NK1.1$^+$ T Cells in the Liver Arise in the Thymus and Are Selected by Interactions with Class I Molecules on CD4$^+$CD8$^+$ Cells[1]

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NK1.1$^+$ T cells represent a specialized T cell subset specific for CD1d, a nonclassical MHC class I-restricting element. They are believed to function as regulatory T cells. NK1.1$^+$ T cell development depends on interactions with CD1d molecules presented by hematopoietic cells rather than thymic epithelial cells. NK1.1$^+$ T cells are found in the thymus as well as in peripheral organs such as the liver, spleen, and bone marrow. The site of development of peripheral NK1.1$^+$ T cells is controversial, as is the nature of the CD1d-expressing cell that selects them. With the use of nude mice, thymectomized mice reconstituted with fetal liver cells, and thymus-grafted mice, we provide direct evidence that NK1.1$^+$ T cells in the liver are thymus dependent and can arise in the thymus from fetal liver precursor cells. We show that the class I$^+$ (CD1d$^+$) cell type necessary to select NK1.1$^+$ T cells can originate from TCR$\alpha^{-/-}$ precursors but not from TCR$\beta^{-/-}$ precursors, indicating that the selecting cell is a CD4$^+$CD8$^+$ thymocyte. 5-Bromo-2$'$-deoxyuridine-labeling experiments suggest that the thymic NK1.1$^+$ T cell population arises from proliferating precursor cells, but is a mostly sessile population that turns over very slowly. Since liver NK1.1$^+$ T cells incorporate 5-bromo-2$'$-deoxyuridine more rapidly than thymic NK1.1$^+$ T cells, it appears that liver NK1.1$^+$ T cells either represent a subset of thymic NK1.1$^+$ T cells or are induced to proliferate after having left the thymus. The results indicate that NK1.1$^+$ T cells, like conventional T cells, arise in the thymus where they are selected by interactions with restricting molecules. *The Journal of Immunology,* 2000, 164: 2412–2418.

T he NK1.1$^+$ T cells are a small subset of CD4$^+$CD8$^-$ or CD4$^-$CD8$^-$ T cells characterized by a mature phenotype and functional capabilities (1, 2). They exhibit an activated phenotype (CD44^{high}, LY6C^{high}, IL-2 receptor β^{high}), express markers associated with NK cells (NK1.1 Ag and Ly49 receptors), and display relatively low levels of the TCR. Their most striking feature is a highly restricted TCR repertoire and a unique specificity. Rather than recognizing conventional MHC molecules, NK1.1$^+$ T cells are restricted by the nonclassical MHC class I-like molecule CD1d (3). Evidence has emerged that the cells recognize glycolipid Ags presented by CD1d (4, 5) and may play a role in immunity to infectious agents and tumors (6, 7).

Development of NK1.1$^+$ T cells, like that of other T cells, requires interactions with the appropriate restricting elements. Essentially, no NK1.1$^+$ T cells develop in mice deficient for β_2-microglobulin (β_2m$^{-/-}$ mice)[4] (8–11) or CD1d (12–14). Although thymic epithelial cells represent the key cell type responsible for selection of conventional T cells, a hematopoietic cell type is responsible for selection of NK1.1$^+$ T cells in vivo (8–11). This conclusion is based on analysis of reciprocal fetal liver chimeras between class I$^+$ and class I-deficient mice. NK1.1$^+$ T cells failed to develop in chimeras where class I$^+$ hosts were repopulated with β_2m$^{-/-}$ fetal liver cells, yet developed normally in chimeras where β_2m$^{-/-}$ hosts were repopulated with class I$^+$ fetal liver cells. These data suggested that class I$^+$ hematopoietic cells are necessary and sufficient for development of NK1.1$^+$ T cells.

Although NK1.1$^+$ T cells represent a minor population in the spleen or the thymus, they are quite abundant in the adult liver and bone marrow. Indeed, there are more NK1.1$^+$ T cells in the livers of young mice than in the thymus (11). NK1.1$^+$ T cells found in the thymus are believed to arise there, since these cells are found in fetal thymuses that have been subjected to extended periods of organ culture in vitro (9). A controversial issue concerns whether NK1.1$^+$ T cells in the periphery arise in the thymus or represent "extrathymic" T cell development. Reports differ as to the extent to which NK1.1$^+$ T cells arise in athymic mice. In some studies, it was concluded that NK1.1$^+$TCR$\alpha\beta^+$ cells are absent or rare in nude mice (15–18). Another study showed that neonatal thymectomy reduced the frequency of liver NK1.1$^+$ T cells (19). NK1$^+$ T cells in the spleen and bone marrow were shown by one group to originate from a grafted thymus (15). In contrast, an early report concluded that NK1.1$^+$ T cells are present in the periphery of nude mice (20). Several other studies have also concluded that NK1.1$^+$ T cells arise in the periphery. In one study, IL-2Rβ^+CD3^{int} cells were examined. In normal mice, a significant fraction of these cells express NK1.1. Based on the finding that IL-2Rβ^+CD3^{int} cells were detected in the periphery of nude mice, it was concluded that IL-2Rβ^+CD3^{int} cells, including NK1.1$^+$ T cells, are thymus independent (21). A subsequent study from this group found enhanced development of peripheral NK1.1$^+$ T cells in nude mice engrafted with a thymus from which thymocytes were depleted. Ancillary studies led the authors to conclude that the effect of the

thymus on peripheral NK1.1$^+$ T cells was indirect, perhaps involving thymic hormones (22). Taniguchi and colleagues (23) provided evidence that rearrangement of the Vα14 gene expressed by NK1.1$^+$ T cells occurs in the periphery of mice, including nude mice, based on the presence of reciprocal rearrangement products in these cells. They concluded that Vα14$^+$ T cells (essentially all of which are assumed to be NK1.1$^+$ T cells) develop extrathymically. Finally, Shimamura et al. (24) showed that fetal liver cells from normal or nude mice can develop into NK1.1$^+$ T cells in vitro under the influence of cytokines and class I$^+$ cells. The authors concluded that NK1.1$^+$ T cells can develop extrathymically.

A related issue concerns the precise identity of the CD1d-expressing hematopoietic cell type that selects NK1.1$^+$ T cells. Shimamura et al. (24) concluded that the selecting cell type is a nonlymphoid cell, since SCID mice could supply the cells in an in vitro system. In contrast, Bendelac (25) showed that β_2m$^{-/-}$ fetal liver cells failed to develop into Vβ8$^+$CD44$^+$ T cells (most of which are NK1.1$^+$ T cells) when engrafted into the environment of class I$^+$ cells found in a SCID mouse . This finding suggested that the class I$^+$-selecting cells are dependent on gene rearrangement. Additional studies suggested that the responsible cells are neither B cells nor mature T cells. The authors proposed that the relevant cell was therefore an immature CD4$^+$CD8$^+$ thymocyte, in line with the notion that development of NK1.1$^+$ T cells occurs in the thymus.

In this paper, we have examined several issues concerning NK1.1$^+$ T cell development, including the role of the thymus and the nature of the class I$^+$-selecting cell. The results provide strong evidence that peripheral NK1.1$^+$ T cells arise in the thymus. Consistent with this conclusion, mixed chimera studies provided evidence that the thymic selecting cells are CD4$^+$CD8$^+$ thymocytes. Despite the thymic origin of NK1.1$^+$ T cells in the periphery, these cells exhibit differences from thymic NK1.1$^+$ T cells in some properties, including turnover rate and expression of Ly49 inhibitory receptors. These differences suggest either that peripheral NK1.1$^+$ T cells are derived from a subset of thymic NK1.1$^+$ T cells or that thymic NK1.1$^+$ T cells change properties once they seed the periphery.

Materials and Methods

Mice

C57BL6/J, C57BL6/J β_2m$^{-/-}$, C57BL6/J A$_\beta$$^{-/-}$ β_2m$^{-/-}$, and C57BL6/J Rag$^{-/-}$ β_2m$^{-/-}$ were bred in a pathogen-free environment at the University of California, Berkeley. C57BL6/J nude, C57BL6/J TCR $\alpha^{-/-}$, and C57BL6/J TCR $\beta^{-/-}$ were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL6/J Rag-1$^{-/-}$ mice, a kind gift from Dr. David Baltimore (California Institute of Technology, Pasadena, CA), were bred in the colony.

Antibodies

PK136 (anti-NK1.1) (26), H57.597 (anti-TCRβ) (27), RL172 (anti-CD4) (28), AD4 (15) (anti-CD8) (29), 500A2 (anti-CD3 (30), and Y3 (anti-K^b) (31) were purified and conjugated according to standard procedures. CT-CD4 (anti-CD4), CT-CD8a (anti-CD8α), and 53-5.8 (anti-CD8β) were purchased from Caltag (South San Francisco, CA); DX5 from PharMingen (San Diego, CA); 53-6 (anti-CD8α) and IM7.8.1 (anti-CD4) from Life Technologies (Rockville, MD); B44 (anti-5-bromo-2′-deoxyuridine (BrdU)) from Becton Dickinson (Mountain View, CA); and streptavidin-conjugated PE from Molecular Probes (Eugene, OR).

Cell preparation

Thymic NK1.1$^+$ T cells were enriched before analysis unless stated otherwise. Immature and CD8$^+$ thymocytes were depleted from a thymocyte preparation with a mixture of anti-heat-stable antigen (J11D), anti-CD8 (AD4 (15)), guinea pig complement (Life Technologies), and rabbit complement (Fisher, Pittsburgh, PA), and viable cells were isolated on a Ficoll (Sigma, St. Louis, MO) gradient.

Liver NK1.1$^+$ T cells were analyzed after initial enrichment (11). The livers were perfused with PBS and dissociated. The single-cell suspension was passed over a nylon wool column to deplete hepatocytes and other adherent cells. The lymphocytes were further enriched using a Percoll (Amhersham Pharmacia Biotech, Piscataway, NJ) gradient; T cells were isolated from the boundary of the 44 and 60% Percoll layers. Because of variability in the cell yield between animals of the same type, and variable contamination of the enriched preparations with hepatic cells, it was difficult to gauge the absolute number of NK1.1$^+$ T cells in the livers. However, no systematic differences in final cell yield between the different experimental groups were detected. Also, the fraction of cells in the final preparation that corresponded to mononuclear cells based on the forward and side scatter gates varied <2-fold between groups in all experiments, suggesting that the number of mononuclear cells does not differ substantially between the different experimental groups.

Immunofluorescence staining and analysis

For all staining experiments, cells were washed three times between steps in 200 μl of staining buffer and analyzed on a Coulter Epics XL (Beckman Coulter, Fullerton, CA). Reanalysis was performed with the WinMDI software (John Trotter, Salk Institute, San Diego, CA). For analysis of NK1.1$^+$ T cells in the thymus, either total thymocytes or enriched thymic NK1.1$^+$ T cells were examined. Suspensions of 10^5-10^6 thymocytes were stained with anti-CD4 (613), anti- CD8 (Tri-Color), anti-NK1.1 (PE), and anti-TCRβ (FITC) in a final volume of 25 μl for 20 min on ice. Staining buffer consisted of PBS, 5% FCS, and 0.02% NaN$_3$. For analysis of liver NK1.1$^+$ T cells, suspensions of 10^5-10^6 enriched liver mononuclear cells (see above) were stained with anti-CD8 (Tri-Color), anti- CD4 (613), anti-NK1.1 (PE), and either anti-TCRβ (FITC) or anti-CD3ϵ (FITC) in a final volume of 25 μl for 20 min on ice.

BrdU-labeled cells were stained with anti-CD8 (Tri-Color), anti-CD4 (613), and anti-NK1.1(PE), followed by fixation in 1% paraformaldehyde and 70% ethanol. The DNA was mildly digested by treatment with DNase 1 (32) and HCl (33). The cells were then stained with anti-BrdU Ab for 30 min at room temperature and analyzed by four-color flow cytometry.

Production of chimeras

Fetal liver chimeras were produced as described previously (10). Briefly, fetal livers from day 16 fetuses were dispersed through an 18-gauge needle. Recipient mice received 980 rad (700 rad in the case of nude mice) 2–4 h before injection with donor fetal liver. A total of 1.5 × 10^7 cells was injected i.v. In the case of mixed fetal liver chimeras, the appropriate fetal liver cells were mixed at the stated percentages and then injected.

Thymus grafting

Fetal lobes from mice at day 16 of gestation were removed and washed in DMEM. The recipient mice were anesthetized with avertin (10 μg/g). The left kidney of the mice was exposed. The fetal thymic lobes were inserted in a small hole made in the kidney capsule. Thymus-grafted mice were sacrificed 8 wk after engraftment for analysis, at which time the presence of engrafted thymi was visually confirmed.

BrdU-labeling kinetics

BrdU-labeling kinetics was determined as described previously (33). Briefly, short-term labeling of mice was performed by i.p. injection of 100 μl of a 9-mg/ml solution of BrdU in PBS. Long-term labeling with BrdU was done by adding BrdU to the drinking water at a final concentration of 0.8 mg/ml. The drinking water was replaced daily. After the labeling period, cell populations were enriched, stained, and analyzed by flow cytometry as described above.

Results

The role of the thymus in development of peripheral NK1.1$^+$ T cells

We initially compared liver lymphocytes from C57BL6/J nude mice and age-matched (1-year-old) control C57BL6/J mice. Older mice were examined because it is known that T cell numbers increase in nude mice as they age (34). Conventional NK cells (CD4$^-$CD8$^-$NK1.1$^+$CD3$^-$ cells) represented a similar percentage of liver mononuclear cells in nude and wild-type mice, as expected (Fig. 1). In contrast, CD4$^+$CD8$^-$NK1.1$^+$ T cells were essentially absent in the nude liver cell population, and there was also a substantial reduction in the percentage of CD4$^-$CD8$^-$NK1.1$^+$ T
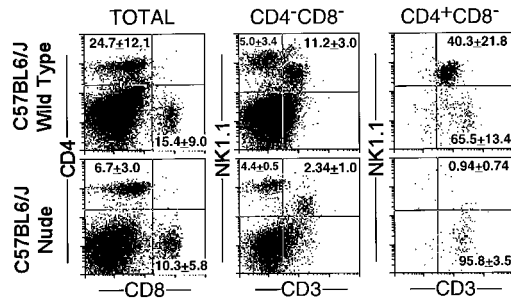
FIGURE 1. Deficiency of NK1.1⁺ T cells in the liver of athymic nude mice. Liver cell suspensions prepared from wild-type or *nu/nu* mice were analyzed by four-color flow cytometry to detect NK1.1⁺ T cells among CD4⁺CD8⁻ or CD4⁻CD8⁻ cells. The numbers indicate the mean percentage ± SD of cells in the quadrant (*n* = three to four mice). Due to compensation settings used in four-color flow cytometry, most of the events in the plots of CD4⁺CD8⁻ cells are present on the baseline (NK1.1⁻) and are therefore invisible.

cells. Interestingly, the reduction in NK1.1⁺ T cells in the nude liver was, if anything, more severe than the reduction in CD4⁺NK1.1⁻ T cells (Fig. 1).

To further investigate the thymus dependence of peripheral NK1.1⁺ T cells, a series of irradiation hematopoietic chimeras were prepared in which fetal liver cells were used to repopulate euthymic or athymic $\beta_2m^{-/-}$ mice. Normal numbers of liver NK1.1⁺ T cells appeared when euthymic $\beta_2m^{-/-}$ mice were repopulated with wild-type fetal liver cells, consistent with previous studies demonstrating that donor class I⁺ hematopoietic cells are necessary and sufficient to stimulate development of NK1.1⁺ T cells (Fig. 2). In contrast, the wild-type donor cells failed to develop into either CD4⁺CD8⁻NK1.1⁺ or CD4⁻CD8⁻NK1.1⁺ liver T cells when transplanted into athymic *nu/nu* $\beta_2m^{-/-}$ hosts (Fig. 2). Since it is possible that *nu/nu* mice exhibit defects in the liver, we also transferred wild-type fetal liver cells to $\beta_2m^{-/-}$ hosts that were surgically thymectomized before transplantation ($\beta_2m^{-/-}$ Tx mice). As with *nu/nu* $\beta_2m^{-/-}$ hosts, development of both CD4⁺CD8⁻NK1.1⁺ and CD4⁻CD8⁻ NK1.1⁺ T cells was severely compromised in the thymectomized hosts. In both nude and thymectomized recipients, the reduction in NK1.1⁺ T cells was more substantial than the reduction in CD4⁺NK1.1⁻ T cells. These results demonstrate that most NK1.1⁺ T cells are thymus-

dependent, but do not prove that the liver NK1.1⁺ T cells are derived from cells that previously resided in the thymus.

To directly address whether thymic cells can give rise to liver NK1.1⁺ T cells, we engrafted wild-type thymic lobes containing T cell precursors into mice that are incapable of producing T cells (RAG-1⁻/⁻ $\beta_2m^{-/-}$ mutant mice). Eight weeks after engraftment, gated CD4⁺ cells from the liver of the engrafted mice and control mice were analyzed for the presence of NK1.1⁺TCRαβ⁺ cells. CD4⁺ cells from the livers of engrafted mice contained a significant percentage of NK1.1⁺ TCR^low cells, well above the percentage in ungrafted $\beta_2m^{-/-}$ mice (Fig. 3). The only source of these T cells is the donor thymus. The donor thymus also gave rise to a significant percentage of conventional NK1.1⁻CD4⁺TCR^high cells in the liver. These data demonstrate that liver CD4⁺NK1.1⁺ T cells can originate from the thymus.

Cell types that select thymic and peripheral NK1.1⁺ T cells

We previously examined irradiation chimeras consisting of $\beta_2m^{-/-}$ hosts that were reconstituted with a mixture of $\beta_2m^{-/-}$ and class I⁺ fetal liver cells. The results showed that class I⁺ hematopoietic cells stimulated neighboring $\beta_2m^{-/-}$ precursor cells to differentiate into CD4⁻CD8⁻TCRVβ8⁺ T cells (most of which are NK1.1⁺ T cells) (8). We have used this mixed chimera system to investigate the identity of MHC class I⁺ hematopoietic cell types that select NK1.1⁺ T cells. Irradiated $\beta_2m^{-/-}$ mice were reconstituted with a mixture of $\beta_2m^{-/-}$ fetal liver cells and class I⁺ fetal liver cells. The class I⁺ fetal liver cells came from various mutant mice (TCRα⁻/⁻, TCRβ⁻/⁻, or Rag 1⁻/⁻) that differ in their capacity to differentiate into defined thymocyte subsets. Using this approach, we could correlate the presence of various class I⁺ cell types in the thymus with successful development of NK1.1⁺ T cells. Each reconstituted animal contained precursor cells ($\beta_2m^{-/-}$) capable of yielding the major thymocyte population (CD4⁺CD8⁺ cells); therefore, the thymuses did not differ significantly in overall composition. Indeed, the overall cellularity of the thymuses in the different groups did not differ substantially (see Fig. 4 legend).

Thymocytes in TCRα⁻/⁻ mice do not develop beyond the CD4⁺CD8⁺ stage, and therefore do not generate mature CD4⁺, CD8⁺, or NK1.1⁺ T cells. We prepared mixed chimeras in $\beta_2m^{-/-}$ hosts with class I⁺ TCRα⁻/⁻ fetal liver cells and $\beta_2m^{-/-}$ fetal liver cells. Thymic CD4⁺NK1.1⁺ T cells developed in near normal numbers in these chimeras, similar to the results with
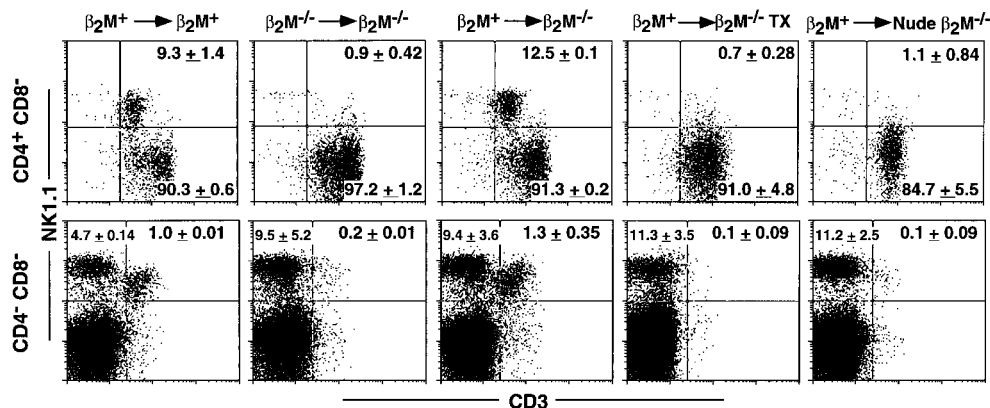
FIGURE 2. Thymus dependence of liver NK1.1⁺ T cells assessed in thymectomized fetal liver chimeras. Host mice that were $\beta_2m^{-/-}$, $\beta_2m^{-/-}$ *nu/nu*, $\beta_2m^{-/-}$, and thymectomized or wild type were irradiated and reconstituted with fetal liver cells from wild-type mice or as a control, $\beta_2m^{-/-}$ mice. After 8–9 wk, liver cell suspensions were examined by four-color flow cytometry. The numbers indicate the mean percentage ± SD of NK1.1⁺ CD3^low cells among either CD4⁺CD8⁻ or CD4⁻CD8⁻ cells as indicated (*n* = three to five individual chimeras).

FIGURE 3. Liver NK1.1$^+$ T cells arise from a fetal thymus graft. Rag-1$^{-/-}$ β_2m$^{-/-}$ mice were engrafted with untreated fetal thymic lobes that had been freshly harvested from normal C57BL/10 fetuses at day 16 of gestation. Eight weeks after reconstitution, liver cells from these mice, as well as from normal B6 and β_2m$^{-/-}$ mice for comparison, were stained and analyzed by four-color flow cytometry. Similar results were obtained in five other experiments. The data represent gated CD4$^+$CD8$^-$ liver cells. CD4$^+$8$^-$ cells represented a similar proportion of liver cells analyzed in the grafted mice (mean, 12.0 ± 1.6%) compared with normal mice (mean, 11.5 ± 2.9%). Liver cell yields were variable, but did not differ systematically between the different groups.

C57Bl/10 (β2m$^+$)Thymus

Gated CD4$^+$CD8$^-$ Liver Cells
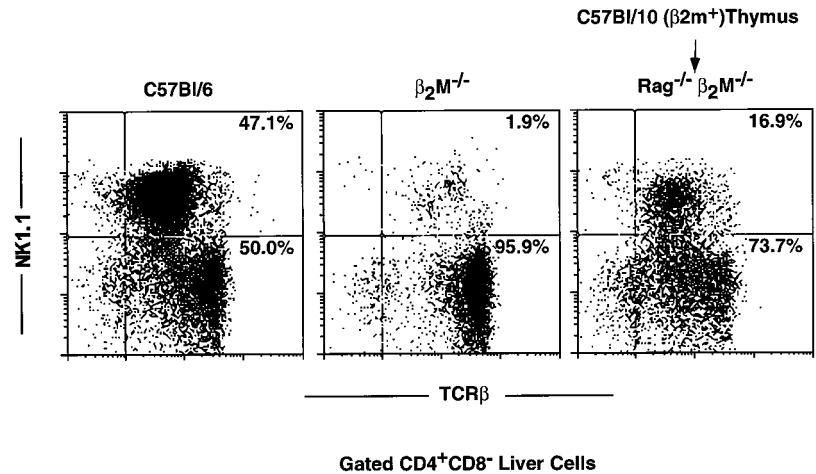
mixed chimeras prepared with class I$^+$ fetal liver cells were from wild-type mice (Fig. 4A). These results indicate that mature T cells expressing class I molecules are not obligatory for stimulating the development of thymic NK1.1$^+$ T cells.

Most thymocytes in TCR$\beta^{-/-}$ mice are arrested at the CD4$^-$CD8$^-$ stage, and therefore generate very few CD4$^+$CD8$^+$ cells. Strikingly, in mixed chimeras prepared with class I$^+$ TCR$\beta^{-/-}$ fetal liver cells and β_2m$^{-/-}$ fetal liver cells, essentially no NK1.1$^+$ T cells developed (Fig. 4A). Since the only known difference between TCR$\beta^{-/-}$ and TCR$\alpha^{-/-}$ cells is in the capacity to generate CD4$^+$CD8$^+$ cells, these data implicate class I$^+$ CD4$^+$CD8$^+$ thymocytes as essential cells for stimulating the development of CD4$^+$ NK1.1$^+$ T cells in the thymus. There are unlikely to be significant differences in thymic architecture in the two types of mixed chimeras, since both contain β_2m$^{-/-}$ fetal liver precursor cells that can generate CD4$^+$CD8$^+$ (albeit class I-deficient) thymocytes. Consistent with the results with TCR$\beta^{-/-}$ precursors, NK1.1$^+$ T cells failed to develop in mixed chimeras in which the class I$^+$ fetal liver cells were from rag-1$^{-/-}$ mice, which also cannot generate CD4$^+$CD8$^+$ or mature thymocytes (Fig. 4A). Importantly, the contribution of class I$^+$ cells to the

hematopoietic compartment (exclusive of T cells) in the chimeras was similar whether the class I$^+$ cells were from wild-type, TCR$\alpha^{-/-}$, or TCR$\beta^{-/-}$ mice. This was assessed by determining the percentage of class I$^+$ B cells in the spleen, which was ~80% of the total in all three types of chimeras (data not shown).

To determine whether CD4$^+$CD8$^+$ cells also play a role in differentiation of liver NK1.1$^+$ T cells, we examined CD4$^+$NK1.1$^+$ T cells in the livers of the same mixed fetal liver chimeras. The results were very similar to the results obtained with thymus cells. Class I$^+$ TCR$\alpha^{-/-}$ cells stimulated the differentiation of significant numbers of CD4$^+$NK1.1$^+$ liver T cells, but class I$^+$ TCR$\beta^{-/-}$ or class I$^+$ rag-1$^{-/-}$ cells did not (Fig. 4B). The higher number of NK1.1$^+$ T cells in hosts with class I$^+$ wild-type cells vs class I$^+$ TCR$\alpha^{-/-}$ fetal liver cells is probably due to the fact that the wild-type cells, unlike the TCR$\alpha^{-/-}$ cells, can themselves contribute to the NK1.1$^+$ T cell compartment. Thus, differentiation of liver NK1.1$^+$ T cells, like that of thymic NK1.1$^+$ T cells, requires class I$^+$ cells that are dependent on TCRβ expression, presumably CD4$^+$CD8$^+$ cells. Since CD4$^+$CD8$^+$ T cells are not found in significant numbers in the liver (data not shown), the data further support a thymic origin of liver NK1.1$^+$ T cells.
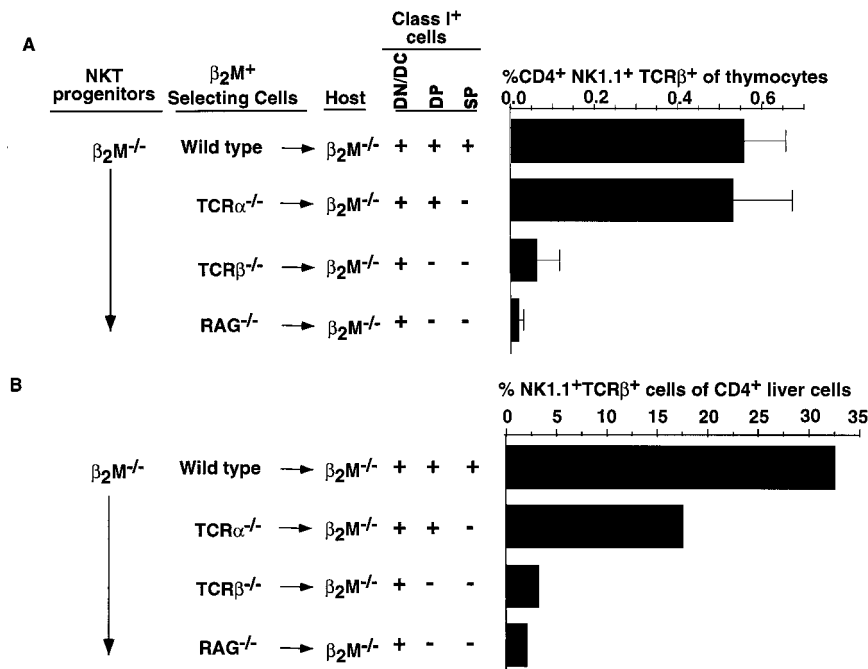
FIGURE 4. Class I$^+$ cells that select NK1.1$^+$ T cells are dependent on TCRβ expression. Capacity of fetal liver cells from mutant mice to provide class I$^+$ cells necessary for selecting NK1.1$^+$ T cells found in the thymus (A) or the liver (B). For all chimeras, fetal liver cells from β_2m$^{-/-}$ mice (20% of input fetal liver cells) were used as a source of cells capable of differentiating into NK1.1$^+$ T cells. The hosts were all irradiated β_2m$^{-/-}$ mice. As a source of class I$^+$-selecting cells (80% of input fetal liver cells), fetal liver cells from the indicated mutant or wild-type mice were employed. TCR$\alpha^{-/-}$ fetal liver cells can provide cells capable of selecting NK1.1$^+$ T cells in both the thymus and the liver, whereas TCR$\beta^{-/-}$ or Rag-1$^{-/-}$ fetal liver cells cannot. A, The data represent means of three to five determinations ± SD. The mean (±SD) cellularities of the thymi in A were 137 ± 16.5, 74.6 ± 1.6, 93.2 ± 23.5, and 125 ± 18.7 million cells for chimeras reconstituted with wild-type, TCR$\alpha^{-/-}$-, TCR$\beta^{-/-}$-, and RAG-1$^{-/-}$-selecting cells, respectively. B, A representative experiment of three that yielded similar results.

FIGURE 5. Labeling kinetics of NK1.1$^+$ T cells in the thymus and liver. *A*, Eight-week-old mice were exposed to BrdU continuously in their drinking water for various periods before analysis of BrdU incorporation in the indicated thymic subpopulations by four-color flow cytometry. The experiment was repeated three times with similar results. *B*, Mice of different ages were exposed to BrdU for 3 days. Thymic NK1.1$^+$ T cells were examined for the percentage of cells that had incorporated BrdU by four-color flow cytometry. Data represent the means of three determinations, and the variation was <10% of the mean. *C*, Young (2.5-wk-old) mice were exposed to BrdU for limiting time periods before determining BrdU incorporation in NK1.1$^+$ T cells and CD4$^+$CD8$^+$ cells in the thymus. The experiment was repeated three times with similar results. *D*, Eight-week-old mice were exposed to BrdU continuously in their drinking water for various periods before analysis of BrdU incorporation in thymic and liver NK1.1$^+$ T cells. Data represent the mean of three determinations, and the variation was <1%.
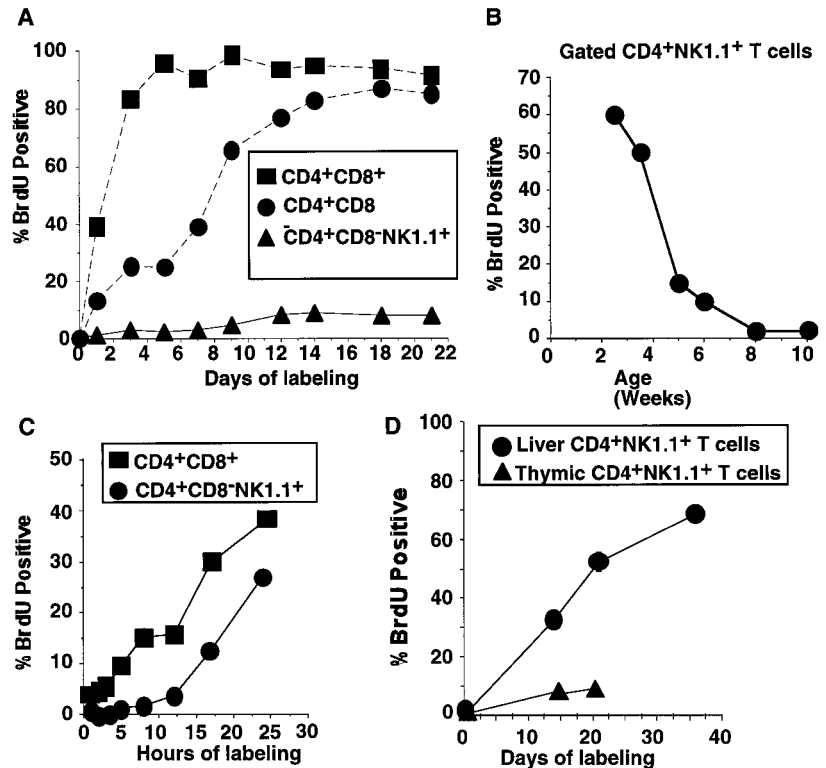
Turnover of thymic NK1.1$^+$ T cells

We employed the BrdU-labeling method to assess the turnover rate of NK1.1$^+$ T cells. We performed continuous labeling experiments with BrdU, in which adult mice (starting at 8 wk of age) were provided with BrdU continuously in their drinking water for varying times, before determining the percentage of BrdU$^+$ NK1.1$^+$ T cells. Strikingly, even after 21 days of continuous exposure to BrdU, <10% of CD4$^+$NK1.1$^+$ T cells were labeled (Fig. 5*A*). In contrast, CD4$^+$CD8$^+$ and conventional CD4$^+$CD8$^-$ thymocytes labeled rapidly, with over half the cells labeling within 2 and 9 days, respectively (Fig. 5*A*). The results indicate that NK1.1$^+$ T cells in the adult thymus represent a largely sessile compartment, unlike CD4$^+$CD8$^+$ and conventional CD4$^+$CD8$^-$ thymocytes.

To investigate the effect of age on the turnover rate of NK1.1$^+$ thymocytes, mice of various ages were provided with BrdU for 3 days, and the proportion of BrdU-labeled CD4$^+$NK1.1$^+$ T cells was determined by flow cytometry. As before, only a very small percentage of adult thymic NK1.1$^+$ T cells were labeled in 3 days (Fig. 5*B*). Strikingly, however, NK1.1$^+$ thymocytes in young mice labeled rapidly. In 2.5-wk-old mice, 60% of the cells labeled in a 3-day labeling period (Fig. 5*B*).

In young mice, the total numbers of thymic NK1.1$^+$ T cells are increasing, raising the possibility that the rapid labeling of these cells during this time period is related to the production of new NK1.1$^+$ cells from proliferating NK1.1$^-$ or TCR$^-$ T cell precursors. If this were the case, most of the BrdU incorporation would be actually occurring in precursor cells, which would subsequently differentiate into NK1.1$^+$ T cells. Alternatively, thymic NK1.1$^+$ T cells may themselves proliferate extensively in young mice, but not in older mice. To address these alternatives, we exposed 2.5-wk-old mice to BrdU for progressively shorter times (Fig. 5*C*). We observed essentially no labeling of thymic NK1.1$^+$ T cells in the first 12 h of labeling, but the cells labeled rapidly in the next 12 h. In contrast, the CD4$^+$CD8$^+$ thymocyte population, known to in-

clude many proliferating cells, began to incorporate BrdU immediately. The delay in labeling of NK1.1$^+$ T cells suggests that most of these cells are not themselves proliferating in young mice, but are derived from a proliferating NK1.1$^-$ and/or TCR$^-$ precursor cell.

The low BrdU incorporation of NK1.1$^+$ T cells in the adult thymus suggests that these cells are produced only at a low rate at the adult stage. One possible explanation for the lower production of these cells at the adult stage is that a feedback process inhibits production of NK1.1$^+$ T cells after the cells have saturated a thymic or peripheral compartment. If so, the number of NK1.1$^+$ T cells in the thymus should be limited by the size of this compartment rather than by the number of progenitor cells. To address this possibility, we prepared chimeras in which increasing numbers of progenitor fetal liver cells capable of differentiating into NK1.1$^+$ T cells (from β_2m$^{-/-}$ mice) were mixed with β_2m$^+$TCR$\alpha^{-/-}$ fetal liver cells as a source of class I$^+$ cells capable of stimulating NK1.1$^+$ T cell development. The fetal liver cell mixtures were employed to repopulate lethally irradiated β_2m$^{-/-}$ recipient mice. The chimeras were examined 8–10 wk after reconstitution.

When the percentage of injected β_2m$^{-/-}$ fetal liver cells was only 2.5% of the total, few NK1.1$^+$ T cells developed (Fig. 6). However, increasing the percentage of injected β_2m$^{-/-}$ fetal liver cells to 5% of the total led to a saturating level of NK1.1$^+$ T cell development, which did not increase significantly further with larger numbers of β_2m$^{-/-}$ input cells (Fig. 6). Thus, once the β_2m$^{-/-}$ precursor cells reach 5% or more of the total, they are saturating for NK1.1$^+$ T cell development. In contrast, the relative number of conventional CD4$^+$ T cells increased continuously as the percentage of β_2m$^{-/-}$ precursor cells was increased to 20% of the input cells. Thus, precursor cell numbers are limiting for the development of conventional CD4$^+$ T cells, but are much less limiting for the development of NK1.1$^+$ T cells. The results support the conclusion that the development of thymic NK1.1$^+$ T cells
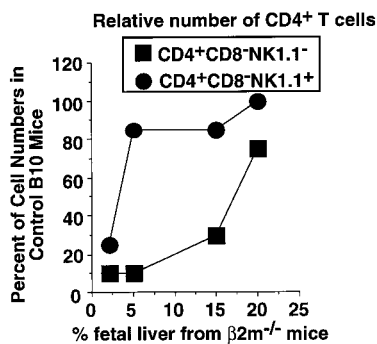
FIGURE 6. NK1.1$^+$ T cell development in the thymus is not limited by precursor cell number. Irradiated $\beta_2m^{-/-}$ hosts were reconstituted with differing ratios of fetal liver cells from $\beta_2m^{-/-}$ mice and class I$^+$ TCR$\alpha^{-/-}$ mice, with a fixed total number of 1.5×10^7 cells/recipient. The class I$^+$ TCR$\alpha^{-/-}$ fetal liver cells serve as a source of selecting cells for the NK1.1$^+$ T cells. Chimeras were analyzed 8 wk after reconstitution. The percentage of class I-deficient cells among splenic B cells closely approximated the percentage of class I-deficient fetal liver cells used to reconstitute the chimeras. The number of CD4$^+$NK1.1$^+$ T cells in the thymus was determined. For comparison, the number of conventional CD4$^+$CD8$^-$NK1.1$^-$ thymocytes was determined in the same chimeras. The data are represented as percentages of the mean cell number of the same type that was observed in normal B10 mice that served as controls. The control cell numbers were 3.6×10^5 CD4$^+$CD8$^-$NK1.1$^+$ T cells/thymus and 7.7×10^6 CD4$^+$CD8$^-$NK1.1$^-$ T cells/thymus.

in normal mice is severely limited by the size of a thymic or peripheral compartment rather than by the number of precursor cells.

Labeling kinetics of peripheral NK1.1$^+$ T cell populations

We next determined the BrdU-labeling kinetics of NK1.1$^+$ T cells in the liver. Adult (8-wk-old) mice were exposed to BrdU for varying intervals, and the percentage of labeled cells was determined among NK1.1$^+$ T cells in the liver. As shown in Fig. 5*D*, nearly 60% of liver CD4$^+$ NK1.1$^+$ T cells labeled in 21 days, compared with <10% labeling of thymic NK1.1$^+$ T cells in the same time period. Thus, adult thymic NK1.1$^+$ T cells, which label slowly, give rise to liver NK1.1$^+$ T cells, which label more rapidly. These data suggest either that NK1.1$^+$ T cells begin to proliferate after emigration from the thymus to the liver, or that the liver NK1.1$^+$ T cells represent a subset of thymic NK1.1$^+$ T cells that cycle more frequently.

Discussion

Thymic development of peripheral NK1.1$^+$ T cells

The results presented here provide several lines of evidence that the large majority of NK1.1$^+$ T cells in the periphery, specifically those in the liver, arise in the thymus. In congenitally athymic nude mice, liver CD4$^+$NK1.1$^+$ T cells are essentially absent, and CD4$^-$CD8$^-$ NK1.1$^+$ T cells are greatly reduced in number. The possibility that the deficit of NK1.1$^+$ T cells in nude mice was due to effects of the nude mutation on nonthymic tissues was ruled out by the finding that NK1.1$^+$ T cells were also severely deficient in fetal liver chimeras prepared with adult thymectomized mice. Since a transplanted wild-type fetal thymus was the source of liver NK1.1$^+$ T cells in a Rag-1$^{-/-}\beta_2m^{-/-}$ host that cannot produce T cells, it cannot be asserted that the thymus plays only an indirect role, by providing signals necessary for NK1.1$^+$ T cell development. These data definitively establish that liver NK1.1$^+$ T cells can arise in the thymus. The experiment does not, however, prove that all NK1.1$^+$ T cells in the livers of normal mice arise in the thymus. However, the additional finding that development of liver

NK1.1$^+$ T cells requires a TCRβ-dependent selecting cell, presumably a CD4$^+$CD8$^+$ thymocyte, does favor the conclusion that liver NK1.1$^+$ T cells develop primarily in the thymus.

Although some authors have concluded that peripheral NK1.1$^+$ T cells develop primarily in the periphery, a review of these studies indicates that there may be little direct contradiction between our results and theirs. It should be emphasized that all other types of T cells are found in the periphery of nude mice to one extent or the other (Ref. 35); Fig. 1). Therefore, finding a few NK1.1$^+$ T cells in athymic mice does not justify the statement that the cells are generally thymus independent. In terms of the specific studies that have concluded that NK1.1$^+$ T cells develop extrathymically, Sato et al. (21) showed that IL-2Rβ^+CD3$^+$ cells are present in the liver and spleen of nude mice, but did not provide evidence that these cells included NK1.1$^+$ T cells. Many IL-2Rβ^+CD3$^+$ cells in normal mice do not express NK1.1 (21). Makino et al. (23) provided evidence for reciprocal rearrangement products of Vα14 genes in the periphery as well as in fetal tissues of nude mice. However, such a finding does not mean that NK1.1$^+$ T cells can mature in the absence of a thymus. Finally, the study showing NK1.1$^+$ T cell development in vitro (24) does not speak directly to what occurs in vivo, since the in vitro conditions may differ substantially from those in vivo.

Cells that select NK1.1$^+$ T cells

The data provide significant new evidence that the NK1.1$^+$ T cells both in the thymus and in the periphery are selected by interactions with class I molecules expressed by CD4$^+$CD8$^+$ thymocytes. This conclusion rests on the mixed chimera experiments, where it was shown that class I$^+$ cells from TCR$\alpha^{-/-}$ mice support the development of NK1.1$^+$ T cells, whereas class I$^+$ cells from TCR$\beta^{-/-}$ mice or Rag-1$^{-/-}$ mice do not. The key difference in these two scenarios is that chimeras prepared with class I$^+$ cells from TCR$\beta^{-/-}$ mice lack most class I$^+$ CD4$^+$CD8$^+$ thymocytes. Our results go beyond previous studies by demonstrating that the lack of class I$^+$ CD4$^+$CD8$^+$ thymocytes also impairs the development of NK1.1$^+$ T cells in the liver. Since CD4$^+$CD8$^+$ T cells are not detected in the liver, these data are in accord with the conclusion that NK1.1$^+$ T cells in the liver arise from the thymus as a result of interactions with class I molecules expressed by CD4$^+$CD8$^+$ thymocytes. In line with this conclusion, CD4$^+$CD8$^+$ cells have been shown to express relatively high levels of CD1d (36). The current experiments do not address the possibility that class I$^+$ mature T cells can also select NK1.1$^+$ T cells, though mature T cells are clearly unnecessary if class I$^+$ CD4$^+$CD8$^+$ cells are present.

Turnover kinetics of the NK1.1$^+$ T cell population in the thymus and liver

The BrdU-labeling experiments suggest that NK1.1$^+$ T cells in the thymus of young mice are derived from a proliferating precursor cell, but do not themselves proliferate extensively. As mice reach 5–6 wk of age, the population stabilizes and undergoes replacement at only a low rate thereafter. The reduced production of NK1.1$^+$ T cells at 5–6 wk of age could reflect an inability of adult precursor cells to differentiate into NK1.1$^+$ T cells. However, we found that adult bone marrow cells were as effective as fetal liver cells in repopulating the NK1.1$^+$ T cell compartment in hematopoietic irradiation chimeras (data not shown). Therefore, it appears more likely that the slow turnover of NK1.1$^+$ T cells in older mice is due to saturation of a thymic compartment and subsequent feedback inhibition events. This view is supported by the chimera studies, which showed that precursor cell numbers are not limiting for the production of thymic NK1.1$^+$ T cells. Even with a very small