Published in final edited form as:

Nat Immunol. ; 12(12): . doi:10.1038/ni.2158.

The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8⁺ T cell subsets

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Abstract

During infection, naive CD8⁺ T cells differentiate into effector cells, which are armed to eliminate pathogens, and memory cells, which are poised to protect against reinfection. The transcriptional program that regulates terminal differentiation into short-lived effector-memory versus long-lived memory cells is not clearly defined. Through the use of mice expressing reporters for the DNA-binding inhibitors Id2 and Id3, we identified Id3^{hi} precursors of long-lived memory cells before the peak of T cell population expansion or upregulation of cell-surface receptors that indicate memory potential. Deficiency in Id2 or Id3 resulted in loss of distinct CD8⁺ effector and memory populations, which demonstrated unique roles for these inhibitors of E-protein transcription factors. Furthermore, cytokines altered the expression of Id2 and Id3 differently, which provides insight into how external cues influence gene expression.

Infection with intracellular pathogens initiates the proliferation of naive CD8⁺ T cells and their differentiation into cytotoxic, cytokine-secreting effector T cells with a short half-life¹. After infection is resolved, most effector CD8⁺ T cells die; however, a small fraction differentiates into memory cells that persist for prolonged periods. After reexposure to pathogen, these memory T cells respond with enhanced proliferation, cytotoxicity and cytokine secretion, which often provides life-long protection against previously encountered pathogens^{2,3}. Given the importance of CD8⁺ T cell memory, the identity of the factors that dictate whether cells will join the memory CD8⁺ T cell pool has become a key question in immunology.

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Accession code. GEO: microarray data, GSE 32675.

Note: Supplementary information is available on the Nature Immunology website.

AUTHOR CONTRIBUTIONS

C.Y.Y. designed and completed the majority of experiments, analyzed and interpreted data, and wrote the manuscript; A.D.S. and R.R.R. generated the reporter mouse lines; J.A.B., J.K., E.Y. and K.C.L. did experiments and discussed and interpreted results; A.K.J. provided technical assistance; H.S.L. and S.S.W. discussed results and provided advice; L.M.D. and C.M. discussed and interpreted results and wrote the manuscript; and A.W.G. directed the study, analyzed and interpreted results, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Memory CD8⁺ T cells are thought to derive from the effector T cell pool^{4–6}. During the effector phase, precursors of long-lived memory T cells have gene expression and cell-surface phenotypes substantially different from those of effector cells^{7,8}. Although it is clear that environmental cues such as exposure to proinflammatory cytokines^{9–13} and the duration or intensity of antigen exposure have a substantial effect on the number and composition of the memory T cell population^{14,15}, the transcriptional connection between the inflammatory environment and the transition to long-lived memory T cells is an area of intense investigation. Indeed many cytokines, such as interleukin 2 (IL-2), IL-7, IL-15, IL-12, interferon (IFN- γ), IFN- α and IFN- β and transforming growth factor- β (TGF- β), influence the transition from effector cells to memory cells, in many cases favoring the generation of effector and short-lived memory cells rather than long-lived memory cells^{9–13}.

Cell surface receptors have proven useful in distinguishing terminally differentiated short-lived effector-memory cells from those more likely to transition into long-lived memory cells. Markers of CD8⁺ T cells with short-lived effector or longer-lived memory potential include the following: CD62L (L-selectin), CD127 (IL-7 receptor α -chain), KLRG-1 (killer cell lectin-like receptor), CXCR3 (chemokine receptor), CD43 (leukosialin) and CD25 (IL-2 receptor α -chain)^{7,16–19}. Many of these surface proteins indicate but are not required for the differentiation of memory CD8⁺ T cells. For example, signaling through the IL-7-receptor is permissive but not ‘instructive’ to the formation of memory CD8⁺ T cells, and KLRG-1 is dispensable for CD8⁺ T cell differentiation^{20,21}. Moreover, loss of IL-2 signaling has little effect initially on the number of developing effector and memory cells, although the composition of the effector subsets is altered and the secondary responses are compromised^{18,22–24}. Therefore, it is of value to explore memory potential beyond a surface phenotypic ‘readout’ and identify the transcriptional programs that control the differentiation of memory CD8⁺ T cells.

Studies have begun to elucidate some of the transcriptional regulators required for the formation of memory CD8⁺ T cells. Both the transcription factor T-bet and the transcriptional repressor Blimp-1 are required for the terminal differentiation of effector CD8⁺ T cells, and loss of either leads to the accumulation of KLRG-1^{lo}CD127^{hi} long-lived memory precursors^{7,25–27}. In contrast, the transcription factors TCF-1 and Eomes both contribute to the maintenance of the long-lived memory precursor subset^{28,29}. Additionally, the transcriptional inhibitor Id2 is essential for the survival of effector CD8⁺ T cells and their memory formation after infection³⁰. However, the interactions among these transcription factors and the signals that drive their expression during the differentiation of memory CD8⁺ T cells remains under investigation.

Id proteins negatively regulate the DNA-binding activity of E-protein transcription factors³¹. Both E proteins and Id proteins have multiple roles in lymphocyte development and homeostasis. Among the four members of the Id protein family in mammals, Id2 and Id3 are the members expressed predominantly in lymphocytes. Deficiency in either leads to many defects in the generation of cells of the immune system. Loss of Id2 impairs development of lymphoid tissue-inducer T cells, natural killer cells, natural killer T cells, CD8 α ⁺ dendritic cells and subsets of intraepithelial lymphocytes^{32–34}. Id2 is involved in CD8⁺ T cell differentiation during infection, which suggests a broader function in the differentiation of mature lymphocytes³⁰. Deficiency in Id3 leads to distinct defects in $\alpha\beta$ thymocyte selection as well as the expansion of subsets of $\gamma\delta$ T cells, follicular-helper-like T cells and innate T cells^{35–39}. However, little is known about the role of Id3 in the CD8⁺ T cell response to infection. Although the signals that control Id2 expression are not understood, it is known that both signaling through the T cell antigen receptor (TCR) and TGF- β can influence Id3 expression and that E-protein transcription factors and Blimp-1 are direct transcriptional regulators of the gene encoding Id3 (*Id3*)^{40,41}.

To delineate the roles of Id2 and Id3 during the CD8⁺ T cell immune response, we generated lines of reporter mice with knock-in of sequence encoding yellow fluorescent protein into the *Id2* locus (Id2-YFP mice) or sequence encoding green fluorescent protein into the *Id3* locus (Id3-GFP mice). With these tools, we found that CD8⁺ effector cells with high expression of Id3-GFP and intermediate expression of Id2-YFP ‘preferentially’ differentiated into KLRG-1^{lo}CD127^{hi} memory precursor cells and survived longer and responded better to secondary challenge than did effector cells that remained Id3-GFP^{lo}Id2-YFP^{hi}. This Id3-GFP^{hi}Id2-YFP^{int} effector population had a gene-expression profile similar to that of long-lived memory cells, before surface expression of known markers of memory CD8⁺ T cells. Both Id3-deficient cells and Id2-deficient cells showed defective CD8⁺ T responses, failing to generate long-lived memory cells (Id3-deficient) or short-lived effector-memory cells (Id2-deficient). Finally, we found that the expression of both Id2 and Id3 was regulated by inflammatory cytokines, which provides a link between the cytokine milieu and regulation of the transcriptional activity that guides CD8⁺ T cell fate during infection.

RESULTS

Dynamic expression of Id2 and Id3 during infection

Id2 supports the survival of CD8⁺ effector T cells³⁰ and Id3 is upregulated in surviving Id2-deficient T cells, but it is not clear if this represents compensatory upregulation and/or the presence of a distinct Id3^{hi} population. To understand better the relationship of the expression of Id2 and Id3 by individual CD8⁺ T cells during infection, we inserted sequence encoding YFP into the first exon of the *Id2* locus, which resulted in a mutated locus (*Id2*^Y) in which YFP expression was under control of the *Id2* regulatory elements (Supplementary Fig. 1a). We used that Id2-YFP reporter mouse line in combination with an Id3-GFP reporter mouse line generated with an identical strategy (but with insertion of sequence encoding GFP into *Id3*; (*Id3*^G)³⁵. Mice heterozygous for these knock-in alleles (*Id2*^{Y/+} and *Id3*^{G/+}) generated CD8⁺ T cell responses equivalent to those of their wild type C57BL/6 littermates (Supplementary Fig. 1b) and had expression of the Id2-YFP and Id3-GFP reporters in many cell types consistent with what we anticipated on the basis of published reports³⁰ and quantitative PCR analysis of mRNA (Supplementary Fig. 2).

Naive polyclonal and OT-I CD8⁺ T cells (with transgenic expression of a TCR specific for ovalbumin (OVA) peptide) had readily detectable expression of both reporters (Fig. 1a and Supplementary Fig. 3a). To study the coregulation of Id2 and Id3 expression, we monitored reporter expression by OT-I or polyclonal CD8⁺ T cells responding to infection. We transferred *Id2*^{Y/+}*Id3*^{G/+} CD45.1⁺ OT-I CD8⁺ T cells into C57BL/6 hosts or directly infected *Id2*^{Y/+}*Id3*^{G/+} mice with recombinant vesicular stomatitis virus or *Listeria monocytogenes* expressing chicken OVA (VSV-OVA or LM-OVA, respectively), both self-limiting infections that were rapidly cleared (Fig. 1 and Supplementary Fig. 3). We monitored Id2-YFP and Id3-GFP in antigen-specific CD8⁺ T cells identified by flow cytometry. *Id2*^{Y/+}*Id3*^{G/+} OT-I CD8⁺ T cell populations responding to VSV-OVA infection *in vivo* showed substantial downregulation of Id3-GFP (mean fluorescent intensity (MFI) about 20% that of naive cells) and more moderate downregulation of Id2-YFP (Fig. 1a,b). Id3-GFP expression was more dynamic, with prominent Id3-GFP^{hi} and Id3-GFP^{lo} subsets emerging just before or coincident with the appearance of KLRG-1^{hi} and CD127^{hi} populations, the contraction of the effector response and the emergence of memory CD8⁺ T cells (Fig. 1a). After infection with LM-OVA, OT-I cells showed similar regulation of the Id2 and Id3 reporters, with a delay in the upregulation of Id3-GFP expression consistent with the generation of more KLRG-1^{hi} effector cells and later reexpression of CD127 (Fig. 1c). By analysis of polyclonal antigen-specific CD8⁺ T cells identified by major histocompatibility complex class I tetramers (H-2K^b-OVA peptide), we found that

reexpression of Id3-GFP occurred at later time points for each infection. However, Id3-GFP expression increased as the frequency of CD127^{hi} cells increased, as with OT-I cells (Supplementary Fig. 3). We sorted OT-I cells on days 0, 7 and 15 of infection and monitored their expression of *Id2* and *Id3* mRNA by quantitative PCR. Notably, expression of the Id2-YFP and Id3-GFP reporters was consistent with mRNA expression (Fig. 1d). We also transferred increasing numbers of OT-I cells into C57BL/6 recipients, thus accelerating the differentiation of CD8⁺ T cells into a long-lived memory phenotype⁴², and found that Id3-GFP was upregulated and Id2-YFP was downregulated more quickly as the number of transferred OT-I cells increased (Fig. 1e). Thus, delayed upregulation of Id3-GFP expression correlated with time points at which memory T cells emerged from the effector population, which suggested a possible role for Id3 late in the differentiation of CD8⁺ T cells into memory cells.

Id3^{hi} phenotype correlates with long-lived memory

We next examined the relationship between the expression of the Id2-YFP and Id3-GFP reporters and a surface phenotype characteristic of precursors to long-lived memory cells (expression of CD62L and CD127) or short-lived effector-memory cells (expression of KLRG-1; Fig. 2a). OT-I CD8⁺ T cells with the highest expression of Id3-GFP were largely CD127^{hi}KLRG-1^{lo}CD62L^{hi} by day 7 of infection, a phenotype associated with long-lived memory cells and their precursors. Conversely, Id3-GFP^{lo} cells had higher expression of KLRG-1 and lower expression of CD62L, a phenotype associated with short-lived effector cells (Fig. 2a). We also observed a similar relationship among phenotypic markers for antigen-specific polyclonal cells on days 7 and 14 of infection, with Id3-GFP^{hi} cells expressing more CD127 and Id3-GFP^{lo} cells skewed to expression of KLRG-1 (Supplementary Fig. 3). We examined cytokine production by Id3-GFP^{hi} effector cells relative to that of Id3-GFP^{lo} cells and found Id3-GFP^{hi} cells produced more IL-2, consistent with the CD127^{hi} KLRG-1^{lo} subset, in addition to producing more IFN- γ and tumor necrosis factor (TNF; Fig. 2b).

Prolonged exposure to IL-2 and higher expression of CD25 are inversely correlated with the differentiation of long-lived memory cells¹⁸. Thus, we also examined CD25 expression by Id3-GFP^{hi} and Id3-GFP^{lo} cells. We observed higher CD25 expression by OT-I cells during the early stages of infection with VSV-OVA or LM-OVA but had difficulty finding bimodal expression of CD25, as reported for infection with lymphocytic choriomeningitis virus¹⁸. Nevertheless, we gated on CD25^{hi} and CD25^{lo} populations and found little difference in expression of the Id2 or Id3 reporter (Supplementary Fig. 4a). T-bet expression was slightly lower in the Id3-GFP^{hi} subset than in Id3-GFP^{lo} cells (Supplementary Fig. 4b). Thus, we observed a correlation between high expression of Id3-GFP in CD8⁺ effector T cells at the peak of infection and the phenotype and cytokine production of memory precursor cells.

Id3^{hi} effectors differentiate into long-lived memory cells

To determine the memory potential of the effector subsets expressing Id3-GFP, we sorted Id3-GFP^{hi} and Id3-GFP^{lo} OT-I effector cells on day 5 of infection with VSV-OVA or LM-OVA and transferred equal numbers of each (1×10^5) into infection-matched hosts. We sorted effector cells devoid of surface KLRG-1, CD127, CD62L and CD25 (Fig. 3a) to ensure we were assessing the memory capacity of cells based on Id3 expression before the upregulation of memory-associated surface receptors. We analyzed the expression of KLRG-1 and CD127 by flow cytometry 3 and 6 d after transfer (effectively days 8 and 11 of infection with LM-OVA) in both spleen and liver (Fig. 3b,c). We observed more differentiation into the CD127^{hi}KLRG-1^{lo} subset by Id3-GFP^{hi} effectors and more differentiation into the CD127^{lo}KLRG-1^{hi} subset by Id3-GFP^{lo} effectors, with over sixfold more Id3-GFP^{lo} than Id3-GFP^{hi} effector cells differentiating into KLRG-1^{hi} cells at day 3.

Conversely, Id3-GFP^{hi} effectors yielded a proportion of CD127^{hi} cells over twofold higher than that yielded by Id3-GFP^{lo} effectors at day 6. To determine if the failure of Id3-GFP^{lo} cells to accumulate was due to lower proliferative capacity, we transferred OT-I cells responding to VSV-OVA into infection-matched host mice treated with the thymidine analog BrdU on days 2–4 after transfer and assessed the proliferation of each subset (Fig. 3d). Id3-GFP^{hi} and Id3-GFP^{lo} populations proliferated equally, which suggested that the difference in the absolute number of cells recovered after transfer was due to differences in survival. Finally, we directly tested the ability of Id3-GFP^{hi} OT-I cells responding to VSV-OVA infection to generate memory by rechallenging hosts with LM-OVA and monitoring the secondary response of both Id3-GFP^{hi} and Id3-GFP^{lo} populations. Notably, the Id3-GFP^{hi} donor cells generated significantly more secondary effectors throughout the infection, with three- to fivefold greater population expansion than that of Id3-GFP^{lo} donor cells (Fig. 3e). These data demonstrated that the Id3-GFP^{hi} effector subset ‘preferentially’ differentiated into long-lived memory cells.

Memory gene-expression program of Id3^{hi} effector cells

As Id proteins influence transcriptional activity, we next sought to determine if the gene-expression profile of Id3-GFP^{hi} and Id3-GFP^{lo} OT-I effector cells foreshadowed their respective fates as long-lived memory cells or short-lived effector cells. We transferred and sorted OT-I cells as described above (Fig. 3) and analyzed the Id3-GFP^{hi} and Id3-GFP^{lo} effector subsets by microarray on day 5 of infection. We found that even at this early stage of infection, Id3-GFP^{hi} effector cells had a gene-expression pattern very different from that of the Id3-GFP^{lo} subset, with nearly 400 transcripts showing a difference in expression of over 1.5-fold; 261 genes were upregulated and 137 genes were downregulated in Id3-GFP^{hi} effector cells relative to their expression in Id3-GFP^{lo} effector cells on day 5 (Fig. 4a). Despite being sorted as negative for the expression of characteristic surface memory T cell markers, the Id3-GFP^{hi} subset showed correlation of gene expression with the CD127^{hi} effector subset at day 8, which has been shown to include memory precursors (published microarray data⁷; Fig. 4b). We also cross-referenced our microarray data with data obtained by chromatin immunoprecipitation followed by deep sequencing in which E2A occupancy at promoter and enhancer regions in T cells was identified⁴³. Here we observed overlap between genes with different expression in Id3-GFP^{hi} and Id3-GFP^{lo} cells and genes with known E2A occupancy in T cells (Fig. 4c), which indicated that Id3 may directly regulate the transcription of target genes by blocking the activity of E proteins at the promoter and/or enhancer regions. Notably, when we compared the gene-expression profiles of OT-I effector cells (day 6) and OT-I memory cells (day 100), we found that 77% of the genes ‘preferentially’ expressed by Id3-GFP^{hi} cells correlated with genes with higher expression by memory cells than by effector cells (Fig. 4d). Conversely, 80% of the genes ‘preferentially’ expressed by Id3-GFP^{lo} cells correlated with genes that showed enrichment in effector cells relative to their abundance in memory cells (Fig. 4d). When we focused on expression of genes of particular interest in CD8⁺ effector and memory subsets, we found that many transcripts associated with memory cells were already expressed by day 5 in Id3-GFP^{hi} effector cells, including *Il7r*, *Sell*, *Il2*, *Bcl2* and *Ccr7*, whereas transcripts associated with terminal differentiation and short-lived effector-memory cells were already downregulated, including *Gzmb*, *Klrg1* and *Prdm1* (Fig. 4e). Furthermore, most of those selected genes demonstrated E2A binding across putative enhancer elements⁴³ (Fig. 4e). We also confirmed by quantitative PCR differences in the expression of a subset of genes (Fig. 4f). Thus, as early as day 5 of infection, differences in the expression of *Id3* mRNA indicate memory potential and cells that have ‘turned on’ the appropriate genetic profile to become long-lived memory cells.

Defective long-lived memory formation with loss of Id3

To investigate whether Id3 was required for the normal generation of memory cells, we generated OT-I mice homozygous for knock-in of sequence encoding GFP into *Id3* (*Id3^{G/G}*), which resulted in Id3-deficient TCR-transgenic donor cells. Id3-deficient mice have a complex phenotype that includes excessive IL-4 production by innate cells^{37,38}. We sought to minimize T cell-extrinsic effects by generating mixed-bone marrow chimeras; for this, we transferred equal amounts wild type and *Id3^{G/G}* OT-I bone marrow into lethally irradiated recipients. As a result, naive *Id3^{G/G}* OT-I cells from the reconstituted chimeras were CD44^{lo} and similar to wild-type cells (Supplementary Fig. 5). We then mixed the *Id3^{G/G}* (CD45.1⁺) and *Id3^{+/+}* (CD45.1⁺CD45.2⁺) OT-I cells at a ratio of 1:1, transferred the mixture into C57BL/6 CD45.2⁺ hosts and monitored the host response to infection with VSV-OVA. We monitored the population expansion of OT-I cells in peripheral blood lymphocyte (PBL) samples over time by flow cytometry (Fig. 5a). In the early expansion phase and at the peak of infection, the *Id3^{G/G}* OT-I cell population expanded and was phenotypically similar to the *Id3^{+/+}* OT-I counterpart (Fig. 5a). However, at later stages, *Id3^{+/+}* cells showed a distinct advantage over the Id3-deficient CD8⁺ T cells. When we examined the ratio of donor cells in PBL samples, *Id3^{+/+}* cells were three- to fourfold more abundant than *Id3^{G/G}* cells 60 d after infection. Notably, the two populations were similar in surface expression of KLRG-1 and CD127 on day 60 (Fig. 5b). However, we recovered considerably fewer *Id3^{G/G}* donor cells than *Id3^{+/+}* donor cells from the spleen by day 60, and the number of cells bearing the phenotype of long-lived memory cells (CD127^{hi} KLRG-1^{lo}) generated by *Id3^{+/+}* donor cells was about three- to fourfold greater than that generated by *Id3^{G/G}* donor cells (Fig. 5b). We obtained similar results with *Id3^{G/G}* and *Id3^{+/+}* OT-I cells after transfer into separate hosts (data not shown). When we examined the *Id3^{G/G}* memory cells present at day 80 in more detail, we found that they underwent normal basal homeostatic proliferation, as measured by BrdU incorporation, and had normal amounts of the intracellular survival factor Bcl-2 (Fig. 5c). The memory cells lacking Id3 expression produced more IFN- γ and TNF after *in vitro* restimulation (Fig. 5d), consistent with the observation that Id3 helps restrain the differentiation of naive T cells into effector phenotypes³⁵. Thus, Id3 is required for the normal development and/or survival of long-lived memory CD8⁺ T cells.

Higher Id3 expression and loss of KLRG-1^{hi} cells without Id2

Published studies have shown that Id2 is essential for the accumulation and survival of effector CD8⁺ T cells³⁰; however, we were interested in how loss of Id2 might affect Id3 expression. We crossed the *Id2^{Y/Y}* and *Id3^{G/G}* knock-in lines to generate *Id2^{Y/Y}Id3^{G/+}*, *Id2^{Y/Y}Id3^{G/+}* and *Id2^{Y/+}Id3^{G/G}* mice. Because of the early lethality of Id2 deficiency, we transferred CD45.2⁺ fetal liver cells into lethally irradiated CD45.1⁺CD45.2⁺ recipients. After 10 weeks of reconstitution, we infected the fetal liver chimeras with LM-OVA and monitored the expression of Id3-FP and Id2-YFP by antigen-specific CD8⁺ T cells identified by the H-2K^b-OVA peptide tetramer in the spleen (Fig. 6). In the absence of Id2 expression (*Id2^{Y/Y}Id3^{G/+}*), Id3-GFP was upregulated at least 100-fold relative to its expression in *Id2^{Y/+}Id3^{G/+}* effector cells (Fig. 6a). In contrast, Id2-YFP was relatively unchanged when Id3 was not expressed. We also examined the phenotype of *Id2^{Y/Y}* antigen-specific CD8⁺ T cells and found that the few Id2-deficient cells that did survive did not express KLRG-1 and were CD127^{hi} (Fig. 6b). Thus, after loss of Id2, Id3-GFP was overexpressed by a small population of cells that bore the phenotype of long-lived memory cells. Together our data suggested that the balance of Id2 expression and Id3 expression controls the differentiation of KLRG-1^{hi} effector and CD127^{hi} memory subsets and that Id2 either directly or indirectly regulates Id3 expression.

Inverse regulation of Id2 and Id3 by cytokines

Many cytokines are known to influence the generation and survival of effector and memory CD8⁺ T cell subsets over the course of infection. To understand how extracellular signals influence expression of Id proteins, we assessed the effect of cytokines on expression of the Id2-YFP and Id3-GFP reporters. We activated *Id2*^{Y/+}*Id3*^{G/+} OT-I splenocytes *in vitro* with irradiated OVA peptide-pulsed splenocytes with or without cytokines and monitored reporter expression by flow cytometry on day 4 of culture. Treatment with IL-2, IL-12 or IL-21 resulted in an MFI of Id2-YFP at least two- to fivefold higher but an MFI of Id3-GFP at least 30% that of cells treated with medium alone (Fig. 7a). Next we examined the effect of IL-2 or IL-12 on the expression of Id2-YFP and Id3-GFP *in vivo*. We sorted naive *Id2*^{Y/+}*Id3*^{G/+} OT-I CD8⁺ cells, transferred them to IL-12-sufficient or IL-12-deficient hosts and monitored reporter expression during the response to infection with LM-OVA (Fig. 7b). In agreement with our *in vitro* data indicating that IL-12 resulted in lower Id3 expression, Id3-GFP was upregulated by antigen-specific CD8⁺ T cells in IL-12-deficient hosts, but we detected no significant difference in Id2-YFP. We consistently observed such upregulation of Id3-GFP throughout the time course (data not shown). To assess the effect of IL-2 signaling on Id2-YFP expression, we generated *Id2*^{Y/+} bone marrow chimeras that were either CD25 sufficient (*Il2ra*^{+/+}) or CD25 deficient (*Il2ra*^{-/-}). We mixed *Id2*^{Y/+}*Il2ra*^{+/+} or *Id2*^{Y/+}*Il2ra*^{-/-} bone marrow with an equal amount of congenically distinct wild-type bone marrow and transferred the cells into wild-type hosts. After reconstitution, we infected the chimeras with VSV-OVA and monitored Id2-YFP expression (Fig. 7c). On days 7 and 12 of infection, Id2-YFP expression was two- to threefold higher when IL-2 signaling by antigen-specific CD8⁺ T cells was intact (Fig. 7c and data not shown). Finally, we sought to determine if CD127-mediated signals controlled the upregulation of Id3 by memory CD8⁺ precursors. Id3-GFP expression was unaltered even when CD127 was blocked throughout the entire infection (Supplementary Fig. 6), which further suggested that Id3 is expressed before and independently of IL-7 receptor expression.

To investigate whether *Id2* expression is directly regulated by cytokines through signals mediated by STAT transcription factors, we examined the *Id2* promoter for STAT-binding sites. Using the VISTA genome browser, we found two potential STAT-binding sites. We examined binding of STAT at the *Id2* promoter by isolating DNA from OT-I CD8⁺ T cells on day 7 of infection, followed by immunoprecipitation of chromatin with antibody to STAT4 (anti-STAT4) or anti-STAT5 (Fig. 7d). STAT-binding sites in the *Id2* promoter were enriched over threefold after immunoprecipitation with anti-STAT4 or anti-STAT5 relative to their abundance after control immunoprecipitation with immunoglobulin G. Thus, Id2 expression may be directly regulated by cytokines such as IL-2, IL-12 and IL-21. Together these data provide evidence of complex codependent roles for Id2 and Id3 proteins in the regulation of effector and memory T cell formation during the immune response.

DISCUSSION

Memory cells originate from effector cells, which requires the induction and careful regulation of a distinct transcriptional program; however, the factors that influence this transition are yet to be fully understood. Progress in this area should identify the basic principles that govern developmental lineage commitment but will also identify molecular pathways with relevance to clinical endeavors such as vaccine design. Our work here has demonstrated that the relative expression of two transcriptional regulators, Id2 and Id3, determined the fate of differentiating CD8⁺ T cells after activation. High expression of Id3 ‘preferentially’ guided the transition to memory T cells, whereas low expression of Id3 led to differentiation into effector T cells. We found that upregulation of Id3 preceded the traditional markers of memory precursor T cells, which indicated that the relative expression

of Id3 and Id2 may be the most relevant phenotypic and functional determinant of memory T cells.

Historically, the confirmation of a phenotypic marker for identifying a population containing memory precursor cells has been the enrichment of memory cell formation when sorted cells are adoptively transferred into a secondary host. Indeed, we found that early after infection, sorted Id3-GFP^{hi} effector cells ‘preferentially’ gave rise to long-lived memory cells, whereas Id3-GFP^{lo} cells differentiated into short-lived effector cells. Id3 was downregulated by CD8⁺ T cells early during the effector phase of the immune response and then was slowly upregulated and maintained in memory cells. The change in Id3 expression occurred before the upregulation of KLRG-1 and CD127 on the cell surface; that is, the sorted Id3-GFP^{lo} and Id3-GFP^{hi} populations in our studies were negative for both KLRG-1 and CD127. Notably, other studies have shown that high expression of CD25 or KLRG-1 negatively correlates with memory potential, whereas those populations of effector cells that express CD127 or CD62L contain more memory precursors^{8,17,18,23,24,44}. The timing and degree of expression of these markers occurs variably during the first week of the immune response, depending on the infection and precursor frequency of responding cells. Here we found that expression of Id3-GFP afforded the ability to identify memory precursors before the differences in expression of surface markers in the context of infection with VSV or *L. monocytogenes*. Effector cells with an Id3-GFP^{hi} phenotype were mainly KLRG-1^{lo}CD127^{hi}CD62L^{hi} and produced more IL-2 (a hallmark property of memory cells) than Id3-GFP^{lo} Id2-YFP^{hi} cells did. Ultimately, we found that as the population expressing Id2 and Id3 accumulated, it ‘preferentially’ converted into the KLRG-1^{lo}CD127^{hi} memory phenotype and responded better to secondary challenge than the Id3-GFP^{lo} population did. In agreement with the observation that Id2 expression was more prominent in short-lived effector-memory CD8⁺ T cells, whereas Id3 expression was more prominent in long-lived memory precursors, we found that Id2 deficiency resulted in a loss of KLRG-1^{hi} cells and Id3 deficiency led to a loss of CD127^{hi} memory cells. Thus, Id2 and Id3 regulate the survival and generation of effector and memory cells differently and their expression can be used to predict which precursors will seed the memory compartment.

How do Id proteins regulate CD8⁺ T cell differentiation? The mechanism may be directly related to their inhibition of E-protein target genes. Our transcriptional profiling analyses found that the gene-expression profiles of Id3-GFP^{hi} cells and Id3-GFP^{lo} cells were similar to those of CD127^{hi} memory precursor cells and CD127^{lo} effector cells, respectively. Notably, many of the signature genes expressed in late-stage memory cells (day 100) were already ‘preferentially’ expressed in the Id3-GFP^{hi} memory precursor cells. Thus, upregulation of Id3 correlates with the establishment of a gene-expression profile associated with long-term memory cells. Many of the genes with different expression in Id3-GFP^{hi} cells and Id3-GFP^{lo} cells have been identified by an independent study as having promoters and enhancers bound by E2A. For example, *Il7r*, *Socs3*, *Cxcr5*, *Ccr7*, *Il2ra* and *Cxcr6*, as well as *Id3* itself, are known E2A targets^{35,43,45}. On the basis of those observations, we suggest that suppression of E-protein DNA-binding activity is essential for the promotion of developmental progression toward the long-term memory cell fate.

It is known that different E proteins have diverse functions in thymocyte development, and our data support the idea that Id2 and Id3 have distinct roles in CD8⁺ T cell differentiation⁴⁶. Indeed, the finding that deficiency in either resulted in a defect in memory cell differentiation indicated that Id2 and Id3 have nonredundant roles in memory cell differentiation. Id3-deficient cells were out-competed by wild-type cells and produced fewer memory cells when transferred into a secondary host. Id2 deficiency also resulted in a smaller memory pool, but the few remaining cells rapidly acquired a KLRG-1^{lo}CD127^{hi} phenotype. This dichotomy may be explained by the notable upregulation of Id3 in Id2-

deficient CD8⁺ T cells. Consistent with that model is the observation that E2A-binding sites are located in putative regulatory regions across the *Id3* locus^{35,43}. Nevertheless, Id3 was unable to compensate completely for the deficiency in Id2-deficient cells, perhaps because Id3 and Id2 bind with different affinities to members of the E-protein family. In such a scenario, cells expressing both Id3 and Id2 may have patterns of gene expression distinct from those of cells expressing only Id3 or Id2. Notably, Id2 expression remained unchanged in Id3-deficient CD8⁺ T cells, which suggested that Id3 is regulated by Id2 but not vice versa.

Our identification of functionally relevant, cell-intrinsic regulators of memory T cell differentiation should enable exploration of the molecular mechanisms by which extrinsic signals influence memory cell differentiation. As an initial step, we examined this issue by monitoring how cytokines known to influence CD8⁺ T cell differentiation affected the expression of Id2 and Id3. IL-2, IL-12 and IL-21 upregulated Id2 expression and downregulated Id3 expression. Those results are consistent with published data suggesting that signaling by IL-2 and IL-12 drive CD8⁺ T cells toward a more terminally differentiated state^{7,18}. Our data indicated that STAT4 and STAT5 bound to putative regulatory elements across the *Id2* locus, which suggested that cytokine-mediated STAT activation may directly modulate Id2 expression. In contrast, those cytokines may indirectly regulate Id3 expression through modulation of Id2 expression. The regulation of Id3 expression is probably complex. In addition to possibly being modulated by the feedback regulation of E-protein activity, the abundance of Id3 mRNA is also modulated by the mitogen-activated protein kinase Erk, as well as by TGF- β pathways^{40,47}. Published studies have shown that Blimp-1 acts to antagonize Id3 expression in lymphocytes⁴⁸. Such regulation is consistent with the idea that small amounts of Id3 promote an effector-like state, whereas abundance of Id3 and small amounts of Blimp-1 activate a long-term memory program of gene expression^{25,26}. In our reporter mice, we observed perturbations in Id2 and Id3 in CD8⁺ T cells lacking T-bet, which suggested possible regulation of Id proteins by T-bet (data not shown). There is also evidence that Id2 and Tbx5 (another member of the T-box family of transcription factors) act in the same molecular pathway during development of the cardiac conduction system⁴⁹. Ultimately, it will be useful to determine how cytokines and transcription factors act in concert with E proteins to modulate the expression of Id2 and Id3 to properly orchestrate the developmental progression from naive cell to the effector or memory cell fate.

In conclusion, our study has identified a previously unknown phenotypic and functional marker associated with the commitment of CD8⁺ T cells to differentiation into long-lived memory cells. Our results, as well as the tools we used to obtain them, lay the foundation for future studies aimed at identifying other factors that regulate Id and E proteins, as well as their downstream targets during both acute and chronic infection. Such information will be useful in the development of effective T cell-specific vaccines and immunotherapy.

ONLINE METHODS

Mice

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. Id3-GFP mice were generated as described³⁵. Id2-YFP mice (generated as described in Supplementary Fig. 1) were backcrossed at least ten times to the C57BL/6 background. Id3-GFP and Id2-YFP mice were crossed with OT-I mice (which have transgenic expression of a TCR that recognizes OVA peptide (amino acids 257–264) presented by H-2K^b). Wild-type male C57BL/6 mice and B6.SJL mice, congenic for the allele encoding CD45.1, were from The Jackson Laboratory and in some cases were crossed to generate F₁ mice expressing both CD45.1 and CD45.2. *Il2ra*^{-/-} and *Il12b*^{-/-} mice were from The Jackson Laboratory.

Adoptive transfer and infection

Mixed-bone marrow chimeras were generated by the transfer of 5×10^6 wild-type and 5×10^6 *Id3^{G/G}* B220⁺CD4⁺CD8⁺ bone marrow cells into lethally irradiated recipient mice (1,000 rads). Wild-type donor cells were CD45.1⁺, *Id3^{G/G}* OT-I donor cells were CD45.2⁺ and recipient mice were CD45.1.2⁺. *Id3^{G/G}* and wild-type OT-I splenocytes from reconstituted bone marrow chimeras were mixed at a ratio of 1:1 (1×10^4 V α 2⁺CD8⁺ T cells each) and were transferred together into the appropriate recipients (as determined by expression of congenic markers). For the generation of fetal liver chimeras, 5×10^6 to 10×10^6 fetal liver cells at embryonic days 14–15.5 were injected intravenously into lethally irradiated recipient mice (1,000 rads). All chimeras were used at least 10 weeks after bone marrow transfer.

For analysis of the primary immune response, mice were infected with 5×10^3 colony-forming units of recombinant LM-OVA or 1×10^5 plaque-forming units of recombinant VSV-OVA. Mice that received adoptive transfer of T cells were infected 1 d after transfer. For secondary challenge, each mouse received 5×10^3 colony-forming units of recombinant LM-OVA 35 d after primary infection.

Flow cytometry

Single-cell suspensions were prepared from spleen, liver or blood. The following antibodies were used (all from eBioscience unless specified otherwise): phycoerythrin-indotricarbocyanine-conjugated anti-CD45.2 (104), anti-CD127 (A7R34), iTAg MHC Tetramer (T03000; Beckman Coulter) and anti-Bcl-2 (556537, BD Biosciences); peridinin chlorophyll protein-conjugated anti-CD45.1 (A20); peridinin chlorophyll protein-cyanine 5.5-conjugated anti-CD8 (53-6.7) and anti-B220 (RA3-6B2); allophycocyanin-conjugated anti-KLRG-1(2F1), anti-CD25 (3C7), anti-IFN γ (XMG1.2), anti-TNF (MP6-XT22), anti-IL2 (6080103022 Miltenyi Biotec), anti-CD44 (IM7), anti-CD4 (L3T4; BD Pharmingen), anti-BrdU (51-9000019AK, BD Biosciences) and anti-CD8 (53-6.7; Biolegend); phycoerythrin-indotricarbocyanine-conjugated anti-CD127 (A7R34) and anti-CD62L (MEL-14); Alexa Fluor 750-conjugated anti-CD8 (104), anti-CD44 (IM7) and anti-CD62L (MEL-14); and Pacific blue-conjugated anti-CD45.1 (A20). Samples were collected on an LSR II, LSRFortessa or FACSARIA (BD Biosciences) fitted with custom mirrors from Omega Filters (510/21 with 502LP or 505LP for GFP; 530/30 with 525LP for YFP) and were analyzed with FlowJo software (TreeStar). For intracellular staining, splenocytes were incubated for a total of 6 h at 37 °C at a density of 4×10^6 cells per well in RPMI-1640 media (Mediatech) containing 10% (vol/vol) bovine growth serum (HyClone) with 10 nM OVA peptide. After 3 h, brefeldin A was added (1 μ l/ml; GolgiPlug; BD Biosciences) and cultures were incubated for an additional 3 h. Cells were collected and stained with antibodies to surface molecules, then were fixed and made permeable in Cytotfix/Cytoperm buffer (BD Biosciences) and then stained intracellularly. For BrdU detection, mice were provided BrdU in their drinking water (0.8 mg/ml) and splenocytes were stained with a BrdU Flow kit (BD Biosciences). For *in vitro* cytokine assays, 1×10^4 *Id2^{Y/+}Id3^{G/+}* OT-I splenocytes were incubated with 5×10^5 OVA peptide-pulsed, irradiated splenocytes (1,200 rads) in 48-well plates in 500 μ l RPMI medium with 10% (vol/vol) FBS, 50 μ M β -mercaptoethanol and L-glutamine–penicillin–streptomycin. All cytokines were added at the start of culture at a concentration of 100 ng/ml, except IL-12 (10 ng/ml). Cells were collected between days 3 and 4.

Quantitative PCR and chromatin immunoprecipitation

Donor cells were sorted, then RNA was extracted with TRIzol reagent (Invitrogen) and treated with DNase (Ambion), and cDNA was generated with a SuperScript III kit (Invitrogen). The abundance of mRNA was assessed by quantitative PCR with the detection

of nonspecific product (SYBR Green; Stratagene) with primers that amplify in a linear relationship with primers for 'housekeeping' genes. Results were normalized to the expression of GAPDH transcripts (encoding glyceraldehyde phosphate dehydrogenase). Chromatin-immunoprecipitation assays were done as described with 10 ng DNA for each immunoprecipitation⁵⁰. Polyclonal anti-STAT4 (C-20; Santa Cruz Biotechnology) or anti-STAT5 (C-17; Santa Cruz Biotechnology) was used for the precipitation of STAT4-DNA or STAT5-DNA complexes, and rabbit immunoglobulin G (2729; Cell Signaling Technology) served as a negative control. Immunocomplexes were bound to protein G–Sepharose beads (Cell Signaling Technology) and were washed four times. DNA was eluted and purified and was analyzed by quantitative PCR for detection of putative STAT-binding sites in *Id2* (ChIP primer sequences, 5'-TGTGCAAACCCCACTAATGA-3' and 5'-CGCTTTTGGGAAGTCAC ATT-3'; quantitative PCR primer sequences, Supplementary Table 1).

Microarray analysis

Donor cells were sorted (Fig. 3), then RNA was extracted with TRIzol and amplified twice with a MessageAmp RNA Amplification kit (Ambion). RNA was labeled with biotin with the BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics) and was purified with an RNeasy Mini kit (Qiagen). The resulting cRNA was hybridized to GeneChip Mouse Gene 1.0 ST arrays and raw cell-intensity files ('.cel' extension) were obtained. Data were normalized and analyzed with the GenePattern software suite.

Statistical analysis

Statistical significance was determined with the two-tailed unpaired Student's *t*-test. *P* values for microarray results were calculated with the χ^2 test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank G. Barton, J. Hamerman, S. Turley, A. Fletcher, M. Rubinstein and members of the Goldrath laboratory for advice and critical review of the manuscript; and L. Shaw for help with figure design. Supported by the Cancer Research Institute, Pew Charitable Trust, the US National Institutes of Health (AI067545 and AI072117 to A.W.G.; and AI073587 to S.S.W.), University of California San Diego (Cellular and Molecular Genetics Training Grant to C.Y.Y.) and the R.E. Bob Smith Education Fund (H.S.L.).

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Nat Immunol. Author manuscript; available in PMC 2013 December 24.

Figure 2.

Expression of Id2-YFP and Id3-GFP during infection correlates with effector and memory precursor subsets. **(a)** Flow cytometry analysis (left) of the expression of KLRG-1 and CD127 (middle row) and of CD44 and CD62L (bottom row) by Id3-GFP^{hi} (Id3^{hi}) and Id3-GFP^{lo} (Id3^{lo}) donor cells (top) from CD45.2⁺ C57BL/6 mice that received Id2^{Y/+}Id3^{G/+} CD45.1⁺ OT-I cells (5×10^4) 1 d before infection with VSV-OVA, assessed on day 7 of infection. Right, quantification of the expression of Id3-GFP and Id2-YFP (top row) and frequency of the KLRG-1^{hi}CD127^{lo}, KLRG-1^{lo}CD127^{hi} and CD44^{hi}CD62L^{hi} subsets (below). **(b)** Flow cytometry (top) and quantification (bottom) of the production of IL-2, IFN-γ and TNF by splenocytes restimulated for 6 h *in vitro* with OVA peptide, assessed on day 7 after VSV infection. Dashed lines, unstained cells. * $P < 0.001$ (two-tailed unpaired Student's *t*-test). Data are representative of four independent experiments with two to three mice per group (error bars, s.e.m.).

Figure 3.

The $Id3-GFP^{hi}$ effector $CD8^{+}$ T cell population includes long-lived memory precursor cells before expression of KLRG-1 or CD127. **(a)** Experimental design: $CD45.2^{+}$ C57BL/6 mice received $Id2^{Y/+}Id3^{G/+} CD45.1^{+}$ OT-I cells (2.5×10^4) 1 d before infection with LM-OVA or VSV-OVA; on day 5 after infection, $CD25^{-}CD62L^{-}CD127^{-}KLRG-1^{-}$ OT-I cells were sorted on the basis of Id3-GFP expression and equal numbers of $Id3-GFP^{hi}$ or $Id3-GFP^{lo}$ cells were adoptively transferred into infection-matched $CD45.2^{+}$ C57BL/6 hosts. **(b,c)** Flow cytometry analysis of the expression of KLRG-1 and CD127 (left) by $Id3-GFP^{lo}$ and $Id3-GFP^{hi}$ $CD45.1^{+}$ OT-I cells from spleen and liver on day 3 **(b)** and day 6 **(c)** after transfer of sorted cells after LM-OVA infection. Middle and right, frequency of $KLRG-1^{hi}CD127^{lo}$ and $KLRG-1^{lo}CD127^{hi}$ subsets (middle) and total donor cells recovered from spleen and liver (right). **(d)** Incorporation of BrdU by sorted OT-I cells responding to VSV-OVA, transferred into infection-matched host given BrdU water for 2 d before collection of cells and analysis 4 d after transfer. **(e)** Kinetics of the population expansion of $Id3-GFP^{lo}$ and $Id3-GFP^{hi}$ $CD45.1^{+}$ OT-I $CD8^{+}$ T cells among PBLs after transfer of OT-I cells responding to VSV-OVA into mice subsequently rechallenged with LM-OVA. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ (two-tailed unpaired Student's *t*-test). Data are representative of three independent experiments with two to three mice per group **(b,c)** or three to five mice **(e)** or two independent experiments with three mice per group **(d)**; error bars, s.e.m.).

Figure 4.

Early Id3 expression correlates with memory potential. Gene-expression analysis of CD8⁺ effector cells in CD45.2⁺ mice that received *Id2*^{Y/+}*Id3*^{G/+} CD45.1⁺ OT-I cells (2.5×10^4) 1 d before infection with VSV-OVA, followed by sorting of CD25⁺CD62L⁺CD127⁺KLRG1⁺OT-I cells based on Id3-GFP expression on day 5 after infection. (a) Gene expression in Id3-GFP^{hi} versus Id3-GFP^{lo} CD8⁺ effector cells. Numbers in corners indicate genes with expression 1.5-fold higher in Id3-GFP^{hi} cells (red) or 1.5-fold higher in Id3-GFP^{lo} cells (blue). (b) 'Volcano' plot of the data in a; colors indicate genes upregulated more than twofold in CD127^{hi} cells (red) or CD127^{lo} cells (blue), and numbers indicate genes that correlate with Id3-GFP^{hi} expression (right) or Id3-GFP^{lo} expression (left). *P* values, χ^2 test. (c) Gene expression in Id3-GFP^{hi} versus Id3-GFP^{lo} cells; green indicates E2A occupancy, identified by chromatin immunoprecipitation followed by deep sequencing. (d) Gene expression by effector (day 6) and memory (day 100) OT-I cells responding to VSV-OVA infection; colors indicate genes upregulated (red) or downregulated (blue) 1.5-fold in Id3-GFP^{hi} cells relative to their expression in Id3-GFP^{lo} cells, and numbers indicate genes up- or downregulated in memory cells. *P* values, χ^2 test. (e) Change in gene expression in Id3-GFP^{hi} versus Id3-GFP^{lo} cells; green indicates E2A occupancy. (f) Quantitative PCR analysis of mRNA in Id3-GFP^{hi} cells, presented relative to expression in Id3-GFP^{lo} cells. ND, not detected. Data are from two independent data sets (a-e; average) or are representative of two experiments (f; average and s.e.m. of triplicates).

Figure 5.

Id3 deficiency results in the defective formation of long-lived memory CD8⁺ T cells. **(a)** Flow cytometry analysis (top) and time course (bottom) of the population expansion of *Id3*^{+/+} (CD45.1⁺) and *Id3*^{G/G} (CD45.1⁺CD45.2⁺) OT-I CD8⁺ T cells among PBLs from CD45.2⁺ mice that received equal numbers of CD44^{lo} *Id3*^{+/+} CD45.1.2⁺ and *Id3*^{G/G} CD45.1⁺ OT-I cells 1 d before infection with VSV-OVA. **(b)** Phenotype (top) and total number (bottom) of *Id3*^{+/+} CD45.1⁺ and *Id3*^{G/G} CD45.1.2⁺ OT-I cells recovered from the spleen on day 60 after infection as in **a**. **(c)** BrdU incorporation (top) and Bcl-2 expression (bottom) of *Id3*^{+/+} CD45.1⁺ and *Id3*^{G/G} CD45.1.2⁺ OT-I cells from mice infected as in **a**, followed by treatment of recipient mice for 7 d with BrdU in drinking water and recovery of cells from the spleen on day 80 after infection. **(d)** Flow cytometry (top) and quantification (bottom) of the production of IL-2, IFN-γ and TNF by splenocytes restimulated for 8 h *in vitro* with OVA peptide on day 80 after infection as in **a**. Dashed lines, unstained cells. **P* < 0.005 (two-tailed unpaired Student's *t*-test). Data are representative of two independent experiments with five to six mice per group (**a,b**; average s.e.m. in **a**) or one experiment with three mice per group (**c,d**; error bars, s.e.m. in **d**).

Figure 6.

$Id2$ -deficient $CD8^+$ T cells upregulate $Id3$ and do not generate short-lived effector-memory cells. **(a)** Reporter expression in splenic antigen-specific $CD8^+$ T cells on day 15 after infection of $Id2^{Y/+} Id3^{G/+}$, $Id2^{Y/Y} Id3^{G/+}$ and $Id2^{Y/+} Id3^{G/G}$ fetal liver chimeras with LM-OVA. **(b)** Flow cytometry analysis of the expression of KLRG-1 and CD127 by $Id2^{+/+}$ or $Id2^{Y/Y}$ splenic antigen-specific $CD8^+$ T cells on day 15 after infection as in **a**. * $P < 0.005$ and ** $P < 0.001$ (two-tailed unpaired Student's t -test). Data are representative of four independent experiments with two to four mice per group (**a**) or three independent experiments with two to five mice per group (**b**; error bars, s.e.m.).

Figure 7.

Id3-GFP expression and Id2-YFP expression are inversely coregulated by cytokines. **(a)** Flow cytometry analysis (left) and quantification (right) of the expression of Id3-GFP and Id2-YFP by *Id2^{Y/+}Id3^{G/+}* CD45.1⁺ OT-I cells cultured for 3–4 d with OVA peptide-pulsed APCs with cytokines (gray) or without cytokines (white; Control). **(b)** Expression of Id3-GFP and Id2-YFP by *Id2^{Y/+}Id3^{G/+}* CD45.1⁺ OT-I cells adoptively transferred into CD45.2 C57BL/6 or IL-12-deficient mice, analyzed on day 15 after infection with VSV-OVA. **(c)** Flow cytometry analysis (left) and quantification (right) of Id2-YFP expression in *Il2ra^{+/+}* and *Il2ra^{-/-}* cells from mixed chimeras infected with VSV-OVA, followed by analysis of antigen-specific splenic CD8⁺ T cells from the donor on day 12 of infection. **(d)** Sequence alignment of human and mouse genes encoding Id2 from the VISTA genome browser (left), and immunoprecipitation of chromatin (with anti-STAT4, anti-STAT5 or immunoglobulin G (IgG)) from wild type OT-I cells on day 7 of infection, followed by quantitative PCR analysis of input or precipitated DNA containing various binding sites (arrows; right). * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ (two-tailed unpaired Student's *t*-test). Data are representative of four independent experiments with three replicates **(a)** or two independent experiments with four to five mice per group **(b)** or three to five chimeras **(c)** or are pooled from three independent experiments with five mice **(d)**; error bars, s.e.m.).