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Id2 Influences Differentiation of Killer Cell Lectin-like Receptor G1\textsuperscript{hi} Short-Lived CD8\textsuperscript{+} Effector T Cells

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CD8\textsuperscript{+} T cells play a crucial role in the clearance of intracellular pathogens through the generation of cytotoxic effector cells that eliminate infected cells and long-lived memory cells that provide enhanced protection against reinfection. We have previously shown that the inhibitor of E protein transcription factors, Id2, is necessary for accumulation of effector and memory CD8\textsuperscript{+} T cells during infection. In this study, we show that CD8\textsuperscript{+} T cells lacking Id2 did not generate a robust terminally differentiated killer cell lectin-like receptor G1 (KLRG1\textsuperscript{hi}) effector population, but displayed a cell-surface phenotype and cytokine profile consistent with cells. We found that deletion of Bim rescued Id2-deficient CD8\textsuperscript{+} cell survival during infection. However, the dramatic reduction in KLRG1\textsuperscript{hi} cells caused by loss of Id2 remained in the absence of Bim, such that Id2/Bim double-deficient cells form an exclusively KLRG1\textsuperscript{lo}CD127\textsuperscript{hi} memory precursor population. Thus, we describe a role for Id2 in both the survival and differentiation of normal CD8\textsuperscript{+} effector and memory populations. The Journal of Immunology, 2013, 190: 000–000.

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CD8\textsuperscript{+} T cells are an essential component of host resistance to viral and intracellular bacterial infections. Upon pathogen recognition in the context of appropriate costimulatory signals, naïve CD8\textsuperscript{+} T cells expand and acquire effector functions, allowing them to kill infected cells and secrete cytokines. Upon resolution of infection, the majority of responding CD8\textsuperscript{+} T cells undergo apoptosis. However, a small number of Ag-specific effector cells remain and establish a heterogeneous memory T cell population that collectively serves to protect against reinfection. Exogenous signals received by responding CD8\textsuperscript{+} T cells early during infection including duration and levels of Ag and inflammatory cytokines are known to influence cell-fate decisions that dictate which cells will die soon after the resolution of infection or develop into memory-precursor cells from which the long-lived memory population is established (1–3). Phenotypic and functional analyses of CD8\textsuperscript{+} effector T cells have used levels of expression of killer cell lectin-like receptor G1 (KLRG1), CD127, CD27, CD43, CXCR3, and CD25 and production of IL-2 to distinguish cells that will go on to become terminally differentiated, short-lived effector memory cells versus long-lived memory cells (4–10). Numerous transcription factors have also been implicated in this cell-fate decision with T-bet, Blimp-1, and Id2 favoring shorter-lived, effector memory cells expressing high levels of KLRG1 and with Eomesodermin, Bcl-6, transcription factor-1, STAT3, and Id3 expression supporting the formation of long-lived memory precursors with high levels of CD127 and CXCR3 (4, 11–22). Intimately intertwined in the formation of these distinct effector and memory populations is the relationship between surviving the contraction phase of the effector population and the initiation of a gene-expression program that supports the phenotypic and functional characteristics of protective memory.

Id2 is a member of the inhibitor of DNA binding (Id) family consisting of transcriptional regulators that act antagonistically on E protein transcription factors to prevent their binding to DNA. Clear roles for E and Id proteins in lymphocyte development are well delineated (23). However, despite relatively high mRNA expression levels of Id2 in mature CD8\textsuperscript{+} effector and memory cells and high Id3 in naive and long-lived memory cells (19–21), the role of these transcriptional regulators in CD8\textsuperscript{+} effector T cell differentiation is still not fully understood. Previously, we showed loss of Id2 resulted in a diminished CD8\textsuperscript{+} effector response and led to fewer remaining memory CD8\textsuperscript{+} T cells, which rapidly acquired a central memory (CD44\textsuperscript{hi}CD62L\textsuperscript{lo}KLRG1\textsuperscript{lo}CD127\textsuperscript{hi}) phenotype (19, 20). This rapid loss of Id2-deficient CD8\textsuperscript{+} effector T cells was due to increased death by effector cells and correlated with high expression of the proapoptotic molecule Bim and low levels of the antiapoptotic molecule Bcl-2. However, in these studies it was not clear if the severe loss of short-lived (CD44\textsuperscript{hi}CD62L\textsuperscript{lo}KLRG1\textsuperscript{lo}CD127\textsuperscript{hi}) effector memory cells was due solely to a failure of those cells to survive or if Id2 also regulated their differentiation. More recently, a role for Id3 expression in predicting memory potential and supporting differentiation of long-lived memory cells has become clear as well, suggesting distinct functions for these two transcriptional regulators in CD8\textsuperscript{+} immunity (20, 21).

One key molecule influencing the contraction phase of the CD8\textsuperscript{+} effector population is the proapoptotic molecule of the Bcl-2...
family of proteins, Bim. Previous studies demonstrated a role for Bim in the induction of death of both CD4+ and CD8+ effector T cells postinfection (24–26). Indeed, Bim-deficient CD8+ T cells failed to undergo apoptosis and continued to accumulate in the spleen, indicating the importance of this protein in modulating the contraction phase of the CD8+ T cell immune response (24, 25). Surprisingly, Bim deficiency appears to rescue both short-lived effector memory and long-lived memory subsets from contraction (27–29). However, the memory cells rescued due to loss of Bim eventually wane, suggesting Bim-independent regulation of long-lived memory subsets (29). Ultimately, although it is understood that Bim is required for the contraction phase of the CD8+ T cell immune response, it is less clear if and how Bim is modulated to produce long-lived T cell memory.

In this article, we elaborate on the role of Id2 in CD8+ T cells during infection. In this article, we elaborate on the role of Id2 in formation of short-lived effector and long-lived memory CD8+ T cells, showing that Id2 influences the development of KLRG1hi effector T cells. We also show that high levels of Bim are responsible for the increased apoptosis observed in Id2-deficient cells responding to infection, likely due to unchecked E protein activity. Surprisingly, Bim deficiency did not rescue the effector T cell differentiation defects observed in the absence of Id2, indicating Id2 controls the CD8+ T cell immune response at two levels: by influencing survival of effector cells and regulating the effector and memory T cell differentiation program.

Materials and Methods
Mice, adoptive transfers, 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. Mice with a mutated Id2 allele (Id2−/−)(Id2<sup>−/−</sup>) were generated as previously described (19) and maintained on the C57BL/6 background. Mice with a mutated Bcl2l11 allele (Bim-knockout (Bim<sup>−/−</sup>)) mice were purchased from The Jackson Laboratory (30). For generation of fetal liver chimeras, 5 × 10<sup>5</sup> RAGKO bone marrow cells were injected i.v. into lethally irradiated (1000 rad), congenically distinct hosts. Chimeras were rested for at least 8 wk after reconstitution of the host. Mixed transfers were conducted as follows: 2 × 10<sup>5</sup> OT-I wild type (WT) cells were mixed with 2 × 10<sup>5</sup> OT-I Id2<sup>+</sup>, Id2<sup>−/−</sup>, or Id2<sup>−/−</sup>Bim<sup>−/−</sup> cells (CD45.2) and transferred into WT CD45.1<sup>+</sup> CD8+ T cell populations during infection. We generated fetal liver or bone marrow chimeras reconstituted with either OT-I TCR-transgenic Id2-sufficient (Id2<sup>+</sup>) or OT-I Id2-deficient (Id2<sup>−/−</sup>) hematopoietic cells. Naive Id2<sup>+</sup> or Id2<sup>−/−</sup> CD8+ T cells recovered postinfection. Thus, we were curious as to whether effector memory populations were absent in the Id2-deficient response due to a failure of cells to survive or properly differentiate. In this study, we investigated how loss of Id2 affected shorter-lived effector-memory (KLRG1hiCD127lo) and longer-lived memory precursor (KLRG1loCD127hi) CD8+ T cell populations during infection. We generated fetal liver or bone marrow chimeras reconstituted with either OT-I TCR-transgenic Id2-deficient (Id2<sup>−/−</sup>) or OT-I Id2-deficient (Id2<sup>−/−</sup>) hematopoietic cells. Naive Id2<sup>−/−</sup> or Id2<sup>−/−</sup> OT-I cells (2 × 10<sup>5</sup>) from chimeras were adoptively transferred into congenically distinct hosts, which were subsequently infected with Lm-OVA. Examination of cell-surface expression of KLRG1 and CD127 by transferred OT-I cells isolated on days 7 and 12 of infection showed a severe paucity of KLRG1hiCD127lo and KLRG1hiCD127hi effector cells in the Id2-deficient population (Fig. 1A). Analysis of absolute cell numbers of Ag-specific CD8<sup>+</sup>KLRG1hi and KLRG1hi populations also showed a severe reduction (~17-fold on day 7 and 37-fold on day 12) in KLRG1hi cells in the Id2-deficient population compared with WT cells. However, the total numbers of KLRG1hi cells were similar between Id2<sup>−/−</sup> and Id2<sup>−/−</sup> cells (Fig. 1A). Id2<sup>−/−</sup> CD8<sup>+</sup> T cells also showed ~3-fold more cells with high CXCR3 expression, consistent with formation of a population of cells with a longer-lived memory phenotype (8). Further, we observed reduced (2- to 3-fold) CXCR6 expression by Id2-deficient CD8<sup>+</sup> effector cells compared with Id2<sup>+</sup> cells (Fig. 1B), a phenotype we previously showed was present in the Id2-deficient NKT population as well (33). Thus, in the absence of Id2, at the peak of infection, CD8<sup>+</sup> effectors do not accumulate as short-lived KLRG1hi effector memory cells.
FIGURE 1. Id2-deficient CD8+ T cells display an altered phenotype during infection. A 1:1 mixture of OT-I Id2-knockout cells (CD45.2) and OT-I Id2-WT cells (CD45.1) were transferred into congenically distinct hosts 1 d before infection with Lm-OVA. (A) KLRG1 and CD127 expression by Id2+ and Id2KO OT-I cells recovered from spleen on days 7 and 12 postinfection. Flow cytometry plots display surface phenotype of CD8+ and CD45.2+ or CD45.1+ gated cells. Numbers indicate percentage of cells in each quadrant (left panel). Average (±SEM) percentage donor CD8+ and total numbers of KLRG1hi and KLRG1lo cells summarizing flow cytometry data (bar graphs, right panel). Data are from two independent experiments and four mice per time point. (B) Histograms of CXCR6 and CD43 expression by donor cells (left panel) and average (±SEM) of mean fluorescence intensity (right panel). Statistical significance was determined using unpaired two-tailed t test. ***p < 0.0005, ****p < 0.0001.

Loss of Bim rescues survival defect in Id2KO CD8+ T cells during infection

We previously showed Bim mRNA levels were increased in Id2-deficient effector T cells (19), and Bim has previously been shown to be a direct target of E proteins (34, 35). The bcl2l11 (Bim) promoter and locus contains >30 conserved E box sites. To determine which E boxes potentially played a role in the regulation of Bim during the CD8+ T cell response, we examined previously published data in which E2A occupancy in A12 T cell lines was assessed by deep sequencing (chromatin immunoprecipitation sequencing [ChIP-Seq]) (34). We observed three E2A-binding peaks containing the E2A-binding motif CANNTG in the Bim locus, and interestingly, H3K4 monomethylation (H3K4me1), a marker of active transcription, was also enriched at these three sites (Fig. 2A). To test whether E proteins bind these specific E box sites in the Bim locus upon CD8+ T cell activation, we isolated Id2+ OT-I cells directly ex vivo or after 24 h of activation with OVA peptide in vitro and performed ChIP. Using an HEB-specific Ab, we observed an increase in E protein binding upon activation at the potential E protein binding sites, with the first site (peak 1, in the promoter region of the Bim locus) showing the greatest increase (Fig. 2A). Together, these data suggest that E proteins can bind DNA at the Bim locus and that Bim expression levels can be regulated directly by E protein transcription factors during CD8+ T cell activation.

To determine if loss of Bim expression could rescue Id2KO CD8+ T cell effector survival and phenotypic development, we crossed Bim-deficient mice to the Id2KO line. We generated fetal liver chimeras reconstituted with OT-I transgenic WT (Id2+Bim+), Bim-deficient (Id2+BimKO), Id2-deficient (Id2KO Bim+), or Id2-deficient Bim-deficient (Id2KO BimKO) hematopoietic cells. Following reconstitution, we transfected these OT-I cells into congenically distinct recipients and infected with Lm-OVA. We analyzed the expansion of the transferred CD8+ T cell populations in recipient mice during the course of infection (Fig. 2B). As expected, the Id2KO Bim+ OT-I T cells did not accumulate during infection, and Id2KO BimKO CD8+ T cells failed to contract to the same extent as WT effector cells following the peak of infection (25). However, Id2KO BimKO CD8+ T cells expanded and contracted to a similar degree as Id2+Bim+ CD8+ T cells, with total cell numbers comparable between Id2+Bim+ and Id2KO BimKO populations (Fig. 2B, 2C). Thus, loss of Bim resulted in rescued of the accumulation defect by Id2KO CD8+ T cells observed during infection, supporting the idea that Id2 expression impacts effector cell survival.

Defects in differentiation of KLRG1hi effecter memory cells by Id2KO BimKO CD8+ cells remain despite rescued survival

Next, we examined the effector and memory phenotypes of Id2KO BimKO CD8+ T cells to determine if the reduction in the short-term effector population in the absence of Id2 was due simply to a failure of those cells to survive. As above, we transferred CD45.1+ OT-I Id2+Bim+, Id2+BimKO, Id2KO Bim+, or Id2KO BimKO cells to CD45.2+ congenic hosts and infected with Lm-OVA. Splenocytes were isolated on day 8 (Supplemental Fig. 1) and day 15 (Fig. 3) of infection, and cell-surface expression of phenotypic markers associated with effector and memory populations was analyzed by flow cytometry. We noted that although the frequency of donor cells was restored to Id2+ levels (Fig. 2), Id2KO BimKO CD8+ T cells displayed a profound defect in the generation of a KLRG1hiCD127lo population (Fig. 3). Id2KO and Id2KO BimKO CD8+ T cells also showed increased frequency of CXCR3hiCD43lo cells compared with Id2+ (Fig. 3, Supplemental Fig. 1), a phenotype associated with long-lived memory cells capable of a strong recall response (8). As we previously reported, the frequency of effector memory cells (CD44hiCD62Llo) was lower and CD27 was higher in Id2KO populations, and this was sustained in the Id2KO BimKO CD8+ T cells at both time points (Fig. 3, Supplemental Fig. 1) (19). CXCR6 expression remained lower in Id2KO BimKO CD8+ T cells compared with WT cells (Fig. 3, Supplemental Fig. 1). Recently it has been reported that CD25, the high-affinity IL-2R α-chain is associated with terminal effector cell differentiation (7, 10). Thus, we analyzed CD25 expression on
Id2-deficient cells on day 4 postinfection and observed that Id2KO cells had lower expression of IL-2Rα relative to WT cells congruent with their long-term memory phenotype, yet this phenotype was not rescued by loss of Bim (Fig. 3).

Examining peripheral blood on day 5 postinfection, we noted that although Id2KOBim+ cells could upregulate KLRG1 expression early in the infection, the frequency of KLRG1hi Id2KOBim+ cells was reduced compared with WT cells (Supplemental Fig. 1A), suggesting that this population does not continue to differentiate or that these cells do not survive after their initial differentiation in the absence of Id2. Furthermore, the frequency of KLRG1hi cells in the absence of both Id2 and Bim was also reduced relative to WT cells, indicating that although loss of Bim can rescue total OT-I cell frequency and number, it could not rescue the survival of KLRG1hi short-lived terminal effector cells (Supplemental Fig. 1A). When we examined splenocytes throughout the infection, we observed comparable expression of KLRG1 between Id2Bim+ cells and Id2BimKO cells or between Id2Bim+ and Id2BimKO cells, once again indicating loss of Bim could rescue overall survival of Id2-deficient OT-I cells responding to infection, but Bim deficiency did not restore the loss of KLRG1hi cell accumulation (Fig. 4A).

Due to the fact that Id2KO CD8+ cells lack a KLRG1hi short-lived effector population at the peak of infection, we also compared the phenotype between Id2+ and Id2KO cells gated only on...
the KLRG1lo population for days 8 and 15 of infection. The phenotypic differences observed in the entire CD8+ population (potentially including effector, effector memory, and memory cells) were maintained in the KLRG1loId2+ and Id2KO populations, although these differences were less pronounced (Supplemental Fig. 2A, 2B). Together, the data supported the more rapid emergence of the long-term memory phenotype among cells in the KLRG1lo subset when Id2 was absent, suggesting Id2 expression regulates gene expression and differentiation within this population.

Previous data show effector subsets, distinguished by KLRG1hi expression, localize to different areas of the spleen during infection (36). When we examined the CD8+ T cell localization in the spleens of recipient mice on day 15 of infection, we noticed that although WT CD8+ T cells were diffusely located throughout the spleen, Id2-deficient CD8+ T cells were concentrated in the T cell zones (Fig. 4C), a phenotype associated with KLRG1lo longer-lived memory precursors (36). As expected, Id2+BimKO/CD8+ T cells localized like WT cells (Fig. 4C). Id2KO/BimKO CD8+ T cells, however, were primarily found in the T cell zones, similar to Id2KO/Bim+ cells (Fig. 4C), again indicating that although loss of Bim rescues survival of Id2-deficient cells, it does not rescue their propensity for developing into shorter-lived effector memory cells.

**Id2 deficiency alters cytokine production by CD8+ T cells during infection**

To assess the functional ability of Id2-deficient T cells in the presence or absence of Bim, we isolated Id2+Bim+, Id2+BimKO, Id2KO/Bim+, and Id2KO/BimKO OT-I cells that had been transferred into recipient mice, which were then infected with Lm-OVA. As we previously observed, Id2KO cells produced IFN-γ at equal levels compared with WT cells on both days 8 (Supplemental Fig. 3B) and 15 (Fig. 5B) of infection. Interestingly, Id2-deficient CD8+ T cells produced more IL-2 (Fig. 5A, Supplemental Fig. 3A), a cytokine that has been associated with memory precursor cells, consistent with their phenotypic alterations (9). CD8+ T cells lacking Id2 produced similar levels of TNF-α compared with WT cells (Fig. 5C, Supplemental Fig. 3C). Loss of Bim alone had no effect on the cytokine profile of CD8+ T cells (Fig. 5). Cytokine production observed in the entire CD8+ population (potentially including effector, effector memory, and memory cells) was maintained in the KLRG1loId2+ and Id2KO populations on both days 8 and 15 of infection (Supplemental Fig. 4).

To examine if E proteins mediated a direct role in regulating cytokine production, we examined the IL-2, IFN-γ, and TNF-α loci for enhanced E2A-binding occupancy using the previously described ChIP-Seq profiles (34). Both IL-2 and TNF-α contained an E box site capable of binding E proteins (Fig. 5D). There were no occupancy peaks present in the IFN-γ locus. ChIP analysis showed minimal HEB binding in naive CD8+ T cells. However, in CD8+ T cells activated for 24 h in vitro with OVAp, we observed increased E protein binding at both the IL-2 and TNF-α E box sites (Fig. 5D). These data indicate a possible role for direct E protein regulation of cytokines during T cell activation.

**Altered gene expression by CD8+ effector cells in the absence of Id2**

To understand further the role of Id2 during the CD8+ T cell immune response, we sorted KLRG1loId2+ and Id2KO OT-I CD8+ T cells on day 6 after Lm-OVA infection and compared their gene-expression profiles by microarray (Fig. 6). We observed that 225 genes were upregulated 1.5-fold and 105 genes were downregulated 1.5-fold in the absence of Id2 compared with WT cells (Fig. 6A, 6B). Interestingly, numerous genes associated with memory formation/function were upregulated in Id2-deficient T cells, including Id3, Il2ra, tcf7, and cxcr3 (Fig. 6C), consistent...
with our phenotypic data. We also isolated Id2\textsuperscript{KO}Bim\textsuperscript{KO} CD8\textsuperscript{+} T cells to compare the gene-expression profile of CD8\textsuperscript{+} T cells in the absence of Bim, with the goal of revealing a distinct profile that would implicate how Id2 promotes differentiation of shorter-lived effector-memory cells (Fig. 6D). We examined the expression of genes up- or downregulated in Id2\textsuperscript{KO}Bim\textsuperscript{KO} cells compared with Id2\textsuperscript{KO} cells and observed that the expression pattern was remarkably similar (Fig. 6D). Genes downregulated in Id2\textsuperscript{KO}KLRG-1\textsuperscript{lo} effectors (Fig. 6A, blue) were also downregulated in Id2\textsuperscript{KO}Bim\textsuperscript{KO} effectors compared with their Id2\textsuperscript{+} counterparts (Fig. 6D, blue) and vice versa (Fig. 6, red). Among the 5–10% of genes with expression that did not correlate between Id2KO and Id2KOBimKO effector cells, there was not significant inverse expression. Thus, a rescue of survival by Id2KO effectors did not reveal an altered expression pattern. We also examined the mRNA levels by qPCR analysis for Id2KO and Id2+ KLRG1\textsuperscript{lo} cells separately isolated for a subset of genes of interest to CD8\textsuperscript{+} memory differentiation (Fig. 6E). These data confirmed the gene-expression profiles determined by microarray (Fig. 6E) and were consistent with a skewing of expression toward differentiation of cells with long-term memory potential. Last, we were interested if the genes that showed altered expression when Id2 was absent were potential targets of E protein transcription factors. Once again, we used published data that evaluated E2A occupancy in T cell lines by ChIP-Seq (34); those genes that we observed to be differentially regulated between Id2\textsuperscript{WT} and Id2\textsuperscript{KO} that were also identified as having significant E2A binding to putative regulatory elements are indicated in green (Fig. 6F). Notably, ∼30–35% of the genes displaying altered expression due to loss of Id2 were also indicated as direct E protein targets. Together, the microarray analyses indicate that Id2 influences the gene-expression pattern in CD8\textsuperscript{+} T cells during infection and that this gene pattern is maintained irrespective of Bim expression and survival, further supporting the conclusion that Id2 plays a key role in differentiation of effector cells to short-term versus long-term effector and memory populations.

### Discussion

The factors controlling the survival of effector CD8\textsuperscript{+} T cells as they undergo contraction, as well as their differentiation to memory cells, has been the subject of intense scrutiny, with transcription factors, survival molecules, and cytokines all being implicated in this process (1–3, 37). Of particular interest is the transcriptional regulation of differentiation leading to the eventual apoptosis of short-lived effector cells during the contraction phase versus the survival of memory-precursor cells that will seed the memory compartment. Although the proapoptotic molecule Bim is a key regulator of contraction of the CD8\textsuperscript{+} effector population, the abundance of memory-precursor cells formed in the absence of Bim is eventually lost, implicating other factors in the regulation of CD8\textsuperscript{+} effector differentiation (29). In this study, we show the transcriptional regulator Id2 influences effector CD8\textsuperscript{+} T cells in...
two ways: by regulating KLRG1hi effector CD8+ T cell differentiation and survival of CD8+ effector T cells (Figs. 1, 2). At all stages of infection, loss of Id2 resulted in impaired formation of the KLRG1hi short-lived effector cell population (Figs. 1, Supplemental Fig. 1A). We found that although loss of Bim led to rescue of the survival defect seen in the absence of Id2, Bim deficiency failed to support formation of terminally differentiated KLRG1hi effector cells by Id2KO CD8+ T cells (Figs. 3, 4). Thus, by uncoupling Bim-mediated CD8+ effector contraction from differentiation and memory formation, we addressed the role of Id2 in these processes.

E protein transcription factors positively regulate expression of Bim; we previously showed upregulation of Bim in Id2KO effector CD8+ T cells, presumably due to unchecked E protein activity, led to defects in survival and subsequent memory formation (19). However, whether Id2 can also regulate CD8+ effector T cell differentiation, irrespective of cell survival, remained unaddressed. In the absence of Id2 alone, when increased E protein activity led to elevated Bim expression, both differentiation of short-lived KLRG1hi effector T cells and survival of effector cells were impacted (Figs. 1, 2). In this study, using mice genetically deficient for both Id2 and Bim, and thus removing the confounding effect of unchecked Bim expression, the CD8+ effector T cell survival phenotype was rescued (Fig. 2). Notably, loss of Bim alone rescued both the KLRG1hi and KLRG1lo subsets when Id2 is expressed. However, differentiation of Id2-deficient CD8+ effector T cells into short-lived effector cells and long-lived memory-precursor cells was still perturbed when Bim was also absent, even though the effector cells accumulated to WT levels, indicating that E proteins together with Id proteins regulate genes necessary for CD8+ effector differentiation as well as genes necessary for survival. As loss of Bim does not favor KLRG1lo cells and gene expression of the Id2KO:BimKO cells were the same, it does not appear likely that the rescued cells are developing aberrantly. We did, however, observe a small Id2KO KLRG1hi population at early time points (day 5; Supplemental Fig. 1A), which was rapidly lost and not rescued to Id2WT levels by Bim deficiency. Thus, we suggest E proteins and Id proteins regulate not only the survival signals required for memory-precursor formation but also influence the gene-expression pattern required for the sustained differentiation of the terminal-effector population.

Aside from regulating Bim expression during CD8+ effector T cell contraction, how do E and Id proteins influence effector

FIGURE 6. Gene expression profile of Id2+ versus Id2KO KLRG1hi cells during infection. Gene expression analysis of WT versus Id2KO effector cells. CD45.1.2” mice received CD45.1 OT-I WT (4 x 10^5) cells mixed with CD45.2 OT-I BimKO, Id2KO, or Id2KO:BimKO (4 x 10^5) cells 1 d before infection with Lm-OVA. KLRG1hi and KLRG1lo cells were sorted on day 6. (A) Fold change plot of WT KLRG1lo versus Id2KO KLRG1hi CD8+ T cells. Numbers in corners indicate genes with a difference in expression of 1.7-fold or more. Genes upregulated in Id2KO KLRG1hi cells are indicated in red; genes downregulated are indicated in blue. Data are pooled from three independent experiments. (B) Volcano plot of expression data in (A). (C) Gene expression of key genes in WT KLRG1hi versus Id2KO KLRG1hi CD8+ T cells. Dotted lines indicate 1.5-fold difference. (D) Gene expression of genes 1.7 fold up- or downregulated in WT KLRG1hi versus Id2KO:BimKO KLRG1hi CD8+ T cells plotted for WT versus Id2-knockout cells. Genes upregulated in Id2KO:BimKO KLRG1hi CD8+ T cells are indicated in red; genes downregulated are indicated in blue. (E) mRNA expression of relevant genes in Id2KO compared with Id2KO KLRG1hi cells on day 6 of Lm-OVA infection. (F) Gene expression in WT KLRG1hi versus Id2KO KLRG1hi CD8+ T cells; green indicates E2A occupancy. Numbers in corners indicate number of genes with E2A occupancy.
T cell differentiation? Our previous data indicate Id2 expression leads to development of short-lived effector cells, whereas Id3 expression marks cells that will contribute to the memory-precursor pool (20). Although microarray analysis revealed up-regulation of Id3 expression in Id2-deficient T cells (Fig. 5C, 5E), Id3 was unable to compensate for either the differentiation or survival defect we observed with loss of Id2. These data support our hypothesis that high Id3 expression marks cells for memory-precursor differentiation and is consistent with our previous data (20). Furthermore, these data imply Id2 and Id3 may have different binding affinities for E proteins or that certain E/Id proteins are expressed at different stages during effector T cell differentiation, thus providing a level of fine-tuning of the CD8+ effector immune response not previously appreciated. Increased expression of Id3 in the absence of Id2 also implies direct or indirect negative regulation of Id3 by Id2, a possibility requiring further investigation.

In our gene-expression analysis, we also observed upregulation of other genes previously associated with memory CD8+ T cell formation including Ccr5, Tcf7, and Cxcr5 (Fig. 5C, 5E). These genes were upregulated in the absence of Id2 and irrespective of Bim expression, thus implicating unchecked E protein activity in genes were upregulated in the absence of Id2 and irrespective of other genes previously associated with memory CD8+ T cell infection. In this study, we explored the role of Id2 in both surviv-ervisors in the control of the effector T cell differentiation during ulation, thus providing a level of fine-tuning of the CD8+ effector T cell differentiation during infection.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Effector phenotype of Id2+ and Id2KO CD8+ T cells on day 8 of infection.  
(A) Flow cytometric analysis of CD127 and KLRG1 expression on donor CD8+CD45.2+ PBL as in Figure 3 but on day 5 of infection. CD45.1+ C57BL/6 mice received OT-I Id2+Bim+, Id2+BimKO, Id2KO Bim+ or Id2KO BimKO (CD45.2+) cells (2 x 10^4) 1 day before infection with Lm-OVA. Gated on CD8+CD45.2+ lymphocytes. (B) Flow cytometric analysis of cell-surface phenotype of donor CD8+CD45.2+ splenocytes as in Figure 3 but on day 8 of infection. CD45.1+ C57BL/6 mice received OT-I Id2+Bim+, Id2+BimKO, Id2KO Bim+ or Id2KO BimKO (CD45.2+) cells (2 x 10^4) 1 day before infection with Lm-OVA. Gated on CD8+CD45.2+ lymphocytes. CXCR6 flow cytometry analysis on day 7 after infection. (C) Bar graphs summarizing average (±SEM) frequency of KLRG1hi or KLRG1lo donor cells among total donor CD8+ cells and total number of KLRG1hi and KLRG1lo donor cells recovered from spleen on day 8 of infection. Data are representative of three independent experiments.

Supplemental Figure 2: Distinct phenotype of Id2KO CD8+ T cells is largely independent of KLRG1 expression. (A) Flow cytometric analysis of CD62L, CD44, CD122 (gated on Ly6C+ population), CD27, CXCR3 and CD43 expression by splenocytes isolated on day 8 after infection. CD45.1+ C57BL/6 mice received OT-I Id2+Bim+, Id2+BimKO, Id2KO Bim+ or Id2KO BimKO (CD45.2+) cells (2 x 10^4) 1 day before infection with Lm-OVA. Gated on CD8+CD45.2+KLRG1lo gated lymphocytes. (A) Flow cytometry as in (A) for donor cells on day 15 post infection.

Supplemental Figure 3: Early functional cytokine production by CD8+ T cells in the absence of Id2 and/or Bim. Flow cytometric analysis of (A) IL-2 (B) IFNγ and (e) TNFα production by OT-I Id2+Bim+, BimKO, Id2KO or Id2KO BimKO (CD45.2+) splenocytes. CD45.1+ C57BL/6 mice received OT-I Id2+Bim+, BimKO, Id2KO or Id2KO BimKO (CD45.2+) cells (2 x 10^4) 1 day before infection with Lm-OVA.
Splenocytes isolated on day 8 post infection were restimulated for 6 h *in vitro* with OVA peptide (solid line) or incubated in media alone (dotted line). Bar graphs indicate average (±SEM) percent cytokine producing donor cells.

**Supplemental Figure 4: Distinct functional cytokine profile is largely independent of KLRG1 expression.** Flow cytometric analysis of IL-2, IFNγ and TNFα production by OT-I Id2⁺Bim⁺, BimKO, Id2KO or Id2KO.BimKO (CD45.2⁺) splenocytes as in Figure 4 and Supplemental Figure 3 gated on KLRG1lo donor CD8⁺ T cells. Splenocytes isolated on day 8 (**A-C**) or day 15 (**D-F**) post infection. Flow cytometric analysis showing representative histograms (left) and bar graphs indicating average (±SEM) percent cytokine producing cells (right).
Supplemental 2: Knell et al.
Supplemental 3: Knell et al.

A

- +OVAp
- -OVAp

IL-2

B

IFNγ

C

TNFα

Supplemental 3: Knell et al.