Id2-Mediated Inhibition of E2A Represses Memory CD8⁺ T Cell Differentiation

Frederick Masson, Martina Minnich, Moshe Olshansky, Ivan Bilic, Adele M. Mount, Axel Kallies, Terence P. Speed, Meinrad Busslinger, Stephen L. Nutt and Gabrielle T. Belz

*J Immunol* published online 27 March 2013
http://www.jimmunol.org/content/early/2013/03/27/jimmunol.1300099

Supplementary Material http://www.jimmunol.org/content/suppl/2013/03/27/jimmunol.1300099_9.DC1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Author Choice Freely available online through *The Journal of Immunology* Author Choice option

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Id2-Mediated Inhibition of E2A Represses Memory CD8⁺ T Cell Differentiation

Frederick Masson,* Martina Minnich, † Moshe Olshansky, † Ivan Bilic, ‡† Adele M. Mount, ‡‡ Terence P. Speed, ‡‡ Meinrad Busslinger, † Stephen L. Nutt,*§ and Gabrielle T. Belz*§

The transcription factor inhibitor of DNA binding (Id)2 modulates T cell fate decisions, but the molecular mechanism underpinning this regulation is unclear. In this study we show that loss of Id2 cripples effector differentiation and instead programs CD8⁺ T cells to adopt a memory fate with increased Eomesoderm and Tcf7 expression. We demonstrate that Id2 restrains CD8⁺ T cell memory differentiation by inhibiting E2A-mediated direct activation of Tcf7 and that Id2 expression level mirrors T cell memory recall capacity. As a result of the defective effector differentiation, Id2-deficient CD8⁺ T cells fail to induce sufficient Tbx21 expression to generate short-lived effector CD8⁺ T cells. Our findings reveal that the Id2/E2A axis orchestrates T cell differentiation through the induction or repression of downstream transcription factors essential for effector and memory T cell differentiation. The Journal of Immunology, 2013, 190: 000–000.

Successful eradication and protection from reinfection by intracellular pathogens such as viruses and bacteria depend on the generation of effector and memory CD8⁺ T cells. Naïve CD8⁺ T cells, on encounter with dendritic cells presenting pathogen Ags, undergo multiple rounds of proliferation and rapidly differentiate into short-lived Ag-specific effector T cells with cytotoxic and cytokine producing capacity. After resolution of the infection, effector CD8⁺ T cell numbers contract significantly, leaving a small (5–10%) residual population of long-lived memory CD8⁺ T cells poised to rapidly respond to a second encounter with the pathogen.

Several transcription factors are essential for the specification and differentiation of peripheral T cells following Ag encounter. These include the basic helix-loop-helix proteins inhibitor of DNA binding (Id)2 and Id3 (1–3), the T-box transcription factors T-bet and eomesoderm (Eomes) (4), B lymphocyte–induced maturational protein 1 (Blimp1) (5–7), Bcl-6 (8), and Tcf7 (also known as TCF-1) (9). T-bet (encoded by Tbx21) and Blimp1 (encoded by Prdm1) direct the terminal differentiation of effector T cells. Ablation of either T-bet or Blimp1 results in a complete loss of KLRG1⁺IL-7R⁻ short-lived effector cells. Furthermore, T-bet drives effector cell formation in a dose-dependent manner at the expense of the formation of memory cells (10). Maintenance of memory cells appears to depend on the induction of Eomes (4). Although our understanding of Eomes regulation is incomplete, recent evidence suggests that Tc7, a critical mediator of the Wnt/β-catenin pathway, regulates CD8⁺ T cell memory by direct binding to the Eomes locus (9). However, the interactions among these transcription factors, as well as the signals that drive effector and memory CD8⁺ T cell fate decisions, are still poorly understood.

It is now established that peripheral T cell differentiation is regulated by the activity of Id proteins (11). Four Id proteins (Id1, Id2, Id3, Id4), which lack a DNA-binding domain, are capable of binding the E proteins, E2A (possessing two isoforms, E47 and E12), HEB, and E2-2. The recent development of reporter mice for Id2 and Id3 has identified that these two transcriptional regulators are expressed in a reciprocal manner and regulate distinct functions in the differentiation of peripheral T cells (3). For example, Id2 is upregulated in effector T cells. In contrast, induction of Id3 reflects the emergence of precursors of long-lived memory T cells, and repression by Blimp1 limits formation of memory CD8⁺ T cells, thus dispelling the notion that Id2 and Id3 are simply redundant (2, 3).

Id2 has multiple essential functions in the hematopoietic system. It is required for the development of CD103⁺ and CD8α⁺ dendritic cells, NK cells, a subset of intraepithelial T cells, and lymphoid tissue inducer cells (12, 13). In Ag-specific CD8⁺ T cells, Id2 has been proposed to act mainly by regulating their survival during
infection (1), but the precise molecular mechanisms downstream of Id2 that determine T cell fate are poorly defined.

To understand this pathway in greater depth we generated mice with a reporter allele encoding GFP under the endogenous Id2 promoter (12) and a conditional allele allowing specific deletion of Id2 in T cells. This enabled us to examine the cellular and molecular pathway resulting from the loss of Id2 and to explore the mechanisms affecting effector and memory T cell fate outcomes in an infection setting. We demonstrated that Id2 was essential for the induction of high levels of Th2x21 and this was required for the generation of short-lived effector CD8+ cells. Loss of Id2 in CD8+ T cells impaired effector T cell differentiation and programmed T cells to adopt a memory cell phenotype with increased Eomes and Tcf7 expression. We also show that induction of Id2 restrains CD8+ T cell memory differentiation by inhibiting E2A-mediated transactivation of Tcf7 expression and that graded expression of Id2 rather than central or effector memory phenotype correlates with CD8+ T cell memory recall capacity. Overall, we reveal that Id2 is a dose-dependent regulator of T cell differentiation by orchestrating the induction or repression of downstream transcription factors critical in effector versus memory differentiation.

Materials and Methods

Mice

Id2^{Gfp/neo} (12), Id2^{fl/fl}LckCre (Id2^{LckCre}), Thx21^{-/-} (14), OT-I (15), Flpe (16), LckCre (17), C57BL/6 (Ly-5.2), B6.SJL-Pipp^c (Pepe)^c/B6J (B6.Ly5.1, Ly5.1^c), Rosa26^{BrdU/Fluc} (18), and Ly5.1 × Ly5.2 (F1) mice were used. Id2^{Gfp/neo} mice were generated as described (19). All mice were bred and maintained under specific pathogen-free conditions in accordance with the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee.

Bacterial and viral infections

Mice were anesthetized with methoxyflurane and then inoculated with 10^4 PFU HKx31 (H3N2) influenza virus. Memory mice were generated by priming with i.p. injection of 10^5 PFU A/PR/8/34 (PR8) influenza virus (20). At the indicated times, mice were sacrificed, spleens, lymph nodes (LN; mediastinal and superficial cervical), and lungs were removed, and single-cell suspension prepared for analysis. For listeria infection, recombinant Listeria monocytogenes encoding OVA (Lm-OVA) was grown in brain-heart infusion broth. Bacterial culture samples were grown to mid-log phase measured by OD (absorbance at 600 nm) and diluted PBS for injection. Mice were inoculated i.v. with 2.5 × 10^5 Rlom-OVA. Injected bacteria numbers were determined by spreading bacterial samples on brain-heart infusion plates followed by incubation overnight at 37°C.

Isolation of CD8+ T cells

CD8+ T cells were enriched from the spleen of Id2^{fl/fl}LckCre^+ or wild-type mice. CD8^+CD19^- T cells were purified either by FACS sorting or by two rounds of negative selection (MACS; Miltenyi Biotec) to obtain purities >95%. CD8+ T cells were then cultured for 3 d in complete RPMI 1640 medium at 5 × 10^5 cells/well in plates coated with anti-CD3 mAb (5 μg/ml) together with anti-CD28 mAb (2 μg/ml) and recombinant human IL-2 (100 U/ml). Cells were then washed once and cultured (5 × 10^5 cells/ml) for 2 d, then replated at 6.7 × 10^4 cells/ml and cultured for an additional 2 d.

Retroviral constructs

Murine stem cell virus (MSCV)-T-bet-internal ribosome entry site (IRES)-GFP (10) has been previously described and MSCV-Id2-IRES-GFP and MSCV-ires-GFP were a gift from Dr. Ross Dickins (Walter and Eliza Hall Institute). Tcf7e2a-ires-GFP and IRES-GFP LMP vectors were a gift from Dr. Ross Dickins (Walter and Eliza Hall Institute).

In vitro T cell culture analysis

CD8+ T cells were enriched from the spleen of Id2^{fl/fl}LckCre^+ mice. CD8^+CD19^- T cells were purified either by FACS sorting or by two rounds of negative selection (MACS; Miltenyi Biotec) to obtain purities >95%. CD8+ T cells were then cultured for 3 d in complete RPMI 1640 medium at 5 × 10^5 cells/well in plates coated with anti-CD3 mAb (5 μg/ml) together with anti-CD28 mAb (2 μg/ml) and recombinant human IL-2 (100 U/ml). Cells were then washed once and cultured (5 × 10^5 cells/ml) for 2 d, then replated at 6.7 × 10^4 cells/ml and cultured for an additional 2 d.

Cell surface staining and FACS analysis

Virusespecific CD8+ T cells were detected by staining with PE-coupled tetrameric H-2D^b MHC class I complexes loaded with epitopes of influenza virus nucleoprotein (NP: D^{NP}_{56-57}; H-2D^b-restricted) or acidic polypeptide (PA: D^{PA}_{24-131}; H-2D^b-restricted) as previously described (20). To block nonspecific binding of Abs, cells were incubated in rat IgG (1 mg/ml) together with anti–FcγR mAb (CD16/32, clone 2-4G21) for 10 min on ice. Samples were then stained with fluorochrome-labeled Abs against CD8α (53-6.7), CD62L (Mel-14), KLKRG1 (2FY), IL-7R (A7R34), Ly5.1 (A20-1.1), Ly5.2 (104), and CD44 (IM7) (all from eBioscience or BD Pharmingen). Viable cells were analyzed by flow cytometry using propidium iodide or Sytox blue exclusion (Invitrogen). Analysis was performed on a FACSCan or a FACS LSRFortessa (BD Biosciences) and data were analyzed using FlowJo flow cytometry analysis software.

Generation of bone marrow chimeras

Mixed bone marrow chimeras were established by reconstituting lethally irradiated (2 × 0.55 Gy) B6.Ly5.1 or Ly5.1 × Ly5.2 (F1) recipient mice with a mixture of Id2^{fl/fl}LckCre^+ (3.4 × 10^6) and wild-type (Ly5.1^c, 1.6 × 10^6) T cell–depleted bone marrow cells and allowed to reconstitute for 6–8 wk (5, 21).

Microarray analysis

D^{NP}_{56-57}CD8+ T cells were purified by flow cytometric sorting (FACSaria flow cytometer, BD Biosciences) from day 9 HKx31-infected PR8–primed Id2^{Gfp/neo} or Id2^{fl/fl}LckCre^+Ly5.1^c mixed bone marrow chimeras. Total RNA was prepared using a Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. DNA microarray analysis of gene expression was performed using Illumina MouseWG-6 v2.0 Expression BeadChip together with the manufacturer’s probe annotation (released in November 2008). The Gene Expression Omnibus referenced accession nos. for these data are GSE44140 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44140) and GSE44141 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44141).

In vitro T cell culture analysis

CD8+ T cells were enriched from the spleen of Id2^{fl/fl}LckCre^+ or wild-type mice. CD8^+CD19^- T cells were purified either by FACS sorting or by two rounds of negative selection (MACS; Miltenyi Biotec) to obtain purities >95%. CD8+ T cells were then cultured for 3 d in complete RPMI 1640 medium at 5 × 10^5 cells/well in plates coated with anti-CD3 mAb (5 μg/ml) together with anti-CD28 mAb (2 μg/ml) and recombinant human IL-2 (100 U/ml). Cells were then washed once and cultured (5 × 10^5 cells/ml) for 2 d, then replated at 6.7 × 10^4 cells/ml and cultured for an additional 2 d.

Retroviral constructs

For detection of intracellular granzyme (Gzm) B, T-bet and Eomes staining was performed directly ex vivo. GzmB was detected using anti-human GzmB Ab (AB12; Invitrogen) using a Cytofix/Cytoperm kit (BD Biosciences). Staining for T-bet (4B10; Santa Cruz Biotechnology) and Eomes (Dan11mag; eBioscience). Staining was carried out on a MyiQ instrument (Bio-Rad). Primers sequences for chromatin immunoprecipitation-PCR analysis

Chromatin immunoprecipitation-PCR analysis

Chromatin immunoprecipitation-Chip was performed with an affinity-purified polyclonal rabbit Ab directed against the N-terminal peptide PRRRAANGLDTQPKKKVRK of the mouse E2A protein. The precipitated DNA was quantified by real-time PCR analysis with SYBR Green, which was carried out on a MyiQ instrument (Bio-Rad). Primers sequences are supplied in Supplemental Table I.

Bio-Chip sequencing

Total thymocytes (∼10^6) from Tcf7e2a^{fl/fl}BirA^+Rosa26^{BrdU/Fluc} mice were used for chromatin precipitation by streptavidin pulldown (Bio-Chip), as recently described in detail (22).

Quantitative RT-PCR

Total RNA was prepared from purified D^{NP}_{56-57}CD8+ T cell populations using an RNaseasy Mini Kit (Qiagen). cDNA was synthesized from total RNA with oligo(dT) and thermoscript reverse transcriptase (Invitrogen). Real-time PCR was performed using the SensiMix SYBR No-ROX Kit (Bioline). Analyses were done in triplicate and mean normalized expression was calculated with Hprt as the reference gene.

Retroviral transduction of OT-I TCR transgenic T cells

Id2^{fl/fl}LckCre^+ or wild-type CD8+ OT-I T cells were enriched from the spleen and cultured in complete RPMI 1640 containing human recombi-
nant human IL-2 (30 U/ml) and 1 μg/ml OVA257–264 peptide for 2 d. Retroviral supernatants were then spun for 1 h at 4000 rpm at 4˚C in 12-well plates coated with 32 μg/ml RetroNectin. Retrovirus supernatant was then carefully removed from the plate and OT-I T cells were then added and cultured with human rIL-2 (30 U/ml) and polybrene (1 μg/ml) in complete RPMI 1640 for 1 d. For adoptive transfer experiments, OT-I T cells transduced with MSCV-T-bet-IRES-GFP, MSCV-Id2-IRES-GFP, or MSCV-IRES-GFP retroviruses were selected in puromycin at 2 μg/ml for 5–7 d. Live cells were then purified by centrifugation on Histopaque density gradient before quantitative RT-PCR analysis.

Statistical analysis
Statistical analyses were performed using Prism software.

Results
Loss of Id2 impairs the differentiation of short-lived effector CD8+ T cells
To map the expression level and the role of Id2 in different T cell populations, we made use of our previously described reporter allele where an IRES-GFP cassette has been inserted into the 3’ untranslated region of the Id2 gene by homologous recombination (12). To track the induction of Id2, wild-type and Id2 reporter mice (Id2fluflu) were infected intranasally (i.n.) with influenza A H5N1. Strikingly, Id2-GFP was rapidly upregulated in all influenza-specific DNP366-and PA224-specific CD8+ T cells regardless of their phenotype or anatomical location (Fig. 1A, 1B).

To examine how Id2 expression affected the emergence of influenza-specific CD8+ effector and memory T cells, we analyzed mice that specifically lacked Id2 in T cells through LckCre-mediated deletion of the entire Id2 coding region (19). To ex-

FIGURE 1. Id2-deficient CD8+ T cells do not differentiate into KLRG1+ effector cells. (A) Id2fluflu and wild-type (+/+) mice were infected i.n. with influenza virus HKx31 and analyzed on day 9. (B) Id2-GFP expression in naive (CD44+CD62L+) and DNP366+ and PA224+ influenza-specific CD8+ T cells from spleen of infected mice. (C) Generation of wild-type and Id2-deficient DNP366-specific CD8+ T cells in Ly5.2LckCre+Ly5.1+ mixed bone marrow chimeric mice 10 d after i.n. infection. Data are representative of 11 mice. (D) Frequency of DNP366-specific CD8+ T cells within the Id20LckCre+ (Ly5.2+) or the wild-type (Ly5.1+) splenic CD8+ T cell compartment from chimeric mice 10 d after HKx31 infection. Data are the means ± SEM from three independent experiments (n = 11). (E) Bone marrow chimeric mice were infected with HKx31 and DNP366+ influenza-specific CD8+ T cells in spleen were analyzed for the indicated markers within the wild-type (Ly5.2+) and Id2-deficient (Id20LckCre+, Ly5.2+) compartments. Data are representative of at least three independent experiments. (F) Frequency of KLRG1+ DNP366+ CD8+ T cells following primary HKx31 influenza infection. Data show the means ± SEM of KLRG1+ influenza virus-specific cells within the CD8+ T cell compartment in indicated tissues. For (E) and (F), data are pooled from three independent experiments (n = 8). (G) OT-I Id20LckCre+ (Ly5.2+) and OT-I wild-type (Ly5.1+Ly5.2+) CD8+ T cells, mixed in a 1:1 ratio, were transferred into wild-type Ly5.1+ mice that were infected i.v. with Lm-OVA 24 h later. (G) Flow cytometric analysis of wild-type (Ly5.1+Ly5.2+) FL1) or Id20LckCre+ (Ly5.2+) OT-I CD8+ T cell population isolated from the spleen 9 d after infection with Lm-OVA. Right panel, Bar graph shows the mean percentage ± SEM of KLRG1+ wild-type or Id20LckCre+ OT-I CD8+ T cell populations in the spleen. (H) Bar graph shows the mean ± SEM of the ratio of wild-type OT-I to Id20LckCre+ OT-I T cells in total OT-I cells and in memory precursors cells (IL-7RhighKLRG1+) Data are pooled from two independent experiments (n = 10). Statistically significant differences were determined using a two-tailed paired Student’s t test (A–G) or a Mann–Whitney nonparametric test (H). *p < 0.05, **p < 0.01, ***p < 0.001.
clude secondary effects due to differing resolution of the infection in intact mice, Ly5.2*Id2\(_{Lck}\) (fl/fl; Lck\(^{Cre\+}\)) and Ly5.1*Id2 (+/+), mixed bone marrow chimeras were generated and infected with influenza virus (HKx31). This revealed that the proportions of virus-specific CD8\(^+\) T cells in Id2\(_{Lck}\) and wild-type compartments were similar in spleen and LNs (Fig. 1C, 1D). However, within the lung and liver, the relative frequency of Id2\(_{Lck}\) D\(^{\text{NP}_{366}^\text{+}}\)- and D\(^{\text{PA}_{224}^\text{+}}\)-specific CD8\(^+\) T cells was reduced, suggesting a defect in the ability to migrate to and/or survive in nonlymphoid tissues (Fig. 1D). KLRG1\(^{+}\)IL-7R \(^{-}\) short-lived effectors were selectively absent from the Id2-deficient CD8\(^+\) T cell compartment in accordance with earlier observations (Fig. 1E, 1F, Supplemental Fig. 1) (3). The unaltered frequency of influenza-specific CD8\(^+\) T cells in Id2-deficient and wild-type compartments within the spleen and LNs was surprising given a previous report that suggested Id2 was critical to control effector CD8\(^+\) T cell survival (1). In this earlier study, Id2-deficient OVA-specific TCR transgenic CD8\(^+\) T cells (OT-I) responding to systemic infection with rLm-OVA were highly susceptible to apoptosis mediated by the proapoptotic molecule Bim. To better understand the factors that underpin the discrepancies between the two studies, we analyzed the quantity and the quality of the response of Id2\(_{Lck}\) (Ly5.2\(^{+}\)) and wild-type (Ly5.1*Ly5.2\(^{+}\) F1) OT-I T cells transferred into Ly5.1\(^{+}\) recipients following i.v. Lm-OVA infection. Similar to previous results, Id2\(_{Lck}\) OT-I T cells were outcompeted by wild-type OT-I T cells at 9 d after Lm-OVA infection (Fig. 1G, 1H) (1). However, closer analysis revealed that following Lm-OVA infection most (\~80%) wild-type OT-I T cells formed short-lived effectors whereas memory precursors represented a smaller proportion of the overall population. This short-lived effector population was absent in the Id2\(_{Lck}\) OT-I CD8\(^+\) T cell compartment (Fig. 1G), but notably the memory precursor compartment was not affected by Id2 deficiency (Fig. 1H). This demonstrated that in the absence of Id2, the strong inflammatory stimulus of Lm-OVA resulted in the selective failure of the short-lived effector (IL-7R\(^{0.0}\)KLRG1\(^{+}\)) CD8\(^+\) T cell subset to differentiate and/or survive. In contrast, memory precursor (IL-7R\(^{0.0}\)KLRG1 \(^{-}\)) CD8\(^+\) T cells were unaffected showing that Id2 loss does not globally impair virus-specific CD8\(^+\) T cell survival.

**Id2 expression restricts memory recall capacity**

Id2 deficiency significantly affected effector T cell differentiation, but it was not clear whether normal memory was formed when Id2 was lacking. To understand the effect of Id2 loss on memory, we quantitated the proportion of influenza-specific memory CD8\(^+\) T cells within the wild-type and Id2\(_{Lck}\) CD8\(^+\) T cell compartments of mixed BM chimeras infected 9 wk previously with HKx31 virus (Fig. 2A, 2B). Neither the frequency of virus-specific memory CD8\(^+\) T cells (Fig. 2A, 2B) nor the proportion of central memory T cells (F. Masson and G.T. Belz, unpublished observations) was significantly different from control cells. To accurately assess the recall potential of Id2-deficient memory T cells, CD8\(^+\) T cells were enriched from the spleens of HKx31-infected mixed chimeric mice and adoptively transferred into naive recipients prior to influenza infection (Fig. 2C). Surprisingly, 10 d after infection, we found that Id2-deficient CD8\(^+\) T cells had significantly increased in proportion compared with wild-type cells (Fig. 2D), indicating that Id2 expression restricts memory recall potential of CD8\(^+\) T cells.

To understand the role of Id2 in the memory T cell compartment in more detail, we assessed the expression of Id2-GFP in Ag-specific CD8\(^+\) T cells during the memory phase of influenza virus infection. Both effector memory and central memory T cells expressed a lower level of Id2-GFP than did acutely activated effector cells (Fig. 3A). Remarkably, approximately half of the

**FIGURE 2.** Id2-deficient memory CD8\(^+\) T cells exhibit increased recall capacity. (A and B) Mixed bone marrow chimeras reconstituted with Id2\(_{600}^\text{+}\)Lck\(^{Cre+}\) (Ly5.2\(^{+}\)) and wild-type (Ly5.1\(^{+}\)) bone marrow were analyzed 9 wk after i.n. HKx31 infection. (A) Representative flow cytometric profiles and (B) cumulative analysis of the persistence of D\(^{\text{NP}_{366}^\text{+}}\)-specific CD8\(^+\) T cells within the Id2\(_{600}^\text{+}\)Lck\(^{Cre+}\) (Ly5.2\(^{+}\)) and the wild-type (Ly5.1\(^{+}\)) CD8\(^+\) T cell compartments. Individual mice in (B) are shown by an open circle (wild-type) or filled square (Id2\(_{600}^\text{+}\)Lck\(^{Cre+}\)). Data are pooled from three independent experiments (n = 13). Statistically significant differences were determined using a paired two-tailed Student t test. (C and D) Analysis of recall responses of Id2-deficient (Ly5.2\(^{+}\)) and wild-type (Ly5.1\(^{+}\)) virus-specific CD8\(^+\) T cells. (C) Schematic diagram showing the experimental approach for analyses of memory T cell recall responses. (D) Relative contribution of Id2\(_{600}^\text{+}\)Lck\(^{Cre+}\) CD8\(^+\) T cells to the D\(^{\text{NP}_{366}^\text{+}}\)-specific CD8\(^+\) T cell population in spleen, LN, and lung. Data were normalized for the proportion of each genotype represented in the input D\(^{\text{NP}_{366}^\text{+}}\)CD8\(^+\) T cell population and are expressed as log\(_2\) of the ratio of Id2\(_{600}^\text{+}\)Lck\(^{Cre+}\)/D\(^{\text{NP}_{366}^\text{+}}\)/wild-type CD8\(^+\) D\(^{\text{NP}_{366}^\text{+}}\) T cells. Data are pooled from 14 to 18 animals from three independent experiments. Statistically significant differences of the test group to 0 were determined using an unpaired two-tailed Student t test. **p < 0.01, ***p < 0.001.
influenza-specific central memory CD8+ T cells had a reduced Id2 expression comparable with naive CD8+ T cells, suggesting that low Id2 expression level is linked to the reported higher recall potential of memory CD8+ T cells in a dose-dependent manner. To extend this analysis, we examined how loss of Id2 affected the induction of the effector transcriptional program. Remarkably, the expression of the cytolytic molecules GzmA and GzmB were significantly reduced in Id2-deficient DNP366-specific CD8+ T cells (Fig. 4A, 4B). Id2-deficient cells also displayed an altered expression of integrins and chemokine receptors such as CD49a, CD103, CX3CR1, and CCR7 likely to impair T cell migration (22–24) (Fig. 4A). This is consistent with our observation that Id2-deficient CD8+ T cell frequency is reduced within nonlymphoid tissues (Fig. 1D). Overall, our data show that Id2-deficient virus-specific CD8+ T cells exhibit an impaired transcriptional program of effector differentiation and consequently fail to appropriately differentiate into short-lived effector CD8+ T cells.

Having established that Id2 was important for memory CD8+ T cells, we speculated that distinct levels of Id2 were deterministic in the transcriptional program of Ag-specific CD8+ T cells. To test this hypothesis, we subjected DNP366-specific effector CD8+ T cells purified according to their differential expression of Id2-GFPlow and Id2-GFPhigh to microarray analysis and compared their gene expression profiles to the 233 DE genes identified by comparing Id2-deficient and wild-type DNP366-specific CD8+ T cells (Supplemental Fig. 2). This analysis revealed that most of the DE genes were strongly dependent on Id2 expression levels. Indeed, 76% of DE genes found to be upregulated in Id2-deficient cells were also upregulated in Id2-GFPint cells compared with Id2-GFPhigh cells. Similarly, 83% of the DE genes observed to be downregulated in absence of Id2 were downregulated in Id2-GFPint cells (Supplemental Fig. 2B). This analysis revealed that most of the DE genes were strongly dependent on Id2 expression levels. Indeed, 76% of DE genes found to be upregulated in Id2-deficient cells were also upregulated in Id2-GFPint cells compared with Id2-GFPhigh cells. Similarly, 83% of the DE genes observed to be downregulated in absence of Id2 were downregulated in Id2-GFPint cells (Supplemental Fig. 2B).

Furthermore, expression of Eomes, Id3, and Tcf7 mRNA was higher on Id2-GFPint cells compared with Id2-GFPhigh T cells, whereas GzmB expression was significantly reduced in Id2-GFPint cells T cells (Supplemental Fig. 2B, 2C). However, despite an increased expression of genes involved in memory formation, Id2-GFPint virus-specific CD8+ T cells did not exhibit increased long-
term survival compared with Id2-GFP<sup>high</sup> virus-specific CD8<sup>+</sup> T cells (Supplemental Fig. 2D, 2E). This indicated that the survival of memory cells is not dictated by the level of Id2 expression, consistent with our previous results (Fig. 2B).

Overall, our data demonstrate that the transcriptional program of CD8<sup>+</sup> T cell differentiation is exquisitely sensitive to the concentration of Id2.

**Id2 controls effector differentiation by inhibiting E2A**

Id2 is a key negative regulator of the E protein transcription factor family but it is less clear which specific E protein Id2 might act on during T cell differentiation. We hypothesized that Id2 controls CD8<sup>+</sup> T cell differentiation by limiting the transcriptional activity of E2A. To investigate this question, we transduced wild-type and Id2<sup>Lck<sup>Cre<sup>+</sup></sup></sup> OT-I T cells with retroviruses encoding either a shRNA against Tcfe2a (encoding E2A) or a control hairpin and then analyzed the expression of key target genes identified as DE in our previous microarray analysis. The Tcfe2a shRNA induced an 80% reduction of Tcfe2a expression, and this reduction was not compensated by an increased expression of the other E protein family members Tcf7, Id3, or Socs3 (Fig. 5A). Remarkably, silencing of Tcfe2a in Id2<sup>Lck<sup>Cre<sup>+</sup></sup></sup> OT-I T cells and, to a lesser extent, in wild-type OT-I T cells resulted in a decrease in the expression of several genes important for the development and/or the maintenance of memory T cells, such as Tbx21, Id3, and Socs3 (Fig. 5A). In contrast, Id2<sup>Lck<sup>Cre<sup>+</sup></sup></sup> OT-I T cells transduced with the Tcfe2a shRNA exhibited an increased expression of genes encoding the effector molecules GzmB and GzmK compared with cells transduced with the control shRNA retrovirus (Fig. 5A). Of note, the expression of Tbx21 or Prdm1 was not affected by Tcfe2a knockdown in vitro, suggesting that they are not direct targets of E2A.

To confirm the involvement of E2A, we then wanted to determine the proportion of genes having E2A occupancy at promoter/enhancer distance among the list of 233 DE genes in the absence of Id2. We took advantage of Tcfe2a<sup>Bio/Bio</sup> mice (I. Bilic and M. Busslinger, unpublished observations), which express an E2A-Bio protein containing a C-terminal biotin acceptor sequence that is efficiently biotinylated in vivo by coexpression of the *Escherichia coli* biotin ligase BirA from the *Rosa26<sup>BirA</sup>* allele. We then determined the genome-wide pattern of E2A binding in total thymocytes from Tcfe2a<sup>Bio/Bio</sup> mice, which in Id2 expression is low (Supplemental Fig. 6), and hence allows high E2A binding to its target genes. Using a stringent p value of <10<sup>−10</sup> for peak calling, we detected 4337 E2A-binding regions, which defined 2541 E2A target genes in total thymocytes. We next used these E2A target genes to cross-reference our microarray data comparing Id2-deficient and wild-type virus-specific CD8<sup>+</sup> T cells. Strikingly, the results from this analysis showed that among our list of genes found to be upregulated in absence of Id2, the proportion of those with an E2A-binding site is significantly higher than among all genes of the genome, consistent with the transactivating function of E2A (Fig. 5B). In contrast, there is no significant difference of enrichment of genes with E2A occupancy among the genes found to be downregulated in absence of Id2 compared with the whole genome, suggesting that they are not directly regulated by E2A (Fig. 5B). Consistent with the Tcfe2a silencing data (Fig. 5A), our Bio-ChIP sequencing analysis identified several E2A-binding sites at the loci of key genes involved in memory T cell differentiation or function such as Tbx21, Gzmk, and Gzmb (F. Masson and G.T. Belz, unpublished observations). Overall, our results suggest that Id2 restrains memory T cell development by inhibiting E2A transcriptional activity.
Id2 controls effector and memory CD8+ T cell differentiation by inhibiting E2A transcriptional activity. (A) Wild-type and Id2Lox/LoxLckCre+ OT-I cells were transduced with shRNA-tcfe2a-GFP and shRNA-control-GFP retroviruses. GFP+ cells (95% positive) were subjected to quantitative RT-PCR for the indicated genes. Graphs show the means ± SEM of the experimental triplicates of the expression relative to Hprt and are representative of two independent experiments. (B) Bio-ChIP sequencing data of total thymocytes from Tcfe2aROSA26BiBirA/BirA mice. Bar graph shows the proportion of genes with E2A-binding sites in the whole genome or among the 233 DE (upregulated or downregulated) genes in Id2-deficient virus-specific CD8+ T cells. Statistically significant differences were determined using a Fisher exact test. ***p < 0.001.

E2A regulates Tcfe2a expression in peripheral CD8+ T cells

Tcfe2a, a critical regulator of memory CD8+ T cell differentiation, persistence, and recall potential (9), was significantly upregulated in Id2-deficient CD8+ T cells (Fig. 4C) and correlated with the dose of Id2 (Fig. 4D). Tcfe2a expression was also downregulated in Id2-deficient T cells in which E2A was silenced (Fig. 5A). Our Bio-ChIP sequencing analysis in total thymocytes identified several E2A-binding sites at the Tcfe2a locus (Fig. 6A). However, because Id2 is not expressed in double-positive thymocytes (27) whereas it is expressed at low level in naive CD8+ T cells (Fig. 1B) and at a high level in effector CD8+ T cells (Fig. 1B), the binding of E2A to the Tcfe2a locus is expected to be inversely correlated to the level of Id2 expression in these different T cell subsets. Therefore, we then investigated whether Tcfe2a was also a direct target of E2A in peripheral CD8+ T cells. Using conventional ChIP-quantitative PCR analysis, we tested whether E2A could bind to these sequences also in naive and in vitro-activated CD8+ T cells. In naive CD8+ T cells, E2A no longer bound to site 2 and interacted weakly with sites 1, 3, and 5 in contrast to thymocytes (Fig. 6B). However, E2A efficiently bound to site 4 (located 33 kb upstream of the Tcfe2a transcription starting start), suggesting that E2A activates the Tcfe2a gene through this upstream enhancer in naive CD8+ T cells. Critically, the interaction of E2A with site 4 and the weaker binding sites was lost following activation and concurrent induction of Id2 expression (Fig. 6B), suggesting that E2A directly regulated Tcfe2a expression in a manner that was inhibited in the presence of Id2.

Id2-dependent control of T-bet expression is required for the differentiation of short-lived effector cells

T-bet is a key transcriptional regulator of effector cell differentiation that is downregulated in the absence of Id2 (Fig. 4B). Because T-bet promotes short-lived effector cell differentiation in a dose-dependent manner (10), we speculated that the reduction of T-bet expression observed in Id2-deficient CD8+ T cells (Fig. 4B), the binding of E2A to the Tcf7 locus (Fig. 6B), suggesting that E2A directly regulated Tcfe2a expression in a manner that was inhibited in the presence of Id2. To test whether downregulation of T-bet expression in Id2-deficient CD8+ T cells was directly responsible for the loss of effector CD8+ T cells, we transduced Id2Lox/Lox OT-I CD8+ T cells with retroviruses encoding T-bet or Id2 or with a control retrovirus. In vitro–activated transduced Id2Lox/Lox and wild-type OT-I T cells were adoptively transferred into Ly5.1+ recipient mice. Mice were infected with Lm-OVA 24 h later. After 8 d, the formation of effector CD8+ T cells from transduced OT-I cells was
Id2 inhibits T cell memory

Discussion

Id2 is a key regulator of CD8+ T cell–mediated immunity, but there is a limited understanding of the molecular mechanisms by which Id2 exerts its effects influencing effector and memory T cell fate decisions. Early studies suggested that the main action of Id2 is to ensure the survival of effector CD8+ T cells following activation (1). In contrast, our study shows the absence of a major defect in the overall survival of virus-specific CD8+ T cell during influenza infection and this has allowed us to dissociate the differentiation defect from the survival defect reported in the former studies. Our data support an alternate model in which Id2 can act as a rheostat to directly regulate the differentiation of naive CD8+ T cells into effector or memory T cells. We demonstrated that Id2 limits T-bet expression partly rescued GzmB expression and this has allowed us to dissociate the differentiation defect from the survival defect reported in the former studies. Our data support an alternate model in which Id2 can act as a rheostat to directly regulate the differentiation of naive CD8+ T cells into effector or memory T cells. We demonstrated that Id2 limits memory CD8+ T cell formation and recall potential in a dose-dependent manner by inhibiting E2A transcriptional activity. In particular, Id2 inhibits E2A-mediated transactivation of Tcf7. Finally, we showed that Id2 is required for the optimal induction of T-bet and thereby for the differentiation of KLRG1+ short-lived effector cells.

Id2 deficiency resulted in a profound alteration of the transcriptional program that drives the differentiation of virus-specific CD8+ T cells. Id2-deficient cells exhibit an increased expression in multiple genes involved in memory T cell differentiation and function (Tcf7, Id3, Socs3, Eomes, Il2, Ccr7) paralleled by a concurrent decrease in the expression of genes associated with an effector phenotype (Tbx21, Prdm1, Ifnar2a, Gzma, Gzmb, Gzmk, Cdl103, Cx3cr1). Remarkably, the comparison of our microarray data analysis with E2A CHIP sequencing data revealed that there was a significant enrichment for genes with E2A-binding sites among the genes upregulated in absence of Id2 compared with the DE genes that were downregulated. This suggests that Id2 represses the memory program of CD8+ T cell...
determination by inhibiting the E2A-mediated transactivation of several key genes involved in the process of memory differentiation such as Tcfr7.

Tcfr7 is a key mediator of the Wnt/β-catenin signaling pathway essential for the generation and the persistence of memory T cells through its control of Eomes expression, as well as for the recall potential of memory CD8+ T cells (9). Tcfr7 is highly expressed in naive CD8+ T cells and is downregulated in effector CD8+ T cells. We showed that both Tcfr7 and Eomes were expressed at higher levels in Id2-deficient effector CD8+ T cells compared with wild-type effector T cells. Using E2A ChIP analyses, we discovered that E2A directly binds to the Tcfr7 locus and this binding is lost following activation concurrent with the induction of Id2. Finally, silencing of Tcfr2ea in Id2-deficient CD8+ T cells resulted in the downregulation of Tcfr7 expression. Taken together, these data suggest that a major pathway by which Id2 restrains the formation of memory precursor cells is by inhibiting the E2A-dependent activation of Tcfr7 in effector CD8+ T cells.

Recently it has been proposed that the repression of Id3 by Blimp1 promoted the development of effector T cells at the expense of memory cell differentiation (2). In our studies, in the absence of Id2, Id3 was strongly upregulated (Figs. 2D, 5A) whereas silencing of Tcfr2ea in Id2-deficient CD8+ T cells reversed this upregulation of Id3 in Id2-deficient CD8+ T cells in vitro without affecting Blimp1 expression (Fig. 5A). From our E2A ChIP sequencing data, we identified several binding sites at the Id3 locus (F. Masson and G.T. Belz, unpublished observations), confirming that Id3 is a direct target of E2A. It has also been reported that Id3-deficient CD8+ T cells did not show any reduction in memory precursor cell frequency (or increase in the frequency of short-lived effector T cells) (2). Thus, repression of Id3 does not appear to influence the lineage choice decision between effector and memory cells at the acute time point of an infection, although it does affect the maintenance of memory cells in the long term. From our studies, we propose that Id3 expression is directly influenced by Id2 expression dynamics, which in turn regulates the E2A-dependent transcriptional activation of the Id3 locus. Thus, Id2 rather than Id3 expression level delineates the effector and memory phenotype during the acute phase of an infection.

Id2 and Id3 are both known to interact with the same basic helix-loop-helix partners (11, 29), but it is not clear whether they bind different E proteins with a similar affinity. Indeed, Id2 and Id3 have clearly different functions in effector and memory cells. This is exemplified by the loss of long-term memory cell absence in Id3 as opposed to the loss of short-lived effector cells observed in the absence of Id2 (1, 3). We have shown in this study that reduction or loss of Id2 expression in endogenous virus-specific memory CD8+ T cells leads to enhanced recall responses. In contrast, Id3 overexpression improved the magnitude of the recall response of memory CD8+ T cells (2). In this latter setting, it is not clear whether this represents the physiological action of Id3. Given the contrasting roles of Id3 and Id2 in T cell differentiation, it will be crucial to determine with which E protein partners each Id protein interacts when expressed at physiological levels. This partner could be E2A or another member of the E protein family (HEB and E2-2). Indeed, HEB has been recently reported to collaborate with E2A in the generation of memory cells (30). However, it may also be another protein unrelated to the E protein family. Indeed, even though E proteins have been described as the main heterodimerizing partners of Id proteins, other proteins such as the myogenic factors PU.1 and Rb have been described to interact with Id2 (31–33).

Our microarray data analysis has shown that many genes involved in effector differentiation and/or functions were down-regulated in absence of Id2. In particular, the expression of T-bet, a key transcriptional regulator of effector T cell differentiation, was also affected. Our results suggest that T-bet expression is indirectly regulated by E2A because no E protein binding sites were detected at its locus using in silico bioinformatical analysis, or ChIP sequencing in whole thymocytes (F. Masson and G.T. Belz, unpublished observations), or in the A12 T cell line (34). It is therefore likely that the decrease in the expression of this key transcriptional regulator is a consequence of the altered differentiation of Id2-deficient CD8+ T cells toward the memory phenotype. Surprisingly, E2A silencing did not affect Tbx21 expression in vitro (Fig. 5A), suggesting that either the level of E2A silencing was insufficient to induce a significant change of T-bet expression or that other E protein family members HEB and E2-2 may compensate for the loss of E2A in the regulation of T-bet expression. Further analyses showed that decreased expression of T-bet was responsible for the loss of KLRG1+ cells in Id2-deficient CD8+ T cells. In support of this finding, haploinsufficiency of Tbx21 (a decrease in T-bet expression comparable to the downregulation induced by Id2 ablation) was sufficient to block short-lived CD8+ effector T cell differentiation (Fig. 7B) (10), and enforced expression of T-bet in Id2-deficient CD8+ T cells rescued short-lived effector T cell differentiation (Fig. 7C, 7D).

In conclusion, our study has identified Id2 as a critical dosage-dependent regulator of E protein transcriptional activity, in particular E2A, which determines whether a naive CD8+ T cell will commit to effector or memory differentiation. We propose a model in which Id2 promotes effector differentiation by restraining the E2A-mediated transactivation of several key genes essential for memory T cell development and function.

Acknowledgments

We thank Annie Xin, Wei Shi, Malou Zuidschroewoude, Philippe Bouillet, Marc Pellegrini, Lynn Corcoran, Ross Dickins, Manabu Sugai, Simon Preston, and Mary Camilleri for provision of reagents, advice, and technical assistance, and Markus Jaritz for bioinformatic analysis.

Disclosures

The authors have no financial conflicts of interest.

References


Id2 INHIBITS T CELL MEMORY

10


