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Loss of E protein transcription factors E2A and HEB delays memory-precursor formation during the CD8⁺ T-cell immune response

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Abstract

The transcription factors E2A and HEB (members of the E protein family) have been shown to play essential roles in lymphocyte development, while their negative regulators, the Id proteins, have been implicated in both lymphocyte development and in the CD8⁺ T-cell immune response. Here, we show that E proteins also influence CD8⁺ T cells responding to infection. E protein expression was upregulated by CD8⁺ T cells during the early stages of infection and increased E protein DNA-binding activity could be detected upon TCR stimulation. Deficiency in the E proteins, E2A and HEB, led to increased frequency of terminally differentiated effector KLRG1^{hi} CD8⁺ T cells in mice during infection, and decreased generation of longer-lived memory-precursor cells during the immune response. These data suggest a model whereby E protein transcription factor activity favors rapid memory-precursor T-cell formation while their negative regulators, Id2 and Id3, are both required for robust effector CD8⁺ T-cell response during infection.

Keywords

Immune response; Memory; T cells; Transcription factors

Introduction

Upon infection, antigen-specific CD8⁺ T cells undergo extensive expansion resulting in both effector T cells with cytotoxic capabilities, which undergo apoptosis following resolution of the infection, and longer-lived memory T cells that survive to protect against reinfection [1]. Recent studies indicate that the effector CD8⁺ T-cell population can be divided into subsets: effector T cells that terminally differentiate and die shortly after infection clearance and a second subset of effector cells with the potential for long-lived self-renewal capacity that will seed the memory compartment [2–4]. Terminally differentiated effector T cells and memory-precursor T cells can be distinguished on the basis of KLRG1, CD127 and CD25 surface expression [2–4]. A number of transcription factors including T-bet and Blimp-1 have been implicated in this cell-fate decision [2, 5–7], both promoting terminal

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Supporting Information available online

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differentiation of effector CD8⁺ T cells. Conversely, the transcription factors TCF-1 and Eomes have been shown to be important for promoting memory-precursor formation [8, 9]. However, despite known developmental functions, a role for the transcriptional regulators the E proteins in the effector CD8⁺ T-cell immune response has not previously been described.

E proteins are transcription factors that bind DNA at specific E box sites where they influence gene expression in diverse cell types such as developing T cells, developing B cells, plasmacytoid dendritic cells and natural killer T cells [10–12]. In mammals, there are four E proteins (E47, E12, HEB, and E2-2), of which E47, E12 (both splice variants of the gene E2A) and HEB are expressed in T cells. E proteins are negatively regulated by the Id proteins, which lack a DNA-binding domain, and prevent E protein DNA binding when heterodimerized with E proteins. Of the four Id proteins expressed in mammals, Id2 and Id3 play roles in T-cell differentiation as well as in the CD8⁺ T-cell effector response to infection and in memory formation [12–15].

Our previous work revealed a role for Id2 and Id3 in regulating the CD8⁺ T-cell response to infection. Id2-deficient T cells were unable to efficiently mount a CD8⁺ T-cell immune response, with responding CD8⁺ T cells proliferating normally but exhibiting a survival defect, thus leading to a deficit in memory T-cell formation [13]. Id2 expression was upregulated at the peak of infection but interestingly, was downregulated early during infection. Furthermore, Id3, a marker of memory-precursor potential, was also downregulated early in the CD8⁺ T-cell immune response [14]. Given that Id proteins negatively regulate E proteins, we postulated that E proteins are expressed early during infection, potentially influencing CD8⁺ T-cell effector fate decisions at this critical juncture.

We show here that the E protein, E2A, is indeed expressed early in the CD8⁺ T-cell immune response and that both the E proteins E2A and HEB influence formation of the distinct memory-precursor compartments during infection. We propose a model in which E proteins and Id proteins work reciprocally to promote both terminally differentiated effector cells and memory-precursor cells during the CD8⁺ T-cell response to infection.

Results

Upregulated E2A expression by activated CD8⁺ T cells

E protein transcription factors are negatively regulated by the Id proteins: Id2 has been shown to play a role supporting CD8⁺ T-cell effector accumulation and Id3 marks cells with memory-precursor potential [13–15]. We previously found that Id2 mRNA expression was downregulated at day 5 of infection and that CD8⁺ T-cell activation led to increased E protein DNA binding in vivo, suggesting that E proteins may serve a function in CD8⁺ T cells during the earlier stages of infection [13]. Using an E2A-GFP fusion protein reporter mouse line [16], we examined E2A protein expression upon CD8⁺ T-cell activation in vitro and during the CD8⁺ T-cell response to antigen in vivo. We isolated CD8⁺ T cells from the spleens of C57BL/6 (B6) and E2A-GFP reporter mice and stimulated these cells in vitro with anti-CD3 and anti-CD28 for 3 days. We observed that E2A-GFP expression was upregulated in CD8⁺ T cells upon TCR stimulation in vitro (Fig. 1A). We next examined E2A-GFP expression in CD8⁺ T cells responding to infection. We infected B6 or E2A-GFP mice with *Listeria monocytogenes* expressing OVA (Lm-OVA) and followed the polyclonal CD8⁺ T-cell response using K^b-OVAp tetramers to identify antigen-specific CD8⁺ T cells. E2A-GFP expression was consistently increased in tetramer⁺ CD8⁺ T cells 6 days after infection and downregulated by day 8 (Fig. 1B). In contrast, tetramer[−] CD8⁺ T cells did not upregulate E2A-GFP, indicating that only antigen-specific CD8⁺ T cells increased E protein

expression during infection (Fig. 1B). Thus, we showed that E2A protein levels are transiently upregulated in activated CD8⁺ T cells during the early immune response.

Activation of CD8⁺ T cells leads to increased E protein DNA-binding activity in vivo

We also previously reported that TCR stimulation in vitro led to increased E protein DNA binding in activated CD8⁺ T cells [13]. Here, we examined the E protein DNA-binding activity during the CD8⁺ T cells response to in vivo activation. CD45.1⁺ OT-I T cells were transferred into CD45.2⁺ B6 recipients. One day later, recipient mice were infected with VSV-OVA and CD45.1⁺ OT-I responding T cells were isolated on days 4 and 6 of infection. We observed that nuclear extracts isolated from OT-I cells on day 4 of infection showed increased E protein DNA-binding activity and that this DNA-binding activity was reduced by day 6 (Fig. 2A). We incubated an E47 blocking antibody with these nuclear extracts and observed loss of specific E47 binding activity (Fig. 2A). This indicated that active E protein dimers, including the E protein E47, bound DNA at E box sites in activated CD8⁺ T cells during infection.

We next examined E protein DNA-binding activity in Id2-deficient OT-I T cells. OT-I T cells from WT or Id2-deficient mice were transferred to congenically distinct hosts and infected with VSV-OVA. On day 6 of infection, nuclear extracts were isolated from OT-I T cells and evaluated as above. E protein DNA-binding activity was still observed in Id2-deficient OT-I T cells relative to WT cells (Fig. 2B), consistent with previous reports that Id2 negatively regulates E protein DNA-binding activity and that it is poised to do so as it is upregulated 6 days after infection [13].

Accelerated generation of short-lived effector cells during infection in the absence of E proteins

Given that we observed E protein upregulation and increased DNA-binding activity in effector CD8⁺ T cells during infection, we next investigated how expression of E proteins impacts the CD8⁺ T-cell immune response. We used a T cell-specific deletion system in which mice carrying floxed alleles for E2A (E2AKO), HEB (HEBKO), or both (double knock-outs, DKO) were crossed to lines expressing Cre recombinase under control of the CD4 promoter (CD4-Cre), which allowed deletion of these proteins specifically in developing double-positive T cells [17]. DKO E protein-deficient T cells were previously reported to lack TCR surface expression [17]. To circumvent this potential caveat in observing the CD8⁺ immune response, we crossed these lines to the OT-I TCR transgenic line. Analysis of E protein-deficient cells prior to transfer to recipient host mice revealed these cells were slightly increased in terms of activation marker CD44 expression relative to WT cells (Supporting Information Fig. 1A). However, DKO cells also expressed higher levels of CD127 and CD62L and equivalent expression of CD69 and KLRG1 relative to WT cells, indicating a naïve phenotype. Analysis of these cells by PCR demonstrated that E2A and HEB were efficiently deleted in OT-I cells isolated on day 5 and day 7 of Lm-OVA infection (Supporting Information Fig. 1B and C). We first tested if E2AKO, HEBKO, or DKO OT-I cells could mount an efficient CD8⁺ T-cell immune response by adoptively transferring either 1×10^4 OT-I WT E2AKO, HEBKO, or DKO OT-I CD45.2⁺ T cells into CD45.1⁺ mice and infecting the following day with Lm-OVA. We monitored the CD8⁺ T-cell response in peripheral blood during the course of the infection (Fig. 3). We observed no substantial differences in the expansion of OT-I cells in the absence of individual E proteins, although the combined loss of E2A and HEB led to a slight but insignificant increase in the expansion of these CD8⁺ T cells (Fig. 3A).

We next examined the phenotype of E protein-deficient effector T cells throughout the course of infection. We observed that although most effector T-cell markers remained

unchanged in the absence of E proteins (data not shown), DKO OT-I effector T cells rapidly acquired expression of KLRG1 relative to WT cells in peripheral blood (Fig. 3B). These DKO effector cells remain KLRG1^{hi} on day 15 after infection, indicating loss of E proteins affected KLRG1^{lo}CD127^{hi} memory-precursor development (Fig. 3B). Observing total KLRG1^{hi} or KLRG1^{lo} OT-I effector cells, we consistently detected higher frequencies of KLRG1^{hi} cells in the absence of E2A and HEB over the infection time course (Fig. 3C). Indeed, at 2.5 months postinfection, although OT-I total population expansion was not different between WT and DKO cells, we observed fewer KLRG1^{lo} memory-precursor cells with loss of E protein expression (Fig. 5D). Deletion of E2A or HEB alone also led to an increase in KLRG1^{hi} cells but the effect was less pronounced than with loss of both E proteins, consistent with a compensatory function of these two E proteins (Supporting Information Fig. 2). We were concerned that loss of expression of E2A and HEB could be compensated for by upregulation of E2-2 expression. However, we could not detect upregulation of E2-2 mRNA expression in DKO OT-I cells on day 5 after infection, relative to WT cells (Supporting Information Fig. 3) and E2-2 deficiency did not lead to defects in the CD8⁺ T-cell immune response (data not shown). Thus, for the remainder of our analysis we focused on the immune response by CD8⁺ T cells lacking both E2A and HEB.

We next assessed how loss of E proteins affected the CD8⁺ T-cell response to infection in the spleen. Adoptive transfer of either 1×10^4 WT or DKO OT-I CD45.2⁺ T cells into CD45.1⁺ mice followed by infection a day later with Lm-OVA indicated no substantial differences in the expansion of OT-I cells in the absence of E proteins (Supporting Information Fig. 4A). However, E protein-deficient effectors in the spleen demonstrated similarly high KLRG1 expression relative to WT cells in terms of both percentage and cell number on days 5, 7, and 15 after infection (Fig. 4A and B). Furthermore, E protein deficiency decreased the frequency and number of KLRG1^{lo} memory-precursor cells on days 5, 7, and 15 after infection (Supporting Information Fig. 4B). Together, these data indicated E proteins affected development of memory-precursor cells; the loss of E proteins delayed the differentiation of the responding CD8⁺ T cells to memory-precursor cells.

E protein-deficient effector T cells compete with WT cells but show altered differentiation

Since we showed the presence of E protein activity early in the CD8⁺ effector T-cell activation (Figs. 1 and 2), we assessed the response of E protein-deficient T cells at very early time points postinfection (day 3–6 of infection). To this end, we transferred WT and E protein-deficient cells into the same recipient host, which allowed us to assess how E protein-deficient T cells competed with WT cells in an identical inflammatory environment. We mixed 0.25×10^6 OT-I DKO splenocytes (CD45.2⁺) and 0.25×10^6 OT-I WT splenocytes (CD45.1⁺), adoptively transferred these cells into CD45.1⁺CD45.2⁺ congenic hosts and 1 day later, infected with Lm-OVA. DKO OT-I cells in the spleen expanded normally in response to infection relative to WT cells (Fig. 5A). Consistent with equal accumulation, BrdU incorporation and Annexin V staining was similar between both cell types during the early days of infection (Fig. 5B and C). E protein-deficient effector T cells were also capable of producing effector cytokines such as IFN- γ and TNF- α upon 6 h restimulation in vitro on day 6 of infection, indicating these cells acquired effector function comparable to that of WT cells (Fig. 5D).

Interestingly, although WT and E protein-deficient cells appear equivalent in terms of expansion, proliferation, survival, and function, E protein-deficiency still affected KLRG1^{hi} effector cell formation, even at the early time points examined after infection (Fig. 5E). Gating on KLRG1^{lo} or KLRG1^{hi} OT-I cells, we could not detect differences in CD25 expression between WT and E protein-deficient cells; overall however we observed greater frequency of KLRG1^{hi} cells in E protein-deficient cells (Fig. 5E and Supporting Information Fig. 4C). Thus, we conclude that E proteins drive effector T cells toward a longer-lived

memory-precursor phenotype, and that in their absence, effector CD8⁺ T cells will exhibit a terminally differentiated shorter-lived effector phenotype during the initial stages of infection.

E protein transcription factors influence gene expression during CD8⁺ T-cell activation

Work by several groups has examined the gene targets of E protein transcription factors in various cell types [11,18], but the gene-expression pattern affected by E proteins in activated CD8⁺ T cells has not previously been assessed. Sorted WT or DKO CD8⁺ T cells were activated in vitro with anti-CD3 and anti-CD28 for 16 h and their transcript profiles were compared by microarray analysis (Fig. 6). Loss of both E2A and HEB in activated CD8⁺ T cells resulted in 167 genes upregulated more than 1.7-fold and 228 genes downregulated more than 1.7-fold relative to WT cells (Fig. 6A). Within these groups, genes associated with effector T-cell activation such as *Eomes*, *Id2*, and *Fyb* were downregulated within the DKO population while others such as *Cd28* and *Cxcl10* were upregulated (Fig. 6B). Using DAVID bioinformatics resources [19, 20], we grouped these genes into those involved in immune effector processes (Fig. 6C) and into groups of cytokine or chemokine genes (Fig. 6D). However, these two groupings are not mutually exclusive suggesting E proteins influence the CD8⁺ T-cell effector response through regulation of transcription factors, cell-surface markers, transcription factors, and cytokine signaling.

We cross-referenced our findings with previously published data, in which the A12 E2A-deficient T-cell line was transduced with retrovirus containing E47 [21]. These E47 overexpressing cells were then assessed for E2A occupancy by chromatin immunoprecipitation combined with deep DNA sequencing (ChIP-Seq) [21]. We observed that many of the genes we identified as differentially regulated upon loss of E proteins also possessed E2A-bound E box sites in close proximity to their transcriptional start site (TSS), strongly suggesting direct regulation by E proteins (Fig. 6E). In the representative examples shown, E2A occupancy at the first intron of *Cd28* can be seen, as well as H3K4 mono-methylation (H3K4me1) at this site, indicative of active transcription. In the example of *Id2*, E2A binding at the 3'-intergenic region is shown, as well as E2A binding approximately 3 kb upstream of the TSS. Thus, at the gene expression level, it appears E protein transcription factors influence numerous genes known to play a role in an efficient effector CD8⁺ T-cell response to infection and that many of these genes are likely directly activated or repressed by the E protein transcriptional regulators.

Discussion

E proteins are widely recognized as key transcription factors in lymphocyte development and differentiation [12]. In this study, we focused on the role of E proteins, E2A and HEB, during the early stages of the CD8⁺ T-cell immune response. We observed that E protein expression is upregulated upon TCR stimulation both in vitro and in vivo, and that E protein DNA-binding activity is increased during the earliest stages of infection. E protein-deficiency led to increased frequency of KLRG1^{hi} terminally differentiated effector T cells, particularly before day 5 of infection. Further, we provide evidence that E proteins directly regulate effector gene expression in CD8⁺ T cells upon activation, again suggesting a role for these proteins during early infection. Overall, we show loss of E protein transcription factors leads to delayed development of KLRG1^{lo}CD127^{hi} memory-precursor cells (Fig. 3D).

Our previous work examined the roles of Id2 and Id3, proteins known to negatively regulate E proteins [12], in the CD8⁺ T-cell immune response. Id2-deficiency led to a reduced CD8⁺ T-cell response to infection at the peak with no KLRG1^{hi} expression, and an accordingly smaller memory T-cell population [13]. Close examination of Id2 mRNA expression

indicated that Id2 was down-regulated on day 5 postinfection. Furthermore, using Id2-YFP and Id3-GFP reporter mice, we recently showed both of these Id proteins were downregulated in effector T cells early in infection [14]. Loss of Id3 also led to defective long-lived memory cell formation [14]. Thus, given our E protein expression data, we propose a scenario in which E proteins are expressed early during CD8⁺ T-cell infection where they regulate genes required for long-lived memory-precursor formation. As the infection proceeds, Id2 proteins are upregulated, preventing E protein DNA-binding activity and leading to development of terminally differentiated effector cells.

A number of previous studies have identified markers that predict if CD8⁺ T cells will form part of the effector- or memory-precursor compartment during infection [2,3,14,22,23]. In particular, cells with high expression of KLRG1 and early high expression of CD25 are more likely to form part of the terminally differentiated cohort of effector cells [2, 3]. We observed that loss of E proteins in CD8⁺ T cells led to an increase in the frequency and absolute number of KLRG1^{hi} effector cells during the early immune response (Fig. 5). Moreover, our gene expression analysis indicates E proteins may modulate CD127 (IL-7R), which marks long-lived memory-precursor cells, as its expression is downregulated at both the mRNA and protein level in the absence of E2A and HEB (Figs. 3 and 6). Although KLRG1 expression on effector T cells has previously been associated with senescence [24] and thus may mark cells for apoptosis during the contraction phase, we could still detect KLRG1^{hi} OT-I cells in the absence of E proteins even 2.5 months after infection (Fig. 3D). We postulate that loss of E proteins E2A and HEB delays KLRG1^{lo} memory-precursor cell differentiation during the course of an infection.

It is likely that E proteins directly influence expression of genes required for this memory-precursor cell differentiation step. Indeed, we observed a number of genes necessary for the effector immune response were up or downregulated in the absence of E proteins. Genes associated with CD8⁺ effector activation and differentiation such as CD28 and Lag3 were upregulated with loss of E protein expression, while genes associated with memory-precursor formation such as IL-7R and Eomes were downregulated. These data indicate E protein-deficient cells acquire a more effector T-cell transcriptional profile during the early stages of the immune response. Interestingly, both Id2 and Id3, which inhibit E protein DNA binding, were upregulated in the absence of E proteins in vitro (Fig. 6). However, loss of E proteins expression did not result in an Id –protein-deficient phenotype, that is, dominant formation of KLRG1^{lo}CD127^{hi} memory-precursor cells [14], indicating E proteins are not required for Id protein expression in vivo. Further, it is likely that Id2 can regulate Id3 protein expression but not vice versa, either directly or indirectly, and that Id2 and Id3 protein expression is strongly influenced by the cytokine milieu [14]. Thus, whether E proteins can directly positively or negatively regulate expression of the Id proteins during effector T-cell differentiation in vivo remains to be fully elucidated. Importantly, it should be noted that E2A and HEB are not interchangeable and can bind DNA either as homo- or heterodimers and that Id2 and Id3 cannot compensate for one another. Thus, it is possible that different Id proteins target different E proteins with distinct affinities. Given the dramatic phenotypes observed in the absence of Id2 and Id3 [13–15], coupled with the relatively subtle phenotype seen here in the absence of E2A and HEB, we postulate that "turning off" E protein transcriptional activity by expression of Id2 and Id3 is an necessary component of an efficient CD8⁺ T-cell response and essential for KLRG1^{lo}CD127^{hi} memory-precursor formation.

Recent studies have also focused on the transcriptional regulators required for cell-fate decisions during the CD8⁺ T-cell immune response. For example, T-bet expression is increased in terminally differentiated effector T cells and loss of T-bet leads to a paucity of these cells during infection with LCMV and Lm-Gp33 [2]. Another transcription factor,

Blimp-1, was also shown to be important for effector T-cell formation [5–7]. Blimp-1 deficiency led to an increase in memory-precursor cells and these cells upregulated CD62L expression and IL-2 production, phenotypes associated with central memory cells [6]. While both of these transcriptional regulators promoted a terminally differentiated effector phenotype, the factors specifically required for long-term memory-precursor formation have been more difficult to identify. Previous data have implicated both TCF-1 and Eomes in this early lineage decision and suggested that both are important for memory-precursor formation [8, 9]. Given the data described here, we believe E proteins are transcriptional regulators that influence memory-precursor formation. However, this effect of E protein transcription factors is only evident at the earliest stages of infection and is largely lost as the infection proceeds, presumably due to Id2 and Id3 protein activity that prevents E proteins-binding DNA at the peak of infection. Interestingly, our microarray gene-expression analysis indicates lower Eomes expression in the absence of E proteins (Fig. 6). It is possible that E proteins positively regulate Eomes transcription early in infection, while TCF-1 regulates this expression at later time points when a memory cohort of cells has been established [9]. Interestingly, E proteins have been previously been shown to regulate TCF-1 expression [25]. We observed no difference in TCF-1 expression levels in the absence of E proteins; however, because our microarray gene expression analysis was performed on recently activated T cells in vitro, it is possible that changes in TCF-1 expression in the absence of E proteins would be observed at later time points in effector T-cell differentiation.

In conclusion, we have shown that loss of E protein expression impacts the CD8⁺ T-cell immune response, leading to increased frequency of terminally differentiated effector T cells. We believe E proteins and Id proteins orchestrate this response so that both terminally differentiated and longer-lived effector and memory cell populations will be formed and will remain for long-term protection against subsequent infection.

Materials and methods

Mice, adoptive transfer, and infection

Mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego. E2A^{KO} and HEB^{KO} mice were a gift from Y. Zhuang, generated as previously described [17]. E2A-GFP mice were a gift from Y. Zhuang and C. Murre. CD4-Cre mice were a gift from C. Murre. CD45.1, CD45.2, and OT-I TCR transgenic mice were obtained from The Jackson Laboratory. C57BL/6J (WT) and E protein-deficient mice with differing CD45 congenic markers were collected for transfer into congenically marked hosts. For mixed transfers, WT and E protein-deficient OT-I transgenic splenocytes were mixed at 1:1 ratio and were transferred into the appropriate recipients (as determined by expression of congenic markers). In analysis of the CD8⁺ T-cell immune response, recipient mice were infected 1 day after T-cell transfer with 5×10^3 colony-forming units of recombinant Lm-OVA. Transferred cells were identified by CD45 congenic markers and were analyzed as described below.

Flow cytometry

Single cell suspensions were prepared from indicated tissues. The following antibodies were used: CD45.1 FITC (A20), CD45.2 FITC (104), V α 2 TCR FITC (B20.1), CD127 FITC (A7R34), CD62L FITC (MEL-14), Ly6C FITC (AL-21), CD43 FITC (1B11), H-2K^b OVA tetramer PE (Beckman Coulter), V α 2 TCR PE (B20.1), CD8 PE (53–6.7), CD45.1 PE (A20), CD45.2 PE (104), EOMES PE (Dan11mag), CD27 PE (LG.7F9), CD25 PE (PC61.5), CXCR3 PE (CXCR3–173), CD62L PE (MEL-14), CD8 PerCP (53–6.7), CD45.2

PerCP Cy5.5 (104), CD45.1 allophycocyanin (A20), CD45.2 allophycocyanin (104), V α 2 TCR allophycocyanin (B20.1), CD44 allophycocyanin (1MF), CD8 allophycocyanin (53–6.7) (Biolegend), KLRG1 allophycocyanin (2F1), CD25 allophycocyanin (3CF), T-bet Alexa Fluor[®] 647 (4B10), CD45.1 PE Cy7 (A20), CD45.2 allophycocyanin Cy7 (104), CD44 Alexa Fluor[®] 780 (1MF), CD8 Pacific Blue (53–6.7), CD45.1 eAlexa Fluor[®] 450 (A20), CD44 Pacific Blue (1MF). All antibodies were purchased from eBioscience unless otherwise specified. For analysis of in vivo proliferation, 1 mg BrdU (Sigma-Aldrich) was injected intraperitoneally into mice 4 h before they were sacrificed. The BrdU Flow kit (BD Biosciences) was used to quantify incorporation of BrdU into the DNA of responding CD8⁺ T cells. For analysis of cell viability, splenocytes were stained with allophycocyanin-conjugated Annexin V according to the manufacturers instructions. For analysis of IFN- γ and TNF- α production, splenocytes were incubated for a total of 6 h at 37°C at a density of 5×10^6 per well in RPMI 1640 media (Mediatech) containing 10% (volume/volume) bovine growth serum (HyClone) alone or with 10 nM OVAp. After 3 h, Protein Transport Inhibitor Cocktail (eBioscience) was added and culture incubated for an additional 3 h. Cells were collected and surface stained before permeabilization with Ebioscience Foxp3 staining kit and intracellular staining with IFN- γ and TNF- α . Samples were collected on FACSCalibur, FACSFortessa, or FACS Aria (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Quantitative PCR

OT-I CD8⁺ T cells were sorted from indicated mice, RNA was extracted using TRIzol (Invitrogen), treated with DNase (Ambion), and cDNA was generated using RT-PCR with SuperScript III RT-PCR kit (Invitrogen). mRNA levels were assessed by qPCR using nonspecific product detection (SYBR Green, Stratagene) using primers that amplify in a linear relationship with the housekeeping gene primers. Samples were normalized to HPRT expression.

Gel-shift assay

DNA-binding activity to consensus E-box-binding site was assessed for nuclear lysates from in vitro or in vivo stimulated OT-I T cells of indicated genotype by gel shift assay as previously described [26]. Nuclear extracts from each time point (10 μ g) were incubated with a ³²P-labeled probe (E-box site 5'-TCGAAGAACCTGCAGCAGCT-3') or octamer DNA-binding sequence as a control [26]) for detection of DNA-binding activity.

Microarray analysis

CD8⁺ T cells from indicated mice were sorted, activated in vitro with anti-CD3 and anti-CD28 for 16 h and resorted for live, activated cells. RNA was extracted with TRIzol and amplified for two rounds using the MessageAmp amplified RNA kit (Ambion). RNA was biotin labeled using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Diagnostics) and purification with the RNeasy Mini Kit (Qiagen). The resulting cRNA was hybridized to GeneChip Mouse Gene 1.0 ST arrays and raw CEL files were obtained. The data presented were normalized and analyzed using the Gene Pattern suite software and DAVID Bioinformatics [19,20]. Microarray data were cross-referenced with ChIP Seq data as reported in ref. [21].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DKO	double knock-out
E2AKO	OT-I E2A-deficient
HEBKO	OT-I HEB-deficient
LmOVA	OVA-expressing <i>Listeria</i>

Figure 1.

E2A-GFP expression is upregulated upon CD8⁺ T-cell activation. Flow cytometric analysis of E2A-GFP expression by CD8⁺ splenocytes (A) after activation in vitro or (B) after infection. (A) Flow cytometric analysis of CD8⁺ T cells stimulated in vitro with anti-CD3 and anti-CD28 and analyzed at indicated time points. Gray-line histogram indicates WT control CD8⁺ T cells and black-line histogram indicates E2A-GFP-expressing CD8⁺ T cells. (B) WT control mice and E2A-GFP-reporter mice were infected with Lm-OVA and CD8⁺ Lm-OVA-specific T cells in spleen were analyzed by flow cytometry on indicated days after infection. K^b-OVAp tetramer⁻ CD8⁺ T cells are shown as controls. Gray-line histogram indicates WT control CD8⁺ T cells and black-line histogram indicates E2A-GFP-expressing CD8⁺ T cells. Data shown are representative of two independent experiments performed with a total of four mice.

Figure 2.

E proteins show increased DNA-binding activity in activated CD8⁺ T cells in vivo. Electrophoretic mobility-shift assay of nuclear extracts prepared from OT-I⁺ T cells stimulated during infection. (A) OT-I⁺ T cells were transferred to recipient mice and the mice subsequently infected with VSV-OVA. One day 4 and day 6 after infection OT-I⁺ T cells were harvested from the spleens and lymph nodes of infected mice and nuclear extracts were isolated. Nuclear extracts were incubated with a radiolabeled probe containing an E-box site for detection of E protein DNA-binding (μ E5) or a control probe containing an octamer-binding site (OCT) for confirmation of the quality of the nuclear extracts. E47 blocking antibody was used to show E47-specific binding to radiolabeled E-box site-specific

probe. (B) WT and Id2-deficient OT-I⁺ T cells were transferred to recipient mice that were then subsequently infected with VSV-OVA. On day 6 after infection OT-I⁺ T cells were harvested from the spleens and lymph nodes of infected mice and nuclear extracts treated as in (A). Data are representative of two independent experiments.

Figure 3.

E protein-deficient CD8⁺ cells develop a KLRG1^{hi} CD127^{lo} effector phenotype during *Listeria* infection. CD45.1⁺-recipient mice received either 1×10^4 OT-I WT (CD45.2) or OT-I E protein-deficient (CD45.2) cells 1 day before infection with Lm-OVA. (A) Percent population expansion of OT-I WT, OT-I E2A-deficient (E2AKO), OT-I HEB-deficient (HEBKO) or OT-I E2A/HEB double-deficient (DKO) T cells over time in peripheral blood is shown. (B) Flow cytometric analysis of KLRG1 and CD127 expression by OT-I WT and OT-I DKO cells on indicated days after infection in peripheral blood. Numbers in quadrants indicate percentage of cells. (C) Percent population expansion of KLRG1^{hi} OT-I WT or OT-I DKO cells and KLRG1^{lo} OT-I WT or OT-I DKO in peripheral blood was measured over

time during Lm-OVA infection. Histograms indicate KLRG1^{hi} and KLRG1^{lo} OT-I cells on day 7 after infection. (D) Flow cytometric analysis of KLRG1 and CD127 expression by OT-I WT and OT-I DKO cells at day 77 (memory) after infection in peripheral blood. Numbers in quadrants indicate percentage of cells. Bar graphs indicate percent population expansion of OT-I WT or DKO cells, KLRG1^{lo} OT-I WT or DKO cells and KLRG1^{hi} OT-I WT or DKO cells in peripheral blood on day 77 (memory) after infection. Data shown are average (+ SEM) with $n = 2-7$ mice per group. Statistical significance was determined using unpaired two-tailed t-test where $**p < 0.005$, $***p < 0.0005$. Data are representative of two independent experiments.

Figure 4.

Splenic E protein-deficient CD8⁺ cells develop a KLRG1^{hi} CD127^{lo} effector phenotype during *Listeria* infection. CD45.1⁺-recipient mice received either 1×10^4 OT-I WT (CD45.2) or OT-I E protein-deficient (CD45.2) cells 1 day before infection with Lm-OVA. (A) Flow cytometry of KLRG1 and CD127 expression by OT-I WT and OT-I DKO cells in the spleen on indicated days after infection. Numbers in quadrants indicate percentage of cells. (B) Percent population expansion of KLRG1^{hi} OT-I WT or OT-I DKO cells and absolute cell number of KLRG1^{hi} OT-I WT or OT-I DKO in the spleen on indicated days after infection. Data are shown as mean + SEM of 2–3 mice per group and are pooled from three independent experiments. * $p < 0.05$, ** $p < 0.005$ (unpaired two-tailed t-test).

Figure 5.

E protein-deficient CD8⁺ cells develop an effector-memory phenotype during early stages of infection. CD45.1.2⁺-recipient mice received a mixture of 0.25×10^6 OT-I WT (CD45.1⁺) and OT-I DKO (CD45.2⁺) cells 1 day before infection with Lm-OVA. (A) Flow cytometric analysis of OT-I T cells in spleen. Numbers beside gated area indicate percent in each. Bar graph indicates percent population expansion of OT-I WT and OT-I DKO cells in spleen from 2 to 3 different mice per group per time point. (B) Infected mice received 1 mg BrdU 4 h before analysis of splenocytes. Bar graph indicates percent BrdU incorporation by OT-I cells from day 3 to day 6 after infection. (C) Viability of OT-I WT and OT-I DKO cells assessed by ex vivo staining with Annexin V from day 3 to day 6 postinfection. (D) Flow cytometry of IFN- γ and TNF- α production by splenocytes restimulated for 6 h in vitro with OVAp or incubated in media alone as unstimulated control on day 6 after infection. Lower flow cytometry plots indicate gated OT-I cells IFN- γ production after in vitro restimulation

with OVAp. (E) Bar graphs indicating KLRG1 expression by OT-I WT and OT-I DKO splenocytes from day 3 to day 6 postinfection. Data are shown as mean + SEM of 2–3 mice per group and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ (unpaired two-tailed t-test).

Figure 6. Gene-expression profile of WT versus E protein-deficient T cells stimulated in vitro. Affymetrix microarray analysis of mRNA from CD8⁺ T cells from WT and DKO mice, stimulated in vitro with anti-CD3 and anti-CD28 for 16 h. (A) Normalized expression values for WT versus DKO CD8⁺ T cells. Numbers in corners indicate genes with a difference in expression of 1.7-fold or more in DKO CD8⁺ T cells versus WT cells (upregulation, red dots; downregulation, blue dots). Data are representative of one experiment with two data sets per group. (B) Gene-expression values of genes of interest with a difference in expression 1.7-fold or more in DKO CD8⁺ T cells versus WT cells (upregulation, red; downregulation, blue). (C) Selected genes involved in immune effector processes that are

activated or repressed in DKO CD8⁺ T cells. (D) Selected cytokine and chemokine genes that are activated or repressed in DKO CD8⁺ T cells. (E) E2A DNA-binding sites as identified using ChIP-Seq. Representative examples of Cd28 and Id2 are shown. UCSC Genome Browser was used to visualize binding patterns. Blue pattern denotes E2A DNA binding. Green pattern denotes H3K4 mono-methylation. Red arrow denotes the transcription start site. The Table indicates selected genes up- or downregulated in the microarray that display E2A occupancy as identified by ChIP-Seq.