Transcription factor ID2 prevents E proteins from enforcing a naïve T lymphocyte gene program during NK cell development

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All innate lymphoid cells (ILCs) require the small helix-loop-helix transcription factor ID2, but the functions of ID2 are not well understood in these cells. We show that mature natural killer (NK) cells, the prototypic ILCs, developed in mice lacking ID2 but remained as precursor CD27+CD11b− cells that failed to differentiate into CD27−CD11b+ cytotoxic effectors. We show that ID2 limited chromatin accessibility at E protein binding sites near naïve T lymphocyte–associated genes including multiple chemokine receptors, cytokine receptors, and signaling molecules and altered the NK cell response to inflammatory cytokines. In the absence of ID2, CD27+CD11b− NK cells expressed ID3, a helix-loop-helix protein associated with naïve T cells, and they transitioned from a CD8 memory precursor–like to a naïve-like chromatin accessibility state. We demonstrate that ID3 was required for the development of ID2-deficient NK cells, indicating that completely unfettered E protein function is incompatible with NK cell development. These data solidify the roles of ID2 and ID3 as mediators of effector and naïve gene programs, respectively, and revealed a critical role for ID2 in promoting a chromatin state and transcriptional program in CD27+CD11b− NK cells that supports cytotoxic effector differentiation and cytokine responses.

INTRODUCTION

Natural killer (NK) cells, the first innate lymphoid cells (ILCs) to be identified, are best known for their ability to kill virus-infected and cancer cells through direct cytotoxicity and their production of inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (1). NK cells are also key mediators of antibody-dependent cellular cytotoxicity and have been implicated in the control of immune system homeostasis through their ability to kill immature dendritic cells and T cells during the declining phase of the immune response (2). In contrast to the helper ILCs, which function as innate counterparts to distinct CD4 T cell subsets, NK cells appear to be the innate counterpart of CD8 T lymphocytes (1). CD8 T cells can be identified as naïve antigen–inexperienced cells, terminally differentiated effector (TE) cells that arise days after encounter with antigen, or memory precursor (MP) and memory cells, which persist long after an infection has been eliminated (3). In contrast, NK cells exist in a chronically “primed” state and can be rapidly induced to produce cytokines or to kill target cells. In humans, NK cells exist in two “flavors”: a cytokotic CD56+CD16− subset and a regulatory or “helper-like” CD56+CD16+ subset that produces large quantities of multiple cytokines but is less efficient in cytotoxicity (4). In the mouse, the majority of NK cells are capable of cytotoxicity, but cytokine only–producing NK cells have been identified in the thymus and as an intermediate in NK cell differentiation (5, 6). Three subsets of mature NK cells have been identified with a precursor-progeny relationship defined by the expression of CD27 and CD11b, progressing from CD27+CD11b− to CD27−CD11b+ to CD27−CD11b+ cells. The CD27−CD11b− subset is highly primed for cytokine production with low cytotoxic potential, whereas the CD27+CD11b+ subset also displays robust cytotoxicity (5). NK cells are also capable of forming memory cells that are similar to CD8 T effector memory cells, and these NK memory cells arise from KLRG1− progenitors, the majority of which are present in the CD27+CD11b+ population (7, 8).

The production of mature NK cells requires the coordinated activity of multiple transcription factors including the T-box transcription factors TBET and EOMES, as well as NFIL3, TOX1, ETS1, and ID2 (9). ID2 is a small helix-loop-helix protein that is expressed in all ILCs and is induced during the development of CD8 TE cells (10, 11). ID2 binds to the E protein transcription factors and prevents their ability to interact with DNA, although other targets of ID2 have been identified (12). The E proteins are essential for normal B and T cell development, and they regulate essential components of the adaptive lymphoid gene program (13, 14). Recently, ID2 was shown to regulate the survival of CD11b+ NK cells by preventing the E protein–mediated induction of the suppressor of cytokine signaling protein SOCS3, thereby promoting responsiveness to the cytokine interleukin-15 (IL-15) (15). However, when ID2 first becomes essential during NK cell development and whether its role is limited to the IL-15 response, we determined the cytokine.

Here, we used multiple models of ID2 deficiency in NK cells combined with gene expression profiling and ATAC-seq (assay for transposase-accessible chromatin with sequencing) to understand the mechanisms by which ID2 supports NK cell development. We found that ID2 limited the expression of genes encoding multiple T cell–associated chemokine receptors, cytokine receptors, and signaling molecules and limited DNA accessibility at E protein binding sites near these genes.
genes. A comparison of ATAC-seq data from ID2-deficient or ID2-sufficient NK cells and CD8 T cell subsets revealed that ID2 maintained a CD8 MP-like chromatin accessibility program in CD27⁺CD11b⁻ NK cells and prevented E proteins from promoting a state akin to that of naïve CD8 T cells. We also found that the ID2-repressed naïve-like state required ID3, a protein that maintains the naïve program in T lymphocytes (16). Our findings reveal a critical role for ID2 in preventing a naïve-like chromatin state in NK cells, a role that may be relevant for other members of the ILC family.

RESULTS

**ID2 promoted the development of CD11b⁺ NK cells**

To gain insight into the functions of ID2 in NK cells, we examined Gzmb-Cre Id2⁻/⁻ mice on a C57BL/6 Rag1⁻/⁻ background (hereafter called RId2⁻/⁻ mice). In these mice, Id2 was broadly deleted in all hematopoietic cells including in the bone marrow (BM), spleen, and hematopoietic stem and progenitor cells, consistent with the ability of GzmB-Cre to delete in hematopoietic stem cells (17, 18). Liver ILC1 and BM ILC2 were absent in RId2⁻/⁻ mice, confirming the broad deletion of Id2 (fig. S1, A to D). Consistent with studies in Id2⁺/⁺ mice (19, 20), the frequency and number of NK cells were markedly reduced in the spleen of RId2⁻/⁻ mice compared with control Rag1⁻/⁻ mice (Ctrl) (Fig. 1, A and B). Liver NK cells were also reduced in the absence of ID2 (fig. 1A). In contrast, BM NK cell frequencies and numbers were similar to Ctrl (Fig. 1, A and B). RId2⁻/⁻ BM NK cells expressed EOMES, confirming that they were NK cells and not ILC1 and they had deleted Id2 as determined by polymerase chain reaction (PCR) of genomic DNA (Fig. 1, C and D).

We next tested whether RId2⁻/⁻ NK cells matured normally by examining CD27 and CD11b. In contrast to Ctrl NK cells, the majority of RId2⁻/⁻ NK cells in the BM, spleen, and liver were CD27⁺CD11b⁻, and there were few cells that were CD27⁺CD11b⁺ or CD27⁺CD11b⁻ (Fig. 1E). The total number of CD27⁺CD11b⁻ NK cells in the BM and spleen was similar to or greater than that in Ctrl mice (Fig. 1F). CD27⁺CD11b⁺ and CD27⁺CD11b⁻ NK cells also failed to develop in chimeric mice in which RId2⁻/⁻ hematopoietic cells were in competition with CD45.1⁺ wild-type (WT) cells, indicating that the arrest in differentiation was intrinsic to RId2⁻/⁻ NK cells (Fig. 1, G and H). RId2⁻/⁻ CD27⁺CD11b⁻ NK cells were present in the BM and spleen of chimeric mice despite the presence of WT competitor cells (Fig. 1, G and H). A similar arrest in NK cell maturation was observed when Id2 was deleted at the mature NK cell stage using Ncr1Cre, regardless of whether the mice were Rag1⁻/⁻ or Rag1⁺/⁺ (fig. S1, D to H). Together, our data indicate that mature NK cells can develop in the absence of ID2, but they have an intrinsic inability to differentiate into CD11b⁺ cells.

**RId2⁻/⁻ BM CD27⁺CD11b⁻ NK cells displayed a differentiation-associated quiescence program but retained IL-15 responsiveness**

We examined gene expression profiles in RId2⁻/⁻ and Ctrl NK cells to gain insight into the role of ID2 in NK cell differentiation. Because CD11b⁺ NK cells were absent in RId2⁻/⁻ mice, we compared gene expression in RId2⁻/⁻ and Ctrl BM CD27⁺CD11b⁻ NK cells. We identified 351 and 1070 probe sets that were differentially expressed by more than 2- or 1.5-fold (P < 0.05), respectively, with a majority showing decreased expression in RId2⁻/⁻ cells (Fig. 2A). The transcripts that were decreased that enriched for the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways “Cell Cycle” (P = 2.5 × 10⁻¹⁶) and “DNA Repair” (P = 3.3 × 10⁻¹¹) as determined by the DAVID Functional Annotation Tool (21, 22). Previously published data revealed a set of cell cycle genes that decreased during NK cell differentiation (5), all of which were decreased in RId2⁻/⁻ CD27⁺CD11b⁻ NK cells, suggesting that this program was intact but was dissociated from CD11b expression (Fig. 2B). One of the genes in this program encodes the cell cycle indicator KI67, and fluorescence-activated cell sorting analysis revealed that fewer CD27⁺CD11b⁻ NK cells from the BM of RId2⁻/⁻ expressed KI67 as compared with Ctrl mice (Fig. 2, C and E). In contrast, there was no difference in the frequency of KI67⁺ cells among NK cells in the spleen (Fig. 2, D and F). The majority of BM and spleen CD27⁺CD11b⁻ NK cells were KI67⁺ 3 days after injection of IL-2 or polyI:C (polynosinic-polycytidylic acid), indicating that RId2⁻/⁻ NK cells were able to enter the cell cycle in response to IL-2 or cytokines elicited from dendritic cells after polyI:C treatment (Fig. 2, C to F).

ID2 was reported to maintain responsiveness to IL-15 in NK cells through repression of Socs3 (15). Our gene expression analysis did not reveal any difference in Socs3 or Mcl1, which codes for a critical anti-apoptotic protein induced by IL-15 (23), in RId2⁻/⁻ CD27⁺CD11b⁻ NK cells, and we confirmed this by quantitative real-time PCR (qRT-PCR; Fig. 2G). To further address whether RId2⁻/⁻ cells could respond to IL-15, we undertook a rigorous analysis of their sensitivity to this cytokine in vitro. Ctrl and RId2⁻/⁻ CD27⁺CD11b⁻ NK cells expanded equivalently, had similar DNA synthesis as measured by 5-bromo-2'-deoxyuridine (BrdU), and showed a similar frequency of KI67⁺ cells at multiple concentrations of IL-15 in vitro (fig. S2A, and Fig. 2, H and I). Activation of the mTOR (mammalian target of rapamycin) pathway, as revealed by pS6K, was also similar in RId2⁻/⁻ and Ctrl CD27⁺CD11b⁻ NK cells in vitro (fig. S2, B and C). Therefore, although ID2 may be required for IL-15–dependent survival of CD27⁺CD11b⁻ NK cells, RId2⁻/⁻ CD27⁺CD11b⁻ NK cells, as well as Ctrl cells, responded to IL-15 in vitro.

**ID2 promoted the acquisition of an NK cell effector gene program**

To further test the hypothesis that ID2 promotes NK cell effector differentiation, we examined our gene expression data for transcripts associated with differentiation and the cytotoxic effector program (5). The RId2⁻/⁻ CD27⁺CD11b⁻ NK cells expressed less mRNA than Ctrl NK cells for many of these genes including Gzma, Gzmb, Gzmk, Spi6, and Serpinb9 (Fig. 3A). We confirmed the decreased expression of Gzma, Gzmb, and Gzmk by qRT-PCR, and we also found that mRNA for PERFORIN (Prf1) and the transcription factor TBET (Tbx21) were decreased (Fig. 3B). Il18r1, encoding a component of the IL-18 receptor, was also decreased, and both TBET and IL18R1 proteins were decreased in RId2⁻/⁻ CD27⁺CD11b⁻ NK cells (Fig. 3, A and C). Klrg1, which encodes an activating receptor associated with effector differentiation, was decreased in RId2⁻/⁻ cells, and we confirmed that KLRC1 was lower on these cells (Fig. 3, A and C). Therefore, ID2 promotes the expression of genes associated with NK cell differentiation and cytotoxic function already in CD27⁺CD11b⁻ NK cells.

**ID2 was required for the expansion of NK cells in response to mouse cytomegalovirus**

NK cells play an important role in virus detection, and their expansion after mouse cytomegalovirus (MCMV) infection is promoted by IL-12, IL-18, and IL-33 (8, 24, 25). Given that RId2⁻/⁻ NK
cells have reduced expression of \textit{Il18r1} and \textit{Il1rl1} (Fig. 3A), we questioned whether they could expand in vivo in response to MCMV.

To address this question, we took advantage of an assay in which equivalent numbers of CD45.2+ Rld2−/− (or Rag1−/− Ctrl) and CD45.1+ WT NK cells were adoptively cotransferred into Ly49H-deficient mice followed by infection with MCMV (26). In mice that received CD45.2+ Ctrl NK cells, there was robust detection of CD45.2+ and CD45.1+ NK cells at day 7 after infection (Fig. 3, D and E). In contrast, in mice that received CD45.2+ Rld2−/− NK cells, only the CD45.1+ NK cell population could be detected 7 days after infection.
ID2 prevented T lymphocyte–associated gene expression in CD27<sup>-</sup>CD11b<sup>-</sup> NK cells

To gain further insight into how deletion of Id2 affected the NK cell expression program, we performed gene set enrichment analysis (28, 29). This analysis revealed that Rld2<sup>−/−</sup> cells were enriched for genes that are increased in naïve as compared with memory CD4 T cells and in memory as compared with effector CD8 T cells, in addition to other T cell–associated programs (Fig. 4A and table S1). KEGG pathway analysis revealed that Rld2<sup>−/−</sup> cells had increased expression of genes associated with “cytokine–cytokine receptor interaction” (P = 3.6 × 10<sup>-3</sup>), “JAK-STAT signaling” (P = 4.8 × 10<sup>-3</sup>), and “T cell receptor signaling” (P = 7.5 × 10<sup>-3</sup>). Metascape analysis identified similar pathways and multiple gene ontology terms, including “T cell differentiation” and “T cell activation” (table S2) (30), categories that included cytokines and cytokine receptors, signaling molecules, and T cell–associated genes (Fig. 4B). We confirmed the up-regulation of Cd3g and Cd3d mRNA by qRT-PCR (Fig. 4C) and CXCR5, CXCR3, IL-4R<sub>α</sub>, and CD27 on Rld2<sup>−/−</sup>CD27<sup>CD11b<sup>-</sup></sup> NK cells by flow cytometry (Fig. 4D). As demonstrated previously, we found a subtle but consistent increase in TCF1 by flow cytometry (Fig. 4E) (15). Together, our data indicate that ID2 limits the expression of multiple genes whose protein products are associated with naïve or central memory T lymphocytes.

ID2 modulated chromatin accessibility near genes associated with chemokine and cytokine receptor and T cell receptor signaling

To determine how ID2 controls the gene expression program of NK cells, we compared chromatin accessibility in Rld2<sup>−/−</sup> and Ctrl CD27<sup>CD11b<sup>-</sup></sup> NK cells using ATAC-seq (31). We identified 76,523 regions of open chromatin in Ctrl and Rld2<sup>−/−</sup>CD27<sup>CD11b<sup>-</sup></sup> NK cells, most of which were overlapping. However, 7070 (9.25%) of these regions showed more pronounced accessibility in Ctrl than in Rld2<sup>−/−</sup> cells, whereas 4419 (5.8%) were more accessible in the Rld2<sup>−/−</sup> cells (twofold q < 0.05). The regions of chromatin that were more accessible in Rld2<sup>−/−</sup> cells generally showed less accessibility in Ctrl CD27<sup>CD11b<sup>+</sup></sup> (CD11b<sup>+</sup>) cells, and vice versa.

(Fig. 3, D and E), despite the fact that CD27<sup>CD11b<sup>-</sup></sup> NK cells generally expand more robustly than CD27<sup>CD11b<sup>+</sup></sup> NK cells in response to MCMV (8, 27).
indicating that these regions are closed with NK cell differentiation (Fig. 5A). K-means clustering of these regions revealed five clusters with decreased accessibility upon differentiation from CD27<sup>+</sup>CD11b<sup>-</sup> to CD27<sup>-</sup>CD11b<sup>+</sup> cells (Fig. 5B). These regions were positively correlated with genes that had increased expression in Rld2<sup>−/−</sup> NK cells compared with Ctrl (Fig. 5C). This correlation was evident even when the accessible DNA was 5 to 100 kb away from the transcription start site but was lost at greater distances (Fig. 5C). Therefore, ID2 limited chromatin accessibility at regions that decreased in accessibility as NK cells differentiated and ID2 repressed expression of the associated genes.

To determine the mechanisms by which ID2 repressed chromatin accessibility, we examined the regions with increased accessibility near (<5 kb) genes that increased expression in Rld2<sup>−/−</sup> cells for enriched transcription factor binding motifs using HOMER, as compared with regions that were accessible in Rld2<sup>−/−</sup> and Ctrl CD27<sup>+</sup>CD11b<sup>-</sup> cells. All of the significantly enriched known motifs shared the canonical CANNTG E box binding motif, with the most significantly enriched motif (P < 1 × 10<sup>−13</sup>) being the classic E protein binding site (Fig. 5D) (32). The de novo HOMER motifs also included a highly ranked...
**Fig. 5.** ID2 represses chromatin accessibility at T cell–associated genes by preventing E protein binding. (A) ATAC-seq was used to identify regions of open chromatin in Rld2−/− (red) and Ctrl CD27+CD11b− NK cells and in Ctrl CD27−CD11b+ (green, labeled as CD11b+) NK cells. The graph shows normalized reads at the indicated distance from the center of open chromatin domains that had increased (left graph) or decreased (right graph) accessibility in Rld2−/− as compared with Ctrl CD27+CD11b− NK cells. (B) Heat map and K-means clustering of the regions analyzed in (A) with increased or decreased accessibility in Rld2−/− as compared with Ctrl CD27+CD11b− NK cells. (C) Fraction of genomic regions that showed increased accessibility (red), decreased accessibility (blue), or no change in accessibility (gray) in Rld2−/− CD27+CD11b− NK cells and were associated with genes that increased (left plot) or decreased (right plot) expression by greater than twofold. Black represents the background/null distribution (all genes in the genome). (D) HOMER motif enrichment at regions with increased accessibility near genes that increased expression in Rld2−/− compared with Ctrl NK cells. (E) Normalized reads for open chromatin (left) and nucleosome differential (right) centered over E box motifs associated with genes that increased expression in Rld2−/− NK cells. (F) Accessible chromatin at the Cxcr3, Cxcr5, and Tgfbr1 genes in Rld2−/− (orange) and Ctrl (blue) CD27+CD11b− NK cells and Ctrl CD11b+ NK cells (green) viewed in IGV. Black arrows indicate regions that are more accessible in Rld2−/− compared with Ctrl. (G) Same as (F) but for the Socs3 gene.
E box motif (Fig. 5D). The five known E box motifs were present in 747 of 1025 (73%) ID2-regulated accessible chromatin regions (table S3). Analysis of chromatin accessibility surrounding the E box motifs confirmed increased accessibility in Rld2−/− cells compared with Ctrl CD27+CD11b− cells, and these regions were less accessible in Ctrl CD27+CD11b− cells (Fig. 5E). Furthermore, analysis based on fragment length revealed that these regions had greater nucleosome depletion in Rld2−/− cells, whereas nucleosomes frequently occupied these sites in CD27+CD11b+ cells (Fig. 5E). KEGG pathway analysis of the genes associated with the E box motifs revealed the categories of “Cytokine-cytokine receptor interaction” (P = 3.6 × 10−13), “JAK-STAT signaling” (P = 5 × 10−15), “TCR signaling” (P = 2 × 10−10), and “MAP kinase signaling” (P = 2.4 × 10−7), overlapping with the categories of genes that increase in expression in the Rld2−/− cells. We confirmed the increased chromatin accessibility at the Ccxc3, Ccxc5, and Tgfbr1 genes, among others, in Rld2−/− NK cells, and in most cases, these regions showed less accessibility in Ctrl CD27+CD11b+ cells (Fig. 5F). Socs3 did not show altered chromatin accessibility in Rld2−/−CD27+CD11b− NK cells (Fig. 5G). Together, our data indicate that ID2 controls the activity of E proteins to limit the expression of multiple T cell–associated genes in CD27+CD11b+ NK cells.

Rld2−/− NK cells required AP-1 activation for IFN-γ production

We also examined the chromatin that showed decreased accessibility in Rld2−/− compared with Ctrl CD27+CD11b− cells and found that these were, on average, only slightly decreased during NK cell differentiation (Fig. 5A). Only one of five K-means clusters (Dd) showed reduced accessibility in CD11b− NK cells, indicating that most of these regions remain accessible during NK cell differentiation (Fig. 5B). The chromatin with decreased accessibility in Rld2−/− NK cells was not significantly enriched at genes that had decreased expression in these cells (Fig. 5C). Despite this, HOMER analysis of the regions that had decreased accessibility within 5 kb of genes that decreased in expression revealed an enrichment for AP-1/Batf binding motifs (fig. S3A). Analysis of these regions centered on the AP-1 motif revealed a subtle decrease in accessibility but no change in nucleosome occupancy in Rld2−/− as compared with Ctrl CD27+CD11b− NK cells (fig. S3B). These observations are consistent with the hypothesis that these AP-1 motifs remain accessible in the absence of ID2 but slightly less so than in Ctrl CD27+CD11b− NK cells, possibly due to less transcription factor binding.

HOMER found AP-1 binding sites in regions of decreased chromatin accessibility at the Ifng gene (fig. S3E). AP-1 was implicated in the regulation of Ifng in NK cells downstream of NK cell receptors that have the immunoreceptor tyrosine-based activation motif, which activates AP-1 via protein kinase C-δ (PKCδ) (33). To determine whether AP-1 activation could promote IFN-γ production in Rld2−/− NK cells, we used phorbol 12-myristate 13-acetate (PMA) to activate PKC-δ. This treatment resulted in the rapid production of IFN-γ from both Rld2−/− and Ctrl CD27+CD11b− NK cells (fig. S3, C and D).

We previously demonstrated that Id2−/− NK cells had a reduced ability to produce IFN-γ in response to IL-2 + IL-12 (29). Because NK cell differentiation stage was not considered in those experiments, we reevaluated whether CD27+CD11b− NK cells from Rld2−/− and Ctrl mice produced IFN-γ under these conditions and found that Rld2−/− NK cells produced substantially less IFN-γ than Ctrl cells (fig. S3). IL-12 promotes Ifng transcription through activation of STAT4 (signal transducer and activator of transcription 4) (34); however, our data raised the possibility that Rld2−/− NK cells needed AP-1 for Ifng transcription. AP-1 is also induced by IL-18, a cytokine required for the primed state of NK cells (35). Treatment of Rld2−/− NK cells with IL-18 and IL-12 resulted in robust IFN-γ production, suggesting that AP-1 was required for IFN-γ production in Rld2−/− NK cells (fig. S3, C and D). These data indicate that Rld2−/− NK cells can produce IFN-γ under conditions that lead to AP-1 activation.

ID2 prevented NK cells from acquiring a chromatin accessibility state associated with naïve CD8 T cells

Our data suggest that ID2 prevents the E proteins from augmenting expression of multiple T cell genes that are associated with naïve or memory CD8 T cells. To further investigate the role of ID2 in NK cells, we compared our ATAC-seq data with recently reported ATAC-seq data from naïve, TE, and MP CD8 T cells isolated from mice transplanted with OT-1 CD8 T cells before infection with Listeria monocytogenes engineered to express ovalbumin (36). We first examined genes that were up-regulated in Rld2−/− NK cells, such as Il4ra, Cd3g, and Cd3d, and identified regions that increased accessibility in Rld2−/− as compared with Ctrl CD27+CD11b− cells and decreased accessibility upon NK cell differentiation (Fig. 6, A and B). When compared with the CD8 T cell populations, Rld2−/− NK cells had chromatin accessibility patterns that were evocative of naïve CD8 T cells. On the basis of these observations, we hypothesized that ID2 promotes a TE- or MP-like chromatin landscape in CD27+CD11b− NK cells and prevents a naïve chromatin context.

To determine whether comparison of these populations could reveal, on a larger scale, relationships between the subsets, we determined whether the accessible chromatin regions in Ctrl CD11b− cells as compared with CD27+CD11b− cells (differing by log2, P < 0.05) revealed an association with CD8 TE, as would be expected. These regions were more accessible in TE as compared with naïve or MP (fig. S4A). In contrast, regions that were more accessible in CD27+CD11b− NK cells were frequently more accessible in naïve and MP cells than in TE cells (fig. S4B). When a similar analysis was performed for regions that were more accessible in Rld2−/− NK cells than in Ctrl CD27+CD11b− NK cells, we found that these regions were more accessible in naïve as compared with MP or TE (fig. 6C). In contrast, regions that were more accessible in Ctrl CD27+CD11b− NK cells compared with Rld2−/− NK cells were more accessible in TE than naïve or MP and in MP as compared with naïve CD8 T cells. These data are consistent with the failure of Rld2−/− cells to activate the CD8 TE-like program and indicate that ID2 prevents E proteins from promoting a naïve-like chromatin landscape in CD27+CD11b− NK cells.

We also note that in all of our comparisons, NK cells had a substantial number of differentially accessible regions that were not differentially accessible between any CD8 T cell subsets. This observation was due, in part, to the stringency of our assignment of changes in accessibility because associations were more striking when we reduced the magnitude of differential accessibility or the statistical significance (fig. S4, C and D). However, even in this less stringent analysis, there were numerous differentially accessible peaks in Rld2−/− NK cells as compared with Ctrl, or Ctrl CD27+CD11b− compared with CD27+CD11b− NK cells that were not differentially accessible between CD8 T cell subsets, consistent with the distinct lineage derivation and functional capacities of NK cells and CD8 T cells (fig. S4).

ID3 was required for development of ID2-deficient NK cells

One of the up-regulated genes in Rld2−/− NK cells was Id3 (Fig. 4B), which codes for an ID2-related protein (12). We identified an E box–containing
chromatin region downstream of Id3 that was more accessible in Rld2−/− NK cells than Ctrl that was not accessible in any T cell population (Fig. 7A), although Id3 mRNA is expressed in both naïve and memory CD8 T cells (16, 37, 38). In T cells, ID3 is most highly expressed in naïve cells and maintains the naïve state (16, 36). To determine whether ID3 was required in Rld2−/− NK cells, we examined NK cells from Il7raCreId2f/f (LId2−/−), Il7raCreId2f/fId3−/− (RId2−/−Id3−/−), and Ctrl (Il7raCre or Id2f/f) BM after transplantation into lethally irradiated CD45.1 mice. We used a transplantation approach for these experiments because we found previously that the γδNKT-like cells present in Id3−/− mice repressed NK cell development, but these γδNKT cells failed to develop in BM chimeras. Using this approach, we found that ID3 was not required for NK cell development, consistent with its exceedingly low expression in Ctrl NK cells (Fig. 7B and fig. S5, A to D) (39). Under these transplantation conditions, LId2−/− BM generated a slightly reduced frequency of Lin−CD122+ cells compared with Ctrl BM, and about half of these cells were donor-derived (CD45.2+) compared with nearly 80% in Ctrl BM transplanted mice. The NK cells that developed in Lld2−/− chimeras were arrested at the CD27+CD11b− stage (Fig. 7C). The percent reconstitution for Rld2−/− NK cells, determined as the percent of CD45.2+ cells among Lin−CD122+ NK1.1+DX5+ cells, was about half of that in mice transplanted with Ctrl BM (Fig. 7D). The percent reconstitution in the spleen was better than in the BM, but Lld2−/− NK cells continued to be arrested at the CD27+CD11b− stage (Fig. 7, E and F). Chimeric mice established with Lld2−/−Id3−/− BM, in contrast to Lld2−/− BM, showed a near complete lack of CD45.2+ NK cells in both the BM and spleen (Fig. 7, C to F). Together, these data demonstrate that ID3 is required for the development of ID2-deficient NK cells.

DISCUSSION

Here, we demonstrated that a major function of ID2 is to maintain an “MP-like” chromatin state and gene program in CD27+CD11b− NK cells when the cells are capable of innate-like cytokine responses and are preparing for differentiation into cytotoxic effector cells. In the absence of ID2, NK cells up-regulated numerous genes associated with naïve CD8 T cells, resulting in a shift in chemokine and cytokine receptor expression and intracellular signaling proteins, and the cells lost their ability to produce IFN-γ in response to IL-2 + IL-12. Our analyses indicate that E proteins are the major ID2 target in NK cells and imply that the observed alterations in chromatin accessibility are due to aberrant E protein–mediated recruitment of chromatin-modifying and chromatin-remodeling proteins such as P300/CBP and Mi-2 to the regulatory regions of genes expressed in naïve CD8 T cells (40–42). We identified multiple putative E protein target genes in CD27+CD11b− NK cells including Id3, and we demonstrated that ID3 was essential for the development of ID2-deficient NK cells. Together, our data support the hypothesis that ID2 enforces the MP-like characteristics of CD27+CD11b− NK cells and support their differentiation into cytotoxic effector cells by limiting transcription of the naïve gene program.

Id2−/− CD8 T cells resemble central memory cells, with increased expression of Cxcr3, Cxcr5, and Tgfbr1, and they fail to differentiate into KLRG1+ effector cells even when their survival is enhanced.
Despite the increased expression of these same genes, our data indicate that the KLRG1− NK cells present in Rld2−/− mice are more closely related to naïve CD8 cells than to MP cells based on a comparison of sites of differential chromatin accessibility in Rld2−/− and Ctrl CD27+CD11b− NK cells with those in naïve, TE, and MP CD8 T cells (36). We also examined the changes in chromatin accessibility as NK cells mature from CD27+CD11b− to CD27−CD11b+ effector cells and found that these populations are most closely related to CD8 MP and TE cells, respectively. However, our analysis revealed many differences between the chromatin landscape of NK cells and CD8 T cells, suggesting that the distinct developmental trajectories of these cells, or their complement of transcription factors and chromatin remodelers, resulted in the use of overlapping and unique chromatin regions to control gene expression. This conclusion is consistent with previous studies comparing mature innate and adaptive lymphoid cell chromatin landscapes (44, 45).

Rld2−/− NK cells also resemble naïve CD8 T cells in that they have reduced accessibility at chromatin regions with potential AP-1 binding sites (46), as exemplified by regions at the Ifng locus. IFN-γ was provoked in Rld2−/− NK cells by factors that activate AP-1 (PMA + ionomycin or IL-18 + IL-12) but not in the absence of AP-1 activation (IL-2 + IL-12). The reduced AP-1 activation may be an indirect consequence of the naïve gene program. Rld2−/− NK cells have reduced expression of IL-18R, which primes NK cells for innate cytokine responsiveness and activates AP-1, as well as alterations in multiple surface receptors and signaling pathways. Although IL-18R expression was low on Rld2−/− NK cells, addition of excess IL-18 allowed for IFN-γ production in response to IL-12; however, the in vivo concentration of IL-18 may be insufficient for priming of NK cells with low expression of IL-18R. Alternatively, Rld2−/− NK cells might not reside in their normal niche and therefore fail to receive signals that activate AP-1. Understanding the mechanisms that link ID2 to the innate cytokine responsive state may reveal mechanisms to control NK cell cytokine production in vivo.

Our study provides insight into the direct targets of E proteins in NK cells. Many of these genes are expressed at the earliest stages of NK cell development, when Id2 mRNA levels are lower than in CD27+CD11b+ NK cells. However, it remains to be determined whether E proteins are required for expression of these genes during normal NK cell development. Cxcr5 appears to be a universal E protein target, because it is directly induced in multiple cell types in which E protein function is elevated (47, 48). Cxcr5 is expressed on subsets of NK cells and may allow these cells to enter germinal centers where they can affect antibody production by B cells, similar to what was recently described for CD8 T cells (49, 50). Id2 mRNA is regulated by multiple cytokines, raising the possibility that CXCR5 expression on NK cells could be a consequence of E protein activity. We note that some well-known E protein targets in early B cell development, such as Ebf1 and Rag2, were not expressed in Rld2−/− NK cells, suggesting...
that these loci were inaccessible to E proteins or that additional transcription factors were needed to induce their expression. In addition, and consistent with the naïve state of these cells, critical CD8+ T cell central memory genes were not up-regulated in Rld2−/− CD27+CD11b− NK cells, including Sell, Ccr7, and Il7ra, indicating that these genes are not direct E protein target genes, at least in NK cells.

It was reported that ID2 promotes NK cell survival by preventing E protein–mediated induction of Socs3. We found no evidence for induction of Socs3 in Rld2−/− NK cells and, despite their otherwise naïve state, Rld2−/− NK cells were able to proliferate in response to IL-2 and IL-15 as well as Ctrl cells. However, we cannot exclude the possibility that ID2 represses Socs3 in differentiated CD11b+ cells. Our conclusions are limited to ID2 function in CD27+CD11b− NK cells because we have not examined the consequences of Id2 deletion in CD11b+ NK cells. Moreover, although our ATAC-seq data demonstrate increased accessibility at E protein binding motifs, they do not directly demonstrate E protein binding and they do not reveal which E proteins bind to these motifs. Another potential caveat to our study is that many of our experiments were performed in Ragi−/− mice, in which NK cells have increased access to IL-2 and IL-15, and may differ from NK cells in Ragi+/+ mice in multiple ways (27). Nonetheless, our basic observations, including alterations in cell surface receptors, induction of IFN-γ by IL-18 + IL-12, and the failure to differentiate into CD11b+ NK cells, were observed in Ncr1−/−Id2−/− mice on a Ragi1−/− background.

In contrast to Socs3, we found increased Id3 mRNA and increased chromatin accessibility near the Id3 gene in Rld2−/− NK cells. Moreover, ID3 was critical for the generation of ID2-deficient NK cells. These findings raise two important questions: (i) Why is chromatin accessibility near E protein DNA binding motifs not inhibited by ID3? (ii) Why is ID3 not able to replace ID2 to promote the NK cell “MP-like” state in CD27−CD11b− NK cells? We propose that the answer to both of these questions lies in the mechanisms of Id2 and Id3 transcription. E proteins likely regulate Id3, and therefore ID3 concentration is limited by its ability to inhibit the E proteins. E protein DNA binding must be balanced by ID3 at a level that favors sufficient E protein DNA binding to promote Id3 transcription. In contrast, ID2 can completely inhibit E proteins with no consequence for its own expression. Therefore, our data demonstrate that NK cell development requires tight regulation of the balance between E and ID proteins to maintain the MP-like state in CD27−CD11b− NK cells and to promote cytotoxic effector maturation.

**MATERIALS AND METHODS**

**Study design**

The aim of this study was to determine how deletion of Id2 affected the development of NK cells. To do this, we created mice in which Id2 was deleted in all hematopoietic cells because the Id2 germline mutation is lethal when it is on a C57BL/6 background. The model we have studied has the genotype Gzmb-Cre Rag1−/− Id2−/−, on a C57BL/6 background, and is referred to as Rld2−/−, although we have confirmed most of our observation in Ncr1−/−Id2−/− and Il7rα−/−Id2−/− mice that are Ragi+/+. We used these mice to investigate NK cell development by flow cytometry, combined with analysis of gene and protein expression, and chromatin accessibility by ATAC-seq. We were careful to compare cells at a similar stage of development, in particular, in CD27−CD11b− NK cells. All experiments were performed independently at least three times as indicated in the figure legends.

**Animals**

Rag1+ Gzmb-Cre Id2−/−, Il7rα−/−Id2−/−Id3−/−, Id3−/−, Ncr1−/−Id2−/−, B6.SJL-Ptprca, and various control mice were housed at the University of Chicago under the guidelines of the University of Chicago Institutional Animal Care and Use Committee (17). Klrb−/− (Ly49H-deficient) and B6.SJL-Ptprca mice were housed at the Memorial Sloan Kettering Cancer Center under the guidelines of their Animal Use Committee (51). All mice were on a C57BL/6 background.

**Antibodies and flow cytometry**

BM cells, splenocytes, and liver cell suspensions were incubated with FcBlock before the addition of biotinylated or fluorochrome-conjugated antibodies (fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PEcy7, peridinin chlorophyll protein (PerCP)–cy5.5, APC-eAF780, and Brilliant Violet 421). Antibodies were purchased from eBioscience, BD Pharmingen, or BioLegend. KL6 and BrdU staining was performed using a FoxP3 Fix/Perm staining kit (BioLegend).

**Cytokine stimulation assays**

DX5+ cells were isolated from the BM or spleen by MACS (Miltenyi Biotec) and cultured overnight in the indicated conditions [IL-2 (1000 IU/ml), IL-12 (10 ng/ml), IL-18 (100 ng/ml), PMA (20 ng/ml), or ionomycin (1 μg/ml) with the inclusion of brefeldin A (1:1000) for the last 5 hours]. In vivo stimulation was performed by injecting 500 IU of IL-2 or 150 μg of polyI:C followed by analysis 3 days later. Cytokines were purchased from R&D Systems and PeproTech.

**BM chimeric mice**

CD45.2+ Rld2−/− BM cells were mixed with CD45.1+ Rag1+/+ BM cells at a 1:1 ratio. A total of 5 × 10⁶ cells were injected into lethally irradiated CD45.1+ hosts through the retro-orbital vein. At 8 weeks after reconstitution, the frequency of CD45.1− NK cells in the BM and spleen was determined. In one of three experiments, Rag1+/+ Ctrl NK cells were also injected into CD45.1+ hosts along with CD45.1+ Rag1+/+ BM cells. For analysis of Id3−/− mice, 2 × 10⁶ BM cells were transplanted into lethally irradiated CD45.1+ or CD45.1−CD45.2+ mice and analyzed between 6 and 8 weeks after transplantation.

**Adoptive transfer and MCMV infection**

Splenocytes were treated with anti-CD4 (GK1.5), anti-CD8 (53.6.72), anti-CD19 (1D3), anti-Ter119, and anti-rat immunoglobulin G–coupled magnetic beads to deplete T cells, B cells, and erythrocytes on magnetic columns (Miltenyi Biotec). Enriched Ly49H− NK cells from B6.SJL (CD45.1+) and Ctrl or Rld2−/− (CD45.2+) donors were mixed in equal numbers, and approximately 3 × 10⁶ total Ly49H− NK cells were cotransferred into adult Ly49H−/− recipients. On the following day, recipients were infected with 750 PFU (plaque-forming units) of MCMV (Smith strain; obtained from the salivary glands of MCMV-infected Balb/c mice) via intraperitoneal injection, as previously described (26). Splenocytes were examined for CD45.1+ or CD45.2+ NK cells by flow cytometry 7 days after infection.

**Microarray analysis**

Five thousand CD27−CD11b− NK cells were sorted from the BM of Ctrl and Rld2−/− mice, and RNA was isolated using the RNeasy Micro Kit (Qiagen). Complementary DNA was prepared to probe Affymetrix MOE 430 2 arrays as previously described (52). Three independent samples of each cell type were examined. Raw data were normalized
using RMAExpress, and heat maps and gene expression were created using dChip (53, 54).

**Assay for transposase-accessible chromatin with sequencing**

ATAC-seq was performed as described (31). Briefly, 50,000 BM CD27+CD11b+ NK cells from Rld2−/− and Ctrl mice and CD27+CD11b+ NK cells from Ctrl mice were sorted and used for each ATAC-seq assay (two assays). Nuclei were isolated by lysing the cells with cold lysis buffer [10 mM tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.1% IGEPA PAL CA-630] followed by centrifugation at 500g for 10 min at 4°C. The supernatant was carefully removed, and the pellet was resuspended in transposase reaction mix [25 μl of 2x Tagment buffer, 2.5 μl of Tagment DNA enzyme (FC-121-1030, Illumina), and 22.5 μl of nuclease-free water] and incubated at 37°C for 30 min. After transposition, the sample was purified with a Qiagen MinElute kit, and library fragments were amplified using Nextera PCR Primers (Illumina Nextera Index kit) and NEBNext PCR Master Mix (0541, New England BioLabs) for a total of 10 to 12 cycles followed by purification with a Qiagen PCR Cleanup kit.

The amplified, adaptor-ligated libraries were size-selected with Life Technologies E-Gel SizeSelect gel system in the range of 150 to 650 base pairs (bp) and quantified with an Agilent Bioanalyzer and via qPCR using the KAPA Library Quantification Kit on the Life Technologies Step One System. Libraries were sequenced on an Illumina HiSeq 2000 to generate 7.5 × 10⁷ to 10 × 10⁷ 50-bp paired-end reads.

**Quality control and DNA alignment**

All raw sequence data were quality-trimmed to a minimum Phred score of 20 with Trimomatic (55). Alignment to reference genome mm10 was done with Bowtie2 (56). PCR duplicates were removed using Picard MarkDuplicates (57), and alignments with an edit distance from the reference of more than two, or those that were mapped to the reference multiple times, were removed.

**ATAC-seq analysis**

Read alignment positions were adjusted according to their strand: +4 bp for + strand alignments, and −5 bp for − strand alignments. We called open chromatin regions using Macs2 with the “—nomodel” option set (58).

For nucleosome positioning, we filtered properly paired alignments by their fragment size. Fragments less than 100 bp in size were considered nucleosome-free and were replaced with a single BED region and used as a background. Those with sizes between 180 and 247 bp were considered mononucleosomes and were replaced with a single BED region; those with sizes between 315 and 473 bp were considered dinucleosomes and were replaced with two BED regions, each spanning half the overall fragment length; and those with sizes between 558 and 615 bp were considered trinucleosomes and were replaced with three BED regions, each spanning one-third of the overall fragment length. The mono-, di-, and trinucleosome regions were concatenated and used as the nucleosome signal. The resulting BED regions were analyzed with DANPOS with the parameters −p 1 −a 1 −d 20 −clonalcut 0 to identify regions enriched or depleted for nucleosomes (59).

Enriched transcription factor motifs were identified using HOMER (60), heat maps and accessibility plots were generated using NGSPlot (61), and data tracks were visualized using Integrated Genome Viewer (IGV) (62).

**Quantitative real-time PCR**

qRT-PCR was performed as described previously (63). Data are presented as mRNA expression relative to Hprt mRNA. Primer sequences are available upon request.

**Statistical analysis**

Statistical differences between groups were calculated using Student’s t test, with P < 0.05 considered significant. All statistics were performed using Prism 7 (GraphPad Software). All error bars are SD. *P < 0.05, **P < 0.01, ***P < 0.001.

**SUPPLEMENTARY MATERIALS**


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Transcription factor ID2 prevents E proteins from enforcing a naïve T lymphocyte gene program during NK cell development

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Establishing NK cell identity

The transcription factor ID2 is required for normal differentiation of all innate lymphoid cells, including natural killer (NK) cells. Zook et al. have characterized ID2-deficient NK cells and investigated how ID2 supports full maturation of NK cells into cytotoxic effectors. They found that ID2 limited chromatin accessibility at multiple lymphocyte-associated genes correlated with a naïve gene program, thereby enabling an effector gene program to take hold. This study provides insight into the series of transcriptional programming steps that underpin normal NK cell differentiation.

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