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

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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 Eomesodermin 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: 4585–4594.

S uccessful eradication and protection from reinfection by intracellular pathogens such as viruses and bacteria depend on the generation of effector and memory CD8⁺ T cells. Naive 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 mem-

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Abbreviations used in this article: Blimp1, B lymphocyte–induced maturation protein 1; CD1d, cholinergic immunoprecipitation; DE, differentially expressed; Eomes, eomesodermin; Gzm, granzyme; Id, inhibitor of DNA binding; i.n., intranasal(ly); IRES, internal ribosome entry site; Lm-OVA, Listeria monocytogenes encoding OVA; LN, lymph node; MSCV, murine stem cell virus; NP, nucleoprotein; PA, acidic polymerase; shRNA, short hairpin RNA.

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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 Th2 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 restraints 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

Id2fl/fl (12), Id2fl/LckCre, Thx21Δ/Δ (14), OT-I (15), Flpe (16), LckCre (17), C57BL/6 (Ly5.2+), B6.SJL-Ptprca Pepcb/129ScPtprc–1(B10.D2-13.1) (Ptprca Pepcb/129ScPtprc–1(B10.D2-13.1), MHC class II (M5/114), and Cd4 (GK1.5) for 30 min on ice. The Ab-bound cells were removed using anti-rat IgG Ab-conjugated magnetic beads (Dynabeads; Dynal).

Bacterial and viral infections

Mice were anesthetized with methoxyflurane and then inoculated with 10⁴ PFU HKx31 (H3N2) influenza virus. Memory mice were generated by priming with i.p. injection of 10⁵ 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 in PBS for injection. Mice were infected i.v. with 2.5 × 10⁵ rLm-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 spleen and LN by generating a single-cell suspension and incubating the cells in a mixture of optimally titrated Abs against CD8 (1×10⁵ cells/well in plates coated with anti-CD3 mAb (2 mU/ml). Cells were then washed once and cultured (5 × 10⁵ cells/ml) for 2 d, then replated at 6.7 × 10⁴ cells/ml and cultured for an additional 2 d.

Retroviral constructs

Mice of Id2fl/fl background were infected i.v. with 2.5 × 10⁷ cells of Lm-OVA. Injected bacterial numbers were determined by spreading bacterial samples on brain-heart infusion plates followed by incubation overnight at 37°C.

Cell surface staining and FACS analysis

Virusspecific CD8+ T cells were detected by staining with PE-coupled tetramer H-2d MHC class I complexes loaded with epitopes of influenza virus nucleoprotein (NP; DNP-366-374, H-2d–restricted) or acidic polypeptide (PA; DPA2-24-131, H-2d–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 FACSCanto or a FACSLSRFortessa (BD Biosciences) and data were analyzed using FlowJo flow cytometry analysis software.

Microarray analysis

DNP366-specific CD8+ T cells were purified by flow cytometric sorting (FACSAria flow cytometer, BD Biosciences) from day 9 HKx31-infected PR8–primed Id2fl/fl or Id2fl/LckCre;Ly5.1+” 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 Id2fl/LckCre+ or wild-type mice. CD8α 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 × 10⁵ 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 × 10⁵ cells/ml) for 2 d, then replated at 6.7 × 10⁴ cells/ml and cultured for an additional 2 d.

Intracellular flow cytometry staining

For detection of intracellular granzyme B (GzmB) protein, T-bet and Eomes staining was performed directly ex vivo. GzmB was detected using anti-human GzmB Ab (AB12; Invitrogen) using a Cytotest/Cytoperm kit (BD Biosciences). Staining for T-bet (B8-10, Santa Cruz Biotechnology) and Eomes (Dan11mag; eBioscience) was performed using the Foxp3 kit (eBioscience).

Chromatin immunoprecipitation-PCR analysis

Chromatin immunoprecipitation (ChIP) analysis was performed with an affinity-purified polyclonal rabbit Ab directed against the N-terminal peptide RPRRAANGGLDTQPKKVRKV 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 (∼10⁶) from Tcf2a+/+ or Tcf2a+/- Rosa26Bio/Alb/- 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 DNP366-specific CD8+ T cell populations using an RNAsafe 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

Id2fl/LckCre+ or wild-type CD8+ OT-I T cells were enriched from the spleen and cultured in complete RPMI 1640 containing human recombi-

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nant human IL-2 (30 U/ml) and 1 μg/ml OVA 57-264 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 Rl-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-Tbet-IRES-GFP, MSCV-Id2-IRES-GFP, or MSCV-IRES-GFP retroviruses were washed and expanded in a 75-cm² culture flask in the presence of IL-15 (20 ng/ml) for 4 d. GFP⁺ cells were purified by flow cytometric sorting prior to adoptive transfer into recipient mice that were then infected the following day with Lm-OVA. OT-I T cells transduced with the short hairpin RNA (shRNA) Tcefa2a-IRES-GFP LMP or IRES-GFP LMP 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 used the expression level of an IRES-GFP cassette inserted into the 3’ untranslated region of the Id2 gene (12). To track the induction of Id2, wild-type and Id2 reporter mice (Id2gfp/gfp) were infected intranasally (i.n.) with influenza A HKx31. 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-
clude secondary effects due to differing resolution of the infection in intact mice, Ly5.2+Id2Lck\textsuperscript{fl/fl} (Lck\textsuperscript{Cre+}) and Ly5.1+Id2\textsuperscript{+/+} mixed bone marrow chimeras were generated and infected with influenza virus (HKx31). This revealed that the proportions of virus-specific CD8\textsuperscript{+} T cells in Id2\textsuperscript{fl/fl} and wild-type compartments were similar in spleen and LNs (Fig. 1C, 1D). However, within the lung and liver, the relative frequency of Id2\textsuperscript{fl/fl} D\textsuperscript{NP}\textsubscript{366-} and D\textsuperscript{PA}\textsubscript{224-}specific CD8\textsuperscript{+} T cells was reduced, suggesting a defect in the ability to migrate to and/or survive in nonlymphoid tissues (Fig. 1D). KLRL1\textsuperscript{IL-7R}\textsuperscript{+} short-lived effectors were selectively absent from the Id2-deficient CD8\textsuperscript{+} T cell compartment in accordance with earlier observations (Fig. 1E, 1F, Supplemental Fig. 1) (3). The unaltered frequency of influenza-specific CD8\textsuperscript{+} 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\textsuperscript{+} T cell survival (1). In this earlier study, Id2-deficient OVA-specific TCR transgenic CD8\textsuperscript{+} 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\textsuperscript{fl/fl} (Ly5.2\textsuperscript{+}) and wild-type (Ly5.1\textsuperscript{+}Ly5.2\textsuperscript{+} F1) OT-I T cells transferred into Ly5.1\textsuperscript{+} recipients following i.v. Lm-OVA infection. Similar to previous results, Id2\textsuperscript{fl/fl} 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\textsuperscript{fl/fl} OT-I CD8\textsuperscript{+} 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\textsuperscript{low}KLRL1\textsuperscript{+}) CD8\textsuperscript{+} T cell subset to differentiate and/or survive. In contrast, memory precursor (IL-7R\textsuperscript{high}KLRL1\textsuperscript{+}) CD8\textsuperscript{+} T cells were unaffected showing that Id2 loss does not globally impair virus-specific CD8\textsuperscript{+} 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\textsuperscript{+} T cells within the wild-type and Id2\textsuperscript{fl/fl} CD8\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cells had significantly increased in proportion compared with wild-type cells (Fig. 2D), indicating that Id2 expression restricts memory recall potential of CD8\textsuperscript{+} 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\textsuperscript{+} 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
Id2-deficient CD8⁺ T cells adopt a gene expression signature characteristic of memory precursor cells

To gain insight into how Id2 regulates the lineage choice between effector and memory CD8⁺ T cells, we compared the gene expression profile of Id2⁺/⁻ and wild-type DⁿP₆₆₆⁺/-specific CD8⁺ T cells purified from spleen of Id2⁺/⁻:Ly5.1 mixed BM chimeras 9 d after HKS31 influenza virus infection by microarray analysis. To ensure that meaningful comparisons could be made between wild-type and Id2⁺/⁻ cells, KLRG1⁺ cells were excluded during FACS sorting. Two hundred thirty-three differentially expressed (DE) genes were identified (Fold change ≥ 2 and p value ≤ 0.05) (Supplemental Table II). Id2-deficient CD8⁺ T cells expressed significantly higher levels of key transcriptional regulators important for memory T cell differentiation including Id3, Tcf7 and Eomes (Fig. 4). In contrast, the expression of T-bet and Blimp1, two important regulators of effector T cell differentiation, were markedly reduced in absence of Id2 (Fig. 4B, 4C).

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 DⁿP₆₆₆⁺/-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). Id2-deficient CD8⁺ T cell frequency is reduced within nonlymphoid tissues (Fig. 4D). 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 DⁿP₆₆₆⁺/-specific effector CD8⁺ T cells purified according to their differential expression of Id2-GFP (Id2-GFP⁺⁰ and Id2-GFP⁺⁻) to microarray analysis and compared their gene expression profiles to the 233 DE genes identified by comparing Id2-deficient and wild-type DⁿP₆₆₆⁺/-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 absence of Id2 were also upregulated in Id2⁻/⁻ cells compared with Id2⁻/⁺ cells. Similarly, 83% of the DE genes observed to be downregulated in absence of Id2 were downregulated in Id2⁻/⁻ cells compared with Id2⁻/⁺ cells. Furthermore, expression of Eomes, Id3, and Tcf7 mRNA was higher on Id2-GFP⁺⁰ cells compared with Id2-GFP⁺⁻ cells, whereas GzmB expression was significantly reduced in Id2-GFP⁺⁰ cells compared with Id2-GFP⁺⁻ cells (Supplemental Fig. 2B, 2C). However, despite an increased expression of genes involved in memory formation, Id2-GFP⁺⁰ virus-specific CD8⁺ T cells did not exhibit increased long-
term survival compared with Id2-GFP\textsuperscript{high} virus-specific CD8\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cell differentiation by limiting the transcriptional activity of E2A. To investigate this question, we transduced wild-type and Id2\textsuperscript{fl/fl} OT-I T cells with retroviruses encoding either a shRNA targeting E2A or a control hairpin and then analyzed the expression of key target genes identified as DE in our previous microarray analysis. The Tcf2e2a shRNA induced an 80% reduction of Tcf2e2a expression, and this reduction was not compensated by an increased of the other E protein family members Tcf12 (encoding HEB) and Tcf4 (encoding E2.2) expression (Fig. 5A). Remarkably, silencing of Tcf2e2a in Id2\textsuperscript{lox/lox} 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 Tcf7, Id3, and Socs3 (2, 3, 9, 25) (Fig. 5A). In contrast, Id2\textsuperscript{lox/lox} OT-I T cells transduced with the Tcf2e2a 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 Tcf2e2a 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 Tcf2e2a\textsuperscript{Bio/Bio} 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\textsuperscript{BirA} allele. We then determined the genome-wide pattern of E2A binding in total thymocytes from Tcf2e2a\textsuperscript{Bio/Bio} mice, in which Id2 expression is low (27) and hence allows high E2A binding to its target genes. Using a stringent p value of $<10^{-10}$ 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\textsuperscript{+} 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 Tcf2e2a 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 Tcf7, Id3, Socs3, or Ccr7, whereas no binding sites were detected at the loci of genes associated with an effector phenotype 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.
E2A regulates Tcf7 expression in peripheral CD8+ T cells

Tcf7, 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). Tcf7 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 Tcf7 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 Tcf7 locus is expected to be inversely correlated to the level of Id2 expression in these different T cell subsets. Therefore, we then investigated whether Tcf7 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 Tcf7 transcription starting site), suggesting that E2A activates the Tcf7 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 Tcf7 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 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. 1B), the binding of E2A to the Tcf7 locus of peripheral CD8+ T cells (Fig. 6B), and the weaker binding sites was lost following activation and concurrent induction of Id2 expression (Fig. 6B), suggesting that E2A directly regulated Tcf7 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 Id2LckCre+ OT-I CD8+ T cells with retroviruses encoding T-bet or Id2 or with a control retrovirus. In vitro-activated transduced Id2LckCre+ 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
evaluated (Fig. 7C). Re-expression of Id2 into Id2-deficient OT-I cells rescued the development of KLRGB1 effector cells. Transduction of Id2-deficient OT-I T cells with the T-bet retrovirus resulted in the rescue of T-bet expression to a level similar to Id2-deficient CD8+ T cells transduced with the Id2 retrovirus (F. Masson and G.T. Belz, unpublished observations). Strikingly, Id2-deficient OT-I cells overexpressing T-bet that were then infected with Lm-OVA were capable of reinducing cell death in CD8+ T cells (28). Collectively, our data indicate that Id2 is required to induce sufficient Tbx21 expression to generate short-lived effector CD8+ T cells.

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 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 KLRGB1+ 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, Il2ra, Gzma, Gzmb, Gzmk, Cd103, Cx3cr1, Cd49a). 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

FIGURE 7. Enforced expression of Tbx21 rescues differentiation of Id2-deficient short-lived effector cells. (A and B) PR8-primed mixed bone marrow chimeras reconstituted with a mix of Tbx21 heterozygote (+/+; Ly5.2) and wild-type (+/+; Ly5.1) bone marrow cells were analyzed 9 d after HKx31 i.n. infection. (A) Histogram (left panel) and mean fluorescence intensity (MFI) (right panel) of expression of T-bet in naive (CD44+, black line) or activated DNP-specific CD8+ T cells gated on wild-type (Ly5.1+, blue line) or Tbx21+/- (Ly5.2+, red line) CD8+ T cell compartments from mixed bone marrow chimeras. Data show the means ± SEM (n = 11 animals pooled from three independent experiments). (B) Representative contour plots showing IL-7R and KLRG1 expression in DNP-specific CD8+ T cells. Statistically significant differences were determined using a paired two-tailed Student t test. (C-F) Wild-type (Ly5.1+) and Id2+/+LckCre+ (Ly5.2+) OT-I cells were transduced with control-GFP, Id2-GFP, or T-bet-GFP retroviruses. One week after activation, GFP+ cells were transferred into recipient mice that were then infected with Lm-OVA. Eight days after infection, mice were sacrificed and OT-I T cell response was analyzed in the spleen. (C) Flow cytometric analysis of KLRGB1 and GFP expression on transduced GFP+Id2+/+LckCre+ (Ly5.2+) OT-I T cells or wild-type (Ly5.1+) OT-I T cells 8 d after infection with Lm-OVA. Data are pooled from five to six animals for each condition from two independent experiments. (D) Analysis of the frequency of KLRGB1+ cells within the GFP+Id2+/+LckCre+ or wild-type OT-I T cells that had been transduced with the indicated vectors. Data show the mean percentage ± SEM for spleen with at least five mice for each group pooled from two independent experiments. (E) Bar graph shows the SEM of the experimental triplicates of the expression relative to Hprt. Data are representative of two independent experiments.

Discussion

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differentiation by inhibiting the E2A-mediated transactivation of several key genes involved in the process of memory differentiation such as Tcf7.

Tcf7 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). Tcf7 is highly expressed in naive CD8+ T cells and is downregulated in effector CD8+ T cells. We showed that both Tcf7 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 Tcf7 locus and this binding is lost following activation concurrent with the induction of Id2. Finally, silencing of Tcf2ea in Id2-deficient CD8+ T cells resulted in the downregulation of Tcf7 expression. Taken together, these data suggest that a major pathway by which Id2 restraints the formation of memory precursor cells is by inhibiting the E2A-dependent activation of Tcf7 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 Tcf2ea 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 cells in absence of 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 downregulated 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.

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Disclosures
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References


