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Mature natural killer cell and lymphoid tissue–inducing cell development requires Id2-mediated suppression of E protein activity

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The Id2 transcriptional repressor is essential for development of natural killer (NK) cells, lymphoid tissue–inducing (LTi) cells, and secondary lymphoid tissues. Id2 was proposed to regulate NK and LTi lineage specification from multipotent progenitors through suppression of E proteins. We report that NK cell progenitors are not reduced in the bone marrow (BM) of Id2$^{-/-}$ mice, demonstrating that Id2 is not essential for NK lineage specification. Rather, Id2 is required for development of mature (m) NK cells. We define the mechanism by which Id2 functions by showing that a reduction in E protein activity, through deletion of *E2A*, overcomes the need for Id2 in development of BM mNK cells, LTi cells, and secondary lymphoid tissues. However, mNK cells are not restored in the blood or spleen of Id2$^{-/-}$E2A$^{-/-}$ mice, suggesting a role for Id2 in suppression of alternative E proteins after maturation. Interestingly, the few splenic mNK cells in Id2$^{-/-}$ and Id2$^{-/-}$E2A$^{-/-}$ mice have characteristics of thymus-derived NK cells, which develop in the absence of Id2, implying a differential requirement for Id2 in BM and thymic mNK development. Our findings redefine the essential functions of Id2 in lymphoid development and provide insight into the dynamic regulation of E and Id proteins during this process.

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Abbreviations used: CLP, common lymphoid progenitor; EBF, early B cell factor; γc, γ chain; i, immature; ICAM, intercellular adhesion molecule; LTi, lymphoid tissue–inducing; m, mature; NKP, NK cell progenitor; PP, Peyer's patch; QPCR, quantitative real-time PCR; VCAM, vascular cell adhesion molecule.

NK cells are a subset of lymphoid cells that play a fundamental role in defense against intracellular pathogens, tumor surveillance, and the production of cytokines that promote adaptive immune responses (1). Over the past 5 yr, major advances have been made in our understanding of how NK cells recognize and kill their targets (2–4). In contrast, much less is known about how these cells develop from progenitors in the BM, the major site of NK cell production. NK cells are the progeny of common lymphoid progenitors (CLPs) that also give rise to B lymphocytes in the BM and lymphoid tissue–inducing (LTi) cells in the fetal liver (5–8). During embryogenesis, NK cells develop in the thymus from a bipotent T/NK cell progenitor, and an analogous progenitor likely exists in the adult thymus (9–14). In the BM, the most immature lineage-restricted NK cell progenitors (NKPs) can be identified by the expression of the β chain of the IL-2/15 receptor (CD122) and the absence of cell surface markers associated with T lymphocytes, such as TCRαβ, TCRγδ, CD3, CD4, and CD8 (15, 16). As NK cells mature, they sequentially acquire the cell surface receptors NK1.1 (NKR-P1B and NKR-P1C), CD94, and the α2-integrin DX5 (CD49b; references 15, 16). CD122$^+$NK1.1$^-$DX5$^-$ NKPs and CD122$^+$NK1.1$^+$DX5$^-$ immature (i) NK cells lack lytic capacity, whereas CD122$^+$NK1.1$^+$ DX5$^+$ mature (m) NK cells can lyse cells after their specificity is fine-tuned by stochastic expression of activating and inhibitory receptors of the Ly49 family (15, 17). In the adult thymus, a subpopulation of NK cells develop through a unique GATA-3 and IL-7–dependent pathway, resulting in NK cells with enhanced ability to secrete TNFα and IFNγ (14). However, little is known about the requirements for development of these "thymic" NK cells.

Development of mNK cells in the BM, and their subsequent accumulation in the spleen,

requires expression of CD122, the common γ chain (γc) for cytokine receptors, IL-15Rα, and IL-15, all of which are necessary for IL-15–mediated expansion of iNK and mNK cells (18–20). However, IL-15 and other γc-dependent cytokines are not required for development of NKPs (18, 21–24). Several Ets family transcription factors, including Ets1, MEF-1, and PU.1, have been implicated in development of mNK cells, but the precise stage at which these factors are required has not been well defined (25–27). In addition, the helix-loop-helix protein Id2 is required for development of mNK cells in the spleen and all $CD122^+$ NK lineage cells in the fetal thymus, leading to the hypothesis that Id2 may be required for emergence of the first committed NKPs from CLPs or an analogous fetal thymic progenitor (28, 29). Interestingly, Id2 is also required for the development of LNs, Peyer's patches (PP), and nasal-associated lymphoid tissues, as well as the LTi cells that are required for formation of these secondary lymphoid organs (28, 30, 31). Because NK and LTi cells both develop from CLPs in the fetal liver, it was suggested that Id2 may regulate development of a common NK/LTi progenitor from CLPs or directly regulate cell-fate specification from CLPs (32, 33).

Id2 is an antagonist of E protein transcription factor activity and functions by binding to E proteins and preventing their association with E box sequences in DNA (34). The E proteins encoded by the E2A gene, E12 and E47, are essential for development of committed B lymphocyte progenitors from CLPs as a result of their role in the induction of the B lineage transcription factor early B cell factor (EBF) and subsequent activation of Pax-5, which commits cells to the B lymphocyte lineage (35–38). Therefore, an attractive hypothesis for the role of Id2 in NK and LTi cell development is that Id2 functions to inhibit E protein activity in a subset of CLPs, allowing these cells to adopt non–B lineage cell fates by preventing activation of EBF and Pax-5 and consequent B lymphocyte lineage commitment (13, 33). However, Id2 has been proposed to have targets in addition to E proteins that could function in NK and LTi cell development, including the Ets family proteins PU.1 and the retinoblastoma protein (39–41). Therefore, a role for excess E protein activity in the lymphoid phenotype of $Id2^{-/-}$ mice remains to be demonstrated. Moreover, a requirement for Id2 in the B lymphocyte versus NK cell fate decision in adult BM has not been directly established.

Here, we demonstrate that Id2 is not required for development of committed NKPs but is required specifically for development of mNK cells in adult BM. We find that all of the E proteins, E2A, HEB, and E2-2, as well as Id2, are expressed at varying levels throughout NK cell development. In contrast, Id3 is highly expressed in NKPs, but it is down-regulated in mNK cells, leaving Id2 as the major Id regulator of E protein activity in these cells. Importantly, we show that lowering E protein activity in $Id2^{-/-}$ mice by deletion of E2A results in the restoration of mNK cells in the BM and LTi cells in the embryo, as well as LNs and PPs. Therefore, excess E protein activity is the major cause of the lymphoid phenotypes in $Id2^{-/-}$ mice. Surprisingly, we find that mNK cells in $Id2^{-/-}E2A^{-/-}$ mice fail to accumulate in the peripheral blood or spleen, suggesting that these cells fail to emigrate from the BM. Moreover, the few mNK cells present in the spleen of both $Id2^{-/-}$ and $Id2^{-/-}E2A^{-/-}$ mice express IL-7Rα and low levels of CD11b and CD43, which are characteristic of thymic mNK cells. Because thymic NK cell development is not perturbed in $Id2^{-/-}$ mice, this observation suggests a thymic rather than BM origin for mNK cells

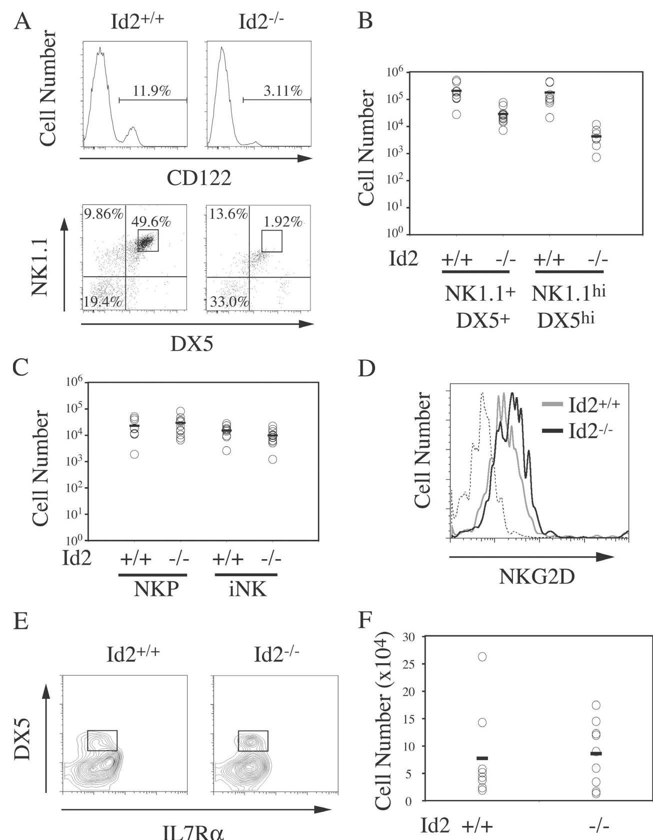

Figure 1. Decreased production of mNK cells, but not NKP, in BM of $Id2^{-/-}$ mice. (A) BM cells from $Id2^{+/+}$ and $Id2^{-/-}$ mice were depleted of Lin^+ cells and stained for CD122, NK1.1, and DX5. NK1.1 versus DX5 (bottom) expression on Lin^-CD122^+ cells (top) is shown. (B) Total number of mNK ($NK1.1^+DX5^+$) and $NK1.1^{hi}DX5^{hi}$ cells in BM of $Id2^{+/+}$ ($n = 9$) and $Id2^{-/-}$ ($n = 12$) mice ($P < 0.002$ for both). Each circle represents the number of cells in femoral BM from one mouse. Bars represent the mean number of NK cells. (C) Total number of NKP and iNK in $Id2^{+/+}$ and $Id2^{-/-}$ BM (NKP, $P = 0.4$; iNK, $P = 0.08$). (D) NKG2D expression on Lin^-CD122^+ $NK1.1^-DX5^-$ BM NKPs from $Id2^{+/+}$ (gray line) and $Id2^{-/-}$ (black line) mice. A negative Ig control (dashed line) is shown for comparison. (E) Thymocytes from $Id2^{+/+}$ and $Id2^{-/-}$ mice were stained for Lin^+ cells, CD122, DX5, and IL7Rα. DX5 versus IL7Rα on Lin^-CD122^+ cells is shown, with the gated population representing "thymic" mNK cells. Positive expression of DX5 and IL7Rα was determined in comparison with samples stained with isotype control antibody. (F) Total number of $DX5^+$ mNK in the thymus of $Id2^{+/+}$ ($n = 9$) and $Id2^{-/-}$ ($n = 9$) mice ($P = 0.7$). Each circle represents the number of cells in one mouse. Bars represent the mean number of NK cells. Mice used in A–C were mixed FVB/NJ × 129/SvJ, and mice used for D and E were backcrossed onto 129/SvJ more than eight times.

in the spleen of these mice. Our data show for the first time that E proteins are the major target of Id2 in vivo and that E protein activity must be suppressed at multiple stages of BM NK cell development. These results provide important insight into the mechanisms controlling NK and LTi cell differentiation and dramatically alter our view of the requirements for Id2 and E proteins in these processes.

RESULTS

Id2 is essential for the development of mNK cells but not NKPs or iNK cells

Mice lacking the Id2 gene have a reduced number of mNK cells in the spleen; however, the requirement for Id2 in BM NK cell development has not been investigated thoroughly. To address this issue, we quantified the number of NKPs, iNK, and mNK cells in the BM of $Id2^{+/+}$ and $Id2^{-/-}$ mice between 4 and 6 wk of age by flow cytometry. All of our analyses included a cocktail of lineage-specific antibodies to exclude T lymphocytes and other cell types (see Materials and methods). Consistent with previous reports, there is a 10-fold decrease in the number of $CD122^+NK1.1^+DX5^+$ mNK cells in $Id2^{-/-}$ BM when compared with $Id2^{+/+}$ BM (Fig. 1, A and B). The $NK1.1^{hi}DX5^{hi}$ population is most severely affected with a >40-fold decrease in $Id2^{-/-}$ compared with $Id2^{+/+}$ BM. Surprisingly however, $CD122^+NK1.1^-DX5^-$ NKPs and $CD122^+NK1.1^+DX5^-$ iNK cells are present in similar numbers in $Id2^{-/-}$ and $Id2^{+/+}$ BM (Fig. 1, A and C). The NK cell receptor NKG2D is expressed on a similar proportion of $CD122^+DX5^-$ cells in $Id2^{+/+}$ and $Id2^{-/-}$ BM, further confirming assignment of these cells to the NK lineage (Fig. 1 D). In addition, $CD122^+DX5^-$ BM cells from $Id2^{-/-}$ and $Id2^{+/+}$ cultured in vitro in c-kit ligand, Flt3 ligand, and IL-2, under limiting dilution conditions, had an identical frequency of cells that were able to proliferate and differentiate into $CD122^+CD94^+$ NK lineage cells (1:9; see Materials and methods). We note that we have examined mice on a mixed FVB/NJ × 129/SvJ background, where the anti-NK1.1 antibody recognizes NKR-P1B, as well as mice backcrossed onto the 129/SvJ background, where the NK1.1 antigen is not expressed. In both sets of mice, we observe a similar decrease in mNK cells ($Lin^-CD122^+DX5^+$) and no decrease in NKP/iNK cells ($Lin^-CD122^+DX5^-$). Therefore, Id2 is not required for the development of NKPs in the adult BM but is required specifically for development of mNK cells.

A subset of NK cells develops in the thymus, where these cells can be distinguished from BM NK cells by expression of IL7Rα (14). Surprisingly, we found that similar numbers of $CD122^+DX5^+$ mNK cells are present in the thymus of $Id2^{+/+}$ and $Id2^{-/-}$ mice and these cells are predominantly $IL7R\alpha^+$ (Fig. 1, E and F). Therefore, Id2 appears to be dispensable for development of mNK cells in the thymus.

$Id2^{-/-}$ splenic NK cells express NK cell receptors and IL7Rα and produce IFNγ

In contrast to the near complete absence of $CD122^+NK1.1^{hi}$ $DX5^{hi}$ mNK cells in $Id2^{-/-}$ BM, these cells can be detected in the spleen, albeit in reduced numbers compared with $Id2^{+/+}$ mice (Fig. 2, A and B; reference 28). Further characterization of $Id2^{-/-}$ splenic mNK cells revealed that they express the Ly49 receptors recognized by the Ly49 antibodies 4E5, 4D11, and 5E6 at frequencies similar to those found in $Id2^{+/+}$ mice, and a cocktail of these antibodies detects essentially all mNK cells in $Id2^{-/-}$ mice (Fig. 2 C and not depicted). A lower frequency of $Id2^{-/-}$ mNK cells express NKG2A/C/E than in $Id2^{+/+}$ spleen, although the level of expression is slightly higher (Fig. 2 C). In contrast, although

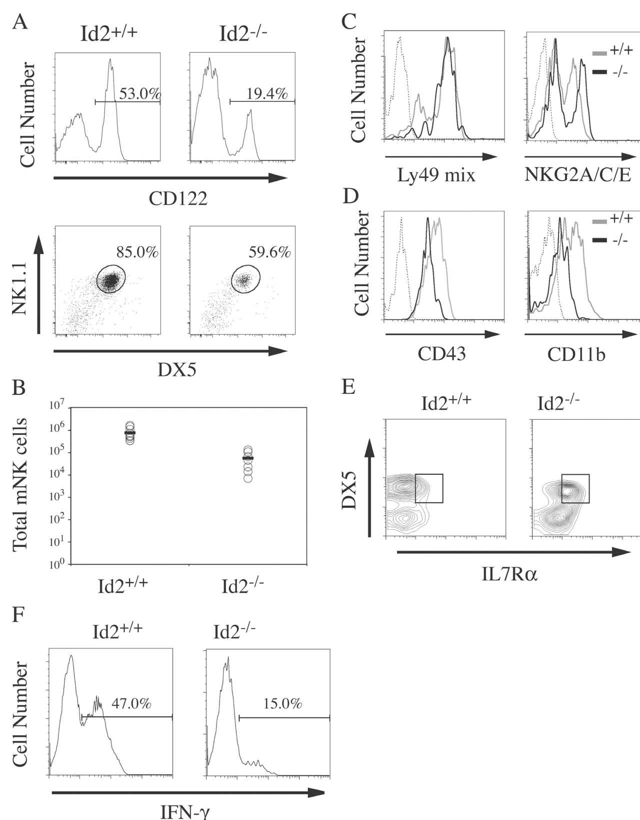

Figure 2. Splenic mNK cells in $Id2^{-/-}$ mice are reduced in number and resemble thymic mNK cells. (A) Splenocytes were stained for Lin^+ cells, CD122, NK1.1, and DX5. NK1.1 versus DX5 (bottom) on Lin^-CD122^+ cells (top) is shown. (B) Total number of $Lin^-CD122^+DX5^+$ mNK cells in spleen of $Id2^{+/+}$ ($n = 10$) and $Id2^{-/-}$ mice ($n = 7$; $P < 0.002$). Each circle represents the number of cells in one spleen. Bars represent the mean number of NK cells. Ly49 receptor (detected with a mix of anti-Ly49 antibodies) and NKG2A/C/E (C) or CD43 and CD11b (D) expression on $Lin^-CD122^+DX5^+$ cells from $Id2^{+/+}$ (gray line) and $Id2^{-/-}$ (black line) splenocytes. Isotype control antibody (dashed line) is shown. Between three and eight mice were analyzed for each marker. (E) IL7Rα and DX5 expression on Lin^-CD122^+ splenocytes from $Id2^{+/+}$ and $Id2^{-/-}$ mice. The boxed area represents $IL7R\alpha^+DX5^+$ cells based on isotype control staining of the same population and is representative of six independent experiments. (F) Lineage-depleted splenocytes from $Id2^{+/+}$ and $Id2^{-/-}$ mice were cultured for 12 h with IL-2 and IL-12. Intracellular IFNγ expression in $CD3^-DX5^+$ cells is shown. Mice used in A and B were mixed FVB/NJ × 129/SvJ, and mice used in C–F were backcrossed more than eight times.

the majority of $Id2^{-/-}$ splenic mNK cells express CD43 and CD11b, these proteins are expressed at lower levels than on $Id2^{+/+}$ cells (Fig. 2 D). Interestingly, we also found that the majority of $Id2^{-/-}$ splenic mNK cells express IL7Rα, a marker of thymus-derived NK cells, raising the possibility that these mNK cells are derived from Id2-independent thymic NK cells rather than from Id2-dependent BM mNK cells (Fig. 2 E). Thymic NK cells also express low levels of CD43 and CD11b, consistent with the phenotype of $Id2^{-/-}$ splenic mNK cells (14). Moreover, we did not observe any differences in in vitro survival or in vivo proliferation between $Id2^{-/-}$ and $Id2^{+/+}$ splenic mNK cells, which is consistent with the hypothesis that the $Id2^{-/-}$ splenic mNK cells do not derive from expansion or survival of a small number of BM-derived mNK cells (unpublished data).

We next tested the ability of $Id2^{-/-}$ splenic mNK cells to produce IFNγ in vitro after overnight culture with IL-2 and IL-12. In WT mice, mNK cells from the thymus produce higher levels of IFNγ under these conditions than splenic mNK cells (14). In contrast, although a small fraction of $Id2^{-/-}$ mNK cells produce IFNγ, they produce less IFNγ on a per-cell basis than $Id2^{+/+}$ mNK cells (Fig. 2 F). This finding is consistent with a previous study showing that $Id2^{-/-}$ splenocytes are able to lyse NK cell targets, although with reduced efficiency compared with $Id2^{+/+}$ splenocytes (28). Collectively, these data indicate that the few mNK cells present in $Id2^{-/-}$ spleen resemble thymic NK cells in that they express IL7Rα and low levels of CD43 and CD11b, but they produce lower levels of IFNγ than WT splenic mNK cells.

Expression of Id and E proteins during NK cell development

To determine why Id2 is required for development of mNK cells rather than at earlier stages of NK cell development, we examined Id and E protein mRNA expression in BM CLP, NKP, and mNK cells by quantitative real-time PCR (QPCR). All of the E proteins are expressed in each of these populations, with the mean ΔC_T values for E47, E2-2, and HEB relative to HPRT (ΔC_TGENE-ΔC_THPRT) in NKPs being 5.1, 3, and 2.35, respectively (unpublished data). E47 mRNA levels are similar in CLPs and NKPs, whereas E2-2 and HEB mRNA decrease during this developmental transition (Fig. 3 A). Additionally, differentiation to the mNK cell stage is associated with a slight decline in E47 and E2-2 mRNA (Fig. 3 A). Therefore, variable levels of mRNA for all of the E proteins are expressed in CLPs and throughout NK cell differentiation, with the lowest levels being observed in mNK cells. By comparison, mRNA for the transcription factor Ets1, which is essential for development of mNK cells, is higher in NKPs and mNK cells compared with CLPs and pro-B lymphocytes (Fig. 3 B).

In contrast to the E proteins, Id2 is expressed at very low levels in CLPs but increases >15-fold in NKPs (mean $\Delta C_T = -2.5$) and mNK cells (Fig. 3 B). Interestingly, Id3 is expressed in CLPs and increases approximately twofold in NKPs but is reduced by 30-fold in mNK cells (Fig. 3 B). Id1 is expressed at lower levels in all NK cell populations and does not change substantially with differentiation stage ($\Delta C_T = 4.39$ in NKPs; unpublished data). Therefore, mRNA encoding all E proteins and Id1-3 are expressed in NK cells

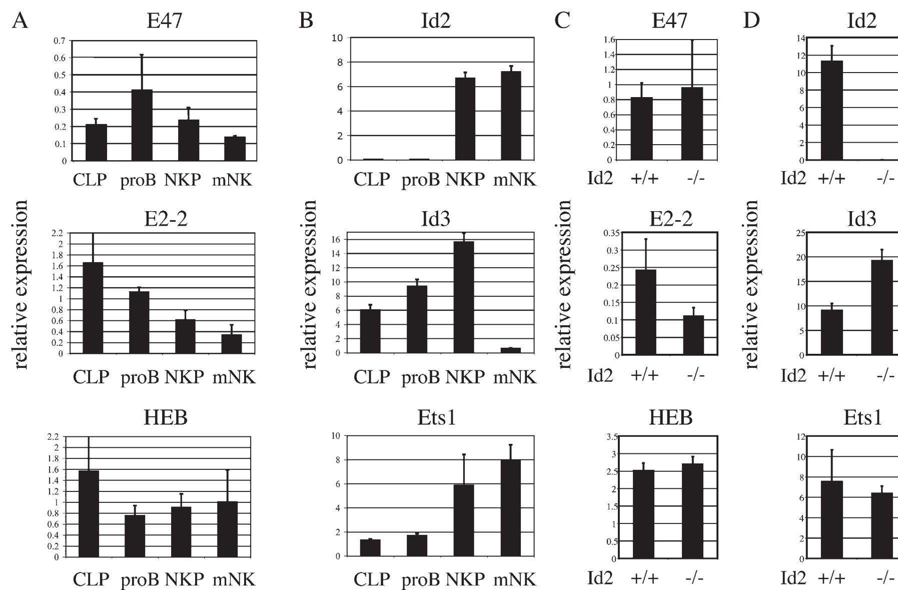

Figure 3. E protein and Id gene expression in NK cell subsets. QPCR analysis of E47, E2-2, and HEB (A) and Id2, Id3, and Ets1 (B) in CLPs, pro-B lymphocytes (proB), and NKP, and mNK cells isolated from BM of C57Bl/6 mice. Similar results were observed using NKP and mNK from FVB/NJ × 129/SvJ mice. QPCR analysis of E47, E2-2, and HEB (C) and Id2, Id3, and Ets1 (D) in $Id2^{+/+}$ and $Id2^{-/-}$ BM NKPs (mixed FVB/NJ × 129/SvJ). E47, HEB, E2-2, and Ets1 ΔC_T values are normalized to the level of expression in 38B9 pro-B lymphocytes (set to 1). Id2 ΔC_T values are normalized to PTL, and Id3 ΔC_T values are normalized to 70Z/3 m/2C1 cells. All samples were run in triplicate, and the standard error is indicated. Experiment is representative of at least three independent amplifications.

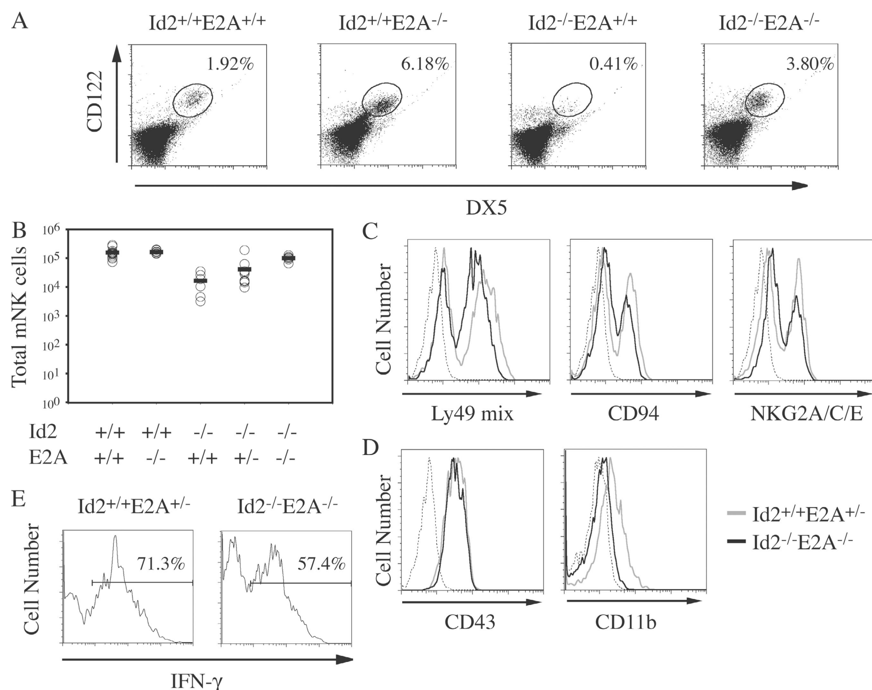

Figure 4. Mature NK cell development is restored in the BM of Id2$^{-/-}$E2A$^{-/-}$ mice. (A) Lin^{-} BM cells from mice of the indicated genotype were analyzed for the presence of CD122^{+}DX5^{+} mNK cells via flow cytometry. (B) Total number of BM mNK cells for mice of the indicated genotype. Each circle represents the number of femoral mNK cells in one mouse, and bars represent the mean number of mNK cells. Id2$^{+/+}$E2A$^{+/+}$, $n = 13$; Id2$^{+/+}$E2A$^{-/-}$, $n = 7$; Id2$^{-/-}$E2A$^{+/+}$, $n = 6$; Id2$^{-/-}$E2A$^{+/-}$, $n = 10$; Id2$^{-/-}$E2A$^{-/-}$, $n = 7$. Id2$^{+/+}$E2A$^{+/+}$ versus Id2$^{-/-}$E2A$^{-/-}$, P = 0.05; Id2$^{-/-}$E2A$^{+/+}$ versus Id2$^{-/-}$E2A$^{-/-}$, $P < 0.05$. Ly49 receptor, CD94, and NKG2A/C/E (C) or CD43 and CD11b (D) expression on Lin^{-}CD122^{+}DX5^{+} BM cells from Id2$^{-/-}$E2A$^{-/-}$ (black line) and littermate control (Id2$^{+/+}$E2A$^{+/-}$ or Id2$^{+/-}$E2A$^{+/-}$; gray line) mice. Data is representative of two to six mice analyzed. (E) Lineage-depleted BM cells from Id2$^{-/-}$E2A$^{-/-}$ and Id2$^{+/+}$E2A$^{+/-}$ mice were cultured for 12 h with IL-2 and IL-12. Intracellular IFNγ expression in CD3^{-}DX5^{+} cells is shown. All mice were mixed FVB/NJ × 129/SvJ.

during development but show dynamic regulation between NKP and mNK cells. Moreover, we observed a dramatic decline in Id3 expression in mNK cells, the stage where Id2 becomes essential for further NK cell differentiation. This finding suggests that NKPs may develop in Id2$^{-/-}$ mice because Id3 is sufficient to compensate for loss of Id2. Consistent with this hypothesis, Id2$^{-/-}$ NKPs show an approximately twofold increase in Id3 mRNA compared with Id2$^{+/+}$ NKPs (Fig. 3 D). Interestingly, E2-2 mRNA was observed to decrease, whereas E47 and HEB mRNA levels remain equivalent, in Id2$^{-/-}$ compared with Id2$^{+/+}$ NKPs (Fig. 3 C). Therefore, alterations in E protein and Id3 expression may allow for NKP development in the absence of Id2.

Loss of E2A rescues mNK cell development in Id2$^{-/-}$ BM

Our data indicate that Id2 is essential for development of mNK cells in the BM, a stage where Id3 mRNA decreases dramatically. This observation suggests that in the absence of Id2, excess E protein activity may prevent further NK cell differentiation. However, Id2 could also function by regulating non–E protein target genes. Therefore, to test the effect of reducing E protein activity on the NK cell phenotype of Id2$^{-/-}$ mice, we created Id2$^{-/-}$ mice with varying levels of E2A and analyzed NK cell development in the BM by flow cytometry. Remarkably, we found that loss of E2A in Id2$^{-/-}$ mice restores the number of CD122^{+}DX5^{+} mNK cells in the BM to levels similar to Id2$^{+/+}$ mice (Fig. 4, A and B). In addition, a partial rescue of mNK cell numbers is observed

Table I. LN development in Id2 and E2A mutant mice

	LNs				
Genotype	Mesenteric	Cervical	Axillary	Brachial	Inguinal
Id2$^{+/+}$E2A$^{+/+}$	30/30	5/5	5/5	5/5	30/30
Id2$^{+/+}$E2A$^{-/-}$	11/11	ND	ND	ND	11/11
Id2$^{-/-}$E2A$^{+/+}$	0/30	0/2	0/2	0/2	0/30
Id2$^{-/-}$E2A$^{+/-}$	24/24	5/5	3/5	1/5	6/24
Id2$^{-/-}$E2A$^{-/-}$	16/16	5/5	5/5	5/5	16/16

in $Id2^{-/-}E2A^{+/-}$ mice, indicating a dose effect for E2A (Fig. 4 B). Importantly, mNK cell numbers are identical in $Id2^{+/+}$ and $E2A^{-/-}$ mice, indicating that loss of E2A does not augment NK cell development when Id2 is present (Fig. 4, A and B). Therefore, a reduction in E protein activity, through loss of E2A, is sufficient to restore mNK cells in $Id2^{-/-}$ BM.

The mNK cells that develop in $Id2^{-/-}E2A^{-/-}$ BM express Ly49 receptors on a comparable frequency of cells as their Id2/E2A heterozygous (het) littermates, whereas slightly fewer cells express CD94 and NKG2A/C/E (Fig. 4 C). Moreover, CD43 expression is indistinguishable between BM $CD122^{+}DX5^{+}$ mNK cells in $Id2^{-/-}E2A^{-/-}$ and het mice, indicating that these cells are fully mature (Fig. 4 D). In contrast, $Id2^{-/-}E2A^{-/-}$ mNK cells do not express CD11b (Fig. 4 D). Decreased expression of CD11b did not inhibit functional activity, as $Id2^{-/-}E2A^{-/-}$ mNKs were able to produce IFNγ in response to IL-2 and IL-12 stimulation in vitro nearly as well as het littermates (Fig. 4 E). Therefore, loss of E2A in $Id2^{-/-}$ mice rescues the development of BM mNK cells, although these cells lack expression of CD11b.

Loss of E2A fails to restore mNK cells in the spleen of $Id2^{-/-}$ mice

Given that loss of E2A rescues mNK cell development in the BM of $Id2^{-/-}$ mice, we next examined $Id2^{-/-}E2A^{-/-}$ mice for the presence of mNK cells in the spleen. Remarkably, the total number of splenic mNK cells was not different between $Id2^{-/-}E2A^{+/+}$ and $Id2^{-/-}E2A^{-/-}$ or $Id2^{-/-}E2A^{+/-}$ animals (Fig. 5, A and B). This surprising result indicates that loss of E2A does not lower E protein activity sufficiently to allow mNK cells to accumulate in the spleen. Indeed, the few mNK cells present in $Id2^{-/-}E2A^{-/-}$ spleen, like $Id2^{-/-}$ splenic mNK cells, expressed IL7Rα and low levels of CD43 and CD11b, suggesting that they may also be derived from thymic NK cells (Fig. 5 C and not depicted). Interestingly however, IFNγ production by $Id2^{-/-}E2A^{-/-}$ splenic mNK cells was closer to that of control (het) mNK cells than previously observed for $Id2^{-/-}$ splenic mNK cells (Fig. 5 D). Therefore, although loss of E2A may not rescue the number of mNK cells in the spleen, these cells respond better to IL-2 and IL-12 than $Id2^{-/-}$ cells with WT levels of E2A.

To determine why BM-derived mNK cells were not present in the spleen of $Id2^{-/-}E2A^{-/-}$ mice, we investigated

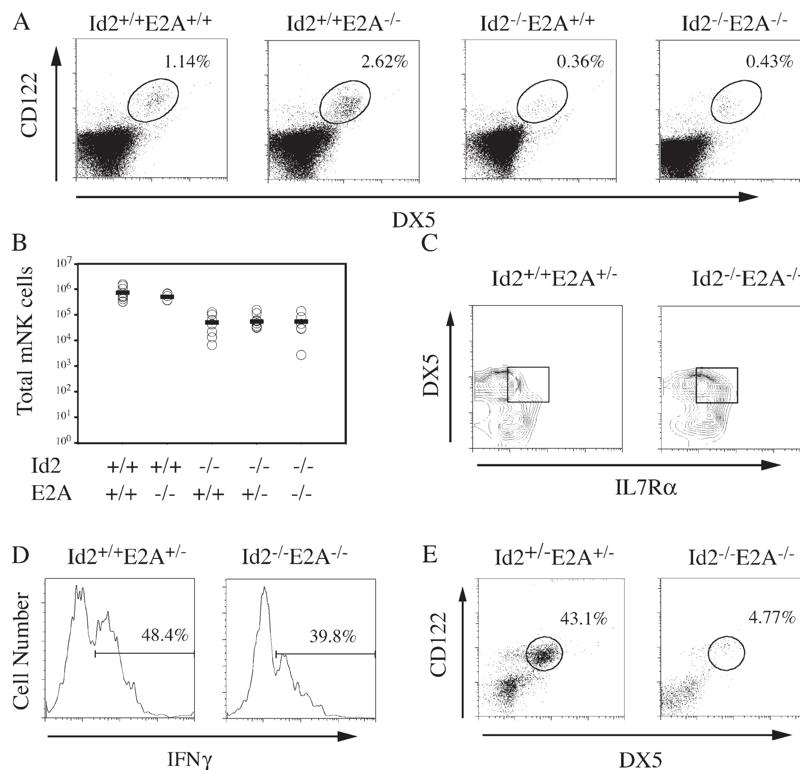

Figure 5. Loss of E2A does not rescue the phenotype of mNK cells in $Id2^{-/-}$ spleen. (A) FACS analysis for CD122 and DX5 on Lin^{-} splenocytes from mice with the indicated genotype. (B) Total number of Lin^{-} mNK cells in the spleen of mice with the indicated genotype. Each circle represents the number of mNK cells in one spleen, and bars represent the mean number of mNK cells. $Id2^{+/+}E2A^{+/+}$, $n = 13$; $Id2^{+/+}E2A^{-/-}$, $n = 7$; $Id2^{-/-}E2A^{+/+}$, $n = 6$; $Id2^{-/-}E2A^{+/-}$, $n = 10$; $Id2^{-/-}E2A^{-/-}$, $n = 7$. (C) DX5 and IL7Rα expression on $Lin^{-}CD122^{+}$ splenocytes from $Id2^{+/+}E2A^{+/-}$ and $Id2^{-/-}E2A^{-/-}$ mice. Data is representative of at least three mice of each genotype. (D) Lin-depleted splenocytes from $Id2^{-/-}E2A^{-/-}$ and $Id2^{+/+}E2A^{+/-}$ mice were cultured for 12 h with IL-2 and IL-12. Intracellular IFNγ expression in $CD3^{-}DX5^{+}$ cells is shown. (E) CD122 and DX5 expression on Lin^{-} peripheral blood from $Id2^{-/-}E2A^{-/-}$ and $Id2^{+/-}E2A^{+/-}$ mice. Data is representative of four separate experiments. All mice were mixed FVB/NJ × 129/SvJ.

whether these cells were accumulating in peripheral blood. Remarkably, the percentage of mNK cells among Lin^- blood cells was reduced by 10-fold in $Id2^{-/-}E2A^{-/-}$ mice compared with het littermates (Fig. 5 E). Therefore, we reasoned that BM mNK cells in $Id2^{-/-}E2A^{-/-}$ mice may fail to emigrate from the BM into the peripheral blood. Regardless, we found no differences in mRNA expression for the chemokine receptors CCR1, CCR5, or CX3CR1 by QPCR in mNK cells isolated from $Id2^{-/-}E2A^{-/-}$ or het BM, indicating that dysregulation of these chemokine receptors is unlikely to be the cause of failed BM emigration (unpublished data).

Loss of E2A rescues LN, PP, and LTi cell development in the absence of Id2

LNs and PPs do not develop in $Id2^{-/-}$ mice because of a failure of LTi cell development during embryogenesis (28). Given that loss of E2A rescues mNK cell development in $Id2^{-/-}$ mice, we examined these animals for the presence of LNs and PPs. Inguinal and mesenteric LNs were readily visible in $Id2^{-/-}E2A^{-/-}$ mice, whereas no LNs were detected in $Id2^{-/-}$ mice, even after in vivo staining with Chicago sky blue (Table I and Fig. 6 A). Further inspection revealed that axillary, brachial, and cervical LNs, as well as PPs, develop in $Id2^{-/-}E2A^{-/-}$ but not $Id2^{-/-}$ mice (Table I and Fig. 6 B). The LNs and PPs developing in $Id2^{-/-}E2A^{-/-}$ mice are smaller that those in WT mice because of the absence of mature B lymphocytes in these animals (unpublished data). Interestingly, mesenteric and cervical LNs were always present in $Id2^{-/-}E2A^{+/-}$ mice, but axillary, brachial, and inguinal LNs developed in only a fraction of these mice, again indicating a dose effect for E protein activity (Table I). B and T lymphocytes areas were observed in $Id2^{-/-}E2A^{+/-}$ LNs that were indistinguishable from those in $E2A^{+/-}$ LNs, whereas only T lymphocytes were detected in LNs from $Id2^{-/-}E2A^{-/-}$ and $E2A^{-/-}$ mice as expected (unpublished data). Therefore, a decrease in E protein activity through loss of E2A is sufficient to overcome the need for Id2 in secondary lymphoid tissue organogenesis.

The presence of LNs and PPs in $Id2^{-/-}E2A^{-/-}$ mice indicates that LTi cells are able to develop during embryonic life. To determine directly whether LTi cells develop in $Id2^{-/-}E2A^{-/-}$ mice, embryonic day 15.5 embryos from breedings of $Id2^{+/-}E2A^{+/-}$ mice were sectioned and stained with antibodies that detect CD4 or CD45, expressed on LTi cells, and intercellular adhesion molecule (ICAM) 1 or vascular cell adhesion molecule (VCAM) 1, which are induced on stromal "organizer" cells through interaction with LTi cells (42). A distinct population of $CD4^+$ cells could be found in association with VCAM-1^+ cells in the cervical region of $E2A^{-/-}$ and $Id2^{-/-}E2A^{-/-}$ embryos but not in $Id2^{-/-}$ embryos (Fig. 6 C). Similarly, $CD45^+$ LTi cells are found in association with ICAM-1^+ cells in the omentum of $E2A^{-/-}$ and $Id2^{-/-}E2A^{-/-}$ embryos but not $Id2^{-/-}$ embryos (Fig. 6 D).

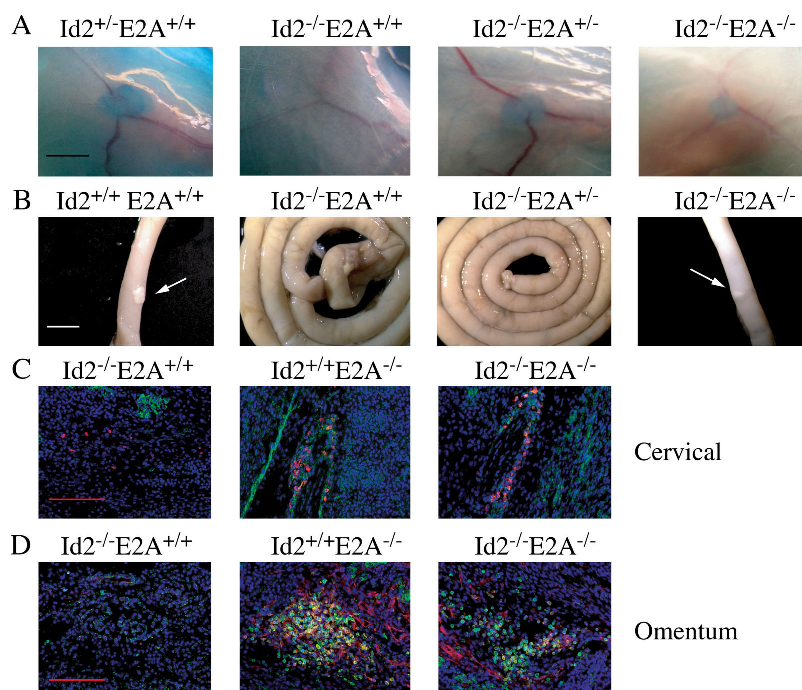

Figure 6. Development of LNs, PPs, and LTi cells in $Id2^{-/-}E2A^{-/-}$ mice. (A) Inguinal LNs from $Id2^{+/-}E2A^{+/+}$, $Id2^{-/-}E2A^{+/+}$, $Id2^{-/-}E2A^{+/-}$, and $Id2^{-/-}E2A^{-/-}$ mice were examined 2 wk after injection of Chicago sky blue. Bar, 0.5 cm. (B) Intestinal PPs were examined in $Id2^{+/+}E2A^{+/+}$, $Id2^{-/-}E2A^{+/+}$, $Id2^{-/-}E2A^{+/-}$, and $Id2^{-/-}E2A^{-/-}$ mice after treatment with acetic acid. Bar, 0.5 cm. (C and D) Cryosections of fixed embryonic day 15.5 embryos were stained with antibodies detecting LTi cells or stromal "organizer" cells. (C) CD4 (red) and VCAM-1 (green) in the cervical region of the embryo and CD45 (D, green) and ICAM-1 (red) in the omentum. All mice were mixed FVB/NJ × 129/SvJ. Bars, 100 μm.

Therefore, loss of E2A is sufficient to restore LTi cells and peripheral lymphoid tissues, in addition to BM mNK cells, in $Id2^{-/-}$ mice.

DISCUSSION

In this study, we have shown that Id2 is required for the development of mNK cells in adult BM, as well as formation of LTi cells, and that it functions by repressing E protein activity. The requirement for Id2 in BM NK cell development coincides with the down-regulation of Id3, suggesting that Id2 becomes the major antagonist of E proteins in mNK cells, whereas Id3 and Id2 may function redundantly in more immature progenitors. All of the E protein genes are expressed during NK cell development, but loss of E2A, through homologous recombination, reduced total E protein activity sufficiently to allow mNK cell development in the absence of Id2. Remarkably, however, a further reduction in E protein activity may be required for accumulation of normal numbers of BM-derived mNK cells in the spleen and peripheral blood. Loss of E2A was also found to be sufficient to restore LTi cells and the peripheral lymphoid tissues that arise from LTi cell activity in $Id2^{-/-}$ mice. Although Id2 has been suggested to interact with non–E protein targets, such as retinoblastoma protein and PU.1 (40, 43), our data indicate that the essential function of Id2 in lymphoid development is modulation of E protein activity. Moreover, our data provide the first genetic evidence for an interaction between an E and Id protein in vivo.

Previous studies have led to the hypothesis that Id proteins function in the B/NK or T/NK lineage decision by inhibiting E protein activity and preventing B or T lymphopoiesis, thereby allowing NK cell development (29, 33, 44). Consistent with this hypothesis, we show that Id2 is dramatically up-regulated during the transition of CLPs to NKPs and remains highly expressed throughout NK cell development. However, we also found that Id2 is not essential for development of NKPs in adult BM. This unexpected finding is likely the result of compensation by Id3, as we found that Id3 mRNA is expressed in CLPs and NKPs and is increased in $Id2^{-/-}$ NKPs. Therefore, in the absence of Id2, NKPs may be able to suppress E protein activity sufficiently via Id3 to allow their development. However, the role of Id proteins in supporting NK lineage specification from CLPs in adult BM requires further investigation.

We also find that Id2 is not essential for development of $CD122^+DX5^+$ mNK cells in the thymus and that the majority of $Id2^{-/-}$ and $Id2^{-/-}E2A^{-/-}$ splenic mNK cells have characteristics of thymus-derived NK cells, including expression of IL7Rα and low levels of CD43 and CD11b (14). Although this observation suggests that the mNK cells present in the spleen of $Id2^{-/-}$ and $Id2^{-/-}E2A^{-/-}$ mice are derived from the thymic NK cell pathway, we cannot fully exclude the possibility that loss of Id2 causes BM-derived mNK cells to acquire the thymic phenotype. A previous study by Ikawa et al. (29) showed that $CD44^+CD25^-CD122^+$ NKPs were absent in $Id2^{-/-}$ fetal thymus. Therefore, the requirements for Id2 in adult and fetal NK cell development may differ. Fetal and adult lymphopoiesis differ in many ways, including distinct relationships between progenitor cells and different cytokine and transcription factor requirements. For example, fetal liver CLPs can give rise to macrophages and LTi cells in addition to NK cells and B lymphocytes (6). In contrast, adult CLPs lack macrophage and LTi potential (42). In addition, commitment to the T/NK cell pathway occurs before thymic colonization in the embryo, whereas this lineage restriction event appears to occur after thymic colonization in the adult (45). Therefore, the differential requirement for Id2 in development of NKPs reported by Ikawa et al. (29) and in this study is likely to be the result of the different origins of these progenitors. Alternatively, the role of Id2 in thymic NK cell development may be strain dependent. Ikawa et al. (29) examined fetal NK cell development from $Id2^{-/-}$ mice backcrossed six generations onto the C57Bl/6 strain, whereas our mice are backcrossed greater than eight generations onto the 129/SvJ strain. However, we also observed no difference in the number of thymic NK cells between heterozygous control and $Id2^{-/-}$ mice on a mixed 129/SvJ $\times$ FVB/NJ background. Nonetheless, the interesting possibility that strain differences exist requires further investigation and may indicate differential regulation of Id or E proteins in different strains of mice, which has not yet been reported.

The thymic NK cells identified by Vosshenrich et al. (14) are better cytokine producers, particularly of TNFα and IFNγ, than splenic mNK cells in response to stimulation with IL-2 plus IL-12. Therefore, given the thymic phenotype of $Id2^{-/-}$ splenic mNK cells, it is notable that these cells produced less IFNγ after cytokine stimulation than heterozygous control splenic mNK cells. This observation suggests that Id2 may have a role in thymus-derived mNK cells that allows for optimal IFNγ production. This function for Id2 in mNK cells may also involve suppression of E protein activity, as splenic mNK cells in $Id2^{-/-}E2A^{-/-}$ phenotypically resemble $Id2^{-/-}$ splenic mNK cells (i.e., they have the thymic phenotype), but they have a greater capacity for IFNγ production. Collectively, these observations raise the possibility that both E and Id proteins play a role in controlling the effector functions of mNK cells. Additional studies will be required to determine the role of these proteins in mNK cells and how their expression is regulated.

In the absence of Id2, essentially no $CD122^+DX5^{hi}$ mNK cells are found in the BM. Notably, this is the stage of NK cell development where Id3 mRNA is substantially reduced, leaving Id2 as the major Id protein in NK cells. That Id2 is functioning through suppression of E proteins is evident from our observation that deletion of E2A from $Id2^{-/-}$ mice restores mNK cells in the BM. However, $Id2^{-/-}E2A^{-/-}$ mNK cells show several differences from heterozygous control BM mNK cells. $Id2^{-/-}E2A^{-/-}$ BM mNK cells appear to be fully mature in that they express the leukosialin CD43, a marker of functional mNK cells (16). However, they fail to express the adhesion molecule CD11b, which is also characteristic of

fully mature NK cells, and they produce less IFNγ than heterozygous control mNK cells. $Id2^{-/-}E2A^{-/-}$ mNK cells are also present at reduced levels in peripheral blood, leading us to suggest that they may fail to emigrate from the BM. Therefore, appropriate regulation of E protein activity may be critical for mNK cells to exit the BM and accumulate in peripheral tissues, such as the spleen. Notably, HEB and E2-2 mRNA levels are equivalent between BM mNK cells from $Id2^{-/-}$ $E2A^{-/-}$ and heterozygous littermate mice, and splenic ($IL7R\alpha^{-}$) mNK cells have higher levels of E2-2 and HEB than BM mNK cells (unpublished data). These findings raise the possibility that loss of E2A may not reduce E protein activity sufficiently in mNK cells to allow these cells to acquire competence to exit the BM. At the present time, very little is known about the mechanisms controlling mNK cell emigration from the BM, and the E protein target genes that regulate this transition remain to be identified.

We have shown that loss of E2A allows progression to the mNK cell stage in $Id2^{-/-}$ BM. This finding is surprising given that HEB and E2-2 mRNAs are expressed at higher levels than E2A mRNA in these cells. There are two possible interpretations of this finding: (a) that E2A specifically is inhibited by Id2 and (b) that loss of E2A decreases E protein activity sufficiently to allow further NK cell development, even though HEB and E2-2 are still present. We favor the second possibility because Id2 has been shown to interact efficiently with all E proteins in vitro (46). However, although E2A mRNA is expressed at lower levels than HEB and E2-2 in NKPs, it is feasible that E2A represents the major functional E protein in NK cells. E47 may have a higher affinity for the consensus E box sequence than the other E proteins, and posttranscriptional or posttranslational modifications of E proteins may affect their protein levels and/or activity (47, 48). Therefore, E2A may be the major antagonist of NK cell differentiation in the absence of Id2.

Another interesting implication of our data is that Id proteins may need only to modulate E protein activity rather than sequester it completely to allow for mNK cell development, as HEB and E2-2 are expressed in BM mNK cells from $Id2^{-/-}E2A^{-/-}$ mice. This finding is intriguing given the range of E protein thresholds previously reported for E protein target genes. For example, in B lymphocyte progenitors, EBF requires a high level of E protein activity for its initial induction, and high levels of E protein activity are required for expression of N-*myc* as compared with c-*myc* (37, 49). Moreover, high levels of E protein activity can inhibit proliferation of lymphocytes, but a reduction of E protein activity also leads to decreased proliferation (37, 50). These observations suggest that E proteins activate distinct target genes depending on their level of expression. Therefore, our findings raise the possibility that low levels of E protein activity function in the regulation of gene expression in NK lineage cells and that Id proteins function to prevent excess E protein activity. Further investigation of a positive role for E proteins in NK cell function will require the ability to knock out multiple E proteins specifically in NK lineage cells.

A variety of transcription factors have been identified as essential for proper NK cell development, including T-bet, Ets1, PU.1, GATA-3, IRF-1, and IRF-2 (51). Of these transcription factors, T-bet, GATA-3, and IRF-2 all display defects in NK cell development that occur at the final stages of maturation in either the BM or spleen, and the NK cells found in these mutant mice all exhibit functional defects. In contrast, the defect in NK cell development in $Id2^{-/-}$ mice occurs earlier than in $T\text{-}bet^{-/-}$, $GATA\text{-}3^{-/-}$, and $IRF\text{-}2^{-/-}$ mice, placing Id2 upstream of these factors. $Id2^{-/-}$ mice resemble mice lacking IL-15 in that $IL\text{-}15^{-/-}$ mice show a decreased number of mNK cells in the spleen that express NK cell receptors and appear to have normal NK function, although iNK cells are also decreased in the BM of $IL\text{-}15^{-/-}$ mice (22). Additionally, unlike $IL\text{-}15^{-/-}$ and $IL\text{-}15R\alpha^{-/-}$ NK cells, the defect in $Id2^{-/-}$ NK cell development is NK cell intrinsic (references 20, 28; unpublished data). The defect in NK cell development defect in $Id2^{-/-}$ mice is unlikely to be due to a failure to respond to γc-dependent cytokines because $Id2^{-/-}$ NKPs proliferate normally in cytokine-supplemented in vitro culture (unpublished data). However, our data are consistent with the hypothesis that, in the absence of Id2, mNK cells either fail to develop or rapidly disappear, and only those few cells that manage to bypass the need for Id2 can be observed and are therefore able to respond normally to cytokines.

We found that loss of E2A in $Id2^{-/-}$ mice rescued development of LNs and PPs, as well as the LTi cells that induce their formation. Although $Id2^{-/-}E2A^{-/-}$ mice developed all LN subsets, $Id2^{-/-}E2A^{+/-}$ mice developed mesenteric and cervical LNs, but axillary, brachial, and inguinal LNs were restored in only a subset of these animals. There are at least two possible explanations for the LN phenotype of $Id2^{-/-}E2A^{+/-}$ mice: (a) that a subset of LTi cells is rescued that is able to induce only mesenteric and cervical LNs but fails to efficiently promote other LN subsets or (b) that only a small number of LTi cells develop in these mice and the signal delivered by these few LTi cells is sufficient only for early LN development. As there is currently no evidence to suggest a qualitative difference in LTi cell subsets, we favor the latter explanation. Lymphoid tissues develop during embryogenesis in a temporal order: mesenteric LN > cervical LN > brachial/axillary LN > inguinal LN > PP. Additionally, distinct requirements for the development of mesenteric and cervical LN subsets are known to exist, perhaps the consequence of unique stromal cell subsets present at these locations (52). LNs are generated by the interaction of $IL7R\alpha^{+}$ and $TRANCE\text{-}R^{+}$ LTi cells, which express LIGHT and $LT\alpha1\beta2$, with stromal "organizer" cells that express LTβR (42). This interaction promotes expression of adhesion molecules VCAM-1, ICAM-1, and MadCAM-1 on the stromal cells, as well as production of chemokines, including CXCL13, CCL19, and CCL21, that recruit greater numbers of LTi cells to the developing LN and stimulate $LT\alpha1\beta2$ and active $\alpha4\beta1$-integrin expression on LTi cells. Interestingly however, mutations in many of these genes lead

to loss of distinct subsets of LNs. For example, CXCL13 and its receptor CXCR5 as well as LTβ are required for the development of most peripheral but not mesenteric or cervical LNs (53, 54). The differential requirements for development of mesenteric and cervical LNs when compared with other subsets may be due to redundancy in signaling pathways or varied signaling thresholds in the stromal organizer cells at these sites. Therefore, a decreased number of LTi cells may be sufficient for development of mesenteric and cervical, but not peripheral, LNs. Alternatively, our findings may be interpreted as evidence of a temporal difference in the ability of developing $Id2^{-/-}E2A^{+/-}$ LTi cells to generate LN subsets. One possibility is that is that E2A protein accumulates during the lifespan of LTi cells, eventually passing a threshold that results in decreased LTi cell survival and failed development of LNs late in ontogeny. Regardless, the development of mesenteric and cervical LNs and, infrequently, other LN populations in $Id2^{-/-}E2A^{+/-}$ mice is consistent with the notion that a partial decrease in E2A rescues only a fraction of the total number of LTi cells in $Id2^{-/-}$ mice. In contrast, loss of E2A in these animals restores LTi cells sufficiently to allow for development of all LN populations, as well as PPs.

In summary, our data indicate that the major function of Id2 in lymphoid differentiation is to control E protein activity. During NK cell development, Id2 and Id3 may cooperatively perform this role, and Id2 becomes essential only at the mNK cell stage, when Id3 mRNA is down-regulated. Future studies will need to address the requirement for modulation of E protein activity in NKPs and whether E proteins play a positive role in any aspect of NK cell development or function.

MATERIALS AND METHODS

Mice and genotyping. Mice were housed at the University of Chicago Animal Resource Center, and experiments were performed in accordance with the guidelines of The University of Chicago Institutional Animal Care and Use Committee. $E2A^{-/-}$ mice were genotyped as described previously (35). $Id2^{-/-}$ mice were genotyped by PCR using the primers Id2-S, 5′-TCTGAGCTTATGTCGAATGATAGC-3′; Id2-AS, 5′-CGTGTTCTCCTGGTGAAATGGCTG-3′; and neo, 5′-TCGTGCTTTACGGTATCGCCGCTC-3′ (28). Mice were maintained on a 129/SvJ or FVB/NJ or mixed background. No obvious differences were detected between these two sets of mice. More recent experiments have been performed on mice backcrossed onto 129/SvJ for eight generations, in which case, NK1.1 was not used as an NK cell marker.

Flow cytometry. Cells were treated with anti-FcγR antibody before staining with antibodies directly conjugated to biotin, FITC, PE, PE-cy5, PE-cy5.5, PE-cy7, and APC, and antibodies specific for the following antigens were used for this study (clone in parentheses): CD49b (DX5), CD122 (TM-β1), CD94 (18d3), NK1.1 (PK136, which recognizes both NKR-P1B and NKR-P1C), CD11b (M1/70), Ly6G (RB6-8C5), CD3 (145-2C11), CD4 (RM4-5), CD8α (Ly-2), Ter119 (Ly-76), B220 (Ra3-6B2), c-kit (2B8), Sca-1 (E13-161.7), IL7Rα (A7R34), CD19 (1D3), NKG2D (CX5), NKG2A/C/E (20d5), Ly49D (4E5), Ly49G2 (4D11), Ly49C/I (5E6), and IFNγ (XMG1.2). Antibodies were purchased from eBioscience and BD Biosciences. Lineage cocktails were as follows: for BM, spleen and peripheral blood, CD3, CD4, CD8, CD19, Ter119, and Ly6G; and for thymus, CD3, CD4, CD8, CD19, Ter119, Ly6G, TCRβ, and CD25.

IFNγ production assay. BM and spleen cells were depleted of Lin^+ cells by magnetic sorting (Miltenyi Biotec). Cells were cultured in Opti-MEM supplemented with 10% FBS; penicillin, streptomycin, and glutamine solution (all from Invitrogen); 2 ng/ml IL-12 (R&D Systems); and 1,000 IU/ml IL-2 (National Institutes of Health). After 8 h, Golgi Plug/Brefeldin A (BD Biosciences) was added, and the cells were left for an additional 4 h. Cells were then stained for CD3 and DX5 surface antigens and placed in Cytofix/Cytoperm solution (BD Biosciences) to prepare for intracellular staining using IFNγ-PE or an IgG-PE control.

Limiting dilution assay. $Lin^-CD122^+DX5^-$ NK progenitors from $Id2^{+/+}$ and $Id2^{-/-}$ mice were sorted directly into cultures containing OPTI-MEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine solution plus a 1:250 dilution of c-kit ligand (from MGF-CHO cells), 50 ng/ml Flt3L, and 1,000 IU IL-2. The cells were sorted at a concentration of 30, 10, and 3 cells (48 wells/cell concentration) and cultured for 10 d before analysis by microscopy and flow cytometry analysis for $Lin^-CD122^+CD94^+$ cells. The frequency of responding NK progenitors was determined by linear regression as the number of input cells resulting in 37% nonresponding wells.

QPCR analysis. QPCR was performed as previously described (37). In brief, total RNA was isolated using Trizol and reverse transcribed using Superscript III (Invitrogen). PCR reactions were set up with first-strand cDNA, gene-specific primers, passive reference dye, and SYBR Green QPCR Master Mix (Bio-Rad Laboratories) according to the manufacturer's instructions. Real-time PCR was performed in triplicate, and fluorometric data were collected at the annealing step of each cycle. A dissociation curve was performed at the end of 40 cycles to confirm specificity of amplification. The primers used for real-time PCR analysis were designed to avoid amplification of genomic DNA. The primers used in this study include Id2-R, 5′-CACAGAGTACTTTGCTATCATTCG-3′; Id2-L, 5′-CCTGAACACGGACATCAGC-3′; E47L, 5′-GCCGAAGAGGACAAGAAGG-3′; HPRT-66F, 5′-ACCTCTCGAAGTGTTGGATA-3′; HPRT-66R, 5′-CAACAACAAACTTGTCTGGA-3′; Id3for, 5′-CACTTACCCTGAACTCAACGCC-3′; Id3rev, 5′-CCCATTCTCGGAAAAGCCAG-3′; Id1for, 5′-TTCAGCCTCCAGAGACTTTGGG-3′; Id1rev, 5′-CCGAGAAGCACGAAATGTGACC-3′; HEBfor, 5′-CGATTAGGAACCCACGAAGG-3′; HEBrev, 5′-GGAAAATGAGCCTCTCTCTGATG-3′; E2-2-98F, 5′-TTGAACCCACCCCAAGACCC-3′; E2-2-98R, 5′-CGCCCTCGTCATCGGATTTG-3′; Ets1for, 5′-CTGACCTCAACAAGGACAAGCC-3′; Ets1rev, 5′-TTCCAGAAGAAACTGCCACAGC-3′; CCR1-F, 5′-CATCATCATACAGGAAGCCAAG-3′; CCR1-R, 5′-GAGGAACTGGTCAGGAATAATAGC-3′; CCR5-F, 5′-TAGCCAGAGGAGGTGAGACATCCG-3′; CCR5-R, 5′-GCAGGGTGCTGACATACCATAATC-3′; CX3CR1-F, 5′-TCCCTTCCCATCTGCTCAGGAC-3′; CX3CR1-R, 5′-ACAATGTCGCCCAAATAACAGG-3′.

Detection of LNs and PPs. LNs were visualized 1 wk after intraperitoneal injection of 100 μl of a 1 mg/ml solution of Chicago sky blue (Sigma-Aldrich). PPs were visualized after incubation of the small intestines in a 7% acetic acid solution on ice for 5–10 min.

Immunofluorescence histology. Embryos were removed from pregnant females at 15.5 d postcoitum and prepared for staining as described previously (28). In brief, embryos were fixed overnight at 4°C in 4% paraformaldehyde, washed, soaked in 30% sucrose in PBS for 1 d, and frozen in OCT (Sakura). Sections were cut at 8 μm, adsorbed onto Superfrost-Plus slides (Menzer Gläser), and stained in PBS supplemented with 1% bovine serum and 0.1% Triton X-100. After 1 h of blocking with 10% bovine serum, sections were incubated overnight at 4°C with directly conjugated monoclonal antibodies, washed, and mounted with Fluoromount G (Southern Biotechnology Associates, Inc.). Images were taken on a microscope (AxioImager M1; Carl Zeiss MicroImaging, Inc.).

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