Defective development of NK1.1\(^{+}\) T-cell antigen receptor \(\alpha\beta^{+}\) cells in zeta-associated protein 70 null mice with an accumulation of NK1.1\(^{+}\) CD3\(^{-}\) NK-like cells in the thymus

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Development of natural killer 1.1\(^{+}\) (NK1.1\(^{+}\)) CD3\(^{-}\) (NK1.1\(^{+}\) T) cells was analyzed in zeta-associated protein 70 (ZAP-70) null (\(^{-/-}\)) mice. Both NK1.1\(^{+}\) TCR\(\alpha\beta^{+}\) and NK1.1\(^{+}\) TCR\(\gamma\delta^{+}\) cell populations were absent in the thymus and spleen. By contrast, the number of NK1.1\(^{+}\) CD3\(^{-}\) cells was increased in these tissues. The NK1.1\(^{+}\) CD3\(^{-}\) thymocytes in ZAP-70\(^{-/-}\) mice had surface phenotypes in common with NK or NK1.1\(^{+}\) T cells. However, some of them were discordant either with NK cells or with NK1.1\(^{+}\) T cells. The NK1.1\(^{+}\) CD3\(^{-}\) cells produced interferon-\(\gamma\) upon stimulation with NK1.1\(^{+}\) cross-linking in the presence of interleukin-2 and exhibited a substantial cytotoxicity against YAC-1 cells. Moreover, the generation of NK1.1\(^{+}\) T cells with invariant V\(\alpha\)14J\(\omega\)281 chains was induced from the NK1.1\(^{+}\) CD3\(^{-}\) thymocytes following stimulation with phorbol myristate acetate and ionomycin in a neonatal thymic organ culture. An introduction of TCR\(\alpha\) and \(\beta\) transgenes to the ZAP-70\(^{-/-}\) mice resulted in generation of an NK1.1\(^{+}\) TCR\(\alpha\beta^{H2}\) population, whereas no substantial CD4\(^{+}\) CD8\(^{-}\) or CD4\(^{-}\) CD8\(^{+}\) population that expressed the introduced TCR\(\alpha\beta\) was generated in the mainstream T lineage. These findings demonstrate that ZAP-70 kinase is indispensable for the development of NK1.1\(^{+}\) T cells and that the unique NK1.1\(^{+}\) CD3\(^{-}\) thymocytes in ZAP-70\(^{-/-}\) mice contain immediate precursors of NK1.1\(^{+}\) T cells. (Blood. 2001;97:1765-1775)
with these engineered mice that development of mainstream T cells was defective. These findings are consistent with a postulate that the same or similar molecular mechanisms operate on the development of both NK1.1+ T cells and mainstream T cells. However, NK1.1+ T cells as well as TCRβ+ cells but not mainstream T cells developed normally in the dominant negative mutant on the Ras/Raf/Mek/Mapk pathway.28 On the other hand, in T-cell factor-1–deficient mice,29 Fyn-deficient mice,30 and pre-TCRβ− mice,26,31 the development of NK1.1+ T cells was selectively abrogated, whereas only minimal defect was observed in mainstream T cells. These findings suggest that certain signaling pathways involved in generation of NK1.1+ T cells are different from those of mainstream T cells.

In the present study, we analyzed development of NK1.1+ T cells in zeta-associated protein (Zap)-70−/− mice. It was shown that Zap-70 tyrosine kinase was essential for the development of mainstream T cells but not for that of NK cells.32 Indeed, NK cells differentiated normally in the peripheral lymphoid tissues and retained the NK activity in Zap-70−/− mice presumably because p72Syk replaced Zap-70 functions in the NK cell population. Herein, we demonstrate that development of NK1.1+ T cells is completely abrogated in Zap-70−/− mice. Instead, a considerable population of NK1.1+ CD3− cells was detected in the thymus as well as in the spleen. Although the surface phenotype of the NK1.1+ CD3− population was quite unique, this population retained intact NK functions. Furthermore, it will be shown that generation of NK1.1+ T cells is induced in the NK1.1+ CD3− thymocytes following stimulation with phosphor myristate acetate (PMA) and ionomycin in vitro. A possible developmental pathway of the NK1.1+ T cells is discussed.

Materials and methods

Mice

Zap-70−/− mice32 were provided by Dennis Y. Loh at the Department of Biology, Nippon Roche Research Center (Kamakura, Japan). These mice were backcrossed with C57Bl/6 (B6) mice for several generations and maintained in the animal facility at the Institute for Genetic Medicine, Hokkaido University, in a specific pathogen-free condition. Zap-70+/+ and Zap-70+/− mice were used as controls for flow cytometric and functional analyses. Zap-70−/−/DO10 TCR transgenic mice were prepared by crossing the Zap-70−/− mice of B6 background with DO10 TCR transgenic mice35 of B10.D2 background. Progenies were screened for the mutant Zap-70− allele and the TCR transgene. C57Bl/6 Rag-1−/− (Rag-1−/−), B6.PL-Thy1.1, and Tcrα−/− and β−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were used for experiments at the age of 14 to 20 weeks.

Antibodies and flow cytometry

Thymocytes and spleen cells were first incubated with unlabelled monoclonal antibody (mAb) 2.4G2 (anti-FcyR) to block nonspecific staining and were then stained with a combination of the following mAb conjugates: biotinylated (biotin)-CD1d (1B1), -CD5 (53-7.3), -CD16 (2.4G2), -CD24 (J1id), -CD25 (7D4), -CD34 (RAM34), -CD44 (IM7), -CD45RB (2C12), -CD62L (MEL-14), -CD69 (H1.2F3), -Ly49A (A1), -TCRβ (GL3), -CD24, -CD5, and -DX5; fluorescein isothiocyanate (FITC)-anti-TCRαβ mAb. The stained cells were sorted into NK1.1+ and NK1.1− populations using FACSVantage (Becton Dickinson). The sorted cells were cultured in the presence of recombinant human IL-2 (1000 U/mL), Pharmaceutical Research Division, Takeda Chemical Industries, Osaka, Japan) and used for functional analyses.

Cytokine enzyme-linked immunosorbent assay

To evaluate IL-4 production, either unsorted (1 × 10^5) or sorted (4 × 10^6) cells from the thymus and spleen were stimulated with immortalized anti-CD3ε mAb (145-2C11; Pharmingen) at 10 μg/mL in a total volume of 50 μL RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 × 10^−5 M 2-mercaptoethanol in a 96-well flat-bottomed plate for 48 hours. IL-4 in the culture supernatants was quantitated with Cytoscreen Immunoassay kit for mouse IL-4 (BioSource International, Camarillo, CA) according to a manufacturer’s protocol. To evaluate interferon (IFN)-γ production, cells were stimulated with immobilized anti-NK1.1 (PK136; Pharmingen) at 50 μg/mL in a total volume of 50 μL RPMI-1640 in the presence of rhIL-2 (1000 U/mL) for 48 hours. IFN-γ in the culture supernatants was quantitated with Cytoscreen Immunoassay kit for mouse IFN-γ (BioSource International).

Assay for cytotoxic activity

Cytotoxic activities were evaluated as previously described.32 In brief, control and Zap-70−/− mice were intraperitoneally administered 200 μg tolirone (2,7-bis[2-(diethylamino)ethyl]9H-fluoren-9-one; Sigma) per mouse 24 hours before collecting thymocytes and spleenocytes. Total thymocytes or spleenocytes depleted of red blood cells were cultured for 7 days in the presence of rhIL-2 (1000 U/mL). The harvested cells were used as effector cells and incubated with 51Cr-labeled YAC-1 or P815 cells (5 × 10^5) at an indicated effectortarget ratios for 4 hours.14 Then 51Cr radioactivity released in the supernatant was quantified with γ-counter (Auto Gamma 5000; Packard, Canberra, Australia). Cytotoxicity was expressed as percent specific lysis, which was calculated as follows: percent specific lysis = [(experimental release − spontaneous release)/maximum release − spontaneous release] × 100. Spontaneous release and maximum release were obtained by incubating target cells alone or with 2 N HCl solution, respectively.

Neonatal thymic organ culture

Thymic lobes were obtained from neonatal BALB/c, Rag-1−/−, or (B6 x B6.Thy1.1)F1 mice within 24 hours of birth and cultured for 5 to 7 days with medium containing 1.35 mM 2′-deoxyguanosine (dGuo; Nakarai-tesque, Osaka, Japan) on the raft of a membrane filter (0.45 μm; Millipore, Bedford, MA) with a sterile sponge (Gelfoam; Pharmacia-Upjohn, Tokyo, Japan). The dGuo-treated thymic lobes were transferred to a Terasaki dish (Nihon Terminex, Osaka, Japan) on the raft of a membrane filter (0.45 μm; Millipore, Bedford, MA) with a sterile sponge (Gelfoam; Pharmacia-Upjohn, Tokyo, Japan). The dGuo-treated thymic lobes were transferred to a Terasaki dish (Nihon Terminex, Osaka, Japan) on the raft of a membrane filter (0.45 μm; Millipore, Bedford, MA) with a sterile sponge (Gelfoam; Pharmacia-Upjohn, Tokyo, Japan). The dGuo-treated thymic lobes were transferred to a Terasaki dish (Nihon Terminex, Osaka, Japan) on the raft of a membrane filter (0.45 μm; Millipore, Bedford, MA) with a sterile sponge (Gelfoam; Pharmacia-Upjohn, Tokyo, Japan).
BLOT/c, RAG-1−/−, or (B6 × B6.Thyl.1)F1 mice in RPMI-1640–based medium in the presence of 1600 nM PMA (Sigma) and 130 nM ionomycin (Sigma).5,40 Five days later the thymic lobes were harvested and minced to obtain a single-cell suspension. Cells were stained with either PE-anti-NK1.1 mAb or PE-control IgG2a (Pharmingen) and FITC–anti-TCRβ mAb and analyzed with FACSscan as described above. The proportion of NK1.1+ TCRβ+ cells was calculated as follows: [percentage of NK1.1+ TCRβ+ cells minus percentage of control IgG2a+ TCRβ+ cells]. Statistical analysis was performed according to the Student t test.

Detection of an invariant Vγ chain and RAG-1 transcripts in induced NK1.1+ TCRβ+ cells

Total RNA was extracted either from neonatal thymic lobes obtained from RAG-1−/− mice or those cultured with sorted NK1.1+ TCRβ+ cells from ZAP-70−/− thymus in the presence or absence of PMA plus ionomycin. Complementary DNA (cDNA) was synthesized using random hexanucleotide (Takara Shuzo, Otsu, Japan) and Moloney marine leukemia virus reverse transcriptase (SuperScript; Gibco) at 37°C for 1 hour in the presence of deoxyribonucleoside triphosphates and ribonuclease inhibitor (RNasin; Promega, Madison, WI). The cDNA products were used as templates in either ordinary or nested polymerase chain reactions (PCRs) for amplification of the following gene products with respective primer pairs (all from Hokkaido System Science, Sapporo, Japan): Vα14 Leader/C-rev1 (for first-round PCR), 5′-ATGAAAGAAGGCGGTATGAC-3′/5′-CAGGAGGATCTGCC-3′ (for first-round PCR), 5′-TAAAGCACAGCATCGACAC-3′/5′-CAATCACTGTAGTGCCACGCTC-3′ (281 transcripts) or a nest/RAG-1 3′/5′-nest (for nested PCR), 5′-CCCAAATCTCGACATCTGACCT-3′/5′-CACATCTGCTTCAAGTGATCC-3′; RAG-1 5′/5′-RAG-1 3′ (for first-round PCR), 5′-GCGGA-3′/5′-nest/RAG-1 3′ (for nested PCR), 5′-CGAAGAAGACAGAAGGAAG-3′/5′-AAGACATCCACAGATGC-3′; and EF-1α 5′/5′-EF-1α 3′ (for first PCR), 5′-CTGCTGATAGTGGGAGAGCT-5′/5′-TTCAGGATACTCCAGAGCA-3′. Thermal cycling was performed with the following programs: 40 cycles of heat denaturation at 94°C for 1 minute, annealing at 53°C for Vα14 Leader/C-rev1, 52°C for Vα14 Leader/C-rev1, or 50°C for EF-1α 5′/EF-1α 3′ for 1 minute, and elongation at 72°C for 2 minutes. PCR products were electrophoresed on either a 3% (for Vα14 Leader/C-rev1 transcripts) or a 1% (for RAG-1 and EF-1α transcripts) agarose ethidium bromide gel according to the length of amplified bands.

Analysis of gene rearrangement of TCR Vγ chain from NK1.1+ TCRβ+ thymocytes in ZAP-70−/− mice

Genomic DNA was extracted from either B6 thymocytes, B6 ear skin, or NK1.1+ TCRβ+ thymocytes of ZAP-70−/− mice and subjected to a PCR-based analysis of gene rearrangements22,23 with slight modifications. PCR was performed using a primer pair of a coding region of Dβ2 (Dβ2-5′) and 3′-downstream region of Jβ2.7 (Jβ2-3′) to detect rearrangements of Dβ2 to Jβ2 cluster. Rearrangement of Vβ8 to Dβ2/Jβ2 was examined by PCR with a primer pair of a coding region of Vβ8.2 (Vβ8-5′) and the same Jβ2 primer as described above. Sequences of PCR primers were: Dβ2-5′, 5′-TAGGCACCCTGTGGAAGAAGCT-3′; Jβ2-3′, 5′-TGGAGGCTGTCTCCTACTATGATT-3′; and Vβ8-5′, 5′-GCATGGCGCTGAGCTGATGCA-3′. Dβ2/Jβ2 probe labeled with 32P was used to identify the presence of Dβ2/Jβ2 transcripts and to identify the presence of Vβ8.1 transcripts. Results were analyzed by Southern blotting analysis or gel electrophoresis andAutoradiography. The presence of Vβ8.1 transcripts was confirmed by Southern blotting analysis. Rearrangement of Vγ chain was examined by Southern blotting analysis of genomic DNA with oligonucleotide Vγ14 (SuperScript; Gibco). Hybridization was performed at 37°C overnight in a 5× standard saline citrate 0.02% sodium dodecyl sulfate–based hybridization solution and washed at room temperature with 5× standard saline citrate 0.1% sodium dodecyl sulfate for 15 minutes once followed by a wash in 0.1× saline citrate for 15 minutes. Then, the membrane was washed several times with NaCl/Tris-based buffers and incubated with antifluorescein antibody conjugated with alkaline phosphatase at 4°C overnight with gentle shaking. The membrane was washed 4 times with 0.4 M NaCl, 0.1 M Tris-HCl, (pH 7.5), incubated with CDP-Star for 1 minute, and exposed to x-ray film (Hyperfilm; Amersham).

Results

Development of NK1.1+ T cells was arrested in ZAP-70−/− mice

To examine development of NK1.1+ T cells in the ZAP-70−/− mice, we performed flow cytometric analysis of the thymocytes and spleen cells and compared the profiles with those of control mice (ZAP-70−/− or ZAP-70+/+) mice. Representative results are shown in Figure 1. NK1.1+ CD3+ or NK1.1+ TCRβ+ cells were totally absent in the thymus of ZAP-70−/− mice (0% or 0.02%, respectively). Instead, a substantial proportion of the NK1.1+ CD3− or NK1.1+ TCRβ− population was detected (0.42% or 0.39%, respectively). The NK1.1+ CD3− or NK1.1+ TCRβ− population was rarely detectable in the thymus of control mice. When the TCRγδ+ cell population was compared, no NK1.1+ TCRγδ+ cells were detected in both ZAP-70−/− mice and control mice. However, a substantial proportion (0.35%) of TCRγδ+ cells was detected in the NK1.1− thymocytes of ZAP-70−/− mice but a quite low proportion in those of ZAP-70−/− mice. Similar results were obtained when spleen cells were analyzed. No NK1.1+ CD3+ or NK1.1+ TCRβ+ cell was detected in the spleen of ZAP-70−/− mice, whereas a markedly high proportion of NK1.1+ CD3− cells was demonstrated in the ZAP-70−/− spleen as compared with that of control mice (Figure 1).

These differences shown in the flow cytometric profiles were again demonstrated when actual cell numbers were compared between ZAP-70−/− mice and control mice (Figure 2). No significant difference in the mean cell numbers of the thymus and spleen...
Thymus

![Thymus graph]

Figure 2. Cell number of NK1.1+ TCRαβ+, NK1.1+ TCRαβ−, NK1.1+ TCRγδ+, and NK1.1− TCRγδ− subpopulations in the thymus and spleen of control and ZAP-70−/− mice. Mean cell numbers of each subpopulation in the thymus and spleen of control (n = 6) and ZAP-70−/− (n = 5) mice were calculated and shown as means and SD.

was observed between ZAP-70−/− (n = 5) and control mice (n = 6) (thymus: 1.38 ± 0.26 × 10^5 in ZAP-70−/− mice and 1.33 ± 0.32 × 10^5 in control mice; spleen: 1.75 ± 0.44 × 10^5 in ZAP-70−/− and 1.58 ± 0.37 × 10^5 in control mice). However, the number (0.028 ± 0.056 × 10^5) of NK1.1+ TCRαβ+ cells in ZAP-70−/− thymus was significantly smaller than that of control thymus (7.01 ± 2.56 × 10^5; P < .001). By contrast, the number of NK1.1+ TCRαβ+ cells in ZAP-70−/− thymus was approximately 3- to 4-fold greater than that of control thymus (ZAP-70−/−, 2.94 ± 0.46 × 10^5; control mice, 0.80 ± 0.18 × 10^5; P < .005).

Although NK1.1+ TCRγδ− cells were negligible in both control and ZAP-70−/− thymi with the flow cytometric analysis (Figure 1), the actual number of NK1.1+ TCRγδ+ cells in control mice was higher than that in ZAP-70−/− mice, as shown in Figure 2 (control, 0.186 ± 0.074 × 10^5; ZAP-70−/−, 0.365 ± 0.163 × 10^5; P < .005). On the contrary, the mean cell number of the NK1.1+ TCRγδ+ population was considerably higher in ZAP-70−/− thymi than that in control thymi (ZAP-70−/−, 5.79 ± 1.66 × 10^5; control, 3.29 ± 0.91 × 10^5; P < .05). These findings suggest that ZAP-70 is less influential to the development of TCRγδ+ cells than to that of TCRαβ− cells.

The marked decrease in the number of NK1.1+ TCRαβ+ cells and significant increase of NK1.1+ TCRαβ− cells were also demonstrated in the spleen of ZAP-70−/− mice (Figure 2). The mean number of NK1.1+ TCRαβ+ cells in ZAP-70−/− mice was significantly smaller than that of control mice (ZAP-70−/−, 0.165 ± 0.176 × 10^5; control, 10.7 ± 5.75 × 10^5; P < .05), and the mean number of NK1.1+ TCRαβ− cells in ZAP-70−/− mice was significantly higher than that of control mice (ZAP-70−/−, 102 ± 28.6 × 10^5; control, 47.4 ± 9.90 × 10^5; P < .05). The small but substantial population of the NK1.1+ TCRαβ+ cells seen in control mice might be attributable to the presence of ordinary NK cells in the spleen. It was also noted in Figure 2 that the number of NK1.1+ TCRγδ+ cells was considerably smaller in ZAP-70−/− spleen than that in normal spleen (ZAP-70−/−, 0.285 ± 0.193 × 10^5; control, 2.53 ± 1.09 × 10^5; P < .05), whereas the number of NK1.1+ TCRγδ+ cells in ZAP-70−/− mice was almost the same as that of control mice (ZAP-70−/−, 12.6 ± 3.60 × 10^5; control, 11.1 ± 2.58 × 10^5; P = .282).

Next, to examine other surface phenotypes of NK1.1+ CD3− cells detected in the thymus of ZAP-70−/− mice using various mAbs that react mainly T cells (Figure 3A), mainly NK cells (Figure 3B), or stem cells (Figure 3C), whole thymocytes obtained from either control or ZAP-70−/− mice were analyzed with flow cytometry. As shown in Figure 3A, the NK1.1+ population in control mice that corresponds mostly to the NK1.1+ T cells (Figure 1) was CD11b−, CD44+, CD5+, CD8−, CD24−, CD25−, CD44+, CD90−, and CD122+ as reported in previous studies. 1,2,14,37,44 As far as expressions of CD8, CD24, CD25, CD90, and CD122 molecules were concerned, NK1.1+ TCRαβ+ cells in ZAP-70−/− mice showed the same staining pattern as that of NK1.1+ T cells in control mice. However, these NK1.1+ TCRαβ+ cells expressed neither CD4 nor CD8 molecules but showed higher CD44 (Figure 3A), CD2, and CD16 (Figure 3B) fluorescence intensity than NK1.1+ T cells in control mice. When markers for the precursor population were analyzed, both NK1.1+ TCRαβ+ thymocytes of ZAP-70−/− mice and the thymic NK1.1+ T cells of control mice were CD11b− and CD34− (Figure 3C).

In addition, the NK1.1+ thymocytes in ZAP-70−/− mice were CD45R−, CD45RB−, CD62L−, CD69−, CD95−, Ly49A−, Ly49C−, and 2B4−, whereas the NK1.1+ T cells of control mice were CD2−, CD45R−, CD45RB−, CD62L−, CD69−, CD95−, Ly49A−, Ly49C−, and 2B4+ (Figure 3B). These profiles of NK1.1+ thymocytes in ZAP-70−/− were similar to those of ordinary NK cells in the peripheral lymphoid organs.

We then examined surface expression of a pan-NK marker, DX5,5 on NK1.1+ TCR− cells in the thymus and spleen of ZAP-70−/− mice and compared it with that of NK1.1+ cells in control mice. As shown in Figure 4, approximately 96% of NK1.1+ TCR− cells in ZAP-70−/− thymi expressed DX5, whereas most NK1.1+ TCR− cells expressed no DX5 in ZAP-70−/− thymi. In the ZAP-70−/− spleen, however, approximately 30% of NK1.1+ TCR− cells expressed DX5. This finding suggests a phenotypic difference between NK1.1+ T cells in the thymus and those in spleen of normal mice. Almost the same proportions expressed DX5 in NK1.1+ TCR− cells of both in ZAP-70−/− and ZAP-70−/− spleens.

Then, to compare the surface phenotype of NK1.1+ TCR− thymocytes in ZAP-70−/− mice with NK1.1+ TCR− cells of B6 and another NKT-deficient strain, RAG−/−, CD8a− HSA− thymocytes of ZAP-70−/− mice, CD8a− HSA− CD3− thymocytes from B6 mice, and whole thymocytes of RAG−/− mice were analyzed for the various surface markers (Figure 5). These populations were enriched for the NK1.1+ CD3− cells and enabled us to compare the surface phenotypes more precisely. Figure 5A shows that CD44, CD45RB, CD69, and CD16 are more highly expressed on the
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NK1.1+ CD3- cells of ZAP-70-/- or RAG-1-/- mice than those from B6 mice. The higher expressions of these molecules on the cells of 2 NKT-defective mutants could not be explained with the cell sizes, because the NK1.1+ CD3- thymocytes from 3 kinds of mice showed almost the same values of forward light scatter. Figure 5B,C shows that 2B4, CD62L, or CD45R is expressed on NK1.1+ CD3- thymocytes from ZAP-70-/- and RAG-1-/- mice but not on those of B6 mice. On the other hand, approximately 25% to 35% of ordinary NK cells of B6 mice were either Ly49A+ or Ly49C+.46 No expressions of Ly49A and Ly49C were detected on NK1.1+ CD3- cells of ZAP-70-/- and RAG-1-/- mice. It was also noted that the expression of CD95 was lower on NK1.1+ CD3- thymocytes of ZAP-70-/- and RAG-1-/- than that of B6 mice.

These results are summarized in Table 1. This table demonstrates that the NK1.1+ T cells in the thymus and spleen of ZAP-70-/- mice share largely common features with ordinary NK cells. However, the NK1.1+ CD3- cells exhibit several distinct phenotypes that represent neither NK1.1+ T cells nor NK cells. In this table, it is of note that NK1.1+ CD3- cells with surface phenotype similar to those of ZAP-70-/- mice are present in RAG-1-/- thymus.

NK1.1+ CD3- thymocytes in ZAP-70-/- mice produced IFN-γ upon stimulation with NK1.1 cross-linking in the presence of IL-2

To examine functions of the NK1.1+ TCRαβ- cells in the thymus of ZAP-70-/- mice, we analyzed the ability to produce cytokines. It has been shown that NK1.1+ T cells produce large amounts of IL-4 and IFN-γ shortly after stimulation with CD3 cross-linking.13,48,49 When thymocytes or splenocytes obtained from either control or ZAP-70-/- mice were stimulated with immobilized anti-CD3ε mAb for 48 hours, these cells from control ZAP-70-/- mice produced considerable amounts of IL-4 and IFN-γ. By contrast, neither IL-4 nor IFN-γ was produced from thymocytes or splenocytes of ZAP-70-/- mice (data not shown). This finding indicates again that NK1.1+ T cells are absent in the thymus and spleen of ZAP-70-/- mice and that TCRαβ- cells present in these populations are unable to quickly respond to the CD3 cross-linking.

We then stimulated thymocytes from control or ZAP-70-/-

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**Figure 3.** Phenotype of NK1.1+ thymocytes from control and ZAP-70-/- mice. Thymocytes were stained with PE-anti-NK1.1 or FITC-anti-NK1.1 and various cell surface markers as described in "Materials and methods." Expressions of NK1.1 (vertical) and T-cell surface markers (A), NK surface markers (B), and stem cell markers (C). Results are representative of 6 independent experiments.

**Figure 4.** DX5 expressions on NK1.1+ thymocytes and splenocytes from control (ZAP-70-/-) and ZAP-70-/- mice. Thymocytes and splenocytes were stained with PE-anti-NK1.1, FITC-anti-TCRαβ, and biotinylated anti-DX5 followed by streptavidin-Red 670. NK1.1+ TCRαβ- or NK1.1+ TCRαβ- cells in the thymus and spleen were electronically gated and analyzed for the expression of DX5 on FACS. Dead cells were electronically gated out with propidium iodide staining. FACS profiles were shown in contour with NK1.1 versus DX5 staining.
mice with immobilized anti-NK1.1 mAb in the presence or absence of rhIL-2. Thymocytes from ZAP-70<sup>−/−</sup> mice produced substantial amounts of IFN-γ upon stimulation with NK1.1 cross-linking in the presence of rhIL-2 (data not shown). To quantify IFN-γ–producing ability of NK1.1<sup>+</sup> TCRαβ<sup>+</sup> cells, we sorted the NK1.1<sup>+</sup> TCRαβ<sup>+</sup> cells from thymi and spleens of ZAP-70<sup>−/−</sup> mice as well as from spleens of control mice. These cells were then stimulated with immobilized anti-NK1.1 mAb in the presence of rhIL-2 (Figure 6). The NK1.1<sup>+</sup> TCRαβ<sup>+</sup> thymocytes and spleen cells of

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- NK cell markers
  - NKR-P1C: + + + +
  - CD2: + + + +
  - CD16: ±/low ±/low int int
  - CD45R: – – int int
  - CD45RB: +/low – + +
  - CD62L: – – +/- +/-
  - CD69: –/low –/low int int
  - CD95: + + –/low –/low
  - Ly49A: (10%) (20%) – –
  - Ly49C: (25%) (60%) – –
  - 2B4: – – + +
  - DX5: – – + +

- Stem cell markers
  - CD34: + + + +
  - CD117: – – – ND

Numbers in parentheses indicate the proportions of cells that express respective antigens; ND indicates not determined.

*The expression of 2B4 is not observed on freshly isolated NK cells. However, 2B4 is expressed on cultured NK cells.*

†Thirty percent of the cells were positive in spleen.
Percent specific lysis was calculated as described in “Materials and methods.”

Figure 7, both thymocytes and splenocytes from either control or ZAP-70 mice showed an intact cytotoxic activity.

To examine cell-mediated functions of the NK.1.1+ TCRβ+ cells in ZAP-70+/− mice, cytotoxic activity was then analyzed. Thymocytes and splenocytes from either control or ZAP-70+/− mice that had been administered thilone were cultured with rhIL-2 for 7 days.13,14 The harvested cells were then analyzed for the killing activity against 51Cr-labeled YAC-1 or P815 cells. As shown in Figure 7, both thymocytes and splenocytes from either control or ZAP-70+/− mice showed significant cytotoxicity against YAC-1 cells but not against P815 cells. The cytotoxicities seen in both thymocytes and spleen cells of ZAP-70+/− mice were consistently higher than those seen in control mice. This result was consistent with the increased proportions of NK.1.1+ CD3+ cells in the thymus and spleen of ZAP-70+/− mice as compared with those of control mice (Figure 1).

When the harvested cells from thymocyte cultures at day 7 were analyzed for NK.1.1 and TCRβ expressions with flow cytometry, almost 100% of cells were CD3+ among NK.1.1+ cells of ZAP-70+/− mice, whereas 40% and 60% of cells were CD3− and CD3+, respectively, among NK.1.1+ cells of ZAP-70+/− mice, as reported previously.15 Similarly, when harvested spleen cells were analyzed, 100% of NK.1.1+ cells were CD3− in ZAP-70+/− mice, whereas 70% and 30% of the NK.1.1+ cells were CD3+ and CD3+, respectively, in ZAP-70+/− mice (data not shown). These findings suggest that no proportional change of CD3− and CD3+ populations was induced during the culture with IL-2.

NK.1.1+ TCRβ+ thymocytes had a germline configuration in TCR−β gene locus

Flow cytometric analyses demonstrated that NK.1.1+ TCRβ− cells expressed neither TCR nor CD3 molecules on the cell surface. To examine a configuration of TCR gene, the rearrangement of TCRβ gene in NK.1.1+ CD3+ cells obtained from the thymus of ZAP-70+/− mice and thymocytes and skin cells of C57BL/6 mice (control) was analyzed with a PCR-based technique. The rearrangement of Vβ9 to Dβ2-Jβ2 was not detected in the TCRβ gene locus of the NK.1.1+ TCRβ− cells from ZAP-70+/− mice (data not shown). In addition, we found no band being generated by Dβ2 to Jβ2 rearrangements in the NK.1.1+ TCRβ− cells of ZAP-70+/− mice (Figure 8). Thus, it was demonstrated that the TCRβ gene locus of the NK.1.1+ TCRβ− cells from ZAP-70+/− mice was in a germline configuration.

Generation of NK.1.1+ TCRβ+ cells was induced from NK.1.1+ TCRβ− thymocytes of ZAP-70+/− mice in hanging-drop culture with PMA and ionomycin

To directly examine the developmental potential of NK.1.1+ TCRβ− thymocytes in ZAP-70+/− mice, a devised induction assay of NK.1.1+ TCRβ+ cells was performed. The NK.1.1+ TCRβ− cells were sorted from the thymocytes of ZAP-70+/− mice (Figure 9A, left panel) and cultured with PMA and ionomycin in dGuo-treated neonatal thymi from BALB/c mice in a hanging-drop setup. As shown in Figure 9A (middle and right panels), a substantial proportion (3.03%) of NK.1.1+ TCRβ+ cells was detected after 5 days of culture. BALB/c mice express no NK.1.1 antigen. Thus, rearrangement of TCR genes appeared to be induced in the NK.1.1+ TCRβ− population of ZAP-70+/− mice by an addition of PMA plus ionomycin. When NK.1.1+ TCRβ− cells of ZAP-70+/− mice were cultured with thymi of RAG−/− mice in the presence of PMA plus ionomycin, small but substantial numbers of NK.1.1+ TCRβ+ cells were detected (data not shown). No NK.1.1+ TCRβ+ cells were detected in this setup. In addition, NK.1.1+ TCRβ+ cells induced in the thymic organ culture of (B6 x B6.Tlyl1)F1 mice were Thy1.1+ (data not shown). These findings again indicated that the induced NK.1.1+ TCRβ+ cells were derived from NK.1.1+ TCRβ− cells of ZAP-70+/− mice, and large NK.1.1+ TCRβ+ populations seen in Figure 9A (middle and right panels) appeared to be derived from the dGuo-treated thymi of BALB/c mice.

Figure 9B summarizes 4 separate experiments with BALB/c thymi. Significant generation of NK.1.1+ TCRβ+ cells was induced in the presence of PMA plus ionomycin as compared with those cultured in the absence of PMA plus ionomycin (P < .05). A small population of NK.1.1+ TCRβ− cells seen in the cultures without PMA plus ionomycin could not be explained.

When NK.1.1+ CD3− thymocytes from ZAP-70+/− mice were cultured in the hanging-drop setup, substantial proportions of both NK.1.1+ TCRβ+ and NK.1.1+ TCRβ− cells were detected (data not shown).
not shown). This finding suggests that the cells in the NK1.1\(^{+}\) TCR\(\alpha\beta\) population differentiate to NK1.1\(^{+}\) TCR\(\alpha\beta\) cells through NK1.1\(^{+}\) TCR\(\alpha\beta\) stage.

**Invariant V\(\alpha\)14J\(\alpha\)281 and RAG-1 transscripts were detected in the induced NK1.1\(^{+}\) TCR\(\alpha\beta\) cells**

We then analyzed TCR\(\alpha\) usage in NK1.1\(^{+}\) TCR\(\alpha\beta\) cells that had been generated from NK1.1\(^{+}\) TCR\(\alpha\beta\) cells of ZAP-70\(^{-/-}\) mice following culture with the neonatal thymus in the presence of PMA plus ionomycin. The sorted NK1.1\(^{+}\) TCR\(\alpha\beta\) cells for the culture were demonstrated in the square of the left panel. Proportions of sorted NK1.1\(^{+}\) TCR\(\alpha\beta\) cells were 98.8% to 99.5%. Collected cells from cultures were stained with either PE-mouse IgG2a (isotype control of PK136)/FITC-anti-TCR\(\alpha\) (left panel) or PE-anti-NK1.1/FITC-anti-TCR\(\beta\) (right panel) and analyzed with FACScan. The proportions of NK1.1\(^{+}\) TCR\(\beta\) cells are indicated. (B) Mean proportion of NK1.1\(^{+}\) TCR\(\alpha\beta\) cells. The NK1.1\(^{+}\) CD3\(^{-}\) (about 5 \(\times\) 10\(^{4}\) cells) or 8 \(\times\) 10\(^{4}\) cells were seeded to the dGuo-treated neonatal thymic lobes and cultured in hanging-drop setup in the absence (left column) or presence (right column) of PMA and ionomycin. Five days later, total cells were stained as described for panel A. The net proportion of induced NK1.1\(^{+}\) TCR\(\alpha\beta\) cells were calculated as follows: [percentage of NK1.1\(^{+}\) TCR\(\alpha\beta\) cells minus percentage of control IgG2a TCR\(\alpha\beta\) cells] \(\times\) mean SD.

**Discussion**

It has been shown that ZAP-70 tyrosine kinase is essential for development of mainstream T cells but not for NK cells.\(^{32}\) Targeted disruption of ZAP-70 led to complete blockade of the development of mature-type T cells both in the thymus and in the periphery. By contrast, the development of ordinary NK cells was intact in the ZAP-70\(^{-/-}\) mice, although ZAP-70 was also expressed in the NK cultures, RAG-1 expression was clearly detected in the culture where the band of invariant V\(\alpha\) was amplified (Figure 10, lane 3). A very faint band was detected in the culture without PMA plus ionomycin (lane 2).

**NK1.1\(^{+}\) TCR\(\alpha\beta\)dim \(\beta\) thymocytes were generated in ZAP-70\(^{-/-}\)/DO10 TCR transgenic mice**

The present results suggested that ZAP-70 deficiency was directly associated with lack of rearrangement of TCR genes in the NK1.1\(^{+}\) TCR\(\alpha\beta\) population. We then asked whether an introduction of rearranged TCR\(\alpha\) and \(\beta\) genes induced generation of a particular NK1.1\(^{+}\) TCR\(\alpha\beta\) population in the ZAP-70\(^{-/-}\) background. To examine this possibility, we crossed ZAP-70\(^{-/-}\) mice with DO10 TCR transgenic mice and analyzed the generation of NK1.1\(^{+}\) TCR\(\alpha\beta\) cells in the thymus of ZAP-70\(^{-/-}\)/DO10 mice. As seen in Figure 11A, the NK1.1\(^{+}\) thymocytes of ZAP-70\(^{-/-}\)/DO10 mouse expressed a substantial level of TCR\(\beta\) molecules compared with those in ZAP-70\(^{-/-}\) thymus. The level of TCR\(\alpha\beta\) expression, however, was low compared with that of an NK1.1\(^{+}\) TCR\(\alpha\beta\) population in ZAP-70\(^{-/-}\)/ DO10 mice. When these thymocytes were stained with a clonotypic antibody, KJ1-26, it was shown that an NK1.1\(^{+}\) KJ1-26\(^{dim}\) population was present in the thymus (Figure 11B). The total RNA was then extracted from either the sorted NK1.1\(^{+}\) KJ1-26\(^{dim}\) or NK1.1\(^{+}\) KJ1-26\(^{\ast\ast\ast}\) cells, reverse-transcribed, and PCR-amplified with specific primers for V\(\alpha\)281 and J\(\alpha\)281. Figure 11B shows that V\(\alpha\)281/J\(\alpha\)281 messages are present in both NK and T-cell populations. Thus, it seemed that the lack of ZAP-70 showed no significant influence on the expression of rearranged TCR gene in the NK1.1\(^{+}\) CD3\(^{-}\) population. The NK1.1\(^{+}\) TCR\(\alpha\beta\)dim population expressed no CD4 but broader ranges of CD8 molecules (Figure 11A). Notably, Figure 11Av shows that development of the ordinary thymocyte was still arrested at the DP stage irrespective of the introduction of TCR\(\alpha\) and \(\beta\) transgenes in ZAP-70\(^{-/-}\)/DO10 mouse.

**Figure 10. Detection of V\(\alpha\)14J\(\alpha\)281 transcripts with RT-PCR in inductive cultures.** Total RNA was extracted from the thymic lobes (RAG-1\(^{-/-}\) in C57BL/6 background) alone (lane 1) or with sorted NK1.1\(^{+}\) TCR\(\alpha\beta\) cells in the presence (lane 3) or absence (lane 2) of PMA plus ionomycin, and RT-PCR was performed as described in “Materials and methods” with primer pairs V\(\alpha\)14J\(\alpha\) Leader/Go- rev1 and V\(\alpha\)14J\(\alpha\)281, RAG-1 5’/3’ and RAG-1 5’ nest3’/nest, or EF-1\(\alpha\)5’/EF-1\(\alpha\)3’ for positive control. RT-PCR was also performed without RNA (lane 4) as control. Results are representative of 3 separate experiments.
Figure 11. Detection of NK1.1+/TCRβdim thymocytes in ZAP-70−/−DO10 TCR transgenic mouse. (A) Thymocytes obtained from ZAP-70−/−, ZAP-70−/+, or ZAP-70+/+DO10 mice were stained with PE-anti-NK1.1FITC–anti-TCRβ (i), PE-anti-NK1.1FITC–anti-CD4 (ii), PE–anti-NK1.1FITC–anti-CD6 (iii), and PE–anti-CD4FITC–anti-CD8 (iv), and analyzed. Proportions of NK1.1+/TCRβdim, NK1.1+/TCRβdim (upper and middle panels), or NK1.1+/TCRβdim populations (lower panel) are indicated in the leftmost panels on the top of each squared region. Results are representative of 3 separate experiments. (B) Flow cytometric analysis and RT-PCR detection of transgenic TCR in NK1.1+/TCRβdim thymocytes in DO10/ZAP-70−/− mice. Thymocytes of DO10/ZAP-70−/− mice were stained with PE-anti-NK1.1 antibody and biotinylated KJ1-26 followed by streptavidin-FITC. Dead cells were electronically gated out with propidium iodide staining. The NK1.1+/TCRβdim thymocytes (NK1.1−) and NK1.1+/TCRβdim thymocytes (T) (demarcated with squares in the left panel) were sorted, and the expressions of DO10-specific TCR chain (VαJα28 and Ca) were examined with RT-PCR.

cells. Thus far, the lineage relationship among T, NK, and NK1.1+ T cells has been unclear.

In the present study, we examined development of the NK1.1+ T cells in the ZAP-70−/− mice and found an arrested development of NK1.1+ CD3+ cells in both thymus and spleen. Neither NK1.1+ TCRγδ− nor NK1.1+ TCRβ− cells were detected in the thymus and spleen. The absence of the NK1.1+ TCRγδ+ population was not due to the defective development of the TCRγδ lineage T cells per se, because significantly large populations of NK1.1+ TCRγδ+ cells were detected in the thymus and spleen of ZAP-70−/− mice as compared with those in control mice. It was reported that the NK1.1+ TCRγδ+ cell population was expanded in CD3ζ−/− mice. Thus, the gene disruption of ζ chain and ZAP-70 led to different developmental defects in NK1.1+ TCRγδ− cells, even though ZAP-70 is located downstream of ζ chain. This difference should be pursued in further studies.

Interestingly, we found markedly increased numbers of NK1.1+ TCRβ− cells in the thymus of ZAP-70−/− mice. The surface phenotype of the NK1.1+ TCRβ− cells summarized in Table 1 suggests that the NK1.1+ TCRβ− cells belong to a unique subpopulation different from ordinary NKT or NK cells. Approximately 25% to 35% of splenic NK cells express either Ly49A or Ly49C in the H-2b background. However, the NK1.1+ TCRβ− thymocytes of ZAP-70−/− mice (H-2b background) express neither Ly49A nor Ly49C. The expression of Ly49 on NK cells is developmentally regulated in a nonrandom manner. Thus, the present findings suggest that the NK1.1+ TCRβ− thymocytes are of distinct cell type or may belong to a precursor population of the NK1.1+ T cells. Indeed, we could show that the latter might be the case. The NK1.1+ TCRβ− thymocytes stimulated by an addition of PMA plus ionomycin in the thymic organ culture developed into NK1.1+ TCRβ+ cells.

Interestingly, the NK1.1+ CD3− thymocytes of B6-RAG-1−/− showed almost superimposable phenotypes to those of ZAP-70−/− mice. Thus, developmental defects that lead to TCR gene rearrangement may generate accumulation of cells of the same type. However, the TCR gene disruption on α or β locus exerted somewhat differential influences on the development of NK1.1+ T cells. In the TCRα−/− mice, an accumulation of NK1.1+ CD3− thymocytes was observed (data not shown). On the other hand, NK1.1+ TCRβ− mice but not NK1.1+ CD3− thymocytes were detected in TCRβ−/− mice (data not shown). Thus, it seems important to elucidate genes and signals vital on certain stages of the development of NK1.1+ T cells in further studies.

In normal B6 mice, a small population of NK1.1+ CD3− thymocytes were detectable, and it was demonstrated that these NK1.1+ D3− cells were phenotypically ordinary NK cells (Figure 5). This finding, however, does not exclude a possibility that some of these NK1.1+ thymocytes may correspond to a putative precursor population seen in ZAP-70−/− mice.

When the NK1.1+ TCRβ− cells were analyzed in the spleen of ZAP-70−/− mice, the number of the NK1.1+ TCRβ− cells also increased as compared with that of control mice. We reasoned that the putative precursor population for the NK1.1+ T cells that could not be readily distinguished from ordinary NK cells might also be present and both the precursor and ordinary NK cell populations were recognized with the increasing number of NK1.1+ TCRβ− cells in the spleen of ZAP-70−/− mice. Although expressions of CD45R, CD62L, and 2B4 molecules were relative characteristics of NK1.1+ TCRβ− thymocytes in ZAP-70−/− mice (Figure 5), these molecules could be induced on the splenic NK cells upon activation. Thus far, no appropriate markers that can definitely distinguish NK cells and the precursor cells in the spleen are available. The precise populations that make up the NK1.1+ TCRβ− cells in the spleen of ZAP-70−/− mice should be examined in further studies to clarify whether the NK1.1+ TCRβ− population in the spleen indeed contains the precursors of NK1.1+ T cells.
We demonstrated that substantial natural cytotoxicity and IFN-γ production upon stimulation via NKR-P1 molecules were demonstrated in the thymic NK1.1+ TCRβ- cells of ZAP-70−/− mice. It was shown that a putative precursor cell population of NK cells in bone marrow expressed CD45R and NK1.1 molecules and exhibited cytotoxicity against YAC-1 target cells.52 Eberl and MacDonald53 reported that the same population in bone marrow with surface markers similar to NK1.1+ TCRβ- thymocytes in ZAP-70−/− mice contained precursors for NK1.1+ T cells. Thus, it seems to us that the putative precursor population (NK1.1+ TCRβ- cells) for either NK or NK1.1+ T cells first acquires NK functions.

The NK1.1+ TCRβ- thymocytes in ZAP-70−/− mice showed a germline configuration in TCRβ gene. After 5 days of culture with PMA plus ionomycin, the NK1.1+ TCRβ+ cells were detected and the canonical Vα14Jα281 transcripts were clearly demonstrated. Sato et al23 demonstrated that a pre-NKT cell population expressing NK1.1, TCRβ, pre-Tα, and RAG1/2 could differentiate into mature CD3+ Vα14+ NKT cells in the presence of IL-15, GM-CSF, and bone marrow–derived stromal cells. Because the TCRβ genes were not rearranged in the NK1.1+ TCRβ- thymocytes of ZAP-70−/− mice, these cells may be precursors for the pre-NKT. However, these results appeared to be in discordance with the stepwise model proposed by DiSanto and Rodewald26 for the development of NK1.1+ T cells. Using γ chain−/− mice, these demonstrated that the NK1.1+ T cells differentiated from T cells that expressed Vα14 and Vβ8 chains. These precursor cells possessed characteristic profiles in cytokine productions but expressed no NK1.1 or Ly49 antigens. The difference may be attributable to the mice studied but should be elucidated in further investigation.

NK1.1+ TCRβ- cells seen in ZAP-70−/− mice lacked both CD34 and CD117 expressions. It was reported that fetal thymic NK1.1+ CD117+ and NK1.1+ CD117− cells at gestational day 15 were capable of generating mainstream T cells, NK1.1+ T cells, and NK cells, whereas NK1.1+ CD117− cells remained CD4−CD8− and committed exclusively to NK cells.54 Although the NK1.1+ TCRβ- cells in the thymus of ZAP-70−/− mice or pre-NKT cells reported by Sato et al23 resemble the NK1.1+ CD117− cells in the fetal thymus, the NK1.1+ TCRβ- cells and pre-NKT cells could differentiate into the NK1.1+ T cells in the presence of PMA plus ionomycin or IL-15 plus GM-CSF in conjunction with stromal cells from either thymus or bone marrow, respectively.

The TCRβ gene loci of NK1.1+ TCRβ- cells in ZAP-70−/− mice were in germline configuration. Because the disruption of ZAP-70 kinase led to no defect in TCR gene rearrangement in mainstream T cells,22,23 the mechanism underlying the lack of TCR expression on the NK1.1+ TCRβ- cells was unclear. In the last experiment, we showed that introduction of rearranged TCR genes to ZAP-70−/− mice resulted in generation of an NK1.1+ TCRβdim population. Thus, disruption of ZAP-70−/− might influence on the TCRβ rearrangement but not on the expression of the rearranged TCR genes. Perhaps the TCR rearrangement in NK1.1+ T-lineage cells is more ZAP-70-dependent than that in mainstream T cells, which cannot be compensated by Syk kinases.

We show herein a unique NK1.1+ CD3− cell population in the thymus of ZAP-70−/− mice, and this population may contain a precursor for NK1.1+ T cells and possesses intact NK cell functions. It seems that NK1.1+ T cells and NK cells share a critical pathway for their differentiation. Detailed single-cell–based analyses of the generation of NK1.1+ T cells from NK1.1+ TCRβ- thymocytes but not from minor contaminants in the dGuo-treated thymic lobes with PMA plus ionomycin are undertaken in our laboratory.

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Defective development of NK1.1+ T-cell antigen receptor αβ+ cells in zeta-associated protein 70 null mice with an accumulation of NK1.1+CD3− NK-like cells in the thymus

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