



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

Markus D. Boos, Yoshifumi Yokota, Gérard Eberl, B. L. Kee

► To cite this version:

Markus D. Boos, Yoshifumi Yokota, Gérard Eberl, B. L. Kee. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity.. Journal of Experimental Medicine, 2007, 204 (5), pp.1119-1130. 10.1084/jem.20061959 . pasteur-00161296

HAL Id: pasteur-00161296

<https://pasteur.hal.science/pasteur-00161296>

Submitted on 25 Oct 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Markus D. Boos,¹ Yoshifumi Yokota,⁵ Gerard Eberl,⁶
and Barbara L. Kee^{1,2,3,4}

¹Committee on Immunology, ²Committee on Cancer Biology, ³Committee on Developmental Biology, and ⁴Department of Pathology, University of Chicago, Chicago, IL 60657

⁵Department of Molecular Genetics, School of Medicine, University of Fukui, Matsuoka, Fukui 910-1193, Japan

⁶Laboratory of Lymphoid Tissue Development, Institut Pasteur, 75724 Paris Cedex 15, France

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.

CORRESPONDENCE

Barbara L. Kee:
bkee@bsd.uchicago.edu

Abbreviations used: CLP, common lymphoid progenitor; EBF, early B cell factor; γ c, 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 TCR $\alpha\beta$, TCR $\gamma\delta$, 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 α 2-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 TNF α and IFN γ (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 (γ c) for cytokine receptors, IL-15R α , 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 γ c-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-7R α 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

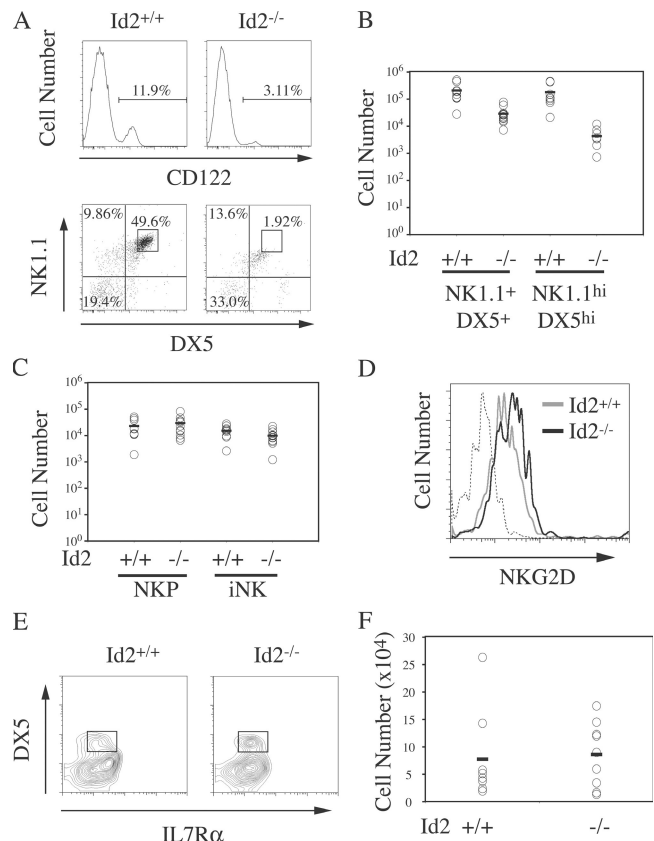


Figure 1. Decreased production of mNK cells, but not NKP, in BM of Id2^{-/-} mice. (A) BM cells from Id2^{+/+} and Id2^{-/-} mice were depleted of Lin⁺ cells and stained for CD122, NK1.1, and DX5. NK1.1 versus DX5 (bottom) expression on Lin⁻CD122⁺ cells (top) is shown. (B) Total number of mNK (NK1.1⁺DX5⁺) 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 IL7R α . DX5 versus IL7R α on Lin⁻CD122⁺ cells is shown, with the gated population representing "thymic" mNK cells. Positive expression of DX5 and IL7R α 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 $\times$ 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 NKP 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 NKP, 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⁻ NKP 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 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 NKP 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 IL7R α (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 α ⁺ (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 IL7R α and produce IFN γ

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

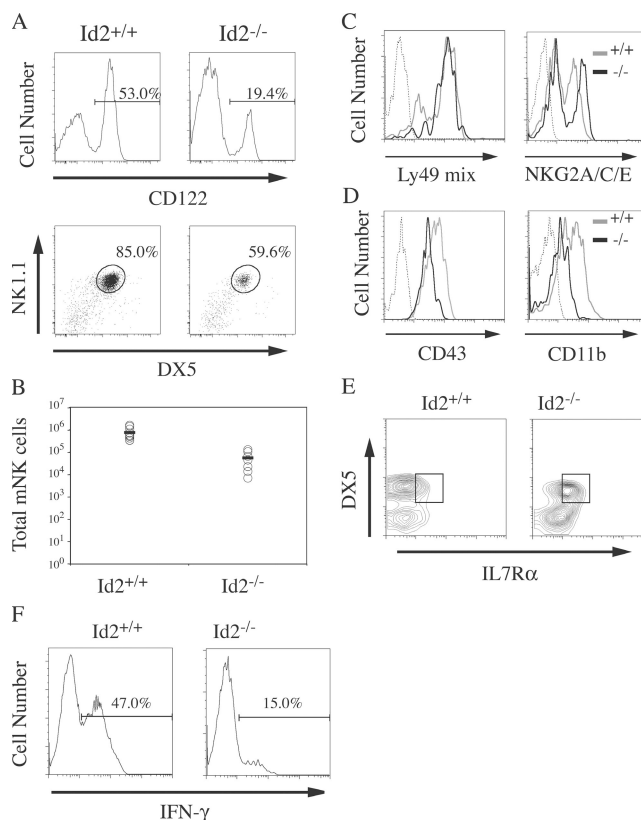


Figure 2. Splenic mNK cells in Id2^{-/-} mice are reduced in number and resemble thymic mNK cells. (A) Splenocytes were stained for Lin⁺ cells, CD122, NK1.1, and DX5. NK1.1 versus DX5 (bottom) on Lin⁻CD122⁺ cells (top) is shown. (B) Total number of Lin⁻CD122⁺DX5⁺ mNK cells in spleen of Id2^{+/+} ($n = 10$) 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) IL7R α and DX5 expression on Lin⁻CD122⁺ splenocytes from Id2^{+/+} and Id2^{-/-} mice. The boxed area represents IL7R α ⁺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 IFN γ 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 $\text{Id}2^{-/-}$ splenic mNK cells express CD43 and CD11b, these proteins are expressed at lower levels than on $\text{Id}2^{+/+}$ cells (Fig. 2 D). Interestingly, we also found that the majority of $\text{Id}2^{-/-}$ splenic mNK cells express IL7R α , 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 $\text{Id}2^{-/-}$ splenic mNK cells (14). Moreover, we did not observe any differences in in vitro survival or in vivo proliferation between $\text{Id}2^{-/-}$ and $\text{Id}2^{+/+}$ splenic mNK cells, which is consistent with the hypothesis that the $\text{Id}2^{-/-}$ 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 $\text{Id}2^{-/-}$ splenic mNK cells to produce IFN γ in vitro after overnight culture with IL-2 and IL-12. In WT mice, mNK cells from the thymus produce higher levels of IFN γ under these conditions than splenic mNK cells (14). In contrast, although a small fraction of $\text{Id}2^{-/-}$ mNK cells produce IFN γ , they produce less IFN γ on a per-cell basis than $\text{Id}2^{+/+}$ mNK cells (Fig. 2 F). This finding is consistent with a previous study showing that $\text{Id}2^{-/-}$ splenocytes are able to lyse NK cell targets, although with reduced efficiency compared with $\text{Id}2^{+/+}$ splenocytes (28). Collectively, these data indicate that the few mNK cells present in $\text{Id}2^{-/-}$ spleen resemble thymic NK cells in that they express IL7R α and low levels of CD43 and CD11b, but they produce lower levels of IFN γ 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 ($\Delta C_{T\text{GENE}} - \Delta C_{T\text{HPRT}}$) 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

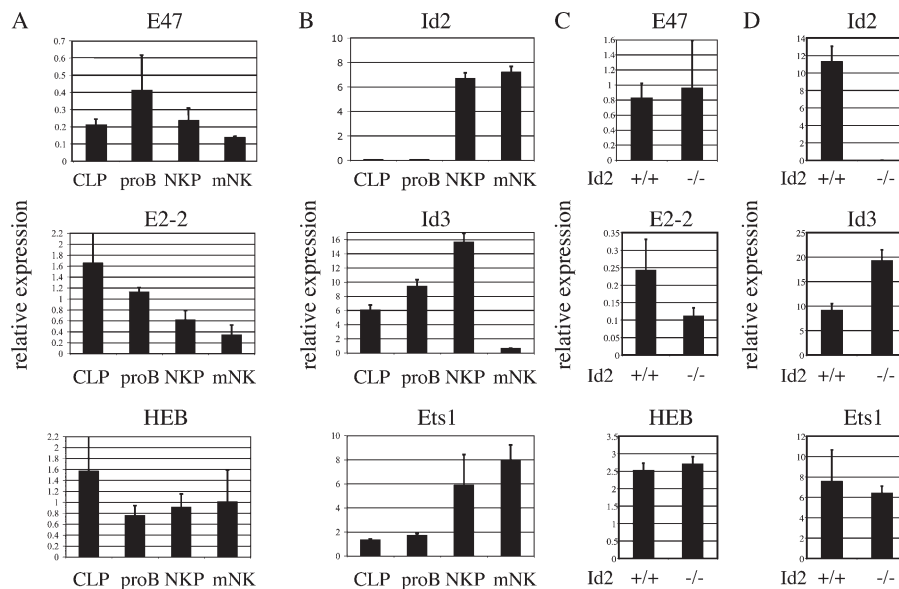


Figure 3. E protein and Id gene expression in NK cell subsets.

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 $\times$ 129/SvJ mice. QPCR analysis of E47, E2-2, and HEB (C) and Id2, Id3, and Ets1 (D) in $\text{Id}2^{+/+}$ and $\text{Id}2^{-/-}$ BM NKPs (mixed FVB/NJ $\times$

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.

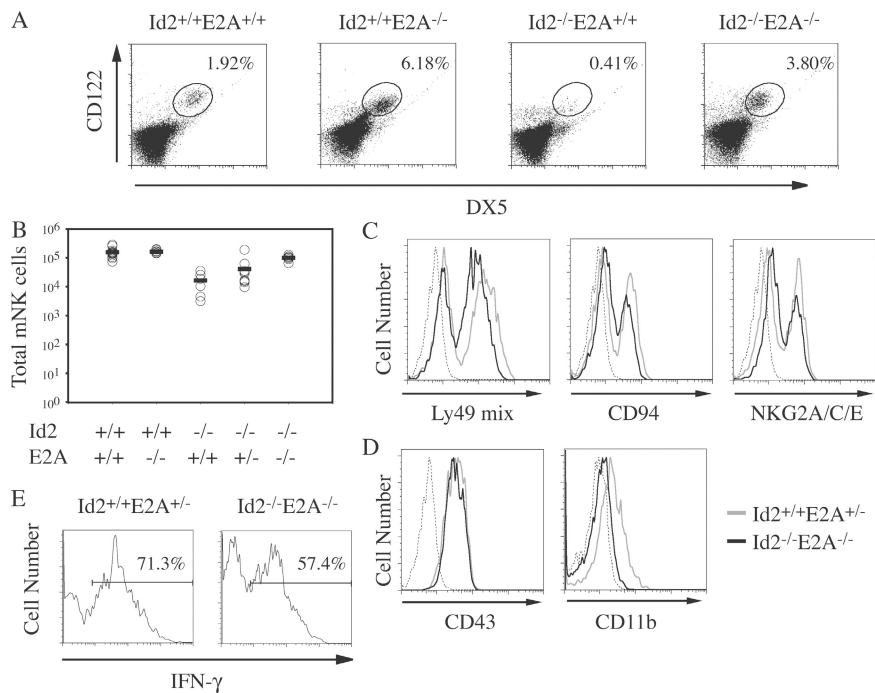


Figure 4. Mature NK cell development is restored in the BM of *Id2*^{-/-}*E2A*^{-/-} mice. (A) Lin⁻ BM cells from mice of the indicated genotype were analyzed for the presence of CD122⁺DX5⁺ mNK cells via flow cytometry. (B) Total number of BM mNK cells for mice of the indicated genotype. Each circle 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 IFN-γ 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

Genotype	LNs				
	Mesenteric	Cervical	Axillary	Brachial	Inguinal
<i>Id2</i> ^{+/+} <i>E2A</i> ^{+/+}	30/30	5/5	5/5	5/5	30/30
<i>Id2</i> ^{+/+} <i>E2A</i> ^{-/-}	11/11	ND	ND	ND	11/11
<i>Id2</i> ^{-/-} <i>E2A</i> ^{+/+}	0/30	0/2	0/2	0/2	0/30
<i>Id2</i> ^{-/-} <i>E2A</i> ^{+/-}	24/24	5/5	3/5	1/5	6/24
<i>Id2</i> ^{-/-} <i>E2A</i> ^{-/-}	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 IFN γ 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 IL7R α 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, IFN γ 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

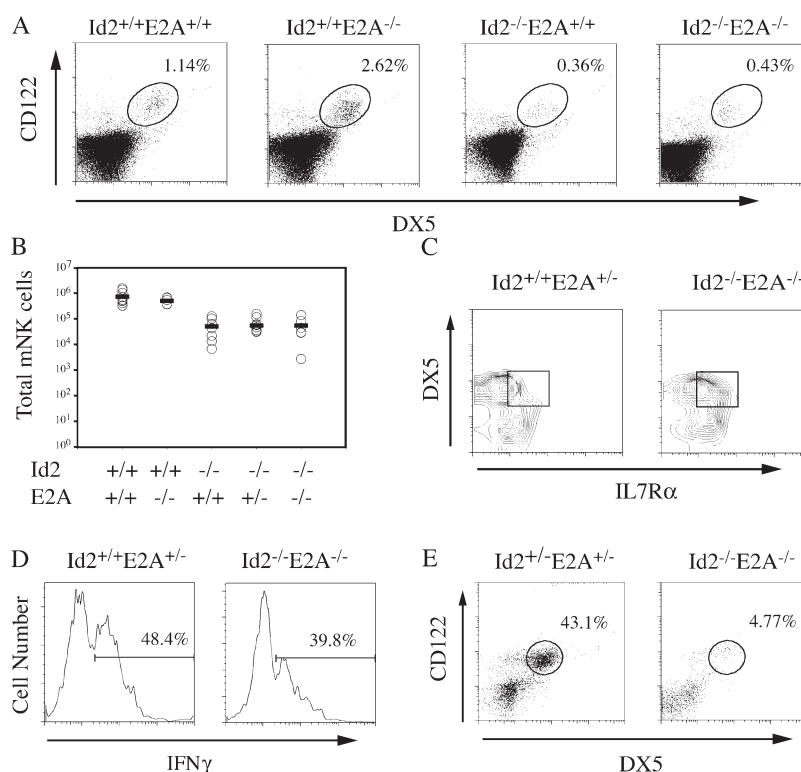


Figure 5. Loss of E2A does not rescue the phenotype of mNK cells in $Id2^{-/-}$ spleen. (A) FACS analysis for CD122 and DX5 on Lin⁻ splenocytes from mice with the indicated genotype. (B) Total number of Lin⁻ mNK cells in the spleen of mice with the indicated genotype. Each circle represents the number 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 IL7R α 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. Intra-cellular IFN γ 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 $\times$ 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 than 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).

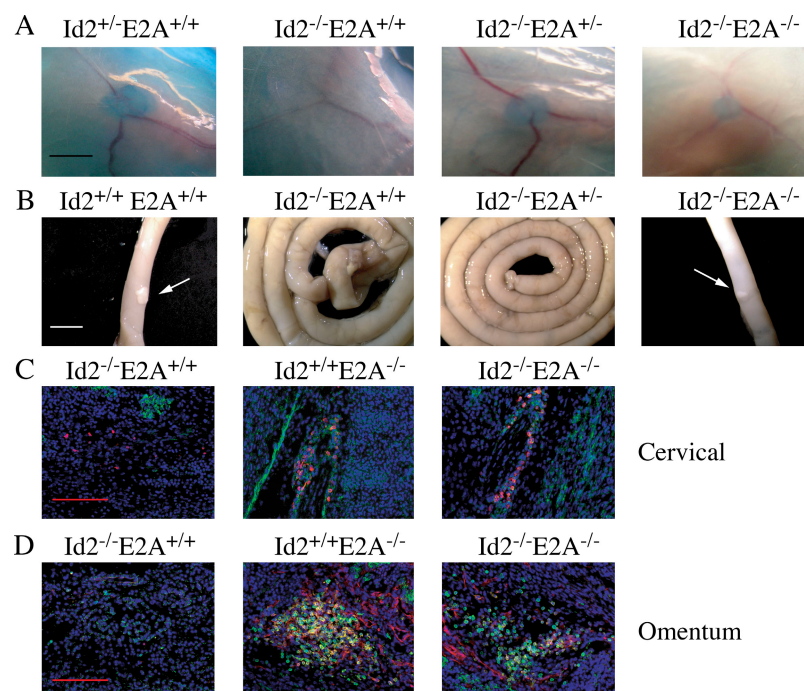


Figure 6. Development of LNs, PPs, and LTi cells in Id2^{-/-}E2A^{-/-} mice. (A) Inguinal LNs from Id2^{+/+}E2A^{+/+}, Id2^{-/-}E2A^{+/+}, Id2^{-/-}E2A^{+/-}, and Id2^{-/-}E2A^{-/-} mice were examined 2 wk after injection of Chicago sky blue. Bar, 0.5 cm. (B) Intestinal PPs were examined in Id2^{+/+}E2A^{+/+}, 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 LT α i 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 LT α i 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 LT α i cells and the peripheral lymphoid tissues that arise from LT α i 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 IL7R α 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 LT α i cells in addition to NK cells and B lymphocytes (6). In contrast, adult CLPs lack macrophage and LT α i 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 TNF α and IFN γ , 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 IFN γ 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 IFN γ 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 IFN γ 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 IFN γ 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 α ⁻) 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-bet^{-/-}, GATA-3^{-/-}, and IRF-2^{-/-} mice, placing Id2 upstream of these factors. Id2^{-/-} mice resemble mice lacking IL-15 in that IL-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-15^{-/-} mice (22). Additionally, unlike IL-15^{-/-} and IL-15R α ^{-/-} 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 γ c-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 α ⁺ and TRANCE-R⁺ LTi cells, which express LIGHT and LT α 1 β 2, with stromal "organizer" cells that express LT β R (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 α 1 β 2 and active α 4 β 1-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 LT β 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 LT α 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 $^{+/-}$ LT α cells to generate LN subsets. One possibility is that is that E2A protein accumulates during the lifespan of LT α cells, eventually passing a threshold that results in decreased LT α 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 LT α cells in Id2 $^{-/-}$ mice. In contrast, loss of E2A in these animals restores LT α 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'-TCTGAGCTTATGTGAATGATAGC-3'; Id2-AS, 5'-CGTG-TTCTCCTGGTGAAATGGCTG-3'; and neo, 5'-TCGTGCTTTACGG-TATCGCCGCTC-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-Fc γ R 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- β 1), 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), CD8 α (Ly-2), Ter119 (Ly-76), B220 (Ra3-6B2), c-kit (2B8), Sca-1 (E13-161.7), IL7R α (A7R34), CD19 (1D3), NKG2D (CX5), NKG2A/C/E (20d5), Ly49D (4E5), Ly49G2 (4D11), Ly49C/I (5E6), and IFN γ (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, TCR β , and CD25.

IFN γ 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 IFN γ -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'-CACAGAGTACT-TTGCTATCATTTCG-3'; Id2-L, 5'-CCTGAACACGGACATCAGC-3'; E47L, 5'-GCCGAAGAGGACAAGAAGG-3'; HPRT-66F, 5'-ACCTC-TCGAAGTGTGGATA-3'; HPRT-66R, 5'-CAACAACAACCTGTCTGGA-3'; Id3for, 5'-CACTTACCCTGAACCTCAACGCC-3'; Id3rev, 5'-CCCATTCGCGAAAGCCAG-3'; Id1for, 5'-TTCAGCCTCCAG-AGACTTTGGG-3'; Id1rev, 5'-CCGAGAAGCACGAAATGTGACC-3'; HEBfor, 5'-CGATTAGGAACCCACGAAGG-3'; HEBrev, 5'-GGAAAA-TGAGCCTCTCTGTATG-3'; E2-2-98F, 5'-TTGAACCCACCCCAA-GACCC-3'; E2-2-98R, 5'-CGCCCTCGTCATCGGATTTCG-3'; Ets1for, 5'-CTGACCTCAACAAGGACAAGCC-3'; Ets1rev, 5'-TTCCAGAAGA-ACTGCCACAGC-3'; CCR1-F, 5'-CATCATCATACAGGAAGCC-AAG-3'; CCR1-R, 5'-GAGGAAGTGGTCAGGAATAATAGC-3'; CCR5-F, 5'-TAGCCAGAGGAGGTGAGACATCCG-3'; CCR5-R, 5'-GCAGGGT-GCTGACATACCATAATC-3'; CX3CR1-F, 5'-TCCCTTCCCCTCTGC-TCAGGAC-3'; CX3CR1-R, 5'-ACAATGTGCCCCAAATAACAGG-3'.

Detection of LNs and PPs. LNs were visualized 1 wk after intraperitoneal injection of 100 μ l 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 μ m, adsorbed onto Superfrost-Plus slides (Menzel 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.).

We thank members of the Immunology Applications Core Facility for expert cell sorting, Sasha Chervonsky for advice on visualizing PPs; Anne Sperling and Tom

Gajewski for advice and reagents for intracellular IFN γ staining; and Reina Mebius, Vinay Kumar, Megan McNerney, Ruth Taniguchi, Jim DiSanto, and Jim Carlyle for helpful discussions. We also thank members of the Kee laboratory for helpful discussions and comments on the manuscript.

This work was supported by a grant from the National Institutes of Health/National Cancer Institute (R01 CA99978) to B.L. Kee.

The authors have no conflicting financial interests.

Submitted: 11 September 2006

Accepted: 27 March 2007

REFERENCES

- Yokoyama, W.M., S. Kim, and A.R. French. 2004. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* 22:405–429.
- Vidal, S.M., and L.L. Lanier. 2006. NK cell recognition of mouse cytomegalovirus-infected cells. *Curr. Top. Microbiol. Immunol.* 298:183–206.
- Stewart, C.A., E. Vivier, and M. Colonna. 2006. Strategies of natural killer cell recognition and signaling. *Curr. Top. Microbiol. Immunol.* 298:1–21.
- McNerney, M.E., and V. Kumar. 2006. The CD2 family of natural killer cell receptors. *Curr. Top. Microbiol. Immunol.* 298:91–120.
- Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 91:661–672.
- Mebius, R.E., T. Miyamoto, J. Christensen, J. Domen, T. Cupedo, I.L. Weissman, and K. Akashi. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3– cells, as well as macrophages. *J. Immunol.* 166:6593–6601.
- Lian, R.H., and V. Kumar. 2002. Murine natural killer cell progenitors and their requirements for development. *Semin. Immunol.* 14:453–460.
- Kouro, T., V. Kumar, and P.W. Kincade. 2002. Relationships between early B- and NK-lineage lymphocyte precursors in bone marrow. *Blood.* 100:3672–3680.
- Rodewald, H.-R., P. Moingeon, J.L. Lucich, C. Dosiou, P. Lopez, and E.L. Reinherz. 1992. A population of early fetal thymocytes expressing Fc γ RII/III contains precursors of T lymphocytes and natural killer cells. *Cell.* 69:139–150.
- Carlyle, J.R., A.M. Mitchie, C. Furlonger, R. Nakano, M.J. Lenardo, C.J. Paige, and J.C. Zuniga-Pflucker. 1997. Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J. Exp. Med.* 186:173–182.
- Ikawa, T., H. Kawamoto, S. Fujimoto, and Y. Katsura. 1999. Commitment of common T/natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thymus revealed by a single progenitor assay. *J. Exp. Med.* 190:1617–1627.
- Lu, M., R. Tayu, R. Ikawa, K. Masuda, I. Matsumoto, H. Mugishima, H. Kawamoto, and Y. Katsura. 2005. The earliest thymic progenitors in adults are restricted to T, NK, and dendritic cell lineage and have a potential to form more diverse TCR β chains than fetal progenitors. *J. Immunol.* 175:5848–5856.
- Spits, H., F. Couwenberg, A.Q. Bakker, K. Weijer, and C.H. Uittenbogaart. 2000. Id2 and Id3 inhibit development of CD34+ stem cells into pre-dendritic cell (pre-DC)2 but not pre-DC1: evidence for a lymphoid origin of pre-DC2. *J. Exp. Med.* 192:1775–1783.
- Vosshenrich, C.A., M.F. Garcia-Ojed, S.I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, E. Corcuff, D. Guy-Grand, B. Rocha, A. Cumano, et al. 2006. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat. Immunol.* 7:1217–1224.
- Rosmaraki, E.E., I. Douagi, C. Roth, F. Colucci, A. Cumano, and J.P. DiSanto. 2001. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur. J. Immunol.* 31:1900–1909.
- Kim, S., K. Iizuka, H.S. Kang, A. Dokun, A.R. French, S. Greco, and W.M. Yokoyama. 2002. In vivo developmental stages in murine natural killer cell maturation. *Nat. Immunol.* 3:523–528.
- Veinotte, L.L., B.T. Wilhelm, D.L. Mager, and F. Takei. 2003. Acquisition of MHC-specific receptors on murine natural killer cells. *Crit. Rev. Immunol.* 23:251–266.
- DiSanto, J.P., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA.* 92:377–381.
- Koka, R., P.R. Burkett, M. Chien, S. Chai, F. Chan, J.P. Lodolce, D.L. Boone, and A. Ma. 2003. Interleukin (IL)-15R α -deficient natural killer cells survive in normal but not IL-15R α -deficient mice. *J. Exp. Med.* 197:977–984.
- Burkett, P.R., R. Koka, M. Chien, S. Chai, D.L. Boone, and A. Ma. 2004. Coordinate expression and trans presentation of interleukin IL-15R α and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *J. Exp. Med.* 200:825–834.
- Kennedy, M.K., M. Glaccum, S.N. Brown, E.A. Butz, J.L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C.R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* 191:771–780.
- Vosshenrich, C.A., T. Ranson, S.I. Samson, E. Corcuff, F. Colucci, E.E. Rosmaraki, and J.P. DiSanto. 2005. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J. Immunol.* 174:1213–1221.
- Suzuki, H., G.S. Dunca, H. Takimoto, and T.W. Mak. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor β chain. *J. Exp. Med.* 185:499–505.
- Lodolce, J.P., D.L. Boone, S. Chai, R.E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity.* 9:669–676.
- Barton, K., N. Muthusamy, C. Fischer, C.N. Ting, T.L. Walunas, L.L. Lanier, and J.M. Leiden. 1998. The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity.* 9:555–563.
- Lacorazza, H.D., Y. Miyazaki, A. Di Cristofano, A. Deblasio, C. Hedvat, J. Zhang, C. Cordon-Cardo, S. Mao, P.P. Pandolfi, and S.D. Nimer. 2002. The ETS protein MEF plays a critical role in perforin gene expression and development of natural killer and NK-T cells. *Immunity.* 17:437–449.
- Colucci, F., S.I. Samson, R.P. DeKoter, O. Lantz, H. Singh, and J.P. DiSanto. 2001. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood.* 97:2625–2632.
- Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature.* 397:702–706.
- Ikawa, T., S. Fujimoto, H. Kawamoto, Y. Katsura, and Y. Yokota. 2001. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc. Natl. Acad. Sci. USA.* 98:5164–5169.
- Fukuyama, S., T. Hiroi, Y. Yokota, P.D. Rennert, M. Yanagita, N. Kinoshita, S. Terawaki, T. Shikina, M. Yamamoto, Y. Kuroki, and H. Kiyono. 2002. Initiation of NALT organogenesis is independent of the IL-7R, LT β AR, and NIK signaling pathways but requires the Id2 gene and CD3–CD4+CD45+ cells. *Immunity.* 17:31–40.
- Eberl, G., S. Marmon, M.J. Sunshine, P.D. Rennert, Y. Choi, and D.R. Littman. 2004. An essential function for the nuclear receptor ROR γ t in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5:64–73.
- Yokota, Y., S. Mori, S.I. Nishikawa, A. Mansouri, P. Gruss, T. Kusunoki, T. Kataki, and A. Shimizu. 2000. The helix-loop-helix inhibitor Id2 and cell differentiation control. *Curr. Top. Microbiol. Immunol.* 251:35–41.
- Kee, B.L. 2005. Helix-loop-helix proteins in lymphocyte lineage determination. *Curr. Top. Microbiol. Immunol.* 290:15–27.
- Sun, X.-H., N.G. Copeland, N.A. Jenkins, and D. Baltimore. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol. Cell. Biol.* 11:5603–5611.
- Bain, G., E.C. Robanus Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintraub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, et al. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell.* 79:885–892.
- Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell.* 79:875–884.

37. Seet, C.S., R.L. Brumbaugh, and B.L. Kee. 2004. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J. Exp. Med.* 199:1689–1700.
38. Nutt, S.L., B. Heavey, A.G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature*. 401:556–562.
39. Iavarone, A., P. Garg, A. Lasorella, and M.A. Israel. 1994. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev.* 8:1270–1284.
40. Iavarone, A., E. King, X.M. Dai, G. Leone, E.R. Stanley, and A. Lasorella. 2004. Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. *Nature*. 432:1040–1045.
41. Stinson, J., T. Inoue, P. Yates, A. Clancy, J.D. Norton, and A.D. Sharrocks. 2003. Regulation of TCF ETS-domain transcription factors by helix-loop-helix motifs. *Nucleic Acids Res.* 31:4717–4728.
42. Mebius, R.E. 2003. Organogenesis of lymphoid tissues. *Nat. Rev. Immunol.* 3:292–303.
43. Lasorella, A., M. Nosedà, M. Beyna, Y. Yokota, and A. Iavarone. 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature*. 407:592–598.
44. Heemskerck, M.H.M., B. Blom, G. Nolan, A.P.A. Stegmann, A.Q. Bakker, K. Weir, P.C.M. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J. Exp. Med.* 186:1597–1602.
45. Carlyle, J.R., and J.C. Zuniga-Pflucker. 1998. Lineage commitment and differentiation of T and natural killer lymphocytes in the fetal mouse. *Immunol. Rev.* 165:63–74.
46. Langlands, K., X. Yin, G. Anand, and E.V. Prochownik. 1997. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J. Biol. Chem.* 272:19785–19793.
47. Sun, X.H., and D. Baltimore. 1991. An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell*. 64:459–470.
48. Miyamoto, A., X. Cui, L. Naumovski, and M.L. Cleary. 1996. Helix-loop-helix proteins LYL1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells. *Mol. Cell. Biol.* 16:2394–2401.
49. Kee, B.L. 2005. Id3 induces growth arrest and caspase-2-dependent apoptosis in B lymphocyte progenitors. *J. Immunol.* 175:4518–4527.
50. Engel, I., and C. Murre. 1999. Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc. Natl. Acad. Sci. USA*. 96:996–1001.
51. DiSanto, J.P. 2006. Natural killer cell developmental pathways: a question of balance. *Annu. Rev. Immunol.* 24:257–286.
52. Cupedo, T., and R.E. Mebius. 2005. Cellular interactions in lymph node development. *J. Immunol.* 174:21–25.
53. Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Forster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. 406:309–314.
54. Cao, X., E.W. Shores, J. Hu-Li, M.R. Anver, B.L. Kelsail, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 2:223–238.