Transcriptional control of cytotoxic lymphocytes

An unexpected journey with Hobit

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The Blimp-1 homologue Hobit identifies effector-type lymphocytes in humans

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Abstract

Human cytomegalovirus (CMV) induces the formation of effector CD8+ T cells that are maintained for decades during the latent stage of infection. Effector CD8+ T cells appear quiescent, but maintain constitutive cytolytic capacity and can immediately produce inflammatory cytokines such as IFN-γ after stimulation. It is unclear how effector CD8+ T cells can be constitutively maintained in a terminal stage of effector differentiation in the absence of overt viral replication. We have recently described the zinc finger protein Homologue of Blimp-1 in T cells (Hobit) in murine NKT cells. Here, we show that human Hobit was uniformly expressed in effector-type CD8+ T cells, but not in naive or in most memory CD8+ T cells. Human CMV-specific but not influenza-specific CD8+ T cells expressed high levels of Hobit. Consistent with the high homology between the DNA-binding Zinc Finger domains of Hobit and Blimp-1, Hobit displayed transcriptional activity at Blimp-1 target sites. Expression of Hobit strongly correlated with T-bet and IFN-y expression within the CD8+ T cell population. Furthermore, Hobit was both necessary and sufficient for the production of IFN-γ. These data implicate Hobit as a novel transcriptional regulator in quiescent human effector-type CD8+ T cells that regulates their immediate effector functions.
Introduction

The CD8+ T cell compartment responds to viral infection through the massive expansion of virus-specific clones from the naïve repertoire. These cells differentiate into cycling effector cells that can efficiently clear viral infection through upregulation of effector molecules such as interferon (IFN)-γ, granzyme B, and perforin. An important aspect of CD8+ T cell differentiation is the generation of memory CD8+ T cells that remain present after viral clearance and that provide enhanced protection against re-infection. Naïve cells can form both effector and memory cells, as evidenced by the transfer of single naïve T cells and molecular bar-coding that have established the pluripotent nature of naïve T cells [1;2]. At early time points during primary infection, memory precursor effector cells (MPECs) that have the potential to generate memory cells separate from short-lived effector cells (SLECs) that lack this capacity [3;4]. MPECs are defined as CD127+KLRG1- cells, whereas SLECs are characterized as CD127-KLRG1+ cells. Although only a fraction of MPECs will form bona fide memory cells, the characterization of CD8+ T cells as MPECs or SLECs has been instrumental in unraveling the roles of transcription factors in effector and memory differentiation of CD8+ T cells. The differentiation of CD8+ T cells into MPECs and SLECs is under the control of a network of transcription factors that are specifically upregulated in either MPECs or SLECs. T-bet [5], Blimp-1 [6;7] and Id2 [8] are enriched in SLECs and are essential for SLEC formation, whereas Id3 [8;9], STAT3 [10], Tcf1 [11] and Eomes [12] are upregulated in MPECs and are required for MPEC differentiation. These transcription factors act as a rheostat that balances the ratio of SLECs and MPECs during primary infection to efficiently combat infection without compromising the formation of memory cells.

During chronic infection, CD8+ T cells can persist as effector-type cells [13;14]. Effector CD8+ T cells in productive infection with chronic viruses such as with HIV-1 in humans and LCMV Docile or Clone 13 in mice display an exhausted phenotype and have limited capacity to produce cytokines such as IFN-γ, TNF-γ and IL-2 [15]. Transcription factors that are expressed in SLECs such as Blimp-1 and T-bet are also important for the generation of effector T cells during productive chronic infection [16;17]. Eomes is highly expressed in chronic effector CD8+ T cells in contrast to SLECs and is important in the regulation of the exhaustive phenotype [16]. During chronic latent infection, such as with murine cytomegalovirus (mCMV) in mice, virus-specific CD8+ T cells are also constitutively maintained, but T cell exhaustion does not occur [13]. The mCMV-specific effector CD8+ T cells remain fully functional and can respond immediately with the production of cytokines [13].

In humans, tetramers have been used to characterize human CMV (hCMV) specific CD8+ T cells [18-20]. These CD8+ T cells typically express CD45RA but lack CD27 and phenotypically resemble long-lived and fully functional effector CD8+ T cells found in mice after mCMV infection [13;18-20]. Human CD45RA+CD27- effector CD8+ T cells maintain high levels of granzyme B protein and produce high levels of IFN-γ immediately upon stimulation [21]. Similar to memory cells these effector-type cells survive in the absence of detectable levels of virus, but in contrast to memory cells they lack expression of CD122 and CD127 and are not maintained on homeostatic cytokines such as IL-7 and IL-15 [22]. The presence of low amounts of antigen during latent infection imposes conditions on the maintenance of effector
CD8+ T cells that are distinct from those during productive infection, suggesting the requirement for unique transcriptional regulation. Previously, we have performed microarray analysis on hCMV-specific CD8+ T cells to establish transcription factors that are upregulated during the differentiation of long-lived effector cells in humans [22]. The analysis revealed that transcription factors that are upregulated in murine SLECs such as T-bet and Blimp-1 are also upregulated in human long-lived effector CD8+ T cells. Moreover, Eomes was also upregulated in human long-lived effector cells. However, as all of these transcription factors are strongly upregulated during primary and chronic infection in the presence of high antigen loads, they cannot solely account for the unique behavior of long-lived effector cells during antigen scarcity. Interestingly, our microarray screening revealed that Homologue of Blimp-1 in T cells (Hobit; alias for ZNF683) was specifically upregulated in long-lived effector CD8+ T cells [22]. Hobit is closely related to the transcriptional repressor Blimp-1 that regulates the terminal differentiation of B cells into plasma cells and CD8+ T cells into cytotoxic effectors [23]. Recently, we have described that Hobit is expressed in NKT cells, and required to maintain NKT cells in a quiescent state of terminal effector differentiation in mice [24]. Hobit also regulates immediate effector functions of NKT cells, such as the production of IFN-γ and granzyme B [24]. Therefore, we hypothesized that in humans, Hobit would act to regulate effector-type CD8+ T cells. Here, we describe the expression profile, expression regulation and function of human Hobit. We found that Hobit was specifically expressed in effector-type lymphocytes within the CD8+ T cell, NK and NKT cell lineages. We show that Hobit acts as a transcriptional repressor at Blimp-1 target sites and regulates effector functions.
Results

Human Hobit is specifically expressed in effector-type lymphocytes

Previously, we have identified Hobit as a transcription factor that is specifically up-regulated late during effector differentiation in a microarray study of hCMV-specific CD8+ T cells [22]. To confirm and extend our microarray data, we used qPCR to analyze the expression of Hobit in a panel of leukocyte subsets. As expected, Hobit was expressed in total CD8+ T cells, but expression was not confined to CD8+ T cells, as shown by the abundant expression of Hobit in NK cells (Fig. 1A). Hobit was not expressed or only at low levels in total CD4+ T cells, regulatory T cells and DCs (Fig. 1A). Using CD27 and CD45RA to separate CD8+ T cells into naive (CD27+CD45RA+), effector (CD27-CD45RA+) and memory cells (CD27+CD45RA+), we found that Hobit was expressed at much higher levels in effector cells than in naive and memory cells (Fig. 1B). Within the NK cell lineage, the expression of Hobit was enriched in the CD56dim population compared with the CD56bright population (Fig. 1C). CD56dim NK cells are terminally differentiated cytotoxic cells with low proliferative capacity. In contrast, CD56bright NK cells have a high proliferative capacity and low cytotoxic potential [25]. Thus, Hobit is specifically found in lymphocytes with immediate effector function.

![Figure 1. Hobit mRNA expression is confined to effector CD8+ T cells and NK cells.](image)

(A) The expression of Hobit was analyzed at the mRNA level using qPCR in the indicated subsets of leukocytes. (B) (Left panel) Dotplot depicts the sorting strategy for naive, memory and effector subsets of CD8+ T cells that were identified by CD27 and CD45RA expression as indicated. (Right panel) Subsets of naive, memory (mem) and effector (eff) CD8+ T cells were isolated and analyzed for Hobit expression using qPCR. (C) Hobit expression was analyzed in CD56bright and CD56dim subsets of NK cells. (A-C) Data are shown as mean ±SEM (n=3 (A and B) or 5 (C)) and are representative of two independent experiments. * p<0.05; ** p<0.01; Anova with post-hoc Bonferroni test.

Protein expression of Hobit identifies effector-type lymphocytes

We generated a monoclonal antibody (Sanquin-Hobit/1) against a truncated form of Hobit to enable analysis of Hobit protein expression by flow cytometry (Fig. 2A). The Hobit specificity of this antibody was verified using NK92 cells that have endogenous Hobit expression and NK92-Hobit knockdown (KD) cells, in which Hobit mRNA expression was reduced using Hobit-targeting shRNA (Fig. 2B). Staining with the antibody was reduced in NK92-Hobit KD cells compared with mock transduced NK92 cells confirming its specificity for Hobit (Fig. 2, C and D). We used this newly developed anti-Hobit antibody to analyze effector differentiation of CD8+ T cells in human
adjacent
contains
identity
Acids
Human
sites
Transcriptional variants of Hobit display distinct activity at Blimp-1 target
lineage.

We observed that KLRG1+ in contrast to KLRG1+ EM CD8+ T cells displayed substantial
expression of Hobit (Fig. 3, C and D). KLRG1 is a receptor associated with terminal
differentiation of effector lymphocytes [26]. Therefore, the correlation between Hobit
and KLRG1 expression within EM CD8+ T cells suggests that Hobit expression
is associated with terminal differentiation of CD8+ T cells. We analyzed the CD8+ T
cell compartment in tonsils to determine Hobit expression within secondary lym-
phoid tissues. As effector CD8+ T cells are nearly absent from tonsils [27], CD8+ T
cells were separated into naive and memory cells and analyzed for Hobit expression.
We observed that Hobit expression was low on naive and memory CD8+ T cells
within tonsils (Fig. 3, E and F), which is in line with the absence of CD8+ T cells with
explicit effector functions from the secondary lymphoid tissues. Staining for Hobit
on virus-specific populations of CD8+ T cells that were identified using tetramers,
showed strong expression in hCMV-specific CD8+ T cells (hCMV-IPS and hCMV-NLV),
intermediate expression in lytic EBV-specific CD8+ T cells (EBV-EPL), but no or low
expression in non-lytic EBV specific (EBV-HPV) and Flu-specific CD8+ T cells (Flu-
GIL, Fig. 3G and Table S1). These results substantiate the expression profile of Hobit
in CD8+ T cell subsets identified by CD27 and CD45RA phenotype, as hCMV-specific
CD8+ T cells are largely maintained as quiescent effector cells and non-lytic EBV
specific and flu-specific CD8+ T cells as memory cells [18;19;28]. The intermediate
expression levels of Hobit in lytic EBV-specific CD8+ T cells are consistent with the
more pronounced effector or effector memory phenotype of these cells compared
with non-lytic EBV specific CD8+ T cells [18;19;28]. Similar to effector CD8+ T cells,
CD56dim NK cells in peripheral blood uniformly expressed Hobit (Fig. 4, A, C and D).
In line with Hobit mRNA expression (Fig. 1, A and C), CD56dim NK cells expressed
more Hobit protein than CD56bright NK cells (Fig. 4, A and D). Consistent with the
lack of Hobit expression in tonsillar CD8+ T cells, CD56dim and CD56bright NK cells
within tonsils did not express Hobit (Fig. 4, B, C and D). In addition to NK cells, we
observed Hobit expression in a considerable fraction of the NKT cell population (Fig.
4, E and F). We did not detect substantial expression of Hobit in B cells, CD4+ T
cells including regulatory T cells, subsets of dendritic cells, monocytes and neutro-
phils (Fig. 4G). Thus, similar to Hobit mRNA expression, Hobit protein expression
is confined to effector-type lymphocytes within the CD8+ T cell, NK cell, and NKT cell
lineage.

Transcriptional variants of Hobit display distinct activity at Blimp-1 target
sites

Human Hobit is a putative zinc finger-containing transcription factor of 504 amino
acids that contains 4 C2H2-type zinc fingers and shares approximately 58% overall
identity with mouse Hobit [24]. The gene encoding human Hobit (ZNF683) con-
tains 6 exons and is located on chromosome 1p36.11 (Fig. 5A). The genomic con-
text, which is conserved with the mouse gene [24], suggests that ZNF683 and the
adjacent gene AIM1L arose through duplication from the region spanning PRDM1
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Figure 2. Clone Sanquin-Hobit/1 specifically recognizes Hobit in NK92 cells. (A) Schematic representation of the truncated Hobit protein that was used for immunization to generate anti-Hobit antibodies. (B) Hobit mRNA expression was determined in NK92 cells that were transduced with a mock or Hobit KD construct by qPCR. (C-D) Sanquin-Hobit/1 specificity was determined using intracellular flow cytometry in NK92 cells that were transduced with a mock or Hobit KD construct. (C) Histogram overlay depicts the expression of Hobit. Numbers in upper right corner show geometric mean fluorescence intensity (geo MFI) of Hobit expression. (D) The geo MFI of Hobit expression in mock and Hobit KD NK92 cells was determined. (B and D) Data are shown as mean ±SEM (n=5) and are pooled data of five independent experiments. * p < 0.05; Two-tailed paired t-test.

(encoding Blimp-1) and AIM1 (Fig. 5B). Human Hobit shares considerable homology with Blimp-1 within the zinc finger region (78% identity and 91% similarity with the 4 most N-terminal zinc fingers of Blimp-1), but lacks the SET domain that characterizes Blimp-1 and the other members of the PRDM family (Fig. 5A). Thus, Hobit resembles the Blimp-1 isoform, which initiates from an intragenic promoter and encodes a protein lacking the SET domain. Sequencing of individual clones after molecular cloning of Hobit from PBMCs resulted in the identification of 3 different transcripts of the ZNF683 gene. These transcripts encode 3 different proteins that we designated as Hobit Large (L), Hobit Extra Large (XL) and Hobit Small (S, Fig. 5A). Compared with Hobit L, Hobit XL contains 20 additional amino acids between zinc finger 2 and 3 that are encoded by an alternative exon in between exons 5 and 6 of Hobit L. Therefore, the zinc finger domains of Hobit L, but not those of Hobit XL, are spatially aligned in the same manner as the first 4 zinc finger domains of Blimp-1 (Fig. 5A). The Hobit S transcript contains a deletion of 20 base pairs compared with Hobit L, which arises from alternative splicing of exon 4 onto exon 5. This deletion
Figure 3. Hobit protein is expressed under steady state conditions in cytotoxic CD8+ T-cells. (A-F) Hobit protein expression was assessed in the indicated CD8+ T cell subsets that were identified using staining for CD27, CD45RA and CCR7 to separate naïve (CD27+CD45RA+CCR7+), total memory (CD27+CD45RA-), central memory (CM; CD27+CD45RA-CCR7+), effector memory (EM; CD27+CD45RA-CCR7-), effector cells (CD27-CD45RA+CCR7-). (A) Histogram overlays depict the expression of Hobit in blood CD8+ T-cells. Numbers in upper right corner show percentage of Hobit+ CD8+ T-cells. (B) Bar graphs display the percentage of Hobit+ cells and the geometric mean fluorescence intensity (geo MFI) of Hobit expression in blood CD8+ T cell subsets. (C) Histogram overlays depict the expression of Hobit...
in KLRG1- and KLRG1+ EM CD8+ T-cells. (D) Bar graphs display the percentage of Hobit positive cells and the geo MFI of Hobit expression in KLRG1- and KLRG1+ EM CD8+ T cells. (E) Histogram overlays depict the expression of Hobit in the indicated subsets of tonsillar CD8+ T cells. (F) Bar graphs display percentage of Hobit+ cells and Hobit geo MFI in tonsil CD8+ T cells. (G) Hobit histogram overlays of the indicated subsets of virus-specific CD8+ T cells that were identified by tetramer binding and staining for CD8. (A, C and E) Data shown are representative of six donors (unpaired samples) or (G) three donors. (B, D and F) Data are shown as mean ±SEM (n=6 (B and F) or 4 (D), from a pool of two independent experiments) experiments. ** p<0.01; *** p<0.001; Anova with post-hoc Bonferroni test.

introduces a frameshift and results in a premature stop codon that truncates Hobit S within the first zinc finger domain (Fig. 5A). The abundance of the Hobit isoforms was determined in subsets of NK cells and CD8+ T cells using a PCR that distinguishes between the 3 transcripts, as verified using transfectants that over-expressed either Hobit XL, L or S (Fig. 5, C and D). The analysis showed that Hobit L was present at higher levels than Hobit XL in EM and effector CD8+ T cells as well as in CD56dim and CD56bright NK cells. The levels of Hobit S were below detection limit. Thus, Hobit L, which displayed the highest similarity with Blimp-1, is the most prominently expressed isoform of Hobit.

Blimp-1 has been reported to primarily function as a transcriptional repressor using target sites that overlap with those of IRF1 and IRF2 [29;30]. As the zinc finger domains confer DNA-binding specificity of Blimp-1 and this region is most highly conserved with Hobit, we analyzed whether Hobit was able to recognize known target sites of Blimp-1. Previously, binding sites of Blimp-1 have been demonstrated within the promoter regions of TAPASIN, BTN3A3 and SP110 [31;32]. Therefore, we generated the Hobit isoforms with an HA-tag to enable supershift. We found that Hobit XL and L, similar to Blimp-1, bound to target sequences present in the promoters of TAPASIN, BTN3A3 and SP110 using EMSA (Fig. 6A). The defect in DNA binding of Hobit S showed that the zinc finger region of Hobit was essential (Fig. 6A). Reciprocally, a construct containing only the zinc finger domains of Hobit showed that the minimal region sufficient for DNA binding activity was located within the zinc finger region (Fig. 6A). Using a luciferase reporter assay, we established that Hobit L similar to Blimp-1 acted as a transcriptional repressor at these sites (Fig. 6B). Furthermore, the zinc finger domains of Hobit were both essential and sufficient for Hobit to suppress expression of the luciferase reporter. Interestingly, although Hobit XL was able to bind to the same target sequences as Hobit L, the Hobit XL isoform lacked transcriptional repressor activity (Fig. 6, A and B). This underlines the importance of the spacing of the zinc fingers in the functional activity of Hobit and suggests that Hobit XL can act as a negative regulator of Hobit L. Thus, the dominant Hobit L isoform functions as a transcriptional repressor that uses similar target sites as Blimp-1, indicating that Hobit and Blimp-1 are transcription factors with overlapping roles.

Hobit is expressed in IFN-γ producing CD8+ T cells and regulates IFN-γ production. Quiescent effector CD8+ T cells in latent hCMV infection have constitutive expression of IFN-γ mRNA that enables them to immediately produce IFN-γ upon antigenic triggering [22]. To identify transcription factors in these cells that displayed a synchronous expression profile with IFN-γ, we compared microarray data [22] from hCMV-specific cells at peak, 1 year post-infection and during latency with naive CD8+
Figure 4. Hobit protein is expressed under steady state conditions in cytotoxic NK and NKT cells. (A and B) Histogram overlays depict the expression of Hobit in (A) blood and (B) tonsil NK cells. Numbers in upper right corner show percentage of Hobit+ cells or (D) the geometric mean fluorescence intensity (geo MFI) of Hobit expression in NK cells. (E) Histogram overlays depict the expression of Hobit in NKT cells. (F) Bar graphs display percentage of Hobit+ cells or geo MFI of Hobit expression in NKT cells (G) Histograms depict the expression of Hobit in the indicated subsets of leukocytes from peripheral blood. Neutrophils were obtained from...
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Figure 5. Alternative splicing results in expression of 3 different Hobit isoforms. (A) Schematic models of the genomic structure of ZNF683 (encoding Hobit, top) and the protein structures of Hobit Large, XL and Small and Blimp-1 (bottom). (B) The orientation of ZNF683 and AIM1L on chromosome 1 and PRDM1 (encoding Blimp-1) and AIM1 on chromosome 6 is depicted. (C,D) The relative expression of Hobit isoforms was analyzed using a PCR that distinguishes between the isoforms in (C) CD8+ T cell subsets and in (D) NK cell subsets. Jurkat cells transduced with Hobit XL, Large (L) or Small (S) were used as controls. Data shown are representative of two independent experiments.
T cells, using nearest neighbor analysis with IFN-γ as a starting point. We found 31 genes that clustered together with IFN-γ, as similar to IFN-γ, they were highly expressed during all stages of hCMV infection, but not in naive CD8+ T cells (Fig. 7A). As expected, T-bet, which is essential to induce IFN-γ expression, associated with IFN-γ in hCMV-specific CD8+ T cells (Fig. 7A). Strikingly, the expression pattern of Hobit and Blimp-1 more closely resembled the expression profile of IFN-γ than that of T-bet (Fig. 7A), suggesting that Hobit and Blimp-1 may be involved in the regulation of IFN-γ expression in hCMV-specific effector CD8+ T cells. We did not find other transcription factors that had an expression profile that matched that of IFN-γ (Fig. 7A). IFN-γ protein expression requires activation, making it impossible to directly analyze for co-expression of Hobit and IFN-γ protein in CD8+ T cells under resting conditions. Supporting the nearest neighbor analysis, Hobit and T-bet were stringently co-expressed in CD8+ T cells using flow cytometry (Fig. 7B). Moreover, Hobit also strictly correlated with T-bet expression in virus-specific CD8+ T cells, as EBV-specific, EBV-specific and CMV-specific CD8+ T cells displayed low, intermediate and high expression of Hobit and T-bet, respectively (Fig. 7C and Table S1). This suggests that Hobit, similar to T-bet, may be involved in the regulation of IFN-γ expression.

The strong correlation of Hobit expression with T-bet and IFN-γ expression prompted us to study the role of Hobit in IFN-γ production. Therefore, NK92 cells were transduced with shRNAs that induced specific knockdown of Hobit expression (Fig. 2, B-D). Mock transduced NK92 cells upregulated expression of IFN-γ mRNA and protein to a higher extent than NK92-Hobit KD cells after stimulation with PMA and ionomycin (Fig. 7, D and E). This shows that Hobit is involved in the regulation of IFN-γ production. To examine whether Hobit was sufficient to induce IFN-γ expression, Jurkat cells that do not express endogenous Hobit were transduced with Hobit L, Hobit XL, or Hobit S or with an empty vector as a control (Fig. 7F). After brief stimulation with PMA and ionomycin, Jurkat cells expressing Hobit L, in contrast to control, or Hobit XL or Hobit S expressing Jurkat cells, produced IFN-γ, as measured by ELISA (Fig. 7F). Thus, Hobit L is the functionally active isoform of Hobit that is both necessary and sufficient to induce production of IFN-γ.
**Figure 6. The Hobit isoforms display distinct binding and transcriptional activity at Blimp-1 target sequences.** (A) EMSA evaluation of Hobit variants is shown on previously identified Blimp-1 binding sites in the promoter sequences of TAPASIN (top panel), BTN3A3 (center panel), and SP110 (bottom panel). Comparable amounts of in vitro translated proteins representing the Extra Large (XL), Large, Small or Zinc Finger region only (ZnF) forms of Hobit were assessed in the absence or presence of an antibody to the HA epitope tag. The position of Hobit XL (closed circle, non-supershifted), Large (closed triangle, non-supershifted), and ZnF (closed arrow, non-supershifted and open arrow, supershifted) proteins are indicated. Supershifted Hobit XL and Large are not visible. (B) The repressive capacity of Hobit and Blimp-1 was analyzed at promoter sequences of TAPASIN (top panel), BTN3A3 (center panel), and SP110 (bottom panel) in HeLa cells transduced with luciferase reporter constructs containing these promoter sequences and the indicated overexpression constructs of Hobit and Blimp-1. Luciferase activity was assessed in the absence or presence of 200 IU/ml IFN-γ. The data are displayed as arbitrary units (AU) with unstimulated cells co-transfected with the empty overexpression vector set at 1. (A and B) Data are representative of two independent experiments using separate plasmid preparations. (B) Data are shown as mean ±SD (n=3, technical replicates). ∗ p < 0.05; Anova with post-hoc Bonferroni test.
Figure 7. Hobit strongly correlates with IFN-γ production in CD8+ T cells and induces IFN-γ expression. (A) The genes that co-cluster with IFN-γ are depicted, using nearest neighbor analysis with IFN-γ as a starting point on microarray data of naïve and hCMV-specific CD8+ T cells at peak of infection, 1-year post-infection and during latency. Shown are the mean changes in transcript levels with respect to naïve CD8+ T cells. (B) Dotplot depicts CD8+ T-cells that were analyzed for intracellular co-expression of T-bet and Hobit. Numbers represent percentage of cells within quadrant. (C) Dot plot depicts expression of T-bet and Hobit in flu-specific (green), EBV-specific (blue) and CMV-specific (red) CD8+ T cell populations. (D-F) IFN-γ production was analyzed by (D) qPCR and (E) ELISA in NK92 cells that were transduced with mock or Hobit KD constructs and cultured in the absence or presence of PMA and ionomycin. (F) IFN-γ production was examined using ELISA in Jurkat cells that were transduced with empty vector, Hobit XL, Hobit Large or Hobit Small and briefly cultured in the presence of PMA and ionomycin. (B and C) Data are representative of six donors from three independent experiments (B) or of two donors from one experiment (C). (D, E and F) Data are shown as mean ±SEM (n=6 (D and E) or 3 (F)) and are pooled of three independent experiments. * p < 0.05; Two-tailed t-test (D and E); Anova with post-hoc Bonferroni test (F).
**Discussion**

The differentiation of CD8+ T cells into effector and memory cells is controlled through a well-established network of transcription factors. Here, we have described that human CD8+ T cells in a resting effector state specifically expressed the Blimp-1 homologue Hobit both at the mRNA and protein level. Consistent with the specific expression in effector-type CD8+ T cells, Hobit was present at higher levels in hCMV- than in EBV- and Flu-specific CD8+ T cells. Hobit was able to function as a transcriptional repressor that recognized the same target sites as Blimp-1, suggesting overlapping roles for the homologous transcription factors. We found that Hobit regulated the induction of IFN-γ production.

Hobit shares high homology with Blimp-1, in particular within the Zinc finger region. In line with the structural similarity, we have shown that Hobit recognized known target sites of Blimp-1 including those within the TAPASIN, BTN3A3 and SP110 promoters [31]. The Zinc fingers of Blimp-1 show extensive evolutionary conservation and conserved target site recognition between evolutionary distant species [32]. The data presented here demonstrate that the conservation of the Zinc finger domains between Hobit and Blimp-1 is reflected in the capacity to recognize similar binding sites. This suggests that both transcription factors may have largely overlapping site recognition in vivo. The 2 most N-terminal zinc finger domains of Blimp-1 are crucial for Blimp-1 to bind its target sequences [33]. Similarly, we found that the Zinc finger domains of Hobit were both required and sufficient to recognize Blimp-1 target sites. Hobit lacks the PR-domain that is typical for Blimp-1 and the other members of the PRDM family [34]. The PR-domain which is similar to the SET domain, confers endogenous methyltransferase activity, but appeared dispensable for Blimp-1 to induce histone modifications [33]. Indeed, a functional isoform of Blimp-1, Blimp-1 a, lacking the PR-domain is observed in human myeloma plasma cells as well as NK cells [35-37]. Rather than directly inducing histone methylation, Blimp-1 has been described to recruit the G9a methyltransferase through the 2 most N-terminal Zinc finger domains [38]. In addition, Blimp-1 recruits the histone deacetylase HDAC2 and Groucho proteins through these Zinc finger domains and a proline rich region directly upstream [39;40]. The high homology between Hobit and Blimp-1 within the Zinc finger domains suggests that the ability to recruit these histone-modifying proteins may be conserved between the transcription factors. Consistent with this notion, we observed that Hobit similar to Blimp-1 acted as a transcriptional repressor. Of the three splice variants of Hobit that we detected, Hobit XL and L in contrast to Hobit S were commonly expressed in human NK cells and CD8+ T cells. Although Hobit XL and L both bound to known Blimp-1 target sites, only Hobit L displayed transcriptional repressor activity, suggesting that Hobit XL may function as a dominant negative Hobit isoform that suppresses the function of Hobit L. Also Hobit S may function as a dominant negative isoform, but its limited expression, suggests that it only has a modest role in human lymphocytes under homeostatic conditions. Thus, Hobit, in particular Hobit L, and Blimp-1 may regulate similar transcriptional programs in effector-type CD8+ T cells and NK cells.

At a functional level the limited available evidence suggests that Hobit and Blimp-1 regulate similar programs, as both have been reported to control the expression of granzyme B and IFN-γ [6;7;17;24;37;41]. We have shown previously that Hobit
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induces granzyme B and represses IFN-γ in murine NKT cells [24] and Blimp-1 has been shown to regulate granzyme B and IFN-γ in a similar manner in murine CD8+ T cells and NK cells [6;7;17;37;41]. A role for human Hobit in the regulation of granzyme B is suggestive, as expression of Hobit is confined to cytotoxic populations of CD8+ T cells and NK cells. However, we did not find evidence for the regulation of granzyme B expression by human Hobit (unpublished observations). We found a strong association between Hobit and IFN-γ and the IFN-γ regulating transcription factor T-bet. In NK92 cells, Hobit was required for the regulation of IFN-γ expression and using Jurkat cells we found that Hobit was sufficient to induce IFN-γ expression. Strikingly, Hobit appears to positively regulate IFN-γ whereas it acts as a transcriptional repressor on known motifs of Blimp-1. Although it is possible that Hobit-driven regulation at the IFN-γ locus is indirect, Hobit similar to Blimp-1 may positively induce expression in a direct manner. Blimp-1 has been shown to directly act on the IL-10 locus, which results in enhanced expression of IL-10 [42]. Interestingly, human Hobit positively regulated IFN-γ expression, whereas murine Hobit and Blimp-1 negatively regulated IFN-γ expression [24;37;43]. The reason for these differential effects of Hobit are unclear, but they may be related to differences in cellular context as Hobit is expressed in different cell types in mouse and man. Overall, Hobit is involved in the regulation of immediate effector functions that include the regulation of IFN-γ expression.

The transcriptional regulation of the differentiation and maintenance of hCMV-specific CD8+ T cells is complex. We have previously shown that transcription factors that are essential for the formation of effector cells such as T-bet, Eomes and Blimp-1 are also expressed in hCMV specific CD8+ T cells during latency and at late time points after primary infection [22]. The expression of these transcription factors suggests that the explicit effector phenotype of hCMV-specific CD8+ T cells can be explained through common transcriptional regulation pathways with early effector cells. The capacity of hCMV specific CD8+ T cells to persist late after primary infection suggests that they require a combination of transcription factors that is not found in short-lived effectors for long-term maintenance. Our findings in hCMV-specific CD8+ T cells suggest a role for Hobit in the transcriptional regulation of these long-lived effector-type cells. The role of Hobit is not confined to hCMV-specific cells and long-lived effector-type CD8+ T cells, as we have also found expression of Hobit in the KLRG1+ subset of EM CD8+ T cells. Long-lived effector cells uniformly express KLRG1 [26], suggesting that Hobit expression is upregulated in terminally differentiated CD8+ T cells that are defined by KLRG1 expression. The expression of Hobit within KLRG1+ EM CD8+ T cells is in line with the capacity of these memory cells to rapidly produce cytokines and to exert direct cytotoxic potential similar to effector cells.

The in vivo study of Hobit in CD8+ T cell differentiation is complicated as, in contrast to human Hobit, murine Hobit is specifically expressed in NKT cells, but not or at low levels in naive, memory and effector subsets of CD8+ T cells [24]. Interestingly, murine NKT cells are maintained after development in the thymus as resting cells in an effector or effector memory stage. Similar to long-lived effector CD8+ T cells, NKT cells are able to generate cytokine responses within hours after stimulation and a thymic subset of mature NKT cells displays constitutive granzyme B expression [44]. Hobit not only regulates the expression of granzyme B and IFN-γ in NKT cells,
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but also is essential for the maintenance of the mature thymic NKT cell subset [24]. Despite the disparity in expression between human and murine Hobit, this suggests that similar to murine Hobit in NKT cells, human Hobit in CD8+ T cells may regulate the maintenance of long-lived effector cells and their capacity to generate immediate effector responses.

The specific expression of the transcription factor Hobit in long-lived effector cells with immediate effector function such as hCMV-specific CD8+ T cells suggests that these cells undergo a unique and late differentiation process that separates them from other effector and memory cells. Long-term maintenance and the immediate capacity to produce effector molecules are a useful, but uncommon, combination of features of CD8+ T cells in adoptive transfer settings of vaccination. Therefore, our findings on the role of Hobit in the differentiation process of CD8+ T cells may form an important first step in the development of vaccine strategies that employ long-lived effector CD8+ T cells.
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Materials and Methods

Structural analysis.
Genomic and amino acid sequences of Hobit and Blimp-1 were derived from the Ensembl project of genome databases. Structural domains in Hobit and Blimp-1 were assigned with the SMART simple modular architecture research tool.

Reagents.
The following antibodies were used: anti-CD3 (BD Biosciences), anti-CD4 (BD Biosciences), anti-CD8 (BD Biosciences and BioLegend), anti-CD16 (Sanquin Reagents), anti-CD19 (eBioscience), anti-CD25 (BD Biosciences), anti-CD27 (Sanquin Reagents), anti-CD45RA (BD Biosciences), anti-CD56 (BD Biosciences), anti-CCR7 (BD Biosciences), anti-granzyme B (Invitrogen), anti-TCR Vβ11 (Immunotech), anti-TCR Vα24J18 (eBioscience), anti-IFN-γ (BD Biosciences), and anti-T-bet (BioLegend). Anti-HA antibodies were purchased from Abcam and Roche. The following tetramers to identify virus-specific CD8+ T cells were used: hCMV-NLV, hCMV-TPR and hCMV-IPS for hCMV, EBV-EPL, EBV-HPL and EBV-GLC for EBV, and Flu-GIL for influenza. Details of these tetramers are provided in Table S1.

Plasmids.
The Hobit isoforms XL, L and S were cloned from cDNA of PBMCs into LZRS pBM-IRES-EGFP retroviral vectors as a XhoI and EcoRI fragment to generate HA-tagged proteins. A bi-cistronic expression vector pIRES2-EGFP (Clontech) encoding full-length (FL) Blimp1 has been described previously [45]. Sequences encoding Hobit isoforms were excised from the LZRS pBM-IRES EGFP vectors and cloned into both pBluescript (Stratagene) and pIRES2 EGFP (Clontech) between EcoRI/XhoI and EcoRI/SalI sites, respectively. Lentiviral pKLO.1 plasmids containing shRNA that target Hobit (TRCN0000162720; CAGAAGAGCTTCACTCAACTT) or that do not target Hobit as a control (MISSION Non-Target shRNA Control SHC002: CCGGCAACAAGATGAGAGCACAACCT) were obtained from Sigma (MISSION shRNA Lentiviral Transduction Particles).

Cell culture.
NK92 cells were maintained in RPMI 1640 (Gibco) containing 10% heat inactivated FCS (ICN Biomedicals), IL-2 (50 U/ml; Biotest), streptomycin (100 ng/ml; Life Technologies), and penicillin (10 U/ml; Yamanouchi, Pharma). NK92 cells were transduced using lentiviruses containing pKLO.1 plasmids with control or Hobit KD shRNA. Transduced NK92 cells were selected on puromycin (Sigma-Aldrich) containing medium. Jurkat cells were grown in Iscove’s modified Dulbecco’s medium (IMDM; Gibco) containing 10% FCS, streptomycin (100 ng/ml), penicillin (10 U/ml) and 0.0004% β-mercapto-ethanol (Merck). Jurkat cells were transduced using retroviruses containing LZRS pBM-IRES-EGFP with Hobit XL, Large or Small on retronectin (Takara Shuzo) coated plates. Transduced jurkat cells were sorted to over 95% purity flow cytometry based sorting using GFP expression as a selection marker.

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 (Milenyi 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. NK cells were isolated using flow-cytometric sorting for CD3, CD56 and CD16 to obtain the CD3-CD16+CD56dim and CD3-CD16-CD56bright NK cell subsets or total CD3-CD56+ NK cells. For activation cells were cultured with PMA (2 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 4 hours.

**PCR and quantitative PCR.**

To detect Hobit XL, L and S splice variants a PCR was performed using the following forward: 5’-TTCAGCTCTGCATCGTTTTG-3’ and reverse: 5’-TCTTTTGGATGCTCTGGT-3’. The amplification products of Hobit XL (260 bps), Hobit L (200 bps) and Hobit S (180 bps) were separated on agarose gel.

RNA was isolated using the Invisorb RNA isolation kit (Invitek) or Trizol reagent (Invitrogen). Then, cDNA was synthesized using RevertAID H Minus Reverse Transcriptase (Thermo Scientific) and random primers (Invitrogen) or poly dT oligos (Invitrogen). Quantitative PCR was performed on a Lightcycler (Roche) or a StepOnePlus RT PCR system (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) and the following primers sets: Hobit (forward: 5’-CATATGGCGAGCTTTGG-3’, reverse: 5’-AGAGCTCTCACTCAACTGCC-3’), Blimp-1 (forward: 5’-CAACAATTTTGCCCTTCTCC-3’, reverse: 5’-GATCATTGGCTGCTTCTC-3’), IFN-γ (forward: 5’-TTAGCTCTGCATCGTTTG-3’, reverse: 5’-CTCTTTGGATGCTCTGGT-3’), and 18S (forward: 5’-GGACACAAAGCTCCTGGAAGA-3’, reverse: 5’-CAAGAATGTCAGCGACGCCCTCTA-3’). Values are represented relative to that of 18S and calibrated relative to naive CD8+ T cells unless indicated otherwise.

**Generation of anti-Hobit antibodies.**

To generate antibodies against Hobit, the non-Zinc finger part of Hobit was cloned from PBMC cDNA into vector p-ET-30a between KpnI and EcoRI restriction sites (Fig. 2A). Truncated and His tagged Hobit protein was purified using Ni2+ columns and imidazole elution. Balb/c mice were immunized 3 times with isolated Hobit protein (25 μg/ml) in montanide adjuvants. Splenocytes were isolated and fused to immortalized SP2/0 myeloma cells using polyethylene glycol 4000 (Merck). Monoclonal hybridomas that produced anti-Hobit antibodies were selected using a flow cytometry-based screening method, in which PBMCs of hCMV+ donors were labeled with hybridoma supernatants and goat anti-mouse Ig (Dako) in the secondary step. We detected one monoclonal antibody (Sanquin-Hobit/1) that specifically stained Hobit, as verified using staining of NK92 cells that were transduced with mock or Hobit KD shRNA constructs (Fig. 2, C and D).

**Flow cytometry.**

Cells were labeled with fluochrome-conjugated or biotinylated antibodies in PBS 0.5% BSA for 30 min at 4°C. In case of biotinylated antibodies, fluochrome-conjugated streptavidin was added in a second step. For intracellular and nuclear staining, cells were fixed and permeabilized using fixation and permeabilization buffers of BD Biosciences and eBioscience, respectively. Expression was analyzed on FACS Canto II, BD LSRFortessa or BD LSR II flow cytometers (BD Biosciences).
EMSAs.
To generate Hobit proteins representative of the different isoforms or the zinc finger region alone, in vitro transcription-translation reactions were performed using mMESSAGE mMACHINE and Retic Lysate IVT kits (Ambion). Protein expression was verified by Western blot. The double-stranded probes used for EMSA contained the following sequences: TAPASIN: \((5’-\text{TGGGAGGAAAGTGAAAGTGAAAGT-3’})\), BTN3A3: \((5’-\text{TGGAAATGAAAGTGAAAGT-3’})\), SP110: \((5’-\text{AGAAAAAAGTGAAAGTCACTTGGGAATTG-3’})\). DNA probes end-labeled with T4 polynucleotide kinase were incubated with in vitro translation products, adjusted for expression levels, in the presence of poly(I:C) (Amersham) for 30 min at room temperature. Supershift was performed by the addition of rabbit anti-HA antibodies prior to mixing with radioactive probe.

Luciferase Assays.
HeLa cells were seeded to reach 80% confluency on the day of transfection and then co-transfected with GeneJuice (Novagen) reagent according to the manufacturer’s instructions with the indicated luciferase reporter constructs and overexpression vectors of Hobit and Blimp-1. The firefly luciferase reporter constructs containing sequences from the promoters for human TAPASIN, BTN3A3 and SP110 have been described previously [31;32]. A control Renilla luciferase vector was used for normalization. Luciferase activity was assessed 6 h after treatment with medium alone or medium containing 200 IU/ml IFN-y. Experiments were conducted using the Promega dual luciferase assay system and analyzed on a Berthold Lumat LB Luminometer.

Microarray analysis.
Microarray data was obtained from naive CD8+ T cells and hCMV specific CD8+ T cells at peak of infection, 1 year after infection, and during latency, as described [22]. Expander software was used to perform nearest neighbor analysis on the microarray data. IFN-y was taken as a starting point and a neighbor joining tree was constructed based on the expression pattern of the genes in the indicated subsets of CD8+ T cells.

ELISA.
Production of IFN-y was measured in the supernatants of cell cultures using PeliPair human IFN-y reagent set (Sanquin Reagents) according to the manufacturer’s protocol.

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).

Study Approval.
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.

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Conflict of interest
The authors declare no financial or commercial conflict of interest.
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References:


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