Commitment to natural killer cells requires the helix–loop–helix inhibitor Id2

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We have previously described how T and natural killer (NK) lineage commitment proceeds from common T/NK progenitors (p-T/NK) in the murine fetal thymus (FT), with the use of a clonal assay system capable of discriminating p-T/NK from unipotent T or NK lineage-committed progenitors (p-T and p-NK, respectively). The molecular mechanisms controlling the commitment processes, however, are yet to be defined. In this study, we investigated the progenitor activity of FT cells from Id2$^{-/-}$ mice that exhibit defective NK cell development. In the Id2$^{-/-}$ FT, NK cells were greatly reduced, and a cell population that exclusively contains p-NK in the wild-type thymus was completely missing. Id2$^{-/-}$ FT progenitors were unable to differentiate into NK cells in IL-2-supplemented-FT organ culture. Single progenitor analysis demonstrated that all Id2$^{-/-}$ fetal thymic progenitors are destined for the T cell lineage, whereas progenitors for T/NK, T, and NK cell lineages were found in the control. Interestingly, the total progenitor number was similar between Id2$^{-/-}$ and Id2$^{+/+}$ embryos analyzed. Expression of Id2 was correlated with p-NK activity. Our results suggest that Id2 is indispensable in thymic NK cell development, where it most probably restricts bipotent T/NK progenitors to the NK cell lineage.

basic helix–loop–helix transcription factor | clonal assay | progenitor | T cell | fetal thymus

Natural killer (NK) and T cells are thought to share common progenitors (1–3). Bipotent T/NK progenitors (p-T/NK) are found in the fetal thymus (FT) (4–7) and fetal liver (8). By modifying the multilineage progenitor assay, which enables us to discriminate various types of progenitors such as T, B, and myeloid lineage-committed cells and even multipotent progenitors (9), we recently established a clonal culture system that supports the development of both T and NK lineage cells (10). This progenitor assay revealed that p-T/NK is present mainly in the earliest thymic cell population and suggested that commitment of bipotent p-T/NK to T or NK lineage-committed progenitors (p-T or p-NK) occurs in FT. The regulation of cell fate determination in the bipotent p-T/NK, however, has not yet been fully elucidated, although transcription factors are likely to play a key role in cell lineage specification.

Recent gene inactivation experiments have demonstrated that the family of helix–loop–helix (HLH) proteins plays fundamental roles in the development of both T and NK cells (11–17). E proteins consisting of HEB, E2-2, and E2A gene products, E12 and E47, form a distinct subgroup within the large family of basic helix–loop–helix (bHLH)-type transcription factors (18). Hetero- or homodimers formed through the HLH region bind E-box DNA sequences, CANNTG, via the basic regions of these factors and regulate gene expression of their downstream targets (18) such as CD4 (19). Another important subgroup of HLH proteins is the family of Id proteins that possess the HLH region but lack the basic region and negatively regulate the functions of E proteins by quenching them by dimer formation (18, 20). E2A-deficient mice show, in addition to impaired B cell development (21, 22), a greatly reduced number of T cells and a partial block at the earliest stage of T cell development (11–13), where bipotent T/NK progenitors are mainly found (10). A similar block in early T cell development is observed in transgenic mice bearing Id1 or Id2 under the control of the lck promoter (23, 24). HEB$^{-/-}$ and transheterozygous E2A$^{+/-}$HEB$^{+/-}$ mice show a defect in transition from CD4$^-$CD8$^-$ to CD4$^+$CD8$^+$ T cells (13, 17). Thus, the amount of E proteins is important in the regulation of early T cell development. On the other hand, mice lacking Id2 display a marked reduction in NK cell population in the adult as well as a lack of lymph nodes and Peyer's patches (14). Conversely, overexpression of Id3 in CD34$^+$ human fetal liver cells and postnatal thymocytes results in the generation of NK cells at the expense of T cell development (25). Furthermore, enforced expression of Id3 in human pre-T cells can induce some part of the NK cell property (26). These results suggest that the regulation of the activity of E proteins seems to be crucial for the developmental fate choice of NK cells.

We investigated FT cells of Id2$^{-/-}$ embryos and found a marked reduction in NK cell number and a lack of a p-NK-enriched population. Single progenitor assay revealed the abrogated activity of Id2$^{-/-}$ FT progenitors in their ability to differentiate into the NK lineage cells, although there was no reduction in the total progenitor number in Id2$^{-/-}$ thymocytes. These results suggest an essential role of Id2 in the commitment of p-T/NK to the NK cell lineage.

Materials and Methods

Mice. All animal procedures described in this study were performed in accordance with the guideline for animal experiments of the Kyoto University Graduate School of Medicine. C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan). B6Ly5.1 mice were maintained in our animal facility. Id2$^{-/-}$ mice (14) were backcrossed to B6 mice for six generations. The NK receptor complex locus was confirmed to be the B6 strain origin by PCR (27). For timed pregnancies, the day of vaginal plug was counted as day 0. Genotyping was performed as described (14).

mAbs, Flow Cytometric Analysis, and Sorting of Progenitor Cells. All mAbs and procedures for flow cytometric analysis have been described previously (10). To isolate CD44$^+$CD25$^-$CD122$^-$ cells in the lineage marker (Lin)$^-$ CD3$^-$CD4$^-$CD8$^-$ (triple negative, TN) population, 14 days postcoitus (dpc) FT cells were stained with biotinylated Lin (anti-TER119, anti-Mac-1, anti-Gr-1, anti-NK1.1, and anti-B220) washed and then stained with a mixture of phycoerythrin-streptavidin (PE-streptavidin), PE-anti-CD4, PE-anti-CD8, PE-anti-CD3, PE-anti-CD122, FITC-anti-CD25, and allophycocyanin-anti-CD44. Cells were subsequently sorted with the use of a FACS Vantage. Statistical

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Abbreviations: HLH, helix–loop–helix; bHLH, basic HLH; dGuo, deoxyguanosine; dpc, days postcoitus; FT, fetal thymus; HOS, high oxygen submersion; Lin, lineage marker; NK, natural killer; p-NK, NK lineage-committed progenitor; p-T, T lineage-committed progenitor; p-T/NK, T/NK progenitor; TN, triple negative; PE, phycoerythrin; TCR, T cell antigen receptor.

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analyses were done with the Mann–Whitney *u* test, and *P* values less than 0.05 were considered significant.

Cytokine-Supplemented High Oxygen Submersion (HOS) Culture. HOS culture, including growth factors, has been described in detail elsewhere (9, 10). Single deoxyguanosine (dGuo)-treated FT lobes (B6Ly5.1) were placed into the wells of a 96-well V-bottom plate (Nalge Nunc International, Rochester, NY), into which progenitors were added. The plates were centrifuged at $150 \times g$ for 5 min at room temperature and placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan), and the air inside was replaced with a gas mixture (70% O_2/25% N_2/5% CO_2). The plastic bag was incubated at 37°C. Culture medium was also supplemented with stem cell factor (10 ng/ml), IL-2 (25 units/ml), and IL-7 (50 units/ml) to evenly support the development of T and NK cells (10). Medium was replaced by half every 5 days.

After 10 days of culture, cells inside and outside the FT lobe were harvested from each well. A quarter of each sample was stained with FITC-anti-Ly5.2, PE-anti-Thy-1, and Cy5-anti-Ly5.1 to be screened for the presence of progenitor type ($Ly5.1^-$) cells and expression of Thy-1 on these cells. Samples containing $Ly5.1^-Thy\text{-}1^+$ cells were selected for further analysis. The remaining three-quarters of cells from the selected samples were divided into three groups. One group was stained with FITC-anti-CD3ε, PE-anti-NK1.1, and Cy5-anti-Ly5.1; the second group was stained with FITC-anti-CD8, PE-anti-CD4, and Cy5-anti-Ly5.1; and the third group was stained with FITC-anti-T cell antigen receptor (TCR)γδ, PE-anti-TCRαβ, and Cy5-anti-Ly5.1. The surface phenotype was analyzed with a FACS Vantage.

Reverse Transcription–PCR. mRNA was isolated from $CD44^+CD25^-$, $CD44^+CD25^+$, $CD44^-CD25^+$, $CD44^-CD25^-$, $CD44^+CD25^-CD122^+$, and $CD44^+CD25^-CD122^-$ cells (3,000 cells each) within 15-dpc Lin^- TN FT cells of B6 with the use of a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia). After conversion of mRNA to cDNA with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD) and oligo(dT) primers, PCR was performed as described (8) using various sets of primers. The sequences of primers used are as follows:

Id1 sense, 5′-TCAGGATCATGAAGGTCGCCAGTG-3′; Id1 antisense, 5′-TGAAGGGCTGGAGTCCATCTGGT-3′; Id2 sense, 5′-TCTGAGCTTATGTCGAATGATAGC-3′; Id2 antisense, 5′-CACAGCATTCAGTAGGCTCGTGTC-3′; Id3 sense, 5′-CCTCTCTATCTCTACTCTCCAACA-3′; Id3 antisense, 5′-TGACCAGCGTGTGCTAGCTCTTCA-3′; Id4 sense, 5′-GCGATATGAACGACTGCTACAGTC-3′; Id4 antisense, 5′-ACTTAGCAGTCTGGTCGACAACAC-3′; E2A sense, 5′-CATCCATGTCCTGCGAAGCCAC-3′; E2A antisense, 5′-TTCTTGTCCTCTTCGGCGTCGG-3′; HEB sense, 5′-GTCAACCAAGCCCCTCCTATGATT-3′; HEB antisense, 5′-ATTGATGGGAGGAGTATGTGAGGC′; E2–2 sense, 5′-TGACCACATGACTAGCAGGGATCT-3′; E2–2 antisense, 5′-AGAGGTGCTGTAATGGTTTGTGCC-3′; β-actin sense, 5′-TCCTGTGGCATCCATGAAACT-3′; β-actin antisense, 5′-GAAGCACTTGCGGTGCACGAT-3′. Amplification was performed for 20 cycles for β-actin and for 30 cycles for all other genes. Fifteen microliters of PCR product was electrophoresed through 1.2% agarose or 5% polyacrylamide gel and stained with ethidium bromide.

Results

$CD44^+CD25^-CD122^+$ Cells Are Missing in $Id2^{-/-}$ FT. 17-dpc $Id2^{-/-}$ fetal thymocytes were analyzed for NK cells by flow cytometry. As shown in Fig. 1*A* (*Upper*), only 0.5% of $Id2^{-/-}$ fetal thymocytes were $NK1.1^+CD3^-$ NK cells, whereas 2.4% and 2.5% were

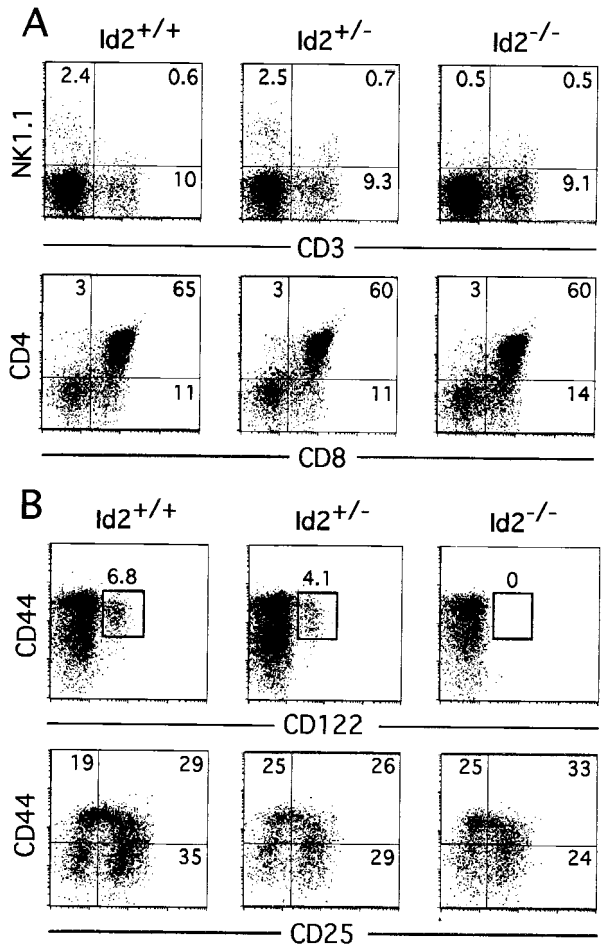

Fig. 1. Selective developmental defect in NK cells of the $Id2^{-/-}$ fetal thymus. (*A*) Cells of 17-dpc FT were stained with CD3-, NK1.1-, CD4-, and CD8-specific mAbs. Total thymocyte numbers were $1.5 \pm 0.9 \times 10^6$, $1.8 \pm 0.6 \times 10^6$ and $0.7 \pm 0.6 \times 10^6$ for $Id2^{+/+}$, $Id2^{+/-}$, and $Id2^{-/-}$ FT, respectively. (*B*) Cells of 14-dpc FT were stained with FITC-conjugated mAbs specific for TER119, Mac-1, Gr-1, B220, NK1.1, CD3, CD4, and CD8. Only FL-1-negative cells were analyzed for expression of CD44 vs. CD122 and CD44 vs. CD25. Total thymocyte numbers were $5.6 \pm 1.4 \times 10^4$, $3.2 \pm 1.4 \times 10^4$, and $1.5 \pm 1.3 \times 10^4$ for $Id2^{+/+}$, $Id2^{+/-}$, and $Id2^{-/-}$ FT, respectively. Numbers represent the percentage of cells in the gated population and in each quadrant in *A* and *B*.

so in wild-type and $Id2^{+/-}$ FT, respectively. This result demonstrated a marked reduction of NK cells in $Id2^{-/-}$ FT to an extent similar to that observed in the adult bone marrow cells and splenocytes of $Id2^{-/-}$ mice (14). On the other hand, no significant difference was detected in T cells among the three genotypes with respect to percentages of $CD3^+$ thymocytes (Fig. 1*A*, *Upper*) and cells expressing CD4 and/or CD8 (Fig. 1*A*, *Lower*), although the total thymocyte number was slightly decreased in $Id2^{-/-}$ FT ($1.5 \pm 0.9 \times 10^6$, $1.8 \pm 0.6 \times 10^6$, and $0.7 \pm 0.6 \times 10^6$ for $Id2^{+/+}$, $Id2^{+/-}$, and $Id2^{-/-}$ FT, respectively). These results indicated that the defect in $Id2^{-/-}$ fetal thymocytes is rather specific to NK cells.

The $CD44^+CD25^-$ cell population within TN cells corresponds to the most immature stage in FT and progresses to the $CD44^+CD25^+$, $CD44^-CD25^+$, and, subsequently, $CD44^-CD25^-$ stages of T cell differentiation (28). We previously reported that bipotent p-T/NK is abundantly found at the $CD44^+CD25^-$ stage, and p-T/NK activity is reduced at the subsequent $CD44^+CD25^+$ stage and confined to the T cell lineage afterward (10). Moreover, the $CD44^+CD25^-$ cell population in 14-dpc FT

can be further divided into two fractions according to CD122 (the IL-2 receptor β chain) expression: $CD122^+$ cells exclusively contain p-NK (10). Based on these observations, the $CD44^+CD25^-CD122^+$ cell population was next investigated in $Id2^{-/-}$ FT at 14-dpc. As shown in Fig. 1*B* (*Upper*), $CD44^+CD25^-CD122^+$ cells were $7.0 \pm 0.3\%$ and $4.2 \pm 0.3\%$ of the cell population in $Id2^{+/+}$ ($n = 5$) and $Id2^{+/-}$ FT ($n = 3$), respectively, showing a slight decrease in the population in $Id2^{+/-}$ FT ($P < 0.05$). In $Id2^{-/-}$ fetal thymocytes, in contrast, no $CD44^+CD25^-CD122^+$ cells were detected. These data suggest that the lack of a $CD44^+CD25^-CD122^+$ cell population is responsible for the impaired NK cell development in $Id2^{-/-}$ FT, supporting the notion that thymic NK cells are derived from $CD44^+CD25^-CD122^+$ cells. In addition, the dosage of the wild-type allele of Id2 seems to correlate with the percentage of the fraction enriched for p-NK, although NK cells were observed at similar percentages in $Id2^{+/+}$ and $Id2^{+/-}$ FT at 17 dpc. Analyses of CD44 and CD25 expression, on the other hand, indicated seemingly normal fetal thymocyte development in both $Id2^{+/-}$ and $Id2^{-/-}$ FT. Slightly delayed T cell differentiation, however, was noted in $Id2^{-/-}$ FT, as the most prominent population was $CD44^-CD25^+$ cells in wild-type and $Id2^{+/-}$ FT, whereas $CD44^+CD25^+$ cells predominated in $Id2^{-/-}$ FT (Fig. 1*B, Lower*). Consistent with this observation, a 4-fold reduction in the total thymocyte number was observed in $Id2^{-/-}$ FT at 14 dpc in comparison with that of wild-type FT (Fig. 1), showing a delayed development of $Id2^{-/-}$ thymocytes with regard to cell proliferation as observed at 17 dpc.

IL-2-Supplemented Fetal Thymus Organ Culture Fails to Induce NK Cell Differentiation from $Id2^{-/-}$ Fetal Thymocytes. To explore the differentiation potential of $Id2^{-/-}$ fetal thymocytes, 100 Lin^- TN $CD44^+CD25^-CD122^-$ cells isolated from 14-dpc FT were seeded into a dGuo-treated normal FT lobe in the presence of IL-2, IL-7, and stem cell factor under HOS conditions, where T and NK cells are evenly supported to differentiate (10). After 10 days, wild-type and $Id2^{+/-}$ fetal thymocytes had generated T and NK cells to a similar extent (Fig. 2, *Upper*). In the $Id2^{-/-}$ fetal thymocyte culture, however, no NK cells were identified, whereas T cells were efficiently induced (Fig. 2, *Upper*). Thus, the $CD44^+CD25^-CD122^-$ cell population in $Id2^{-/-}$ FT is impaired in generating NK cells even under highly supportive culture conditions. We found no significant difference among thymocytes of $Id2^{+/+}$, $Id2^{+/-}$, and $Id2^{-/-}$ genotypes with respect to expression of TCR$\alpha\beta$, TCR$\gamma\delta$, CD4, or CD8 (Fig. 2).

Abrogated Commitment of $Id2^{-/-}$ Fetal Thymic Progenitors to NK Cells. We further investigated the commitment status of $CD44^+CD25^-CD122^-$ fetal thymocytes of $Id2^{-/-}$ mice with the use of a progenitor assay at the single-cell level. $CD44^+CD25^-CD122^-$ fetal thymocytes at 14 dpc were sorted and cultured with a dGuo-treated FT lobe in the presence of stem cell factor, IL-2, and IL-7 at one cell per lobe under HOS conditions. After 10 days, cells generated were collected and analyzed by flow cytometry to determine the potential of the progenitors. As previously reported (10), wild-type $CD44^+CD25^-CD122^-$ fetal thymocytes contained all three types of progenitors, p-T/NK, p-T, and p-NK (Fig. 3, *Upper*). $Id2^{-/-}$ fetal thymocytes, in contrast, contained p-T only. No p-T/NK or p-NK were detected (Fig. 3, *Lower*), demonstrating that $Id2^{-/-}$ fetal thymocytes are devoid of NK lineage progenitor activity. Remarkably, the number of p-T in the $CD44^+CD25^-CD122^-$ fetal thymocytes of $Id2^{-/-}$ mice (17.5 ± 0.7, $n = 4$) corresponded to the total number of p-T/NK, p-T, and p-NK of wild type (17.0 ± 0.9, $n = 4$) (Fig. 3). This correspondence indicates that the number of cells bearing a progenitor activity in $Id2^{-/-}$ FT at the $CD44^+CD25^-CD122^-$ stage is similar to that of $Id2^{+/+}$ FT, although the commitment of these cells is different.

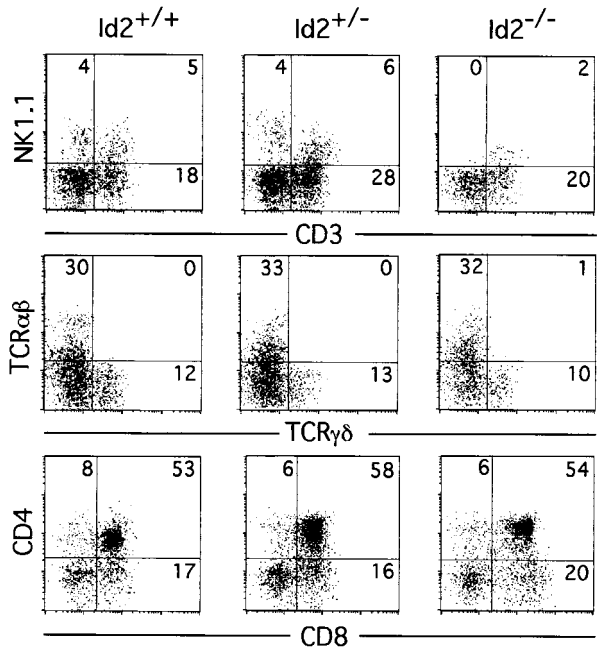

Fig. 2. $Id2^{-/-}$ fetal thymocytes give rise to T cells but not NK cells in IL-2-supplemented fetal thymus organ culture. $Lin^-CD44^+CD25^-$ FT cells at 14 dpc were sorted, and 100 cells were cultured with a dGuo-treated FT lobe (B6Ly5.1) in the presence of IL-2 (25 units/ml), IL-7 (50 units/ml), and stem cell factor (10 ng/ml). After 10 days of culture, cells in each well were analyzed by flow cytometry. Representative flow cytometric profiles for expression of indicated markers on $Ly5.1^-$ cells were shown. Cell recovery was 2.4×10^4, 3.0×10^4, and 2.0×10^4 cells for $Id2^{+/+}$, $Id2^{+/-}$, and $Id2^{-/-}$, respectively.

Expression of Id2 in TN Fetal Thymocytes Is Closely Related to p-NK. To investigate the molecular events that regulate NK lineage specification in FT, we examined the gene expression of Id and E proteins in subfractions of FT cells of normal fetuses. mRNA was isolated from 15-dpc FT cells fractionated according to the expression of CD44, CD25, and CD122, and reverse transcription–PCR was performed. As shown in Fig. 4*A*, Id2 expression was readily detectable in the $CD44^+CD25^-$ fraction that contains p-T/NK, p-T, and p-NK, whereas expression was reduced to a barely detectable level at later stages of fetal thymocyte development. On the other hand, Id1 was expressed in $CD44^+CD25^+$ cells, with faint expression in $CD44^+CD25^-$ and $CD44^-CD25^+$ cells. Id3 and Id4 were barely detectable in all cell populations analyzed. The E proteins E2A and HEB are targets of Id proteins and were consistently detected. No expression of E2–2 was observed in any cell population analyzed. Expression of Id2, E2A, and HEB was further analyzed in subfractions defined by expression of CD122. Id2 was abundantly detected in $CD122^+$ cells that exclusively contain p-NK (10), whereas lower expression was observed in $CD122^-$ cells that include p-T/NK, p-T, and p-NK (Fig. 4*B*). A reciprocal pattern of expression was noted for E2A and HEB. Taken together, these results indicate the correlation of Id2 expression and progenitor activity for the NK cell lineage. Given that the function of Id proteins is to suppress the activity of E proteins, these results suggest that negative regulation of E2A gene products and/or HEB by Id2 is required for the generation of thymic NK cells.

Discussion

Using a newly established assay system, we have shown that the regulation of E protein function is critical for the cell fate choice

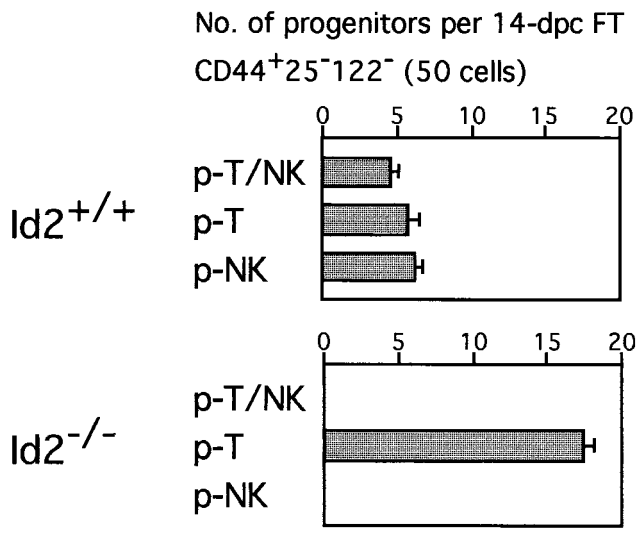

Fig. 3. Abrogated NK cell development of Id2$^{-/-}$ fetal thymocytes. Single CD44^{+}CD25^{-}CD122^{-} FT cells at 14 dpc were picked up under microscopical visualization and were seeded into wells containing a dGuo-treated FT lobe. After 10 days of culture under HOS conditions, the numbers of the different types of progenitors detected among 50 CD44^{+}CD25^{-}CD122^{-} cells were scored. Error bars indicate SEM ($n = 4$ in both genotypes). The flow cytometric profiles of cells generated from p-NK, p-T, and p-T/NK were essentially same as those presented in our previous report (10).

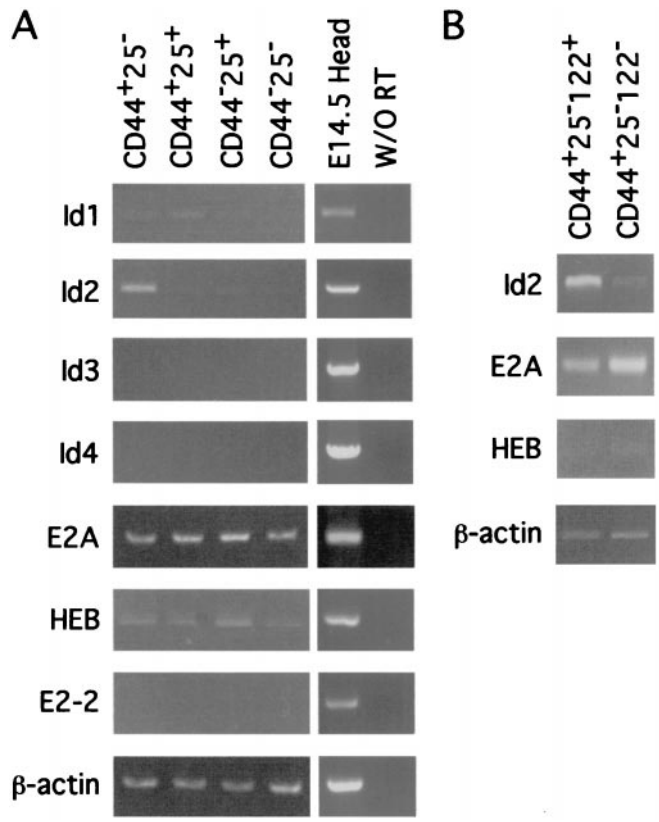

Fig. 4. Reverse transcription–PCR analysis for the gene expression of Id and E proteins in subpopulations of fetal thymus. Wild-type thymocytes at 15 dpc were fractionated, based on cell surface phenotype, into developmental subsets. Lin^{-} TN CD122^{+} cells in FT are exclusively NK lineage committed, as previously reported (10). mRNA was prepared from sorted cells (as indicated at the *Top* of the figures) and reverse-transcribed. The cDNA was amplified with specific primers for each gene indicated. Reverse transcription–PCR and PCR products of 14-dpc embryonic head total RNA served as positive and negative controls, respectively, in each reaction.

by bipotent T/NK precursors of the NK lineage. Id2$^{-/-}$ embryos are virtually devoid of the CD44^{+}CD25^{-}CD122^{+} p-NK-enriched population. The abrogated NK potential of Id2$^{-/-}$ FT cells was explicitly revealed by this single progenitor assay. In contrast, the absence of Id2 does not seem to affect T cell development in FT. These findings suggest an intriguing role for Id2 at the bifurcation point at which bipotent T/NK precursors differentiate into T and NK lineage cells.

Id proteins negatively regulate cell differentiation as well as positively control proliferation (20). Inhibition of differentiation is observed in numerous model systems such as myogenesis (29) and myelopoiesis (30), which is mediated by functional inactivation of bHLH transcription factors by Id proteins at the protein level (29, 31). Conversely, cell cycle stimulator properties of Id proteins have been observed in cultured cells such as mammary epithelial cells (32), erythroleukemia cells (33), and osteosarcoma cells (34), with forced overexpression of Id proteins. Antagonization of the retinoblastoma protein and the suppression of *p21*^{WAF1} transcription induced by bHLH factors have been reported (34–36), although the detailed mechanisms are still unclear. Arrest in cell differentiation and enhanced cell proliferation are usually seen simultaneously in the same experimental systems (32–34, 37). Because terminal cell differentiation is generally accompanied by exit from the cell cycle, it is conceivable that Id proteins are valuable tools for the investigation of the mechanisms controlling cell differentiation and proliferation.

Based on the proposed functions of Id proteins, there are two possible roles for Id2 in the generation of thymic NK cells. One is the direct involvement of Id2 in commitment of bipotent p-T/NK to p-NK through the inactivation of E protein function. Another is the essential requirement of Id2 in the maintenance of progenitor activity of p-NK after commitment, thus prohibiting the survival of Id2$^{-/-}$ p-NK.

We favor the former possibility, inasmuch as cell fate choice in bipotent p-T/NK for T or NK cell lineages seems to be regulated by the activity of E proteins, as demonstrated by gain-of-function and loss-of-function experiments. A schematic representation of Id2 function in cell fate decision is presented in Fig. 5. E proteins lead to the T cell lineage, whereas their functional inactivation by Id directs differentiation to the NK cell lineage (11–17, 21–26). As demonstrated in this work, loss of Id2 leads to a defect in NK cell development but does not result in the reduction of the total progenitor number within the CD44^{+}CD25^{-}CD122^{-} thymic cell population. Thus it is conceivable that the loss of Id2 fails to support the lineage specification of p-T/NK to p-NK, and, as a consequence, all bipotent p-T/NK are destined for the T cell lineage. Considering that Id2 gene expression correlates well with p-NK activity, we propose that Id2 is a key player in NK cell development *in vivo*, although the importance of a negative regulator of bHLH factors in NK cell development has been demonstrated by enforced expression of Id3 in *in vitro* experimental systems (25, 26). This notion is supported by the very recent report by Spits *et al.* (38).

As for the possibility that Id2 plays a role in the survival of NK cells, we need to assume that Id2$^{-/-}$ p-T possesses a higher proliferation activity than that of wild-type p-T to explain the finding that progenitor numbers in the CD44^{+}CD25^{-}CD122^{-} cell population are the same for Id2$^{-/-}$ and wild-type fetal thymocytes. In this context, Id2 should have dual effects on cell cycle control, depending on the cell type: suppression of cell growth in p-T and promotion in p-NK. Overexpression of Id3, functioning in a manner similar to that of Id2, does not seem to confer an advantage on developing human NK cells (25).

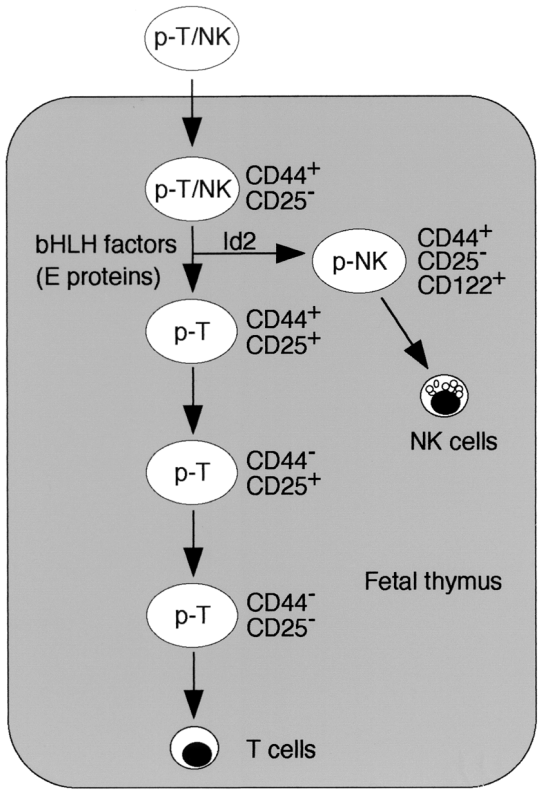

Fig. 5. Schematic representation of Id2 function in the lineage restriction of NK cells. Bipotent T/NK precursors first colonize the fetal thymus. At the stage of $CD44^+CD25^-$, some FT cells start to express Id2, which inactivates bHLH factors, probably E proteins such as HEB and E2A gene products. These cells are committed to the NK cell lineage and express CD122/IL-2Rβ. At present, the order of CD122 expression and NK cell commitment is uncertain, although our data suggest that commitment precedes CD122 expression.

Furthermore, total cell numbers in $Id2^{-/-}$ FT were reduced at 14 and 17 dpc, compared with those of $Id2^{+/-}$ and wild-type embryos. These findings suggest that a possible role of Id2 in fetal thymocyte survival is less likely, although Id2 functions indeed as a cell cycle stimulator and a survival factor in mammary epithelial cells during pregnancy (39). A resolution of this issue requires the development of tools to identify respective progenitors within fetal thymocytes and analyses of their proliferation in FT.

Id2-null mutant mice are virtually devoid of $CD122^+$ FT cells. CD122/IL-2Rβ is a component not only of IL-2 but also of IL-15 receptors (40, 41). In mice, both the administration of a mAb against CD122 and gene inactivation of CD122 result in severely impaired NK cell development (42, 43), demonstrating its essential role for NK cell development. Based on our observation that the $CD122^-$ subfraction of $CD44^+CD25^-$ FT cells of wild-type mice contains pT/NK and pNK (ref. 10 and Fig. 3), we think that CD122 expression occurs after cell fate determination of NK lineage and that the signaling involving CD122 is required for the proliferation and/or maturation of NK lineage cells. However, we cannot exclude the possibility that CD122 expression is a prerequisite for NK cell development. One intriguing speculation is a direct link between Id2 and the induction of CD122 gene expression in bipotent T/NK precursors. Given a bHLH factor that suppresses the gene expression of CD122 in bipotent T/NK precursors, the Id2 protein expressed in a subset of the precursors may release the suppression, directly or indirectly, and lead the cells to a developmental pathway to NK lineage via a signal involving CD122.

The name of Id is derived from the ability of these proteins to inhibit DNA binding as well as differentiation (20, 31). As mentioned above, Id proteins inhibit cell differentiation in several *in vitro* systems (29, 30). In the cell fate choice of bipotent T/NK precursors, however, Id2 is required for the commitment of bipotent FT cells to the NK lineage, and its deficiency results in the impaired development of NK cells. The situation is the same for the lymphotoxin-expressing $CD4^+CD3^-$IL-7 receptor-α^+ cell population that is indispensable for the organogenesis of peripheral lymphoid organs as previously reported (14). The absence of Id2 leads to the severely disturbed differentiation of the cell population, which results in the alymphoplasia phenotype of Id2 null mutant mice (14). In this regard, Id2 can be a stimulator of cell differentiation for some cell types, particularly at a bifurcation point from which bipotent precursors differentiate into two distinct cell lineages. Thus it might be appropriate to view Id proteins as regulators, not simply as inhibitors of cell differentiation.

Our present work reinforces the indispensable role of Id2 in cell fate determination of NK lineage and highlights the $CD44^+CD25^-CD122^+$ cell population as a distinct and very early developmental stage on the differentiation pathway to NK cells. Id2-deficient mice show defective NK cell development not only in the adult bone marrow (14) but also in the embryonic thymus. This defective development suggests that these organs share, at least to some degree, regulatory mechanisms for NK cell development. Our understanding of how NK cells are generated would be facilitated by further developmental dissection of cells at the $CD44^+CD25^-$ stage and by identification of signals that induce the expression of Id2 in early fetal thymocytes.

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