from these \( \text{T}_{\text{EM}} \) cells. An observation that contradicts this model is that memory CD8^+ T cells are measured at the peak of the response. Indeed, these IL7Rα expressing memory precursor cells are already present when the Ag driven response is at its peak. In addition, immunization with mature peptide coated DCs in the absence of inflammation induces formation of memory cell as early as 4 days after immunization. These memory cells possess all the functional characteristics of conventional memory cells and are equally capable of proliferation after challenge.

In a second model named **divergent differentiation**, naïve cells directly divide into either an effector or memory cell, bypassing the effector stage (Fig. 1). An elegant study by Chang et al. showed that the unequal partitioning of key proteins at the first cell division can imprint the future faith of the effector and memory cell. Direct contact between a T cell and a DC preceding the first cell division after priming, leads to an asymmetric segregation of T cell proteins present in the synapse. The distal daughter cell contained more CD8 and effector molecules GzmB and IFNγ, was larger in size, and showed higher expression of CD69, CD43, CD25, and CD44 while CD62L expression was low. Proximal daughter cells were less granular, contained more IL7Rα mRNA and expressed high levels of CD62L but lower levels of CD69, CD43, CD25, and CD44.

The divergent differentiation model in which priming of naïve cells leads to the formation of two distinctive daughter cells that form the effector and memory pool can be modified...
into a slightly more realistic model that still incorporates the observations by Chang et al. Instead of a sharp separation between $T_{\text{Eff}}$ and $T_{\text{Mem}}$ after the first cell division, the memory potential of a cell is elevated or decreased in a graded fashion by re-localization of specific molecules\textsuperscript{101,104,105}. In this manner, cells can inherit a range of phenotypes with explicit effector or memory traits at the extreme ends. This adjusted model leaves room for a range of differentiation states that are induced by the strength of the ‘signal 3’ mediators that the T cell encounters during the initiation of the immune response\textsuperscript{17,106}. It would be intriguing to test if the presence of certain cytokines alters the rate of symmetric versus asymmetric cell divisions after priming. Recently, by single-cell qPCR, Arsenio et al. have shown that the transcriptome of the proximal and distal daughter cell (respectively nearest and farthest away from the APC) are predictive of respectively an effector and memory fate\textsuperscript{107}. Asymmetric

Figure 1. Three proposed models of CD8\textsuperscript{+} T cell differentiation: A) Linear differentiation model B) Divergent differentiation model C) Decreasing potential model. Each model describes the transitions that CD8\textsuperscript{+} cells undergo after initial priming and prolonged exposure to antigen.
partitioning of IL2Rα during mitosis represented an early molecular switch towards terminal differentiation and could be discerned as early as the first division.

Using single cell transfer and barcoding experiments the outgrowth of one naive TCR transgenic CD8+ T cell could be monitored to assess if the environmental cues that trigger the first expansion determine the SLEC or MPEC fate. This individual cell can give rise to both effector and memory populations\textsuperscript{98,108}, opposing against a model that the initial signals provided to the naive cells generate different memory subsets. The strength of the TCR and peptide bound MHC complex did not seem to be the most important determinant of the total T cell population, since the summation of the clonally expanded individual T cells was equivalent to the polyclonal response that is generally observed after adoptive transfer\textsuperscript{109,110}. This opposes against asymmetric division being the sole driver of the heterogeneous CD8+ T cell population and favors the decreasing potential model.

Finally, in the decreasing potential model (also called the progressive differentiation model) the strength of the signals during the expansion phase is the main determinant of the degree of effector cell differentiation (Fig. 1)\textsuperscript{17,99}. T cells that encounter little antigen or that are stimulated for a short period during priming undergo a small amount of differentiation and become MPECs. Cells that have undergone prolonged TCR signaling and cytokine or antigen stimulation become more terminally differentiated and develop into SLECs. They also become more susceptible to apoptosis, implicating that the memory pool that remains after contraction is larger after a short stimulation than after a prolonged stimulation (Fig. 1).

The decreasing potential model predicts distinct lineages of effector and memory T cells. Bannard et al. irreversible marked CD8+ T cells that have gained effector function to test whether the effector stage is indeed required for memory formation. To this purpose, they constructed a transgenic mouse, of which the T cells that at any point in time transcribed the \textit{gzmB} gene remained YFP positive\textsuperscript{111}. This model enabled measurement of the responses without requirement for adoptive transfer, which may alter the dynamics of memory differentiation\textsuperscript{112}. Virus specific CD8+ T cells were negative for GzmB expression, but YFP+ was readily detectable in a sizable portion of these memory cells. Thus, influenza specific CD8+ T cells that express GzmB early in the response generate memory cells, which is not in line with the divergent differentiation model in which T\textsubscript{Eff} and T\textsubscript{Mem} are distinct lineages\textsuperscript{103}. In contrast, this observation fits with the absence of a clear separation between T\textsubscript{Eff} and T\textsubscript{Mem} differentiation at early time points.

**Memory Stem cells**

Recent evidence has shown that a small percentage of the memory T cells have the capacity to self-renew and display the molecular characteristics of stem cells\textsuperscript{113,114}. These memory stem cells (T\textsubscript{SCM}) activate telomerases to maintain their proliferative capacity and have a transcriptional profile that to a large extent resembles HSCs. Antigen experienced cells all express IL2Rβ and IL15Rβ which allow for homeostatic proliferation and contain high amounts of co-stimulatory or pro-apoptotic molecule FAS (CD95). These markers were found in cells with a naive phenotype (CXCR3, CD44+CD62L+ in mice and CD45RA+CD62L+ in ...
in human), although the activation markers and presence of TRECs indicated that several rounds of proliferation had taken place\textsuperscript{115}.

In a series of \textit{in vivo} clonogenic adoptive transfer assays Graef \textit{et al.} has proven that not only the small subset of T\textsubscript{SCM} cells have functional hallmarks of stemness, cells belonging to the T\textsubscript{CM} compartment also display multipotency, self-renewal and the ability to reconstitute the T cell compartment in an immunocompetent host\textsuperscript{116}. After infection with \textit{Listeria monocytogenes}, a single-epitope specific T\textsubscript{CM} cell gave rise to a diverse progeny consisting of T\textsubscript{CM} and T\textsubscript{EFF} similar to naive T cells, thereby protecting the immunocompromised host. During three single-cell adoptive transfers, the single naive T cell, single primary T\textsubscript{CM} and single secondary T\textsubscript{CM} were equally efficient in supplementing the T cell compartment. Unfortunately, naive-like CD44\textsuperscript{-} CD62L\textsuperscript{+} cells could not be detected, possibly due to the detection limit of the assay. The authors state that a major role for these T\textsubscript{SCM} cells is not expected since at least 20\% of the individually transferred cells are able to re-expand and ensure full immunocompetence. That T\textsubscript{CM} cells can propagate T cell memory and have stem cell-like properties is in contrast to data presented by Gattinoni \textit{et al.} who attributes this potency to the CD44\textsuperscript{-} CD62L\textsuperscript{+} stem cell subset. In order to reconcile both models, Gattioni postulates that T\textsubscript{SCM} could out-perform the T\textsubscript{CM} cells in terms of progeny numbers in lymphoid tissue and the periphery.

It is clear that in the thymus, not one signal is responsible for programming CD8\textsuperscript{+} T cells to adopt a SLEC or MPEC fate. Furthermore, it has been shown that effector and memory cell formation is not determined by asymmetric division with the daughter cell proximal to the antigen presenting APC receiving stronger signals and is driven towards a more terminally differentiated state. Instead, CD8\textsuperscript{+} T cell fate is determined by extrinsic stimuli such as the strength of TCR triggering, duration of Ag presentation and the presence of inflammatory cytokines such as IL-12, Type-I interferons, and cytokines such as IL2, IL7, IL15, IL27. The model that is best reflective of CD8\textsuperscript{+} T cell differentiation is a combination of multiple models, with T\textsubscript{SCM} fitting more in the linear differentiation model, where only stem cells can generate all memory subsets and effector cells. Moreover, ample evidence is observed underlining various aspects of the divergent and decreasing potential. Therefore, a gradient model was proposed in which a wide spectrum of differentiated T cells are formed with SLEC on one side and MPEC fate on the other end, with in between intermediate effector cells\textsuperscript{117}. This model provides plasticity to the T cell to transit through differentiation states depending on the signal encountered.

\textbf{Effect of ‘signal 3’ cytokines on T-bet and Eomes expression in CD8\textsuperscript{+} T cell differentiation}

More recently, it has become clear that the relative T-bet expression may determine whether primed naïve CD8\textsuperscript{+} T cells become SLECs or MPECs\textsuperscript{17}. Inflammatory signals such as IL12 present during CD8\textsuperscript{+} T cell priming determine T-bet levels\textsuperscript{118}. High T-bet levels lead to a more terminally differentiated SLEC phenotype, whereas cells with low T-bet adopt an MPEC fate. Because the IL7R is downregulated, SLECs have reduced survival potential\textsuperscript{17}. MPECs have
high IL7Rα expression, which allows the cells to homeostatically proliferate in response to the cytokine IL7. Indeed, in T-bet deficient mice the generation of T_{EM} CD8^+ T cells was compromised, which resulted in excessive production of T_{CM} CD8^+ T cells^{119}. In support of this, effector CD8^+ T cells from IL12^{-/-} mice have reduced amounts of T-bet and generate more memory CD8^+ T cells^{118,120}.

As depicted in figure 2, SLEC cells are characterized by high KLRG1 and low IL7Rα expression, while MPEC cells are IL7R^{hi}, KLRG1^{lo}. Re-expression of T-bet in T-bet deficient mice by retroviral transduction resulted in lower IL7Rα mRNA expression, indicating that T-bet can repress transcription of IL7Rα, either directly or indirectly. Thus, inflammatory signals like IL12, during priming regulate T-bet expression in a dose-dependent manner. High T-bet expression induces SLECs, while low levels promote MPEC formation.

Figure 2. The differentiation from naïve CD8^+ cells into SLEC or MPEC and respectively long lived effector cells and memory cells, is governed by T-bet, Blimp-1 and Eomes. These cells all produce the cytokines IFNγ and IL2, albeit at different quantities. Ultimately, exhaustion can be induced. These cells produce little cytokines and are not able to control infection.

IL12 not only induces T-bet expression during L. monocytogenes infection, but at the same time suppresses Eomes, thereby promoting effector function at the cost of memory generation^{118,121}. Addition of IL12 results in robust IFNγ production and cytolytic activity in OT-1 cells. By enhancing and maintaining mTOR kinase activity IL12 provides the deterministic signal for CD8^+ effector maturation^{122,123}. mTOR has the ability to sense the metabolic state of cells, the extracellular nutrient availability and the presence of growth factors/cytokines. Moreover, this protein controls key cellular processes that govern cell fate including apoptosis, proliferation and cell growth^{124,125}. Inhibition of mTOR blocked persistent T-bet expression and promoted memory-precursor generation. At the same time,
IL12 and mTOR inhibit Eomes, in part through inhibition by Forkhead O transcription factor FOXO1\textsuperscript{126-128}. Thus, mTOR functions as a molecular switch that depending on the nature and the intensity of the signals received, determines the induction of either T-bet or Eomes that will lead to either effector or memory cell formation, respectively.

The group of Steven Reiner has elegantly shown that Eomes-deficient memory CD8\textsuperscript{+} T cells have defects in long-term persistence and secondary expansion post challenge, two hallmark properties of memory CD8\textsuperscript{+} T cells\textsuperscript{129}. These cells also were less capable in populating the bone marrow niche. Together, emerging evidence has shown that T-bet and Eomes can act redundantly, but can also have a reciprocal function in promoting terminal differentiation\textsuperscript{130} versus memory differentiation (Eomes) of Ag-specific CD8\textsuperscript{+} T cells.

Like IL2, IL21 can promote CD8\textsuperscript{+} T cell expansion and effector function\textsuperscript{60;62;63}. However, IL2 induced Eomes expression, whereas IL21 repressed Eomes leading to less differentiated cells, as shown by the retention of human differentiation markers CD45RA, CD62L, CD28, CD27, and IL7Rα on the CD8\textsuperscript{+} cell surface\textsuperscript{131}. Remarkably, these cells have a high proliferative capacity with increased antitumor activity compared to cells primed and expanded in other γc cytokines\textsuperscript{132}. Thus, by inhibiting effector cell formation and exhaustion, IL21 maintains the cells in a stem cell like state, associated with high proliferative potential and long term survival\textsuperscript{132;133}. In contrast, Sutherland \textit{et al}. show that IL21 induces T-bet which results in upregulation of cytolytic molecules Perforin and GzmB\textsuperscript{134}. However, the transient expression may be an important prerequisite for the development of the memory CD8 phenotype that is commonly observed.

Another type 3 cytokine that alters the expression of T-box genes and therefore has an impact on the extent of memory formation is IL27. This member of the IL12 family cytokine is produced by activated APCs\textsuperscript{135;136}. Signaling through this pathway markedly induces expression of T-bet, GzmB and IL12Rβ2 in CTLs\textsuperscript{137}. This allows IL27 to synergize with IL12 in IFNγ production and to augment proliferation. \textit{In vivo}, \textit{Influenza virus} or \textit{T. gondii} infection showed that the IL27R rather than the IFNyR is critical for T-bet dependent IFNγ production in CD8\textsuperscript{+} T cells\textsuperscript{138}. Thus, IL12, IL21 and IL27 can all be grouped as cytokines that can induce effector function via T-bet. The differences in T-bet kinetics, such as the rapid but transient induction by IL21, opposed to the slow but sustained induction by IL12, leads to qualitative differences leading to the generation of functionally different CD8\textsuperscript{+} T cells.

Two groups have linked differential IL2 signaling with transcriptional events that regulate terminal effector CD8\textsuperscript{+} T cell formation \textit{in vivo}\textsuperscript{58;139}. Pipkin \textit{et al}. investigated the effects of IL2 signaling and inflammatory signals such as CpG and IL12 on T-cell differentiation\textsuperscript{139}. In a cell culture in which the stimuli could be tightly regulated, they showed a strong induction of Eomes and effector molecules such as GzmB and Perforin after administration of IL2. Furthermore, memory cell formation was diminished at the expense of effector cells. Strikingly, during weak stimulation of the TCR, inflammatory signals were required in combination with IL2 to induce the effector cell program necessary for expression of the cytotoxic effector molecules. When a strong TCR signal was applied, IL2 was sufficient for formation of GzmB and Perforin expression, bypassing the requirement for inflammatory signals. These findings were confirmed in an \textit{in vivo} experiment in which IL2Rα deficient CD8\textsuperscript{+}
T cells were studied after infection with LCMV. The CD8+ T cells showed reduced Perforin and GzmB expression and defective killing. Furthermore, the IL2Ra deficient cells expressed less T-bet and KLRG1 and more Bcl-6, indicative of memory cell formation. Inflammatory stimuli enhanced expression of IL2Ra and T-bet and countered late Eomes and Perforin induction. Thus, IL2 and inflammatory cytokines are key in inducing terminal differentiation of effector T cells at the expense of the emergence of memory T cells.

The findings by the group of Ahmed were consistent in that LCMV-specific CD25hi CD8+ T cells compared to CD25lo CD8+ T cells showed a more explicit effector-like phenotype (KLRG1+, T-bet, Blimp-1, GzmB and Perforin), proliferated more extensively and had a diminished persistence when adoptively transferred into infection matched congenic hosts. Kalia et al. described the kinetics of CD25 expression in detail. Early after infection all CD8+ T cells upregulate IL2Ra when virus is abundant. After 3-4 days when Ag levels were decreasing and CD8+ T cell expansion had reached its peak, CD25 expression became more heterogeneously expressed by these Ag specific cells. Around day 6 when Ag was largely cleared from the system, CD25 was either uniformly downregulated or alternatively the CD25hi cells were relocalized to niches rich in Ag, which enabled the CD25hi T cells to persist. Thus, they hypothesized that heterogeneity in CD25 leads to divergent developmental programs, such that cells that maintain high levels of CD25 expression receive comparatively more IL2 signals and are more likely to differentiate into effector CD8+ T cells than CD25lo cells. Kalia et al. stated that their data is in favor of a linear model in which early effector cells differentiate into (non-)lymphoid memory cells or terminally differentiated effector cells.

Next to the TCR triggering and signal 3 molecules, other factors can alter T-bet expression and therefore influence IFNγ expression. Ma et al. showed in response to viral infection, miR-29 expression is lowered compared to naive cells to allow for IFNγ production in a manner independent of T-bet and Eomes. Although Steiner et al. found that mIR-29 was indeed involved in regulation of IFNγ, this involved the simultaneous targeting of both T-bet and Eomes.

In conclusion, while T-bet can be readily induced in CD4+ cells by different cytokines (IL12, IL15, IL27), IL12, IL21 and IL27 are the only cytokines known to date to regulate T-bet expression in CD8+ T cells. Furthermore, IFNγ has been shown to augment T-bet expression via STAT1 to promote CD8+ T cell expansion. Thus, exposure to inflammatory signals plays a critical role in T cell expansion and acquisition of effector function. Future research is required to show whether other cytokines have an effect on T-bet or Eomes levels thereby altering the memory potential. It is important to unravel the influence of the timing of T cell encounter with these cytokines and persistence of the inflammatory cytokines, as this may determine the extent of SLEC and MPEC formation.

**Effect of T-bet on IFNγ expression**

How T-bet affects IFNγ expression is a well-studied question because production of IFNγ is an important aspect of the effector phenotype of CD8+ T cells. Usually full methylation of conserved promoter regions inhibits the transcription of the target gene. However, T-bet can override repressive epigenetic modification in the presence of CpG methylation at that
site. Instead, T-bet causes decreased loading of the co-repressor Sin3A on methylated DNA, thereby transactivating the \textit{Ifn}y promoter \textsuperscript{74}. T-bet can also recruit a histone demethylase (JMJD3) creating an open chromatin structure that allows IFN\gamma expression\textsuperscript{143}. Thus, T-bet can induce expression of IFN\gamma by altering the chromatin structure even in silenced chromatin. If direct T-bet binding to the \textit{Ifng} promoter is essential for IFN\gamma production is still a matter of debate. Ursui \textit{et al.} has shown that in developing T\textsubscript{H}1 cells the principle role of T-bet is to negatively regulate GATA3 expression instead of positively regulating the \textit{Ifng} gene\textsuperscript{144}. T-bet maintains histone hyperacetylation at the \textit{Ifng} promoter\textsuperscript{145,146}. Even in T-bet\textsuperscript{-/-} mice the \textit{Ifng} promoter can become accessible by histone H3 acetylation when naive cells are stimulated under the right conditions. This suggests that IFN\gamma production is facilitated by an accessible promoter region and is not absolutely dependent on T-bet\textsuperscript{144}. However, the use of \textit{in vitro} reporter assays and retroviral constructs expressing T-bet or a dominant-negative form of T-bet indicated that this transcription factor is involved in early acquisition of accessibility at the \textit{Ifng} promoter\textsuperscript{77,147}. Recently, ER fusion constructs in which T-bet could either be retained in the cytoplasm or be located to the nucleus were studied. This showed that in T-bet\textsuperscript{+/-} T\textsubscript{H}1 cells there is a constant need for T-bet activity to induce IFN\gamma\textsuperscript{82}. Despite rapid nuclear translocation, demonstrable effects on IFN\gamma and CD122 were delayed by 12-48h, indicating that perhaps other factors are required. T-bet might bind to the IFN\gamma locus and in turn recruit other factors such as NFAT or Runx3\textsuperscript{1,148} enabling their DNA binding.

The mechanism of how T-bet promotes IFN\gamma production was elucidated in T\textsubscript{H}1 cells and this may be exemplary for other cell types including CD8\textsuperscript{+} T cells\textsuperscript{149}. CTCF is a protein that alters the looping of the IFN\gamma chromatin. This looping helps to drive the juxtaposition of T-bet-binding enhancers and the flanking CTCF-binding elements to the \textit{Ifng} locus and promotes robust expression of IFN\gamma. This occurs in naïve CD4\textsuperscript{+} cells that differentiate into T\textsubscript{H}1 effectors, although the sites occupied by CTCF are found in most cell types. Therefore, this altering of the chromatin architecture by CTCF to bring together cis enhancer regions together with transcriptional regulators to the IFN\gamma locus seems to be universal and applicable to CD8\textsuperscript{+} T cells\textsuperscript{150}, but to date has not been verified yet.

**TFs that interact with T-bet and Eomes to induce effector molecules in CTLs**

In CD4\textsuperscript{+} T cells it has been shown that T-bet is upregulated early after priming and induces Runx3. This is a member of the Run family of three DNA binding TF that control thymocyte differentiation and CD4/CD8 lineage decision\textsuperscript{151,152}. T-bet and Runx3 cooperate to induce IFN\gamma and to silence IL4, thereby stably endorsing T\textsubscript{H}1 differentiation\textsuperscript{153}. Recently, the function of Runx3 in CD8\textsuperscript{+} T cell formation was elucidated. Mice deficient in Runx3 fail to express Eomes, while T-bet expression was unaffected. These CD8\textsuperscript{+} T cells are strongly impaired in the production of effector molecules such as Perforin, GzmB and IFN\gamma\textsuperscript{+}. In contrast to CD4\textsuperscript{+} T cells, naïve CD8\textsuperscript{+} T cells already express Runx3 before activation\textsuperscript{154}. Runx3 represses Runx1 and positively regulates Eomes, GzmB, Perforin and IFN\gamma. The direct binding of Runx3 to the promoters of these effector molecules suggests that the transcription factor has a
direct effect on the expression of GzmB, Perforin and IFNγ. The authors present a model in which only Runx3 is capable of inducing all three cytokines and Eomes does not induce GzmB expression. However, earlier reports already showed that Eomes is also capable of binding to the promoter sites of GzmB, Perforin and IFNγ. Future experiments in which Runx3 is introduced into conditional Eomes deficient mice are required to show whether the binding of both TFs is necessary to induce effector molecule expression or that Runx3 alone is sufficient. As mentioned above there is a temporal difference in T-bet (early) and Eomes (late) expression, to induce production of IFNγ. Therefore, Runx3 might synergize at different time points with either T-bet and/or Eomes to maintain effector CTL differentiation.

Recently, the formation of SLECs by IL2 was shown to be in part licensed by the cell surface receptor Notch. After cleavage of the Notch intracellular domain it translocates to the nucleus where it acts as a transcriptional activator. A large portion of the SLEC expression profile such as GzmB, Perforin, IFNγ, CD25 (IL2Rα), T-bet as well as Akt and mTOR activity are all regulated by Notch as shown by an influenza virus model in Notch1-2-KO mice. Expression of Notch1 is also induced in naïve cells by several of these factors and regulates the gene coding for Eomes. Backer et al. propose that Notch senses the severity of the infection and drives a positive feedback loop that allows full differentiation into SLECs.

**Blimp-1**

B-lymphocyte induced maturation protein-1 (Blimp-1) is a zinc finger containing protein that has been shown to be a major regulator of differentiation of B-lymphocytes into immunoglobulin (Ig) secreting plasma cells (see also Fig. 2). Blimp-1 was first named by Davis and co-workers who described a gene that was rapidly induced during plasma cell differentiation of B-cells in mice. Blimp-1 was later identified as the murine homolog of the human repressor PRDI-BF1 that was described three years earlier by Keller and Maniatis. PRDI-BF1 binds to the positive regulatory domain I (PRDI) site in the interferon-beta (IFN-β) promoter and therefore was named PRDM1 binding protein (PRDI-BP) although the name was later shortened to PRDM1. Recently, human reports have also been referring to PRDM-1 as Blimp-1. Because this name is more widely used we will name the protein that is encoded by the gene Prdm1, Blimp-1, irrespective of the species.

**Blimp-1 in B-cell differentiation**

Blimp-1 is expressed in plasmablasts and plasma cells, but Blimp-1 expression is not detected in naïve and memory B-cells. Blimp-1 is found in increasing levels from low expression in the plasmablast stage, in which cells are able to secrete antibody and proliferate rapidly, up to high expression in long lived plasma cells that are high antibody producers but have no proliferation potential. Enforced expression of Blimp-1 in primary B cells as well as in B-cell lines can induce differentiation into Ig secreting cells. To achieve this, Blimp-1 extinguishes expression of many genes which encode for a mature B-cell identity. Micro-array analysis has shown that Blimp-1 represses hundreds of target genes which are involved in a large variety of
molecular programs in B cells such as cell proliferation (e.g. c-Myc\textsuperscript{168}), immunoglobulin class switching (AID, Ku70, Ku86, DNA-PKcs, STAT6) and intracellular signaling (Spi-B, ID3). The direct and indirect targets of Blimp-1 and their effect on humoral responses have been comprehensively reviewed\textsuperscript{169-173}. Many targets are also transcription factors themselves, implicating a cascade of transcriptional control governed by Blimp-1.

**Domains of Blimp-1**

Blimp-1 belongs to the PRDM family of genes which both comprise a Krüppel-type Zn-finger region and a PR domain (Fig. 3). The Zn-finger region confers DNA-binding capacity to Blimp-1, and only the first two of the five Zn-fingers are required for sequence specific recognition of PRDM locus in the \textit{Ifnβ} promoter\textsuperscript{174}. However, \textit{in vivo} binding to the \textit{Ifnβ} promoter is not completely abolished when these two Zn-fingers are deleted, indicating that association with DNA may also occur via the other Zn-fingers perhaps through a weaker association, or involving other co-factors\textsuperscript{175}. The function or binding specificity of Zn-fingers 3-5 is currently unknown.

![Figure 3](image)

**Figure 3.** RNA transcript and protein encoding Blimp-1/PRDM-1. Next to the untranslated region, Blimp-1 contains four domains that confer specific functions to the full length protein. Below, three splice variants are depicted in which one or multiple motifs are not part of the expressed protein, altering its function.

The PR domain is a subclass of the SET domain found in transcriptional repressors such as the PRDM family. PR domain-containing proteins can methylate lysine residues on histones, thereby silencing gene expression by making the chromatin less accessible. However, although the PR domain of Blimp-1 is closely related to known histone methyltransferases (HMT), Blimp-1 has no HMT activity itself. By performing ChIP analysis omitting specific domains Gyory \textit{et al.} showed that Blimp-1 recruits a HMT to target promoters such as \textit{IFNβ} through a domain consisting of the first three Zn-fingers\textsuperscript{175}. Surprisingly, the Zn-finger
region and not the PR domain is crucial for silencing gene transcription by recruiting HMTs. Nevertheless, the PR domain is important as earlier reports by the same group showed that the Blimp-1β isoform in which the PR domain is abrogated displays only a fraction of the repressive function of the full length protein. However, Blimp-1b maintains normal DNA-binding activity, nuclear localization and association with HDACs. Thus, the PR domain of Blimp-1 is important for its repressive activity, although it does not display any HMT activity.

**Isoforms of Blimp-1**

Naturally occurring splice variants have proven to be helpful in determining the function of Blimp-1. Most transcripts give rise to the full-length protein (Blimp-1α). An internal promoter site, which lies between exon 3 and 4, gives rise to Blimp-1β (Fig. 3). This alternative start site results in a truncated protein which has a disrupted PR domain but leaves the Proline rich region and Zn-finger motif intact.

Blimp-1β has been detected in human NK cells, in higher levels than the full-length isoform. Blimp-1β is prominently expressed in myeloma cell lines and T cell lymphoma. Blimp-1β positive cells displayed higher c-Myc levels and abnormal Blimp-1β expression was correlated to poor disease outcome. Repression of the truncated Blimp-1 protein has been accomplished *in vitro* and *in vivo* by treating patients with Bortezomib, a proteasome inhibitor that has been reported to be effective in T cell lymphoma. Zhao *et al.* showed that Bortezomib downregulated IRF-4 and c-Myc, a prohibitir of cellular growth, and that this was preceded by downregulation of Blimp-1β. Mice that lack the PR domain arrest at embryonic day 10.5 like loss-of-function mutants despite abundant expression of the truncated protein. Heterozygous mice carrying full-length and truncated Blimp-1 develop normally, despite the failure to bind to DNA and interact with HDAC1 and G9a. Therefore, truncated Blimp-1 did not behave as a dominant negative regulator in mice, although species specific differences are expected.

T-blimp1 is a truncated protein devised by the Calame lab, which lacks the N-terminus but has an intact Zn-finger (Fig. 3). A T-Blimp-1 transgenic mouse was generated, that confirmed the *in vitro* experiments which showed that this truncated form inhibited Blimp-1 function. As expected T-Blimp-1 transgenic mice exhibited a developmental block at the plasma cell stage and exclusively memory B-cells were generated. However, to date no inhibitory function in T cells has been described.

**Blimp-1 is involved in the formation of effector T cells**

Since Blimp-1 deficient mice are embryonically lethal, two alternative mouse models have been developed to study Blimp-1 deficiency. Martins *et al.* crossed a mouse with *loxP* flanked *Prdm1* with a mouse expressing a Cre transgene under control of the *Lck* proximal promoter. Since Lck assists signaling from the TCR complex, these mice had a specific Blimp-1 deletion in all T lymphocytes. The group of Steven Nutt placed the green fluorescent protein (GFP) in the Zn-finger region of Blimp-1, thereby destroying the DNA binding capacity of the protein. The expression of GFP allowed them to monitor Blimp-1 expressing cells in
heterozygotes, while fetal cells of homozygous mutants could be harvested to reconstitute the immune system of irradiated wildtype mice. While the first approach is T cell specific, the latter approach eliminates Blimp-1 from the entire lymphoid and myeloid system.

Results from the GFP knock-in mouse showed that Blimp-1 expression is absent in naïve cells but is induced in antigen-primed CD4+ and CD8+ T cells both after in vivo stimulation and after in vitro TCR stimulation together with αCD28 and IL2. This supports earlier data which shows that both in human and in mice, Blimp-1 levels are elevated in effector memory (CD62L−CD44+) and central memory (CD62L+CD44+) T cells.

Deletion of Blimp-1 led to a striking increase in the in vivo number of effector cells predominantly in the CD8+ compartment and to a lesser extent of CD4+ cells, leading to severe auto-immune pathologies. Creney et al. has shown that the lack of IL10 production by Treg cells is the main contributor to the pathology of Blimp-1 deficient mice. Blimp-1 expression is restricted to a distinct population of effector Treg cells, which produced large quantities of IL10, highly expressed ICOS and tend to localize to non-lymphoid tissues such as the gastrointestinal tract and the lungs. Within these tissues Blimp-1 restricted the number of effector Treg cells by limiting the expression of Bcl2 and CCR6, which mediate the migration of Th17 cells and Treg cells to sites of inflammation and non-lymphoid tissue. Blimp-1 acted together with IRF4 to regulate the expression of IL10 and CCR6 by altering the accessibility of the genes by altering the chromatin structure. Surprisingly, Creney et al. showed that Blimp-1 directly bound to a conserved region in intron 1 of the Il10 gene, which suggests that Blimp-1 acted as a transcriptional activator. This indicated that Blimp-1 may not be solely a transcriptional repressor.

Further evidence that Blimp-1 is required for differentiation into functional CTLs was found by three groups that published their findings in the same issue of Immunity. Rutishauser et al. and Shin et al. used reporter mice in an LCMV infection model in which the gene for yellow fluorescent protein (YFP) was placed after the Blimp-1 promoter. Blimp-1 was upregulated in the majority of the LCMV specific effector cells during the expansion phase and progressively declined, both in the number of cells that expressed YFP as well as in the expression level per cell. Analysis of the CD8+ T cell subsets revealed that SLECs (KLRG1+IL7Rα−) contained more Blimp-1 than MPECs (KLRG1−IL7Rα+) (Fig. 2). This was in agreement with earlier micro-array results in which day 8 effectors were found to contain more Blimp-1 than memory CD8+ T cells. Furthermore, infection of the reporter mouse with two different strains of LCMV that initiate acute or chronic infection also showed that Blimp-1 expression increases during the CD8+ T cell expansion phase. At this time point of infection most antigen specific CD8+ T cells are SLECs, confirming that Blimp-1 is primarily expressed in those cells.

To analyze the effect of Blimp-1 expression in primed T-cells, loxP flanked Prdm1 mice were crossed with mice containing Cre recombinase driven under the GzmB promoter. These Prdm1−/− mice allow normal Blimp-1 expression until 1-2 days after T-cell activation when the GzmB promoter becomes activated. Results showed that LCMV infection in mice lacking Blimp-1 induced profoundly fewer effector cells with a SLEC phenotype. This corresponded with a reciprocal increase in cells with a MPEC or T CM phenotype in various
Subsequent micro-array analysis showed that deletion of Blimp-1 resulted in downregulation of genes with an effector cell signature (Perforin, GzmA, T-bet, ID-2) and upregulation of memory cell molecules (CCR7, IL7R). This is consistent with the notion that Blimp-1 is a terminal repressor and removal will result in upregulation of its target genes. Micro-array analysis in which differentially regulated genes in Prdm1−/− mice after LCMV infection were compared with MPECs and SLECs, shows that the majority of genes in Blimp-1 deficient mice follow the same trend as MPEC cells. In addition, Prdm1−/− CD8+ T cells showed an enhanced survival, a higher proliferative capacity and elevated IL2 production after LCMV infection compared to wt CD8+ T cells. In conclusion, Blimp-1 induces the formation of SLECs and suppresses the formation of MPEC and subsequent memory cells, in particular Tcm cells.

Shin et al. further elucidated the effects of Blimp-1 deletion on acute versus chronic (Clone 13) LCMV infection187,189. Clone 13 infection induced substantially elevated Blimp-1 expression levels in CD8+ T cells during the chronic phase, especially in clones directed against the NP396 epitope, which undergo exhaustion and deletion early during chronic infection. This suggests that Blimp-1 is associated with T cell dysfunction during chronic infection. Furthermore, the YFP reporter mouse further showed that Blimp-1 expression correlates with the elevated expression and coexpression pattern of inhibitory receptors on exhausted CD8+ T cells. To further assess the role of Blimp-1 in T cell exhaustion, the GzmB-Cre Prdm1−/− mouse model described above was used. Conditional deletion of Blimp-1 restored key aspects of memory cell differentiation such as expression of CD127 and CD62L. Furthermore, terminally differentiated populations such as CD8+ NP396 specific cells that were deleted during chronic infection, partially survived. In concert with earlier data that Blimp-1 regulates GzmB expression, it was hypothesized that the Blimp-1 deficient mice would be better equipped to clear persistent infection. However, full knockout mice were not able to control viremia. Interestingly, conditional haploinsufficient mice were indeed able to control viral titers faster than wt mice. Thus, although high levels of Blimp-1 are associated with T cell exhaustion, lower Blimp-1 levels are necessary for full effector CD8+ T cell differentiation and the eventual control of chronic infection.

Kallies et al. sought to determine the effects of Blimp-1 deletion on acute antiviral CD8+ T cell responses190,191. A challenge with Influenza A virus (IAV) revealed that chimeric mice which lack functional Blimp-1 in their immune system are not able to control infection. Therefore, chimeric mice containing a mix of Prdm1+/− (wt) and Prdm1gfp/gfp (Blimp-1 deficient) bone marrow were generated. These mice do not suffer from lethal pulmonary pathology as the full knockout mice while Blimp-1 deficient and wt cells could be examined within the same host. Challenge with IAV revealed that Prdm1gfp/gfp cells are mainly found in the lymph node and show diminished migration to the site of infection. Since CCR7 was elevated in Blimp-1 deficient cells, Blimp-1 might repress CCR7 expression to induce exit from the lymph node. Furthermore, antigen specific Blimp-1 deficient cells produced more IFNγ, TNFa and IL2, while Granzyme B expression was virtually absent. The absence of Granzyme B resulted in CD8+ T cells with impaired cytotoxic potential. Genes encoding other cytotoxic proteins like GzmK and Perforin were only partially impaired which could explain
the residual killing activity of Blimp-1 deficient IAV specific CD8+ T cells. These data show that Blimp-1 is essential for the cytolytic function of the antigen specific T cells.

Only a handful of genes involved in terminal T cell differentiation have been found to be directly repressed by Blimp-1. Next to Bcl6, Il2 and Fos, Blimp-1 directly targets the promoter of Id3. Repression of id3 by Blimp-1 induces a pro-apoptotic program that down regulates numerous genes necessary for the maintenance of genomic integrity in proliferating cells, through the increase of E2a transcriptional activity. Therefore, a way to increase the SLEC pool is Blimp-1 dependent repression of inhibitor ID3, thereby limiting their capacity to enter the memory T cell pool.

Another mechanism by which Blimp-1 induces a decrease in CD8+ T cell numbers was described in a recent article by Shin et al. where Blimp-1 was shown to silence IL2Ra (CD25) and Cd27. T cells expressing CD25 and the member of the TNF receptor family CD27, preferentially survive after acute viral infection. In response to infection with LCMV, Blimp-1 deficient CD8+ cells showed increased proliferation and remained responsive to cytokines due to failure to silence these two genes. Furthermore, constitutive expression by retroviral transduction enhanced CD8+ T cell survival and shRNA silencing of CD25 and CD27 further decreased the frequency of Blimp-1 deficient CD8+ T cell response. Thus, Blimp-1 normally functions to down-regulate cytokine receptor expression, thereby promoting death of the effector cells. Chromatin immunoprecipitation experiments (ChIP-Seq) revealed that Blimp-1 recruits histone methyltransferase G9a and deactetylase HDAC2 to the IL2Ra and Cd27 loci, silencing these genes. This epigenetic closure of loci takes place at the peak of the anti-viral response, after viral clearance and cessation of IL2 production. Then, CD25 and CD27 become susceptible to Blimp-1 regulation. Therefore, Blimp-1 operates not only by direct binding to the promoters of target genes, but serves as a epigenetic regulator that enhances the SLEC population while suppressing the MPEC progenitor cells.

More than one mechanism can act simultaneously on the same target as was shown by the Blimp-1 mediated repression of inhibitory receptor PD1. Blimp-1 can directly bind the PD-1 gene, thereby repressing transcription by deposition of repressive histone trimethylation marks. Secondly, it inhibits the expression of the transcriptional activator NFATc1. Lastly, Blimp-1 removes NFATc1 from the PD-1 locus that is induced after TCR stimulation.

In conclusion, Blimp-1 is essential for the migration and differentiation of effector T cells and of efficient recall of memory CD8+ T cells. Moreover, Blimp-1 promotes terminal differentiation into short-lived cytotoxic T lymphocytes rather than MPEC and TCM cells. The mechanisms by which Blimp-1 initiates this developmental program is diverse and its role in epigenetic regulation is just being unraveled.

Scope of this thesis

As described in the previous sections, the field that investigates the influence of transcriptions factors on CD8+ memory cell formation is rapidly evolving. Many papers describe the expression and function of the well-established and essential regulators of transcription T-bet, Eomes and Blimp-1 in CD8+ T cells in mice. In this thesis we focus on these proteins
in human. Moreover, the list of TFs that govern CD8\(^+\) cell fate decisions and other cytolytic cells in mice and men is ever growing. The aim of this thesis was to identify and characterize (novel) transcription factors that regulate effector functions in long lived cytolytic human lymphocytes.

In this first chapter a comprehensive overview of the literature to date of the transcription factors that play a prominent role in CD8\(^+\) T cell differentiation has been given. In chapter 2 we describe general transcriptional changes underlying the program that determines the differentiation status of CD8\(^+\) T cells in a chronic viral model system in humans (Chapter 3). In this study we introduce a novel transcription factor that we have named Hobit for homologue of Blimp-1 in T cells. Chapter 3 and 4 describe the putative function of Hobit in men and mice, respectively. Chapters 5 will address the expression of Hobit and other transcription factors in Large Granular Lymphoma cells. In the chapter 6 we have explored a novel way to phenotypically and functionally distinguish NK cells. In the discussion our findings will be summarized and placed in a broader perspective.
Reference List


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GENERAL INTRODUCTION


