TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver

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Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a key effector molecule expressed by natural killer (NK) cells and has been shown to prevent tumor initiation, growth, and metastasis. Here we demonstrate that TRAIL is the dominant cytotoxic effector molecule expressed by NK cells in fetal mice. On birth and with age, NK cells develop full functional capacity, including the ability to secrete interferon γ (IFN-γ) and interleukin 13 (IL-13) and mediate perforin- and Fas ligand-mediated cytotoxicity. However, interestingly, a phenotypically immature TRAIL$^+$ NK cell subpopulation is retained in the liver of adult mice, and its retention is dependent on IFN-γ but not dependent on host IL-12, IL-18, or endogenous host pathogens. Adoptive transfer of either adult liver or neonatal TRAIL$^+$ NK cells resulted in the appearance of TRAIL$^-$ NK cells with a mature phenotype, suggesting that these TRAIL$^+$ NK cells were indeed a precursor. Although inducers of IFN-γ stimulate TRAIL expression on mature NK cells, data indicated that constitutive TRAIL expression was a hallmark of immature cytotoxic NK cells. This study is the first to describe the concomitant maturation of NK cell effector function with surface phenotype in vivo and implicates an important defensive role for NK cell TRAIL in the developing immune system. (Blood. 2005; 105:2082-2089)

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a type 2 transmembrane protein belonging to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in most normal cells.[1,2] We recently reported that a subpopulation of natural killer (NK) cells in adult mouse liver constitutively expressed TRAIL in an interferon (IFN-γ)–dependent manner.[3] These liver NK cells were partially responsible for the natural antimetastatic function against TRAIL-sensitive tumor cells.[3,4] We have also demonstrated that IFN-γ–mediated TRAIL induction on NK cells played a substantial role in the IFN-γ–dependent antimetastatic effect of IL-12 and α-galactosylceramide (α-GalCer).[5] Given that both NK cells and TRAIL have been implicated in natural protection against primary tumor development,[6,7] TRAIL may be a major responsible for the NK cell-mediated and IFN-γ–dependent mechanisms of tumor surveillance in the adult.[8,9]

Despite a recent explosion in our knowledge concerning NK cell receptors,[10,11] the ontogeny development of mouse NK cell effector function is still poorly defined. It has been suggested that a CD122$^+$ NK1.1$^-$DX5$^-$ population contains the early NK cell-committed precursor,[12] but subsequent developmental changes, including the acquisition of cytotoxic effector molecules and cytokine-producing function, are not well understood.[13] The presence of a high frequency of phenotypically immature NK cells in mouse liver was shown in a recent report.[14] In addition, the selective expression of TRAIL on immature human NK cells was reported using in vitro culture.[15] In this study, we report the first analysis of the development of NK cell effector function from fetal to adult life. We have discovered that TRAIL$^+$ NK cells are phenotypically immature NK cells, which are predominant in fetal and neonatal mice, and are functionally distinct from the mature NK cell subset in adult mice. TRAIL is expressed at the earliest stages of NK cell development and in the fetus appears the predominant effector pathway. A coincident expression of NK cell receptors occurs in young mice as the NK cell repertoire acquires additional effector functions. Some phenotypically immature TRAIL$^+$ NK cells specifically remain in the adult liver, affording the adult continued TRAIL-mediated surveillance capacity in the liver.

Materials and methods

Mice

C57BL/6 (B6) and BALB/c mice were obtained from Charles River Japan (Yokohama, Japan) and The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). RAG-2–deficient (RAG-2$^{-/-}$), TRAIL-deficient (TRAIL$^{-/-}$), IFN-γ–deficient (IFN-γ $^{-/-}$), IL-12–deficient (IL-12$^{-/-}$), and IL-12– and IL-18–deficient (IL-12$^{-/-}$IL-18$^{-/-}$) B6 mice and perforin-deficient (perforin$^{-/-}$), TRAIL-deficient (TRAIL$^{-/-}$), and perforin and

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TRAIL–deficient (perforin$^{-/-}$TRAIL$^{-/-}$) BALB/c mice were derived as described previously.[4,5,16,17] 129/J mice were obtained from Japan SLC (Hamamatsu, Japan). All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University and Peter MacCallum Cancer Centre. Germ-free BALB/c mice were kindly provided by Yakult Central Institute for Microbiological Research (Tokyo, Japan). To obtain timed pregnant mice, mice were mated for 15 hours and the fetuses were removed at fetal day 17 (plug date = day 0). Some mice were treated with 2 μg α-GalCer (Kirin Brewery, Gumma, Japan) 3 days before the analyses.

Flow cytometric analysis

Mononuclear cells (MNCs) were prepared from the spleen, liver, and bone marrow as described.[3,5] To avoid the nonspecific binding of monoclonal antibodies (mAbs) to FcγR, antimouse CD16/32 (2.4G2) mAb (BD PharMingen, San Diego, CA) was added to the mAb mixture. Surface phenotype of B6-derived NK cells (gated on NK1.1$^+$ CD3$^-$ cells) was analyzed by 4-color immunofluorescence on a FACSCaliber (BD Bioscience, San Jose, CA). Cells were stained with fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)–, or allophycocyanin (APC)–conjugated antimouse NK1.1 mAb (PK136) and FITC-, Cy-Chrome–, or APC-conjugated antimouse CD3 mAb (145-2C11) as previously described.[3,5] In addition, cells were incubated with FITC- or biotin-conjugated antimouse CD11b mAb (M1/70), PE-conjugated antimouse pan NK cells mAb (DX5), FITC- or biotin-conjugated antimouse CD94 mAb (18d3), PE- or biotin-conjugated anti-mouse TRAIL mAb (N2B2), FITC-conjugated anti-mouse Ly49 mAbs (antimouse Ly49A ^{B6} [A1], antimouse Ly49C and I mAb [5E6], antimouse Ly49D mAb [4E5], antimouse Ly49G2 mAb [4D11], antimouse Ly49I mAb [YL1-90]), PE- or biotin-conjugated anti-NKG2D (CX5), isotype-matched control mAbs (G155-178, MOPC-31C, R35-95, [illegible] R4-22, A19-3, G235-2356, and Ha4/8), streptavidin-PE, streptavidin-Cy-Chrome, and streptavidin-APC. Antibodies and staining reagents were purchased from eBioscience (San Diego, CA), except [illegible]P-Cy5.5, Cy-Chrome–conjugated antibodies, anti-Ly49 mAb, isotype-matched control mAbs, and streptavidin-Cy-Chrome (BD PharMingen).

Cell preparation and in vitro stimulation

Freshly isolated hepatic and splenic MNCs derived from wild-type or RAG-2$^{-/-}$ B6 mice were stained with biotinylated anti-NK1.1 mAb, and NK1.1$^+$ cells were enriched by autoMACS using streptavidin microbeads (Miltenyi Biotec, Bergisch Glabach, Germany) according to the manufacturer's instructions. Enriched NK1.1$^+$ cells were stained with PE-conjugated anti-TRAIL mAb and Cy-Chrome–conjugated antimouse CD3 mAb or PE-conjugated anti-DX5 mAb, FITC-conjugated anti-Ly49 mAbs, and Cy-Chrome–conjugated antimouse CD3 mAb, then purified (98%-99%) subpopulations were obtained by cell sorting on a FACS Vantage (BD Bioscience). Purified NK cells and TRAIL$^+$ or TRAIL$^-$ NK cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 25 mM NaHCO$_3$ in humidified 5% CO_2 at 37°C as previously described.[18] NK cells (10^5) were stimulated with immobilized anti-NK1.1 mAb (PK136; 10 μg/mL; BD PharMingen), isotype-matched control immunoglobulin (10 μg/mL; BD PharMingen), IL-12 (100 ng/mL; BD PharMingen), IL-4 (100 ng/mL; BD PharMingen), or IL-18 (200 ng/mL; MBL, Nagoya, Japan) in the presence of IL-2 (2 ng/mL; BD PharMingen), or phorbol myristate acetate (PMA; 50 ng/mL; Sigma Chemical, St Louis, MO) and ionomycin (500 ng/mL; Sigma Chemical) on 96-well flat-bottom culture plate (Costar, Cambridge, MA). Cell-free supernatants were harvested 24 hours later. The mAbs in phosphate-buffered saline (PBS) were immobilized on culture plate by overnight incubation at 4°C.

ELISA

IFN-γ or IL-13 levels in the culture supernatant were evaluated by using a mouse IFN-γ–specific enzyme-linked immunosorbent assay (ELISA) kit (OptEIA; BD PharMingen) or IL-13–specific ELISA kit (Quantikine M; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity of NK1.1$^+$ cells was assessed against TRAIL- and Fas ligand (FasL)–sensitive L929 cells or FasL-sensitive, but TRAIL-resistant, YAC-1s cells by a standard 4- to 6-hour ^{51}Cr-release assay as previously described.[3,5] In some experiments, the indicated NK1.1$^+$ NK cell subpopulations were purified from liver or spleen MNCs of wild-type or RAG-2$^{-/-}$ B6 mice by pre-enrichment using an autoMACS and cell sorting with FACS Vantage as described (see "Cell preparation and in vitro stimulation" and "Flow cytometric analysis"). In some experiments, the cytotoxicity assay was performed in the presence of anti-mouse FasL (MFL4 mAb (10 μg/mL), antimouse TRAIL (N2B2) mAb (10 μg/mL; eBioscience), and/or concanamycin A (CMA; 50 nM; Kyowa Pure Chemicals, Tokyo, Japan). Specific cytotoxicity was calculated as previously described.

Experimental metastasis model and tumor

Adult (6-8 weeks old) and young (2.5 weeks old) BALB/c mice were inoculated with 10^4 or 1×10^5 FasL-sensitive renal carcinoma Renca tumor cells intravenously or intravenously for liver or lung metastasis, respectively.[5] The mice were killed on day 14 after tumor inoculation, and metastatic nodules on the liver and liver were counted under a dissecting microscope (Olympus, Tokyo, Japan).

Adoptive transfer

TRAIL$^+$ NK1.1$^+$ and TRAIL$^-$ NK1.1$^+$ cells were isolated from the livers of 6-week-old RAG-2$^{-/-}$ B6 mice or the liver and spleen of newborn RAG-2$^{-/-}$ B6 mice by sorting after the staining with FITC-conjugated anti-NK1.1 mAb and PE-conjugated anti-TRAIL mAb. Purity of the sorted cells was always more than 97%. The isolated cells (4×10^5) were then intravenously injected into 129/J (NK1.1$^-$H-2^b) mice that had been sublethally irradiated (400 rad). After 7, 10, or 14 days, MNCs from the spleen and liver of the recipient mice were subjected to flow cytometric analysis for donor-derived NK1.1$^+$CD3$^-$ cells.

Statistical analysis

Data were analyzed using a 2-tailed Student t test. $P < .05$ was considered significant.

Results

Phenotype and function of TRAIL-expressing liver NK cells in adult mice

We have previously reported that adult (over 6 weeks old) liver NK cells contained a TRAIL$^+$ subpopulation, which contributed to antitumor surveillance in vivo.[3] However, the origin, surface phenotype, and effector functions of these hepatic NK cells had not been fully explored. Here, using flow cytometry and functional analysis we have further characterized this adult (8-12 weeks old) liver TRAIL$^+$ NK cell subpopulation (Figure 1). In all experiments using B6 mice, NK cells were first identified by their CD3$^-$NK1.1$^+$ phenotype and then expression of other cell surface antigens was analyzed. Clearly, DX5^{dim}Ly-49$^-$ NK cells were characterized as TRAIL$^+$ CD11b^{dim}, whereas DX5^{high} NK cells were TRAIL$^-$ CD11b^{high} (Figure 1A), and TRAIL-mediated cytotoxicity was only demonstrated by DX5^{dim}Ly49$^-$ NK cells, but not DX5^{high} or Ly49$^+$ NK cells (data not shown). Whereas hepatic NK cells were equivalently CD94^{high} or CD94^{dim}, DX5^{dim}Ly-49$^-$ TRAIL$^+$ NK cells were predominantly CD94^{high} (88.4% ± 9.6%; Figure 1A). A high level of NKG2A/C/E expression was similarly predominant in

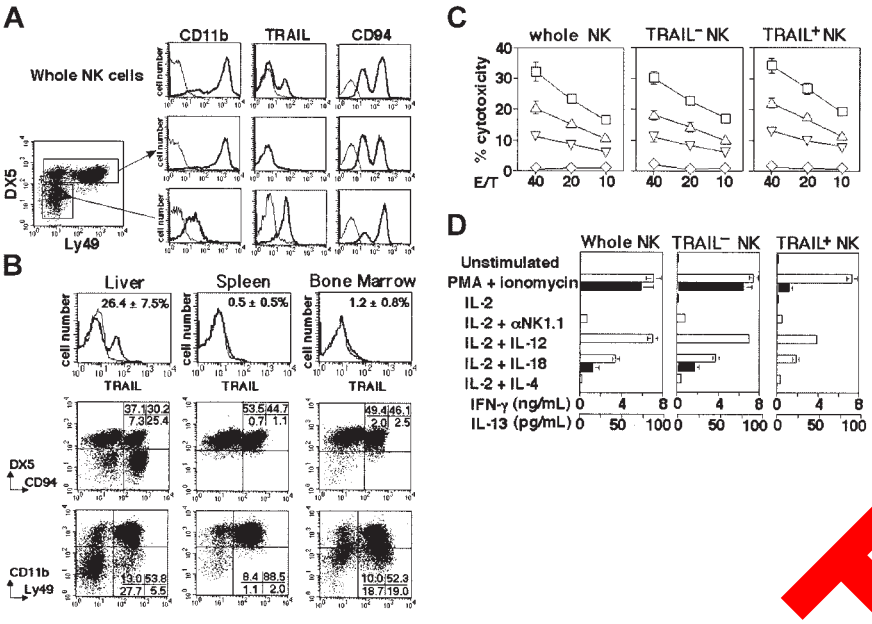

Figure 1. Characterization of TRAIL$^+$ adult liver NK cells. (A) Expression of TRAIL, CD11b, CD94 on DX5^{high} and DX5^{dim}Ly49$^-$ liver NK cell subpopulations. Thick lines indicate the staining with respective mAb, and thin lines indicate the staining with isotype-matched control immunoglobulin. (B) Expression of TRAIL and the indicated molecules was analyzed on NK1.1$^+$CD3$^-$ NK cells freshly isolated from the liver, spleen, and bone marrow of wild-type B6 mice. Thick lines indicate the staining with anti-TRAIL mAb, and thin lines indicate the staining with isotype-matched control immunoglobulin. Quadrant gates for DX5, CD94, and CD11b were set between high- and dim-expressing cells. The numbers represent the percentages of cells in each quadrant. Data are representative of 3 indepe nts. (C) Cytotoxic activity of whole, TRAI TRAIL ells was tested against TRAIL-resis erforin- and Fas sitive YAC-1s target cells in the pr of control imm globulin (□), anti-FasL–neu izing m), CMA (r anti-FasL–neutralizing and CMA ta are esented as the mean of triplicate sa lar results were obta from 3 i ndent exp ents. (D) IFN-γ and IL- uctio whole, TRAIL$^+$, or TRAIL$^-$ NK cells. NK ll po were inc d with various stimuli as ndicated. esents and ■ represents IL-13. Data are represe ean ± SD of triplicate culture amples. Simila were obtained from 3 independent eriments.

TRAIL$^+$ NK cells (data not shown). Similar results were obtained using B6 RAG-2$^{-/-}$ mice (data not shown).

Consistent with the selective distribution of TRAIL$^+$ NK cells in the liver (Figure 1B), DX5^{dim} NK cells and CD11b^{dim}Ly49$^-$ NK cells were found in the liver, but rarely in the spleen. The NK cell population in the bone marrow, which contains few TRA cells, was also distinguishable from that in the liver and s en, with DX5^{dim} NK cells rare and approximately half of the CD1 m NK cells expressing Ly49. In addition, the of CD expressed on Ly49$^-$CD11b^{dim} NK cells was gher th that on liver TRAIL$^+$ Ly49$^-$CD11b^{dim} N ells. Si ar result were obtained using B6 RAG-2$^{-/-}$ mice (not results indicated a distinct distributio NK c populations in the liver, spleen, and bone marrow further tha TRAIL$^+$ NK cell subpopulation in the r was DX CD11b^{dim} Ly49$^-$CD94-NKG2^{high}.

We next analyzed the otoxic activi TRAIL$^+$ or TRAIL$^-$ NK cells in adult (8- eeks old) liver ag t TRAIL-resistant, perforin- and FasL sitive Y -1s cells. The sorted TRAIL$^+$ NK cells were DX5^{dim} 1b y49$^-$CD94-NKG2^{high} as determined by flow cytometry (d t show Both TRAIL$^+$ and TRAIL$^-$ NK cells ated p in d FasL-dependent cytotoxicity, which s com tely abr ed by a combination of perforin inhi CMA i-FasL neutralizing mAb (Figure 1C). Both TRAIL TRAIL K cells expressed NKG2D, which was reported t tribute to their cytotoxicity against YAC-1s (data not shown).[19] Th RAIL$^+$ NK cells were able to exert both perforin- and FasL-dependent cytotoxicity as well as TRAIL-dependent cytotoxicity.

Unsorted whole liver NK cells, sorted TRAIL$^-$ NK cells, and sorted TRAIL$^+$ NK cells, prepared from adult mice (8-12 weeks old), all produced IFN-γ when activated by PMA and ionomycin (Figure 1D). However, levels of IFN-γ production by TRAIL$^+$ NK cells were significantly lower than levels produced by total NK cells or TRAIL$^-$ NK cells when these populations were stimulated with a combination of IL-2 and IL-12 or IL-2 and IL-18. As previously described,[20] IL-13 production was induced in total NK cells when stimulated by either PMA and ionomycin or IL-2 and IL-18; however, TRAIL$^+$ NK cells were comparatively poor secretors of IL-13. Combinations of IL-2 with IL-4 or i bilized anti-NK1 mAb were less effective in triggering IFN-γ and IL-13 secretion.

TRAIL$^+$ NK cells predominate in fetal and neonatal mice

Given AIL$^+$ adult liver NK cells expressed a surface phenotype previously described as "immature,"[13,14] we examined uch NK cells were prevalent earlier in the development of the immune system. We found that the proportions of TRAIL$^+$ NK cells in the liver of young mice (3-4 weeks of age; Figure 2A) were increased compared to adult mice (> 6 weeks of age; Figure 1B), and the proportion of TRAIL$^+$ NK cells in the liver and spleen decreased with age in both B6 wild-type and B6 RAG-2$^{-/-}$ mice (data not shown). In particular, the majority of NK cells in both the liver and spleen of fetal and neonatal mice expressed TRAIL, whereas a distinctly TRAIL$^-$ population was observed in young and adult mice. It was previously suggested that fetal NK cells do not express Ly49s (with exception for Ly-49E), but do express high levels of CD94/NKG2.[21-24] Therefore, we further analyzed the surface phenotype of NK cells of RAG-2$^{-/-}$ mice from fetal age to 3 to 4 weeks of age (Figure 2B). The majority of fetal liver NK cells were TRAIL^{dim}DX5^{dim}CD11b^{dim}Ly49$^-$CD94^{high}NKG2D^{dim} population. At birth, TRAIL$^+$ DX5^{dim}CD11b^{dim}Ly49$^-$CD94^{high}NKG2D^{high} cells began to appear. Within the first 2 weeks of life, TRAIL$^-$ DX5^{high}CD11b^{high}Ly49$^+$CD94^{dim} NK cells also began to appear and proportions increased into adult life. The changes in DX5, CD11b, Ly49, CD94, and NKG2D expression on spleen NK cells were comparable to those observed on liver NK cells. However, significantly fewer DX5^{dim} NK cells expressed TRAIL in the spleen, and they rapidly disappeared after birth with the appearance of DX5^{high}CD11b^{high}CD94^{dim} TRAIL$^-$ NK cells. Similar results were obtained using B6 wild-type mice (data not shown).

Fetal NK cells predominantly use the TRAIL effector molecule

We next analyzed the cytotoxic capacity of liver and spleen NK cells isolated from fetal and neonatal B6 RAG-2$^{-/-}$ mice against perforin-, TRAIL- , FasL-, and NK cell-sensitive L929 target cells (Figure 3A). Fetal liver and spleen NK cells predominantly exerted TRAIL-dependent cytotoxicity as demonstrated by neutralization by anti-TRAIL mAb. Hepatic NK cells from neonatal mice exerted

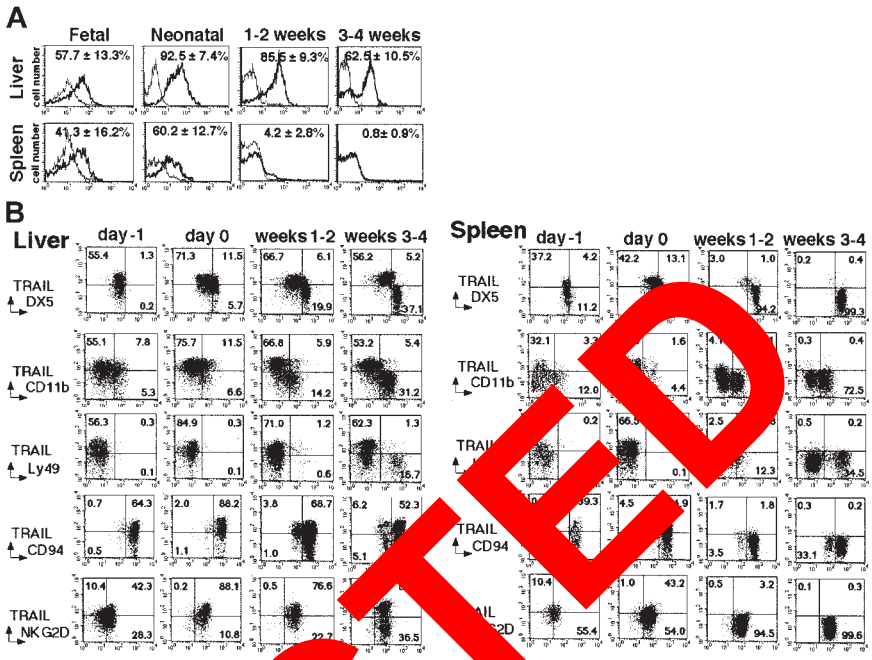

Figure 2. Phenotype of TRAIL$^+$ NK cells in fetal and neonatal mice. (A) TRAIL expression on hepatic and splenic NK cells from wild-type B6 mice at various ages. Thick lines indicate the staining with anti-TRAIL mAb, and thin lines indicate the staining with isotype-matched control immunoglobulin. The numbers indicate the mean ± SD of 5 mice. (B) MNCs were freshly isolated from the liver and spleen of RAG-2$^{-/-}$ B6 mice at the indicated ages, and expression of the indicated molecules was analyzed on NK1.1$^+$CD3$^-$ NK cells. Quadrant gates for DX5, CD11b, and CD94 were set between high- and dim-expressing cells. The numbers represent the percentages of cells in each quadrant. Data are representative of 3 independent experiments.

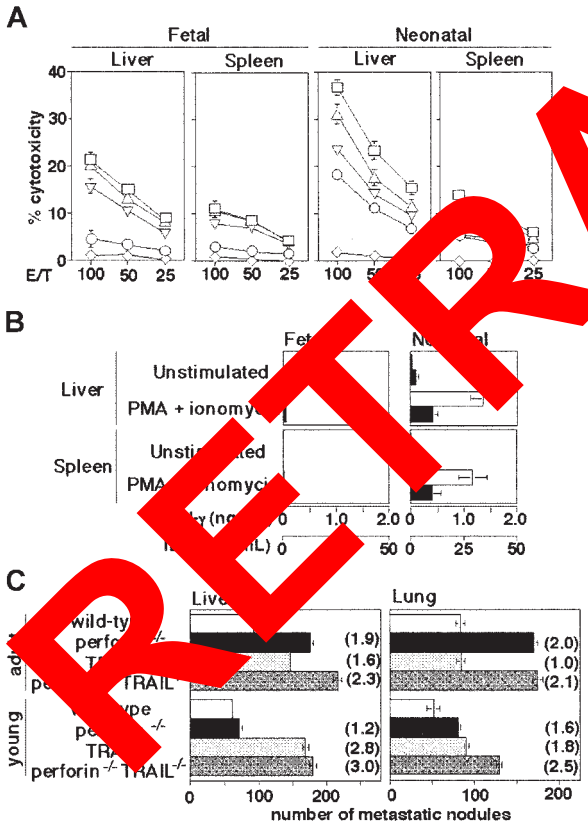

Figure 3. Function of TRAIL$^+$ NK cells in fetal and neonatal mice. (A) Cytotoxic activity of hepatic and splenic NK cells isolated from fetal and neonatal RAG-2$^{-/-}$ mice was tested against TRAIL-, FasL-, and perforin-sensitive L929 target cells in the presence of control immunoglobulin (□), anti-TRAIL–neutralizing mAb (○), anti-FasL–neutralizing mAb (△), CMA (▽), or anti-TRAIL–neutralizing mAb, anti-FasL–neutralizing mAb, and CMA (◇). Data are represented as the mean ± SD of triplicate samples. Similar results were obtained from 3 independent experiments. (B) IFN-γ and IL-13 production by fetal and neonatal NK cells. Isolated NK cell populations were incubated with PMA and ionomycin. □ represents IFN-γ and ■ represents IL-13. Data are represented as the mean ± SD of triplicate culture samples. Similar results were obtained from 3 independent experiments. (C) Preferential TRAIL-dependent tumor surveillance in young mice. Data are represented as the mean ± SD of 5 mice. Relative times increase in metastases compared to wild-type mice are indicated in parentheses. Similar results were obtained from 2 independent experiments.

T[illegible]IL-, FasL-[illegible]nd perforin-dependent cytotoxicity, whereas sp[illegible]ic NK cell[illegible]om neonatal mice demonstrated TRAIL- and per[illegible]-depend[illegible] cytotoxicity, but negligible FasL-dependent cytoto[illegible]ably, the cytotoxic activity of spleen NK cells was [illegible]ignificantly lower than that of hepatic NK cells in both fetal and [illegible] mice. Similarly, fetal and spleen NK cells also mediated [illegible]educed cytotoxicity against YAC-1s target cells (data not shown). We then analyzed the cytokine production by liver and spleen NK cells from fetal and neonatal mice stimulated with PMA and ionomycin (Figure 3B). Fetal NK cells did not produce significant amounts of IFN-γ or IL-13 following stimulation; however, neonatal NK cells produced IFN-γ and some IL-13. Similar profiles of cytotoxic activity and cytokine production were also observed using NK cells from B6 wild-type mice (data not shown). Thus, these results suggested that fetal NK cells predominantly displayed TRAIL effector function; however, on NK cell development and NK cell receptor acquisition, NK cells developed perforin- and FasL-dependent cytotoxicity and the capacity to produce IFN-γ and IL-13.

We have reported that TRAIL expressed on an adult hepatic NK cell subpopulation plays a partial role suppressing tumor metastases in the liver.[3-5] Here, we compared the relative importance of TRAIL and perforin effector pathways in controlling tumor metastasis to the lungs and livers of young (14-17 days old) and adult mice (6-8 weeks old). Consistent with our previous report,[5] in adult mice, the number of lung metastases was significantly increased in perforin$^{-/-}$ mice, but not TRAIL$^{-/-}$ mice, whereas the number of hepatic metastases was significantly increased in both perforin$^{-/-}$ mice and TRAIL$^{-/-}$ mice (Figure 3C). However, in young mice, suppression of lung metastases was additionally TRAIL-dependent and clearance of liver metastases was entirely TRAIL-dependent. These data further supported a functional dependence of NK cells on the TRAIL effector pathway in the developing immune system.

Development of TRAIL$^-$ NK cells from neonatal and adult TRAIL$^+$ NK cells in vivo

Our data suggested that TRAIL$^+$ adult liver NK cells represented a phenotypically immature DX5^{dim}CD11b^{dim}Ly49$^-$CD94-NKG2^{high}

NK cell subpopulation that fails to persist in spleen. Further it was possible that TRAIL$^+$ NK cells in the adult liver might be capable of developing into mature TRAIL$^-$ NK cells. To address these issues, we performed adoptive transfer experiments with purified TRAIL$^+$ and TRAIL$^-$ NK cell populations that were isolated to 98% to 99% purity from adult and neonatal livers and neonatal spleen of RAG-2$^{-/-}$ mice (Figure 4). Ten days after transfer into sublethally irradiated 129/J mice (which do not express the NK1.1 alloantigen), the transfer of adult hepatic TRAIL$^+$ NK1.1$^+$ NK cells resulted in an increase in both TRAIL$^+$ DX5^{dim}CD11b^{dim}Ly49$^-$NK1.1$^+$ NK cells and TRAIL$^-$ DX5^{high}CD11b^{high}NK1.1$^+$ NK cells in the liver, similar to the transfer of neonatal spleen or liver TRAIL$^+$ NK1.1$^+$ NK cells. In the spleen, the transfer of adult hepatic TRAIL$^+$ NK1.1$^+$ NK cells or neonatal liver or spleen TRAIL$^+$ NK1.1$^+$ NK cells predominantly resulted in an increase in TRAIL$^-$ DX5^{high}NK1.1$^+$ cells. By contrast, the transfer of adult TRAIL$^-$ NK cells or neonatal liver or spleen TRAIL$^-$ NK cells only led to the appearance of TRAIL$^-$ DX5^{high}CD11b^{high}NK1.1$^+$ NK cells in both liver and spleen. Ly49 was most highly expressed on NK1.1$^+$ cells in the recipient mice injected with adult liver TRAIL$^-$ NK cells compared to the transfer of neonatal spleen or liver TRAIL$^-$ NK cells, which might indicate that TRAIL$^-$ NK cells are also developing into more mature NK cells and adult TRAIL$^-$ NK cells are more mature than neonatal TRAIL$^-$ NK cells. Similar results were obtained at 7 or 14 days after adoptive transfer, although the number of TRAIL$^-$ NK cells in mice receiving TRAIL$^+$ NK cells was less at 7 days compared to 14 days after adoptive transfer (data not shown). A comparable number of donor-derived NK cells were found in the corresponding tissues of recipient mice after transfer of either adult or neonatal liver NK cells, suggesting that there was no preferential expansion or survival of either population. These results clearly indicated that TRAIL$^+$ NK cells in adult liver are the precursors of TRAIL$^-$ NK cells.

Requirements for maintenance of TRAIL$^+$ adult liver NK cells

We wished to establish what factors were [illegible] maintenance of TRAIL$^+$ adult liver NK cells. To fi[illegible] evaluate[illegible] importance of TRAIL in NK cell differentiation, [illegible]nalyzed NK[illegible]ll subsets in the liver of TRAIL$^{-/-}$ mice ([illegible]gure [illegible] Although[illegible]patic NK cells of TRAIL$^{-/-}$ mice did[illegible] express [illegible]IL[illegible] contained similar proportions of D[illegible]^{im} or [illegible]D11b^{di}[illegible]$^-$ NK cells, corresponding to TRAI[illegible]K ce[illegible]n the liver of wild-type mice (Figure 1A). Simila[illegible]umb[illegible]epatic [illegible] cells were obtained from TRAIL$^{-/-}$ [illegible] wild-typ[illegible]e ([illegible] not shown). As previously reporte[illegible] cells isolate[illegible] TRAIL$^{-/-}$ mice demonstrated perf[illegible]- an[illegible]L- dependen[illegible] cytotoxicity as well as IFN-γ production, similar to[illegible] cells from wild-type mice (data not sho[illegible]se results ind[illegible]d normal NK cell differentiation and h[illegible]ostasis in TRAIL$^{-/-}$ mice.

[illegible]o evaluate [illegible]ther cytokines regulated TRAIL expression on li[illegible]NK cells, w[illegible]nalyzed TRAIL expression on NK cell subsets in t[illegible]vers of[illegible]ult IFN-γ$^{-/-}$, IL-12$^{-/-}$, and IL-12$^{-/-}$IL-18$^{-/-}$

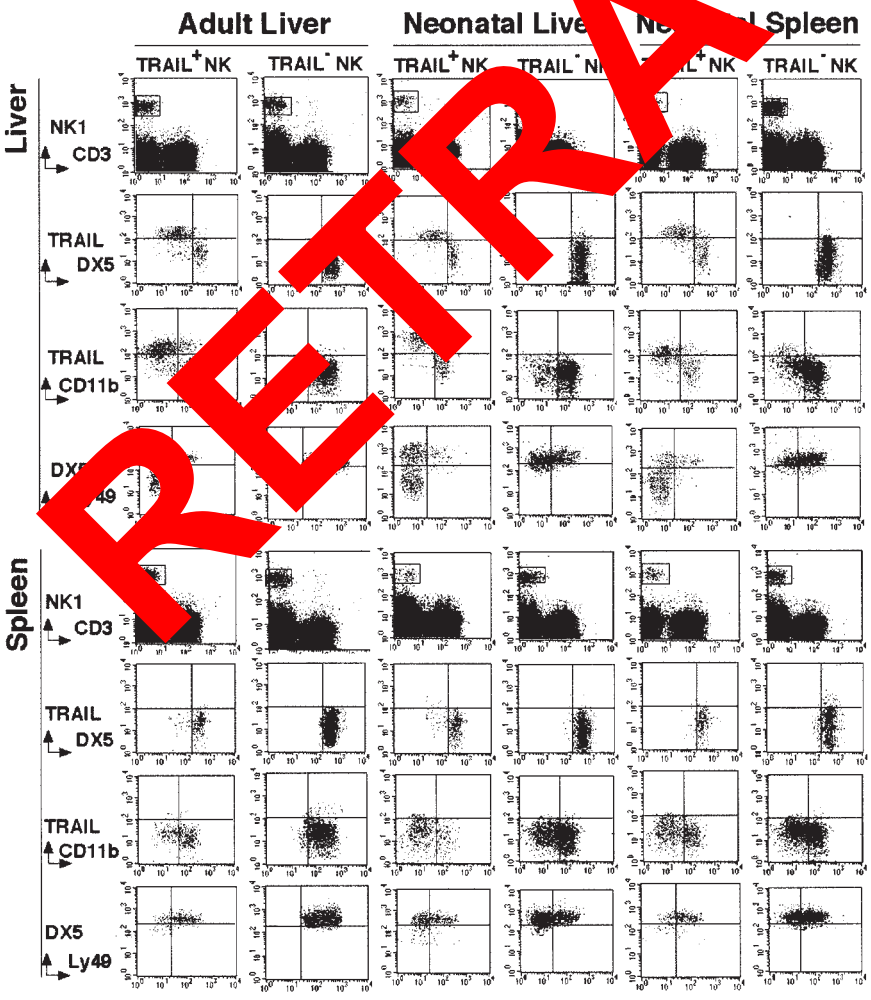

Figure 4. TRAIL$^-$ NK cells develop from TRAIL$^+$ NK cells. TRAIL$^+$ or TRAIL$^-$ NK cells were isolated from adult liver or neonatal liver and spleen of RAG-2$^{-/-}$ B6 mice (NK1.1$^+$, H-2^b) and adoptively transferred into sublethally irradiated 129/J mice (NK1.1$^-$, H-2^b). On day 10, liver and spleen MNCs of the recipient mice were isolated and stained for the indicated molecules. TRAIL, DX5, CD11b, and Ly49 expression was analyzed on the gated NK1.1$^+$CD3$^-$ cells. Quadrant gates for DX5 and CD11b were set between high- and dim-expressing cells. Data are representative of 3 independent experiments.

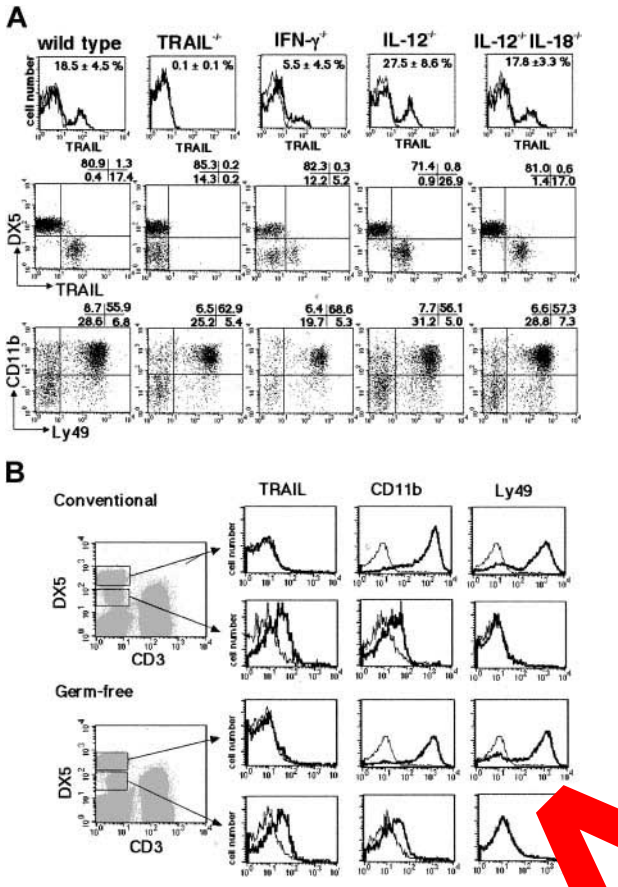

Figure 5. Partial IFN-γ–dependent maintenance of TRAIL+ [illegible] liver NK [illegible]
(A) Hepatic MNCs were freshly isolated from wild-type or the [illegible]ene-targe[illegible] B6 mice, and expression of TRAIL, DX5, Ly49, and [illegible]1b wa[illegible]nalyzed [illegible] NK1.1+CD3− NK cells. Thick histograms indicate the [illegible]ining with [illegible]-TRAIL mA[illegible] and thin histograms indicate the staining with isotype-[illegible]d co[illegible]by [illegible]lin. The numbers represent the mean ± SD of [illegible]UL+ [illegible] mice. [illegible]ant gates for DX5 and CD11b were set betwee[illegible]gh- and di[illegible]ressing cells. The numbers represent percentages of cells in [illegible] quadrant. Data [illegible]epresentative of 3 independent experiments. (B) Consti[illegible] expression o[illegible]tic immature phenotype NK cells in germ-free m[illegible] Hepa[illegible]Cs were freshly isolated from conventional or germ-free BALB/[illegible]mice, and expr[illegible] of TRAIL, CD11b, and Ly49 was analyzed on DX5highCD3[illegible] DX5dimCD3− NK [illegible] Thick histograms indicate the staining with respecti[illegible]Ab and thin histogram[illegible]icate the staining with isotype-matched contro[illegible]munoglob[illegible] Data are representative of 3 independent experiments.

mice (Fi[illegible] Simi[illegible]rs of hepatic NK cells were obtain[illegible]rom I[illegible]-γ−/−, I[illegible]−/−, IL-12−/−IL-18−/−, and wild-type[illegible] (dat[illegible]wn). Hepatic NK cells from IFN-γ−/− mice contain[illegible]icantly [illegible]reased proportions of TRAIL+ NK cells, which wa[illegible]nsistent with the finding that DX5dim TRAIL+ or CD11bdimLy4[illegible]K cells were significantly decreased in IFN-γ−/− mice. TRAIL+ DX5dim and CD11bdimLy49− hepatic NK cells were resident in the liver of IL-12−/− or IL-12−/−IL-18−/− mice similarly to wild-type mice. This pattern was also observed when comparing hepatic NK cells isolated from 2- to 4-week-old mice (data not shown).

Because the constitutive TRAIL expression was unique to hepatic NK cells, we examined whether endogenous bacterial components might regulate TRAIL expression using germ-free mice (Figure 5B). Similar numbers of hepatic NK cells were obtained from BALB/c germ-free mice and control mice (data not shown). Hepatic NK cells from BALB/c mice were divided into 2 populations, DX5high NK cells and DX5dim NK cells. TRAIL expression was restricted to the DX5dim hepatic NK cell population in either conventional or germ-free mice, and these NK cells were identified as a CD11bdimLy49− subpopulation. Taken together, TRAIL expression was not critical for the development of NK cells, and TRAIL expression was partly dependent on IFN-γ, but not dependent on endogenous IL-12, IL-18, or bacterial components.

IFN-γ also induces TRAIL expression on mature NK cells

We have previously reported that the administration of IFN-γ–inducing agents, such as the invariant NKT cell-specific ligand, α-GalCer, or IL-12, induced TRAIL expres[illegible]pleen NK cells in vivo.[5] Our present finding of TRAIL [illegible]X5[illegible]1bdimLy49− immature phenotype NK cells in nor[illegible]iver sugges[illegible] possibility that IFN-γ might induce the emerg[illegible] of TRAIL[illegible] immature NK cells in the spleen rather th[illegible] expres[illegible]f TRA[illegible]on mature NK cells. Therefore, we [illegible]yzed the ph[illegible] of NK cells freshly isolated from [illegible]-type [illegible]e 3 day[illegible] after α-GalCer treatment (2 μg/mouse). [illegible]IL [illegible]K cells [illegible]ere increased in the liver of α-GalCer-[illegible]ated mi[illegible]ompare[illegible] untreated or vehicle-treated B6 mic[illegible]gure 6). TR[illegible]ession was still observed on DX5dim, [illegible]or Ly49− N[illegible]ls isolated from the liver of α-GalCer–treated mi[illegible]milar to those isolated from control mice. Substa[illegible]proportions [illegible]X5high, CD11bhigh, or Ly49+ NK cells als[illegible]pressed TRAIL in[illegible]e liver of α-GalCer–treated mice. T[illegible]IL expression was also clearly induced on spleen NK cells by α[illegible]lCer treatm[illegible] and the majority of TRAIL+ spleen NK cells we[illegible]X5high, C[illegible]1bhigh, or Ly49+ NK cells. Notably, TRAIL+ DX5[illegible], or Ly49− NK cells also emerged in the spleen after α-GalCer injection. Thus, the phenotype of spleen NK cells [illegible]ite similar to those of hepatic NK cells after α-GalCer [illegible]jecti[illegible]. Similar results were obtained in IL-12–treated or IFN-γ–treated wild-type and RAG-2−/− B6 mice (data not shown). These results indicated that exogenous administration of IFN-γ or IFN-γ–inducing agents induced TRAIL expression on mature NK cells as well as the expansion and/or immigration of TRAIL-expressing immature NK cells in the liver and spleen.

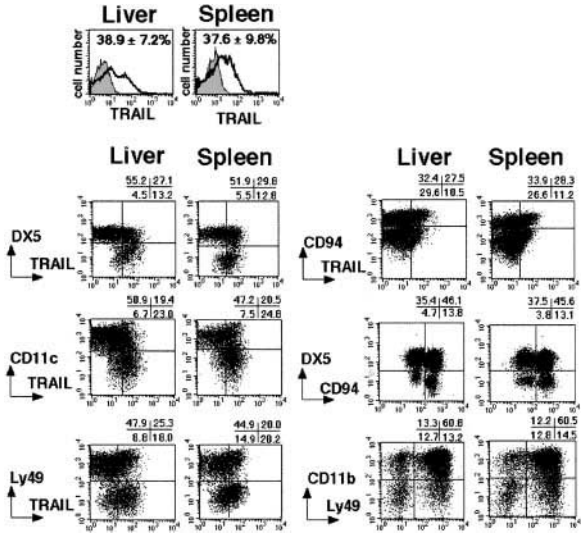

Figure 6. TRAIL+ NK cell subpopulations in the liver and spleen of mice injected with α-GalCer. Hepatic and splenic MNCs were freshly isolated 3 days after α-GalCer treatment of wild-type B6 mice. Expression of TRAIL and the indicated cell surface molecules was analyzed on NK1.1+CD3− NK cells. Open histograms indicate the staining with anti-TRAIL mAb and filled histograms indicate the staining with isotype-matched control immunoglobulin. The numbers indicate the mean ± SD of TRAIL+ cells from 5 mice. Quadrant gates for DX5, CD11b, and CD94 were set between high- and dim-expressing cells. The numbers represent percentages of cells in each quadrant. Data are representative of 3 independent experiments.

Discussion

In this study, we have demonstrated that the predominant DX5^{dim}CD11b^{dim}Ly49^{-}CD94-NKG2^{+} NK cell population that exists in fetal and neonatal mice uses TRAIL as an effector molecule and persists in adult mouse liver. Fetal NK cells demonstrated TRAIL-dependent cytotoxic activity only, and acquisition of other cytotoxic effector molecules (perforin- and FasL-) and cytokines (IFN-γ and IL-13) was concomitant with NK cell receptor acquisition and NK cell maturation. Consequently, antitumor surveillance in infant mice, as opposed to adult mice, was largely dependent on TRAIL-mediated apoptosis rather than perforin-mediated killing. Whereas both newborn and adult liver TRAIL^{+} NK cells developed into TRAIL^{-} NK cells, TRAIL expression itself did not appear to play a critical role in NK cell development. Moreover, TRAIL^{+} NK cells were significantly reduced in IFN-γ$^{-/-}$ mice, but not IL-12$^{-/-}$, IL-12$^{-/-}$IL-18$^{-/-}$, or germ-free mice, suggesting an important role for IFN-γ in maintaining TRAIL expression and immature liver NK cells.

We have shown that TRAIL is a distinctive functional marker to define a unique stage of NK cell differentiation. It has been previously reported that the DX5^{dim}CD11b^{dim} Ly49^{-}CD94-NKG2^{+} immature NK cell population developed from the bone marrow (acquiring CD94-NKG2 and Ly49), and that fetal NK cells of the Ly-49^{-}CD94/NKG2^{+} phenotype were functionally distinct from those observed in adult mice.[12-14,21-26] These cells have long been suspected to be functionally immature and thus unable to cause self-tissue damage despite their lack of Ly49 molecules. It has been suggested that NK cells rely mainly on inhibitory receptors specific for nonclassical major histocompatibility complex (MHC) class I molecules (eg, Qa-1) to maintain tolerance during the first few weeks of life.[21-23] Therefore, the high-level expression of CD94-NKG2A might negatively regulate IL-2^{+} NK cells to prevent antiself-tissue cytotoxicity in vivo. A critical role for such negative regulation of immature NK cell-mediated cytotoxicity was also suggested. A previous report that human immature perforin- and granzyme B-positive NK cells did not lyse target cells although they conjugated with target cells.[15] However, our study would suggest that another mechanism of regulation of self-tissue damage might be the availability of effector pathways. It has been reported that TRAIL induces apoptotic cell death in a wide variety of transformed cells, but not in most normal cells,[1,2] and that TRAIL plays a substantial role not only in tumor surveillance but also in elimination of infected cells in vivo.[9] Thus, TRAIL may be the preferred effector molecule pathway for potential reactive NK cells when they still lack many MHC class I-binding inhibitory receptors (eg, Ly-49).

Our present results are consistent with a previous report that showed that an immature DX5^{dim}CD11b^{dim}Ly49^{-}CD94-NKG2^{+} NK cell population predominantly resided in the liver of adult mice.[14] Since it was shown that hepatic CD11b^{dim} NK cells were less cytotoxic and did not produce IFN-γ after poly-IC treatment,[14] TRAIL^{+} IFN-γ–producing NK cells might increase their CD11b expression level after responding to IFN-α/β. Moreover, these TRAIL^{+} NK cells might express a low level of IL-18 receptor because these NK cells produced significantly lower levels of IFN-γ and IL-13 in response to IL-18. The distinct expression of cytokines receptors, chemokine receptors, and/or adhesion molecules on TRAIL^{+} NK cell subpopulation may enable their retention in the liver because both cytokines and chemokines critically contribute to NK cell development.[26] Alternatively, the microenvironment of the liver might be suitable for the maintenance of TRAIL^{+} NK cells, because it has been suggested that accessory stromal cells supported NK cell differentiation by engaging NK cell receptors or cytokines.[26,28-30] The specific retention of TRAIL^{+} immature NK cells in the adult liver appears to be advantageous in allowing the elimination of transformed cells. Whether the retention of these NK cells also plays additional roles in adult host defense against liver specific pathogens or in tolerance responses to food and other antigens arriving from the gut via portal vein remains to be established. Further characterization of hepatic TRAIL^{+} NK cells in adult and [illegible] may reveal additional key molecules in NK cell development, maintenance, and distribution in vivo.

TRAIL expression on liver NK cells was reduced in IFN-γ$^{-/-}$ mice, but not in IL-12$^{-/-}$, IL-18$^{-/-}$ (data not shown), IL-12$^{-/-}$IL-18$^{-/-}$, or germ-free mice. In addition, GalCer treatment not only induced TRAIL expression on DX5^{dim}CD11b^{high}Ly49^{+} mature NK cells, but also increased immature TRAIL^{+} NK cells in the spleen; thus, IFN-γ might be important for TRAIL expression on NK cells during all developmental stages. However, a substantial TRAIL^{+} NK cell population was still resident in the liver of IFN-γ$^{-/-}$ mice, suggesting that some molecules other than IFN-γ were also involved in regulating TRAIL expression on immature NK cells. Our preliminary assessment of IFN receptor 1–deficient mice suggested that IFN-α/β was not solely critical for TRAIL expression (data not shown). However, a combination of type I and II IFN may be required because TRAIL can be induced on mouse NK cells by IFN-α/β or poly-IC.[31] We could not detect specific expression of TRAIL on DX5^{dim}CD11b^{dim}Ly49^{-}CD94-NKG2^{+} populations developing in vitro with IL-15 from the bone marrow of RAG-2$^{-/-}$ mice (data not shown). Further experiments addressing mechanisms for the selective TRAIL expression on immature NK cells might reveal the critical factor for NK cell development.

The cytotoxic activity of NK cells was dramatically increased after the birth. Up-regulation of effector molecules in NK cells would be one important alteration and indeed TRAIL expression was higher on neonatal NK cells than on fetal NK cells. Similar to in vitro studies with human NK cells,[15] our present data clearly demonstrated that during mouse NK cell development, TRAIL was the effector molecule that was acquired first. Moreover, increased expression of activating NK cell receptors may also play a critical role because increased NKG2D expression on newborn NK cells was concurrently observed. This increased NKG2D expression on NK cells in newborn mice might be due to loss of NKG2D ligand expression because a high expression of NKG2D ligands has been reported in fetal mice.[32] Down-regulation of NKG2D expression on NK cells and impaired perforin-dependent cytotoxicity may occur in fetal mice when NKG2D ligands are expressed, in a similar mechanism as previously reported for adult nonobese diabetic (NOD) mice.[33]

Previous reports suggested that mouse NK cells might differentiate in a manner similar to human NK cells,[12,26] and a NK1-NK2 paradigm was proposed for both human and mouse NK cells.[34,35] It was proposed that immature human NK cells produced IL-13 but not IFN-γ, and thus were defined as type 2 NK cells expressing TRAIL.[15,36-38] Differentiation to an intermediate stage of human NK cells (defined as type 0) led to both IL-13 and IFN-γ production and the expression of TRAIL, FasL, and perforin.[36] Therefore, TRAIL-expressing mouse hepatic NK cells might correspond to human type 0 intermediate NK cells, and fetal TRAIL^{+} NK cells might correspond to human immature NK cells, because they principally exert TRAIL-dependent cytotoxicity and display no IFN-γ production. Consistent with this notion, TRAIL^{+} hepatic NK cells produced IFN-γ and to a

lesser extent IL-13 in responding to PMA and ionomycin. Further experiments addressing the mechanisms by which TRAIL is selectively expressed on immature and intermediate NK cells and similar analysis of human hepatic NK cells might better correlate the process of NK cell development between these species.

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