Transcriptional control of cytotoxic lymphocytes

An unexpected journey with Hobit

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Hobit but not Blimp-1 maintains cytotoxicity in memory CD8 T cells

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Abstract:

Human cytomegalovirus (HCMV)-specific CD8 T cells are maintained in a unique differentiation stage that resembles that of effector cells after primary infection. In contrast to primary effector cells, HCMV-specific cells are non-cycling and maintained long-term in the absence of overt virus replication. HCMV-specific effector cells can directly mount cytotoxic responses through the constitutive production of granzyme B and perforin similar to primary effector cells. The recently identified resident memory T cells (Trm) have also been found to maintain granzyme B expression at the protein level. The transcriptional regulation of cytotoxicity in these quiescent lymphocytes remains unclear. We here show that Blimp-1, an important transcriptional regulator of cytotoxicity in primary effector cells, was not expressed at the protein level in quiescent effector and memory CD8 T cells. Here, we demonstrate that Hobit (ZNF683), a transcription factor related to Blimp-1, maintains granzyme B expression and immediate cytotoxic potential in quiescent effector CD8 T cells and in Trm. These findings indicate that Hobit and Blimp-1 are important during different stages of CD8 T cell differentiation with an early role for Blimp-1 in controlling effector functions during primary infection and a late role for Hobit during quiescence.
Introduction:

CD8 T cells provide an important line of immune defense in viral infection through their ability to eliminate infected cells in an antigen-specific manner. Naïve CD8 T cells that recognize viral antigens are triggered to proliferate and differentiate into effector cells within the lymph nodes. The effector cells migrate to the site of infection, where they up-regulate the expression of pro-inflammatory cytokines including IFN-γ and TNF-α and cytotoxic molecules that assist in clearance of infected cells. Effector CD8 T cells engage two major pathways to induce killing of target cells that are mediated through surface-expressed death receptors such as the FasL/Fas and Trail/Trail-R ligand/receptor pairs and through secretory granules containing perforin and granzymes [1;2]. Recognition of antigen in the context of MHC class I molecules triggers CD8 T cells to establish an immunological synapse with target cells that enables the directional release of cytotoxic granules and results in killing of infected, but not of uninfected cells. Granule-dependent cytotoxicity is driven by the pore-forming protein, perforin, which enables the release of proteolytic granzymes into the cytoplasm of target cells. Granzymes consist of a family of serine proteases, of which granzyme A and B have most clearly been associated with the induction of cytotoxicity [3]. Granzyme B induces pro-apoptotic pathways through the cleavage of BID into active truncated BID in humans and pro-caspases into active caspasases in mice to establish target cell death [4]. Other granzymes engage alternative pathways of cell death or directly impair components of the viral replication machinery [5]. In mice, the importance of granule-mediated cytotoxicity has been shown by targeted disruption of perforin, which impairs viral clearance after infection with lymphocytic choriomeningitis virus (LCMV) and increases the incidence of carcinogen-induced and spontaneous cancers [6;7]. The effects of deficiency in granzyme A or B are similar, but less dramatic than those of perforin ablation, suggesting substantial redundancy between members of the granzyme family [8].

After resolution of the infection, the effector population contracts into long-lived memory populations to enable more powerful responses upon secondary challenge. Central memory (Tcm), effector memory (Tem) and CD45RA+ effector memory cells (Temra) have been identified as separate long-lived CD8 T cell subsets that are maintained during quiescence in human peripheral blood [9;10]. The peripheral tissues including the epithelial layers of skin, lungs and intestine harbor tissue-resident memory CD8 T cells (Trm) that form distinct populations from the circulating memory cells [11]. Memory subsets of Tcm, Tem and Trm have been found to develop at late time-points after infection from a subset of effector cells in in vivo models using herpes simplex virus (HSV), influenza virus, and lymphocytic choriomeningitis virus (LCMV) infection [12-14]. The ontogeny of Temra cells is less clear, as these cells do not arise in mouse infection models. In contrast to effector cells, memory cells are maintained long-term in the absence of antigenic and inflammatory stimuli, which places constraints on the continuous production of effector molecules. Granzyme B-driven cytotoxicity is strongly down-regulated in memory cells compared to effector cells [15]. Tcm and the majority of Tem do not express granzyme B or perforin at the protein level, although epigenetic remodeling of the granzyme B locus suggests that it is more easily accessible in these circulating memory cells compared to naive cells.
Indeed, Tcm and Tem maintain elevated expression of granzyme B mRNA compared to naive cells, but require up-regulation of granzyme B protein to establish cytotoxic responses [15]. In contrast, Temra have been shown to express granzyme B and perforin at the protein level [9] and can execute antigen-specific cytolysis (ref Gamadia). Similarly, Trm that develop in the small intestine and brain after acute infection with LCMV have been reported to express granzyme B during quiescence [18;19]. Thus, a substantial fraction of the long-lived CD8 T cell populations has the potential to immediately engage granzyme B-driven cytotoxicity.

For the improvement of CD8 T cell therapies, it is highly relevant to understand how the immediate cytotoxic potential is maintained into the memory phase. However, at present, the regulation of granzyme B-driven cytotoxicity in Temra and Trm populations is unclear. Previously, we have demonstrated that Hobit is a transcriptional regulator that drives the expression of granzyme B in NKT cells [20]. Interestingly, the expression of Hobit perfectly aligns with long-lived cytotoxic populations, as Hobit is specifically upregulated in Trm and Temra cells within the CD8 T cell lineage [21;22]. Hobit is highly homologous to the transcription factor Blimp-1, which has previously been shown to induce terminal differentiation in the B and T cell lineage [23]. Effector CD8 T cell differentiation is under the control of a set of transcription factors that collaboratively regulate the separation of the short-lived effector and memory precursor lineages. Blimp-1 essentially contributes together with T-bet, Id2 and Notch to the development of short-lived effector cells at the expense of memory precursors [24-28]. Blimp-1 is also involved in the acquisition of the full repertoire of effector functions, as it induces the production of granzyme B in CD8 T cells during the peak of the primary response [26;27]. Therefore, here, we have addressed the role of Hobit and Blimp-1 in the instruction of cytotoxicity during CD8 T cell effector and memory differentiation. Strikingly, we found that Hobit is exclusively important for the maintenance of granzyme B expression during the memory phase.
Results

**Blimp-1 protein is not maintained in the absence of antigenic stimulation**

Blimp-1 is induced in naive CD8 T cells during effector differentiation and expression is maintained into the memory phase, as evidenced using Blimp-1 reporter mice [26,27]. To study Blimp-1 expression during human CD8 T cell differentiation, we setup an in vitro culture protocol (Fig. 1A). Human memory and quiescent effector CD8 T cells expressed Blimp-1 mRNA in contrast to naive CD8 T cells (Fig. 1B), as previously reported [29]. Despite the abundant presence of Blimp-1 mRNA, we observed that Blimp-1 protein expression was nearly absent in memory CD8 T cells (Fig. 1C). Although activation using anti-CD3/28 antibodies in combination with IL-2 did not further increase expression of Blimp-1 mRNA in memory CD8 T cells (Fig. 1B), Blimp-1 protein was strongly induced in these cells (Fig. 1C). These findings suggest that Blimp-1 activity is regulated at the protein level and requires antigenic stimulation in a pro-inflammatory environment. To study the maintenance of Blimp-1 expression after activation, we initially stimulated naive human CD8 T cells using anti-CD3/28 and IL-2, before resting them in the presence of the homeostatic cytokine IL-15 (Fig. 1A). Naive CD8 T cells upregulated Blimp-1 mRNA and protein after stimulation (Fig. 1, B and C). After resting of the cells, Blimp-1 mRNA expression was largely maintained (Fig. 1D), but Blimp-1 protein expression was rapidly lost to near completion (Fig. 1E). The longitudinal analysis indicated that Blimp-1 persists at the mRNA level, but not at the protein level after removal of antigenic stimuli, suggesting that Blimp-1 activity is confined to the effector phase.

**Antigenic stimulation down-regulates Hobit expression**

To establish whether the Blimp-1 homologue Hobit is also regulated after antigenic stimulation, naive CD8 T cells were stimulated with anti-CD3/28 and IL-2 for 3 days and Hobit expression was analyzed. Antigenic activation of naive CD8 T cells did not induce Hobit expression at the mRNA or protein level (Fig. 2, A-C), suggesting that Hobit in contrast to Blimp-1 is not induced during effector differentiation of CD8 T cells after primary infection. We have previously shown that Hobit is expressed at the mRNA and protein level in long-lived effector cells and a subset of memory cells during quiescence [30]. To establish the regulation of Hobit expression in effector CD8 T cells, we stimulated the cells in vitro with anti-CD3/28 and IL-2. In contrast to Blimp-1, Hobit was strongly down-regulated in effector CD8 T cells after 3 days of stimulation with anti-CD3/28 and IL-2 (Fig. 2, A-C). To examine whether the down-regulation of Hobit was a direct effect of T cell activation, we briefly cultured effector CD8 T cells in the presence of PMA and ionomycin. Hobit mRNA was strongly down-regulated in effector CD8 T cells as early as 4 hours after activation with PMA and ionomycin (Fig. 2D). Hobit protein was also down-regulated in effector CD8 T cells after 4 hrs of PMA and ionomycin stimulation, but the down-regulation was not complete at this early time-point (Fig. 2, E and F). To determine whether the expression regulation of human Hobit was similar in vivo, we followed HCMV- and EBV-specific CD8 T cells over time after primary HCMV and EBV infection in kidney transplant recipients. HCMV-specific CD8 T cells (HCMV-TPR) and EBV-specific CD8
T cells (EBV-EPL) were divided into proliferating and non-proliferating cells using the proliferation-associated marker Ki67 and analyzed for Hobit expression (Fig. 2G). Ki67high CD8 T cells had reduced expression of Hobit compared to Ki67low CD8 T cells (Fig. 2G), underlining that recent antigen encounter suppresses Hobit expression in vivo. Similar results were obtained in a longitudinal study of a second kidney transplant patient (unpublished observations).

Hobit is expressed in granzyme B producing CD8 T cells under resting conditions

Long-lived effector CD8 T cells have direct capacity to lyse infected target cells,

**Figure 1: Blimp-1 expression is not maintained at the protein level in human memory CD8 T cells.** (A) Schematic representation displays the culture protocol of CD8 T cells. (B,C) CD8 T cells were sorted based on expression of CD27 and CD45RA into naive (CD27+CD45RA+), memory (CD27+CD45RA-) and effector populations (CD27-CD45RA+). Isolated CD8 T cells were not activated (control) or activated with anti-CD3/CD28 antibodies and IL-2 for 3 days. (B) Blimp-1 mRNA expression was analyzed in naive (left panel), memory (center panel) and effector CD8 T cells (right panel) under the indicated conditions. (C) Blimp-1 protein expression (top lane) was analyzed by Westernblot in naive (left panel), memory (center panel) and effector CD8 T cells (right panel) under the indicated conditions. β-actin was used as a loading control (bottom lane). (D,E) Naive CD8 T cells (CD27+CD45RA+) were activated and then rested in IL-15 for 7 days. (D) Blimp-1 mRNA was analyzed using qPCR and (E) Blimp-1 protein was analyzed using Westernblot at the indicated time-points after stimulation. Data displayed in (B) represents five donors from two independent experiments. Data in (C, D and E) display one representative experiment out of at least two independent experiments. The representative experiment in (D) contained four donors. * p < 0.05 (two-tailed t-test).
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Figure 2. Activation induces downregulation of Hobit expression at the transcriptional level in human CD8 T cells. (A-C) Naive (CD27+CD45RA+) and effector CD8 T cells (CD27-CD45RA+) were isolated using cell sorting and not stimulated (control) or stimulated with anti-CD3/CD28 antibodies and IL-2 for 3 days. The expression of Hobit was determined using (A) qPCR and (B) flow cytometry in naive (left panels) and effector CD8 T cells (right panels) under the indicated conditions. (C) Histograms display binding of secondary antibodies (control; filled grey) and expression of Hobit (black line) in naive and effector CD8 T cells under the indicated conditions. Numbers in upper right corner display geometric mean fluorescence intensity (geo MFI) of Hobit expression. (D-F) Isolated effector CD8 T cells were left unstimulated (control) or were briefly stimulated with PMA and ionomycin. The expression of Hobit was determined using (D) qPCR and (E) flow cytometry. (F) Histogram displays Hobit expression of effector
CD8 T cells under the indicated conditions. Numbers in upper left corner display geo MFI of Hobit expression. (G) The expression of Hobit was determined in the Ki67+low and high fractions of hCMV (TPR) and EBV (EPL) specific CD8 T cells at the indicated time points after transplantation of a hCMV+ and EBV+ kidney into an hCMV- and EBV- recipient. Dotplots display tetramer binding and Ki67 expression in CD8 T cells at the indicated weeks (Wk) after transplantation (Tx). Numbers on top of gates represent percentage of cells. Histograms depict Hobit expression in Ki67+low and Ki67+high tetramer+ CD8 T cells. Numbers in upper left corner represent geometric mean fluorescence intensity (geo MFI) of Hobit expression. Data in (A) displays five donors from two independent experiments. Data in (B) displays one representative experiment with three donors out of at least three independent experiments. Data in (C) shows one representative donor from the data displayed in (B). Data in (D and E) displays at least six donors from three separate experiments. Data in (F) shows one representative donor from the data displayed in (E). Data in (G) displays one representative patient out of two. * p < 0.05 (two-tailed t-test).

as they express high levels of granzyme B and perforin protein under steady state conditions [31]. Interestingly, granzyme B clustered together with Hobit and Blimp-1 in microarray analysis of subsets of memory and effector CD8 T cells, suggesting a causal relation of these transcription factors in the regulation of cytotoxicity [29;30]. To address these findings at the protein level, we co-stained CD8 T cells from human peripheral blood for Hobit and granzyme B. Underlining the transcriptional profiling, a strong association between Hobit and granzyme B was found at the protein level. The majority of Hobit+ CD8 T cells co-expressed granzyme B and vice versa the majority of granzyme B+ CD8 T cells co-expressed Hobit (Fig. 3, A and B). CD8 T cells that expressed both Hobit and granzyme B, also contained high amounts of perforin (Fig. 3, C and D), suggesting that they have the machinery for immediate cytotoxicity. Interestingly, Hobit+ CD8 T cells that did not express granzyme B contained low amounts of perforin (Fig. 3, C and D). These cells expressed high amounts of granzyme K instead of granzyme B (Fig. 3, C and D), suggesting that Hobit identifies separate cytotoxic populations that express either granzyme B or K. As predicted, despite the strong overlap between Hobit and granzyme B expression under steady state conditions, naive CD8 T cells expressed granzyme B in the absence of Hobit expression after in vitro activation (Fig. 3E). In contrast, Blimp-1 protein was not co-expressed with granzyme B in peripheral blood CD8 T cells under resting conditions, but was induced in granzyme B+ cells after TCR stimulation (Fig. 3F). Thus, Hobit was specifically expressed in CD8 T cells with cytotoxic potential under resting conditions in contrast to Blimp-1 that was upregulated in cytotoxic CD8 T cells after activation.

**Resident memory maintains expression of cytotoxic molecules and cytotoxicity**

In contrast to human CD8 T cells in peripheral blood, circulating populations of murine memory CD8 T cells do not maintain granzyme B expression at the protein level [15]. The expression regulation of granzyme B and other cytotoxic molecules in Trm populations within the peripheral tissues at late time-points after infection has not been examined in detail. Comparison of memory populations after LCMV infection using RNA sequencing showed that cytotoxicity-associated molecules including granzyme B and C, FasL and Trail were strongly upregulated in gut and liver Trm compared to circulating memory populations (Fig. 4A). To further study the
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Figure 3: Hobit is co-expressed in human CD8 T cells with granzyme B. (A,B) The co-expression of Hobit and granzyme B was analyzed in CD8 T cells of human peripheral blood. (A) Dot plot depicts Hobit and granzyme B expression in CD8 T cells of a representative donor. Numbers represent percentage of cells within quadrant. (B) The percentage of CD8 T cells expressing only Hobit, only granzyme B or both Hobit and granzyme B was quantified. (C) Histograms and (D) bar graphs depict the expression of perforin (left panels) and granzyme K (right panels) of Hobit-granzyme B- (blue), Hobit+granzyme B- (red) and Hobit+granzyme B+ (black) CD8 T cells. (E,F) The expression of (E) Hobit and granzyme B and (F) Blimp-1 and granzyme B was determined in isolated naive (grey dots) and effector CD8 T cells (black dots) under steady state (control) and after activation for 3 days with anti-CD3/CD28 antibodies and IL-2. Data in (A and C) show a representative example from data in (B and D), respectively. Data in (B) displays the pooled results of nine donors from two experiments and data in (D) displays data of 4 donors from one representative experiment out of two. Data in (E and F) are representative of five donors from two independent experiments. *** p < 0.001 (1-way ANOVA).

expression regulation of these cytotoxicity-associated molecules during primary CD8 T cell responses, virus-specific CD8 T cells were followed over time after acute LCMV infection. The expression of granzyme B protein was induced in effector CD8 T cells at day 8 post infection (p.i.) in spleen, liver and gut (Fig. 4, B-D). Expression of granzyme B protein was maintained within LCMV-specific Trm from liver and gut, but not within LCMV-specific Tem from spleen and liver (Fig. 4, B-D). Similar to granzyme B, Fasl protein was enriched on liver Trm compared to Tem (Fig. 4E). In contrast, protein expression of Trail was not detectable on these memory populations in comparison to resident NK cells (Fig. 4F). Granzyme B and Fasl mediate important pathways that enable cytotoxic T cells to eliminate target cells [1;2], suggesting that Trm
Figure 4: Trm specifically upregulate cytotoxic molecules to maintain immediate cytotoxic potential. (A) Heatmap displays the expression of the indicated cytotoxicity-associated genes in virus-specific circulating (Tcm and Tem) and resident memory CD8 T cells (Trm) of WT mice after infection with LCMV, as determined using RNA sequencing. (B-D) The expression of granzyme B was analyzed in virus-specific CD69-CD62L- (Tem) and CD69+CD62L- CD8 T cells (Trm) in spleen, liver and gut of WT mice at day 8, 15 and day 30+ after infection with LCMV-WE and LCMV-Armstrong, as indicated. (B) Histograms depict expression of granzyme B in the indicated populations of CD8 T cells. (C,D) The percentage of granzyme B+ CD8 T cells was quantified in (C) Tem and Trm of liver and (D) in Tem of spleen and Trm of small intestine (SI). (E,F) Histograms display expression of (E) FasL and (F) Trail in virus-specific Tem and Trm within liver of WT mice at 30+ days after infection with LCMV. For control purposes, TRAIL expression was also analyzed in circulating (cNK) and tissue-resident NK cells (trNK) of the liver. (G,H) The killing of peptide-loaded EL4 target cells was analyzed at (G) 4 and (H) 24 hours of co-culture in the indicated effector/target ratios with virus-specific Tem (left panels) or Trm (right panels) of LCMV-infected mice.
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To determine the cytotoxic potential of the circulating and resident memory populations, we analyzed the ability of LCMV-specific CD8 T cells to mediate lysis of EL4 target cells loaded with cognate peptide. The virus-specific Trm population displayed cytotoxic activity within 4 hours after stimulation in contrast to the virus-specific Tem population (Fig. 4G). Both Tem and Trm killed target cells after 24 hours (Fig. 4H), suggesting that the main difference between both memory populations is in the kinetics of acquiring cytotoxic effector function. These findings indicate that Trm in contrast to circulating memory cells maintain expression of cytotoxic molecules at the protein level to enable the direct elimination of infected cells after secondary infection.

**Blimp-1 drives granzyme B expression during effector phase and Hobit during the memory phase**

In mice, Hobit expression in the CD8 T cell lineage is restricted to Trm, whereas Blimp-1 is broadly expressed in effector and memory CD8 T cells [21]. In line with previous findings [26;27], Blimp-1, but not Hobit, was required for granzyme B expression in effector CD8 T cells at day 8 after LCMV infection (Fig. 5, A and B). In contrast, the expression of granzyme B in Trm of liver and gut at day 30+ p.i. was largely dependent on Hobit (Fig. 5, C and D). The contribution of Blimp-1 to the regulation of granzyme B expression in the memory phase could not be addressed, as Blimp-1 deficient mice were unable to clear LCMV infection (unpublished observations). We observed that expression of granzyme B was compromised at the RNA level in Trm of Hobit KO mice, suggesting that Hobit was essential in the transcriptional regulation of granzyme B (Fig. 5E). The expression of other molecules involved in cytotoxicity including perforin was not dependent on Hobit (Fig. 5F). Granzyme B is stored together with perforin in secretory vesicles that contain the lysosomal protein LAMP-1/CD107a [15]. Peptide re-stimulation of virus-specific cells at day 30+ after LCMV infection induced normal degranulation of CD107a+ vesicles in liver-derived Trm in the absence of Hobit (Fig. 5, G and H), indicating that Hobit controls cytotoxicity specifically through the transcriptional regulation of granzyme B expression. These findings show that Blimp-1 and Hobit-driven regulation of granzyme B in CD8 T cells occurs at early and late time-points after infection, respectively. Thus, Hobit rather than Blimp-1 appears important for the long-term maintenance of granzyme B expression in memory CD8 T cells.

Total CD62L-CD69–CD8 T cells (Tem) and CD62L+CD69+ (Trm) fractions of CD8 T cells at day 30+ of infection were taken from liver of WT mice for use as effectors in the killing assays. Data in (B) displays representative examples from data in (C and D). Data in (C and D) displays the results from one representative experiment out of two with three or four mice per group. Data in (E and F) is representative of five to eight mice from two independent experiments. Data in (G) displays results of seven mice from two pooled experiments and data in (H) displays data of two mice from one representative experiment out of two. *** p < 0.001 (1-way ANOVA).
Figure 5: Blimp-1 induces granzyme B expression in effector CD8 T cells and Hobit in memory CD8 T cells after LCMV infection. (A) Histograms depict granzyme B expression in WT (filled grey) and mutant (solid line) virus-specific CD8 T cells of Blimp-1 KO (left panel) and Hobit KO mice (right panel) at day 8 after infection with LCMV. Numbers indicate percentage of granzyme B expressing CD8 T cells. (B) The percentage of effector CD8 T cells that express granzyme B was quantified in WT and Blimp-1 KO
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mice (left panel) and in WT and Hobit KO mice (right panel). (C,D) Similarly, the expression of granzyme B was analyzed in virus-specific memory CD8 T cells of WT and Hobit KO mice at day 30+ after infection with LCMV. (C) Histograms depict granzyme B expression in the indicated populations of CD8 T cells. (D) The percentage granzyme B+ CD8 T cells was quantified in the indicated populations of CD8 T cells. (E,F) The expression of granzyme B mRNA and perforin was determined using qPCR in virus-specific CD62L-CD69- Tem and CD62L-CD69+ Trm of WT and Hobit KO mice at day 30+ after LCMV infection. (G,H) The degranulation of LCMV-specific CD8 T cells from liver of WT and Hobit KO mice was analyzed at day 30+ after infection. (G) Histograms and (H) bar graphs display CD107a labeling after brief stimulation with gp33 peptide in LCMV-specific CD8 T cells that were identified using intracellular labeling of IFN-γ. CD62L-CD69- (Tem) and CD62L-CD69+ fractions (Trm) of virus-specific CD8 T cells of WT and Hobit KO mice were separately analyzed. Data in (A, C and G) display representative results from at least four mice from two independent experiments. Data in (B) displays results from one independent experiment out of two with at least three mice per group. Data in (D) displays one representative experiment out of two with four or five mice per group. Data in (E and F) displays pooled results from two experiments with six to eleven mice per group. Data in (G) shows a representative example from data in (H). Data in (H) displays results from one representative experiment out of two with five mice per group. *** p < 0.001 (two-tailed t-test or 1-way ANOVA).
Chapter 5

Discussion:

In this report, we describe that the expression regulation of Hobit and Blimp-1 occurs in opposite directions during CD8 T cell differentiation. In CD8 T cells, Blimp-1 is up-regulated by activation, whereas Hobit is downregulated by activation. Blimp-1 mRNA, but not Blimp-1 protein, is maintained in long-lived effector and memory populations of CD8 T cells, indicating that Blimp-1 is repressed by post-transcriptional mechanisms in CD8 T cells. These findings suggested that Blimp-1 did not contribute to the regulation of cytotoxicity in CD8 T cells at late time-points after infection. Indeed, we established that Hobit was an essential transcriptional regulator for the maintenance of granzyme B in CD8 T cells during the memory phase. Thus, the Hobit and Blimp-1-driven transcriptional regulation of cytotoxicity appears temporally separated in CD8 T cells with an early role for Blimp-1 in primary effector cells and a late role for Hobit in memory cells.

The Blimp-1 GFP reporter has been a highly valuable tool in the assessment of the expression pattern of Blimp-1 in B and T lymphocytes [26;32]. The reporter has been shown to truthfully reflect Blimp-1 expression at the mRNA level during B and T cell differentiation, but does not necessarily take into consideration the posttranscriptional regulation that may occur at the level of translation or at the level of proteasomal degradation. We have observed a discrepancy between the expression of Blimp-1 mRNA and protein during quiescence in memory CD8 T cells, suggesting that in these lymphocyte populations posttranscriptional regulation is relevant to acquire Blimp-1 protein. The mechanisms underlying posttranscriptional regulation of Blimp-1 are unclear. It is possible that sumoylation plays a role, as this process has previously been reported to target Blimp-1 for degradation by the proteasome [33]. The expression of Blimp-1 protein has not been directly examined in other Blimp-1+ populations such as regulatory T cells and plasma cells. Functional analyses using conditional deletion of Blimp-1 suggest that Blimp-1 protein activity remains during quiescence in plasma cells, although such analyses do not rule out that more subtle differences in protein expression occur [34]. As regulatory T cells and plasma cells in contrast to CD8 T cells are unable to up-regulate Hobit under homeostatic conditions, we speculate that persistence of Blimp-1 protein and acquisition of Hobit are alternative strategies of these separate lineages to maintain the Hobit/Blimp-1 transcriptional module during quiescence.

Hobit does not appear to share the posttranscriptional regulation with Blimp-1, as the expression of Hobit mRNA perfectly aligned with Hobit protein within human CD8 T cells and NK cells [22]. The functional analyses of Trm using granzyme B regulation as a readout for cytotoxic activity also indicate that murine Hobit remains expressed at the protein level, as it maintains the killing capacity of these cells during the memory phase. Currently, the full spectrum of the signaling pathways acting upstream of Hobit expression are unknown. In line with expression during the resting phase, we have found that the homeostatic cytokine IL-15 is involved in the induction of Hobit expression in Trm [21]. Consistent with an exclusive role during quiescence, we have shown that Hobit was downregulated within hours after T cell activation at both the mRNA and protein level. Indeed, Hobit is not found in short-lived effectors
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during primary infection, but is present in Temra and Trm cells that display direct effector function and that persist during quiescence [21;22]. The rapid kinetics of the decrease in expression after triggering with PMA and ionomycin indicates direct involvement of TCR signaling pathways in Hobit downregulation. In contrast to Hobit, Blimp-1 appears to be induced in response to inflammatory signals. Blimp-1 expression markedly increases during effector differentiation of CD8 T cells in vivo [35-37], and the transcription factor is also upregulated after antigenic stimulation in combination with cytokines such as IL-2, IL-12 and IL-21 in vitro [38-41]. Similarly, human NK cells up-regulate Blimp-1 expression after stimulation in vitro with IL-12 and IL-18 [42]. The expression regulation suggests that despite the overlap in transcriptional targets, Hobit and Blimp-1 have non-redundant roles in immune regulation, as they are expressed under resting and inflammatory conditions, respectively.

Hobit and Blimp-1 are homologous transcription factors that display the highest degree of similarity in the Zinc Finger domains [20;22]. This region in both Hobit and Blimp-1 is essential for the binding to “GAAAG”-containing DNA sequences, providing rationale for their highly overlapping direct target genes in CD8 T cells [21;22]. Blimp-1 directly bound within the granzyme B locus, suggesting direct involvement in the transcriptional regulation of cytotoxicity [21]. In contrast, we were unable to find evidence for direct binding of Hobit at these sites. Technical reasons relating to sensitivity may underlie the difference, as the total number of target genes was about ten-fold lower for Hobit than for Blimp-1 [21]. Previously, we have shown in NKT cells that Hobit is involved in the regulation of granzyme B expression at the transcript level [20]. Here, we have established an essential role for Hobit in the transcriptional regulation of granzyme B in Trm of liver and gut. As Blimp-1 similar to Hobit positively regulates granzyme B expression and granzyme B-driven cytotoxicity in effector CD8 T cells during primary infection [26;27], it appears that both factors instruct the same transcriptional program to establish cytotoxicity. At odds with the induction of granzyme B expression, Blimp-1 has been described as a transcriptional repressor that silences the expression of target genes through the recruitment of co-repressors including the histone H3K9 methyltransferase G9a, Groucho family proteins and histone deacetylases [43-45]. Recently, it has become clear that during plasma cell differentiation Blimp-1 also associates with putative co-activators such as the chromatin-remodeling BAF complex to establish active histone marks in its target genes [46]. The epigenetic landscape of the granzyme B locus has been studied in human and mouse circulating memory cells and evidence for the presence of active hyperacetylation at H3K9 and the absence of repressive hypermethylation at H3K27 has been found [16;17]. At this stage it is unclear whether these epigenetic marks are preserved in Trm and Temra cells or whether these cells contain unique epigenetic marks. Thus, our findings suggest that Blimp-1 contributes to the transcriptional regulation of cytotoxicity during productive infection and Hobit after clearance of infection, but the potential role of these transcription factors in the establishment of these epigenetic marks remains unexplored.

In contrast to Trm that are formed in liver and gut after LCMV infection, Trm that develop in skin after HSV-1 infection and in lungs after infection with respiratory viruses do not express granzyme B protein [47;48]. As HSV-1-specific Trm within the skin maintain higher expression of granzyme B mRNA than circulating memory pop-
ulations [21], it appears that these Trm also display improved retention of cytotoxic capacity, although not at the level of granzyme B protein expression. The differences in the maintenance of granzyme B expression between Trm populations may relate to tissue-specific adaptations that arise between Trm located at different sites. In support of tissue-specific regulation of granzyme B expression, Trm within the dorsal root ganglia that co-arise with skin-resident Trm after infection with HSV-1 express granzyme B at the protein level [48]. As Hobit is expressed in Trm throughout tissues including the skin and lungs [21], Hobit expression does not appear to be sufficient for the induction of granzyme B protein in all of these Trm populations. We have previously observed that Hobit is essential for granzyme B protein expression in NKT cells located in the thymus, but is not sufficient to induce granzyme B protein in NKT cells residing in spleen and liver under steady state conditions [20]. However, after activation with type I IFN liver-resident NKT cells up-regulate granzyme B in a Hobit dependent manner [20]. Previously, it has been shown that stimulation with type I IFN up-regulates protein expression of granzyme B and granzyme B-driven cytotoxicity in antigen-specific memory CD8 T cells within the lungs [47;49]. Therefore, it is conceivable that Hobit is involved in the acquisition of granzyme B expression in Trm throughout tissues, but that the induction of granzyme B protein in skin and lungs requires stimulation with pro-inflammatory cytokines.

Long-lived CD8 T cells with direct cytotoxic potential are superior compared to other memory cells in that these cells are able to respond directly without the need of proliferation and differentiation, which enables them to mount immune responses at an accelerated pace. Here, we have identified that Hobit rather than Blimp-1 is an essential transcriptional regulator in the long-term maintenance of granzyme B-driven cytotoxicity of memory CD8 T cells. Other transcription factors such as T-bet, Eomes, Runx-3 and Notch, which have all been shown to establish cytotoxicity in effector CD8 T cells through the induction of granzyme B and or perforin [24;50;51], may contribute to the regulation of cytotoxicity in memory cells. In contrast to Hobit, these transcription factors are not exclusively expressed in memory cells with direct cytotoxic potential, suggesting that they do not have a specific role in the long-term maintenance of cytotoxicity. Moreover, T-bet and Eomes are downregulated in granzyme B+ Trm populations compared to granzyme B+ circulating populations of memory cells [52]. Thus, our findings have established a Hobit-driven transcriptional network underlying the maintenance of cytotoxicity in CD8 T cells that may provide essential clues to the future use of memory cells with immediate killing capacity in adoptive therapies in patients.
Material and Methods:

Human material
Human PBMCs were obtained from fresh heparinized blood or buffy coats of healthy donors using Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. Total, naive, effector and memory CD8+ T cells were isolated using magnetic sorting with CD8 microbeads (Miltenyi Biotec) and then flow-cytometric sorting for CD27 and CD45RA on a FACS Aria (BD Biosciences) to obtain CD27+CD45RA+ naive, CD27-CD45RA+ effector and CD27+CD45RA- memory cells. We have used blood samples from a kidney transplant patient who was EBV and hCMV seronegative before transplant and who underwent a primary EBV and hCMV infection after receipt of a kidney from a EBV+ and hCMV+ donor. The patient received an immunosuppressive regimen that included prednisolone, cyclosporine A and mycophenolate mofetil. All donors gave written informed consent prior to inclusion in the study and the study was approved by the Amsterdam Medical Center institutional medical ethics committee.

Mice
WT, Zfp683/- (Hobit KO) [20] and Prdm1flox/flox x Lck Cre (Blimp-1 KO) mice [26] were maintained on a C57Bl/6 background. Mice were bred under SPF conditions and animal experiments were performed according to national and institutional guidelines.

Antibodies
The following anti-mouse monoclonal antibodies for flow cytometry were purchased from eBioscience, BD Biosciences, Invitrogen or Biolegend: anti-CD3 (145-2C11), anti-TCRβ (H57-597), anti-CD4 (RM4-5, GK1.5, and MT4), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-Ly5.1 (A20), anti-Ly5.2 (104), anti-IFN-γ (XMG1.2), anti-TRAIL (N282), anti-human Granzyme B (GB-11) and anti-FASL (MFL3). The following anti-human monoclonal antibodies for flow cytometry were purchased from Sanquin Reagents, eBioscience and BD Biosciences: anti-CD8 (RPA-T8), anti-CD27 (O323), anti-CD45RA (HI100) and anti-CD3 (SK7). Antibodies against human Hobit (Sanquin-Hobit/1) were made in house, as previously described [22]. The following antibodies were used for western blotting: anti- Blimp-1 (6D3; eBioscience), anti-β-actin (AC-15; Sigma-Aldrich) and conjugated goat anti-mouse secondary antibodies (DAKO).

Tetramers
To detect LCMV-specific CD8 T cells, MHC class I Db restricted tetramers for the viral epitopes GP33-41 and NP396-404 were produced as described [53].

LCMV infection
Mice were infected intraperitoneally with 30 plague-forming units of the LCMV strain WE. At the indicated time points after infection mice were sacrificed and organs were collected for analysis of CD8 T cell responses.

Cell preparation
Spleen, liver, and small intestine were isolated and ground over 70 µM nylon cell
strainers (BD Biosciences) to obtain single-cell suspensions in PBS containing 0.5% BSA. Small intestine and liver lymphocytes were separated from the other cell fractions via Percoll (GE Healthcare) gradient centrifugation. Liver-cell preparations were resuspended in 44% Percoll solution and pelleted lymphocyte-enriched fractions were collected after centrifugation. Erythrocytes in spleen and liver cell suspensions were lysed (155 mM NH4Cl, 10 mM KHCO3 and 1 mM EDTA) for removal of red blood cells. For the isolation of intraepithelial lymphocytes (IELs) the small intestine was cleared of fat tissue, Peyer’s patches, and fecal content. Then, 1 cm2 pieces of small intestine were incubated for 30 minutes at 37°C in Ca2+ and Mg2+ Free Hank’s buffer (Gibco) containing 5mM EDTA and 1 mM DTT. IELs were separated from the other cell fractions via density gradient centrifugation using 44% and 66% Percoll that enable enrichment of IELs at the interface of these layers. Cells were counted with an automated cell counter (CaseyCounter (Innovatis)).

**In vitro T cell stimulation**

Human CD8+ T cells were activated in 24 or 96-wells plates (Costar) coated with 3.5ug/ml goat anti-mouse IgG antibodies (Jackson Immunoresearch) and 5 µg/ml anti-CD3 (HIT3a; eBioscience) in the presence of 1 µg/ml anti-CD28 (CD28.2; eBioscience) and 50 U/ml IL-2 (Peprotech) for the indicated time. For short-term activation, CD8+ T cells were cultured with PMA (2 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) for 4 hours. For the degranulation assay LCMV-specific CD8+ T cells were activated in 24 well plates in the presence of 5 µg/ml GP¬-33-41 and incubated for 4 hours in the presence of CD107a antibodies (eBio1D43; eBioscience). Brefeldin A (eBioscience) and Monensin (eBioscience) were added to enable intracellular capture of IFN-γ. For the cytotoxicity assay, EL-4 cells were labeled with Cell Trace Violet (Thermo Fisher Scientific) according to the manufacturer’s protocol and loaded with 5 µg/ml GP33-41. The percentage of killing was measured using the viability dye Near-IR (Thermo Fisher Scientific) as a readout.

**PCR and quantitative PCR**

RNA was isolated using Trizol Reagent (Invitrogen). cDNA synthesis was performed on a Verity 96 well Fast Thermo Cycler (Applied Biosystems) using the iScript RT PCR kit (Bio-rad). Quantitative PCR was performed on a StepOnePlus system (Applied Biosystems) using the FAST SybrGreen mix (Applied Biosystems). The following primersets were used: murine Hobit (forward: 5’-TCTCCCACTCTCATCTCCAA-3’, reverse: 5’-CAGACCCACTGCTGCTCAT-3’), murine Blimp-1 (forward: 5’-GACGGGGGTACCTTTCTGCA-3’, reverse: 5’-GGCATCTTGGGAACTGTTG-3’) Granzyme-B (forward: 5’-AAAGTGCTCTTCTCTCGG-3’, reverse: 5’-GAAACTATGCCTGAGCCACT-3’), HPRT (forward: 5’-TGAGAGCTACTGTAATGCTAC-3’, reverse: 5’-AGCAAGCTGAGAGCTTGAACCA-3’), Perforin (forward: 5’-GGAGCTGAAAGACTTATCAGGAC-3’, reverse: 5’-TCTGAGCGCTTTTTAAGCTC-3’), human Hobit (forward: 5’-CATGAGGGGCAAGACGCTTTG-3’, reverse: 5’-GGCAAGTTGAGTAAAGCTCT-3’, human Blimp-1 (forward: 5’-GTTCAGAACGGAGTAGAAACA-3’, reverse: 5’-CAGAGCTGCTTTAGAGCTG-3’), 18S (forward: 5’-GGCAGAACGGCGGTGAAA-3’, reverse: 5’-CAGAGCTGCTTTAGAGCTG-3’). Values are represented relative to that of 18S or HPRT and calibrated relative to naive CD8+ T cells unless indicated otherwise.
Flow cytometry
Cells were stained for 30 min at 4°C with fluorochrome-conjugated antibodies in PBS 0.5% BSA. Intracellular stainings were performed after fixation and permeabilization with the FoxP3 transcription factor staining set (eBioscience) or with the cytofix/cytoperm kit (BD Biosciences). Tetramer labeling was performed at room temperature. Samples were measured with an LSR Fortessa or a Canto II flow cytometer (BD Biosciences) and expression was analyzed using FlowJo software (Tree Star).

Western Blotting
Cells were lysed in buffer containing 2% SDS, 66 mM Tris pH 7, 16% β-mercaptoethanol and 1% protease inhibitor (Calbiochem). Proteins were separated on a NuPAGE 4-12% Bis-Tris gel (Novex, Life Technologies), after which proteins were transferred onto an iBlot Nitrocellulose Gel Transfer Stack (Novex, Life Technologies) using an iBlot or iBlot 2 (Life Technologies). Membranes were stained using the Pierce ECL Western Blot substrate kit (Life Technologies) and proteins were visualized on Fuji Medical X-ray Film using a Medical Film Processor (Konica Minolta Medical & Graphic. Inc, SRX-101A).

Statistics
Values are expressed as mean ± SD or SEM as indicated. Differences between two groups were assessed by Student’s t test. Differences between more than two groups were assessed using one-way ANOVA followed by a Bonferroni post-hoc test. A p-value of less than 0.05 was considered statistically significant (* = p<0.05; ** = p<0.01; *** = p<0.001).
References:


20. van Gisbergen, K.P., Kragten, N.A., Hertoghs, K.M., Wensveen, F.M., Jonjic, S., Ha-


