

# Cytotoxicity of Fresh NK1.1<sup>+</sup> T Cell Receptor $\alpha/\beta$ <sup>+</sup> Thymocytes against a CD4<sup>+</sup>8<sup>+</sup> Thymocyte Population Associated with Intact Fas Antigen Expression on the Target

By Hisashi Arase,\* Noriko Arase,\* Yuko Kobayashi,†  
Yoshiko Nishimura,† Shin Yonehara,† and Kazunori Onoé\*

From the \*Section of Pathology, Institute of Immunological Science, Hokkaido University, Sapporo 060; the †Pharmaceutical Basic Research Laboratories, JT Inc., Yokohama 236, Japan

## Summary

Recent studies have revealed that 10–20% of CD4<sup>+</sup>8<sup>-</sup> or CD4<sup>-</sup>8<sup>-</sup> thymocyte populations contain NK1.1<sup>+</sup> T cell receptor (TCR)- $\alpha/\beta$ <sup>+</sup> cells. This subpopulation shows characteristics that are different from NK1.1<sup>-</sup> CD4<sup>+</sup> or NK1.1<sup>-</sup> CD8<sup>+</sup> T cells and seems to have developed in a manner different from NK1.1<sup>-</sup> T cells. Although extensive studies have been performed on the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes, the physiological role of the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes has been totally unclear. In the present study, we found that freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes, but neither whole thymocytes nor lymph node T cells, directly killed CD4<sup>+</sup>8<sup>+</sup> thymocytes from normal syngeneic or allogeneic mice by using a long-term cytotoxic assay in which flow cytometry was used to detect the cytotoxicity. However, only weak cytotoxicity was detected against thymocytes from *lpr* mice on which the Fas antigen that transduces signals for apoptosis into the cells is not expressed. Furthermore, the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes exhibited high cytotoxicity against T lymphoma targets transfected with *fas* genes as compared with the parental T lymphoma targets or target cells transfected with mutated *fas* genes, which lack the function of transducing signals. On the other hand, NK1.1<sup>+</sup> effector thymocytes from *gld* mice that carry a point mutation in Fas ligand did not kill thymocyte targets from normal mice. The present findings, thus, consistently suggest that the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes kill a subpopulation among CD4<sup>+</sup>8<sup>+</sup> thymocytes via Fas antigen and in this way regulate generation of T lineage cells in the thymus.

Thymocytes consist of four major CD4<sup>-</sup>8<sup>-</sup>, CD4<sup>+</sup>8<sup>+</sup>, CD4<sup>+</sup>8<sup>-</sup>, and CD4<sup>-</sup>8<sup>+</sup> populations. CD4<sup>-</sup>8<sup>-</sup> thymocytes are thought to be potent progenitors that develop into mature CD4<sup>+</sup>8<sup>-</sup> or CD4<sup>-</sup>8<sup>+</sup> thymocytes through CD4<sup>+</sup>8<sup>+</sup> thymocytes. However, in adult mice of most strains, ~10–20% of the CD4<sup>-</sup>8<sup>-</sup> thymocytes express low levels of TCR- $\alpha/\beta$  (TCR- $\alpha/\beta$ <sup>low</sup>) (1, 2). The CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> population has a phenotype similar to that of memory T cells (i.e., CD44<sup>+</sup>, intercellular adhesion molecule 1<sup>+</sup>, MEL-14<sup>-</sup> Thy-1<sup>+</sup>, CD5<sup>+</sup> and heat-stable antigen [HSA]<sup>-1</sup>) (3, 4). Interestingly, ~40–50% of the CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes express V $\beta$ 8.2 TCR (1, 2, 4, 5), whereas only ~10–20% of cells in ordinary CD4<sup>+</sup>8<sup>-</sup> or CD4<sup>-</sup>8<sup>+</sup> population bearing high levels of TCR- $\alpha/\beta$

(TCR- $\alpha/\beta$ <sup>high</sup>) express the V $\beta$ 8.2 TCR. The CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocyte population can proliferate and secrete several kinds of lymphokines upon stimulation via the TCR (6–8). Furthermore, it has been reported that the CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes express NK1.1 antigens in mice of NK1.1 type (4, 9). The NK1.1<sup>+</sup> antigens are not expressed on major classical T cells and have been thought to be a specific marker for NK cells (10, 11). The number of CD4<sup>-</sup>8<sup>-</sup> NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes increases with age and they become a substantial population at relatively late stages in life (1, 5). On the other hand, we have recently found a NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> subpopulation in a CD4<sup>+</sup>8<sup>-</sup> thymocyte population (12, 13). The NK1.1<sup>+</sup> CD4<sup>+</sup>8<sup>-</sup> thymocytes show characteristics similar to those of NK1.1<sup>+</sup> CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes in their surface phenotype, TCR V $\beta$  usage, lymphokine production profile, and ontogeny, although lineage correlation between these two subpopulations has remained unclear.

In our subsequent study (3), we demonstrated that

<sup>1</sup> Abbreviations used in this paper: HSA, heat-stable antigen; LN T, lymph node T cells.

NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes express IL-2 receptor  $\beta$  chain and proliferate upon stimulation with recombinant IL-2. Furthermore, after stimulation with recombinant IL-2 the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes acquired cytotoxic activity against both normal thymocytes and several kinds of tumor cells. Therefore, the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes seemed to be of functional T cell lineage. However, when freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes were evaluated in a 4-h cytotoxic assay, we could not detect significant cytotoxicity. Thus, physiological function of the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes was not uncovered.

On the other hand, it has been demonstrated that most of the thymocytes express considerable Fas antigens that transduce signals for apoptosis into cells (14, 15). The Fas antigen is encoded by the gene at the locus of the mouse lymphoproliferation mutation *lpr* (16). Since mice carrying mutations homozygous at the *lpr* locus and actually lacking the Fas expression have lymphadenopathy and autoimmune diseases, it seems that the expression of the intact Fas antigen is related to the physiological development of T cells in normal mice. However, the mechanism how the Fas antigen is involved in the normal T cell development has been unclear. Identification of cells expressing Fas ligand, if present, in the thymus seems important to clarify the direct function of the Fas antigen in physiological conditions.

In the present study, to elucidate the function of NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes, we analyzed the cytotoxicity of freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes against thymocyte targets by using a long-term cytotoxic assay. We found that the freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes exhibited considerable cytotoxicity against CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Furthermore the cytotoxicity was directly correlated with the intact Fas expression and Fas ligand expression on the targets and effector cells, respectively. Thus, the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocyte population seems to represent a population that regulates thymocyte development via Fas antigen and Fas ligand interaction.

## Materials and Methods

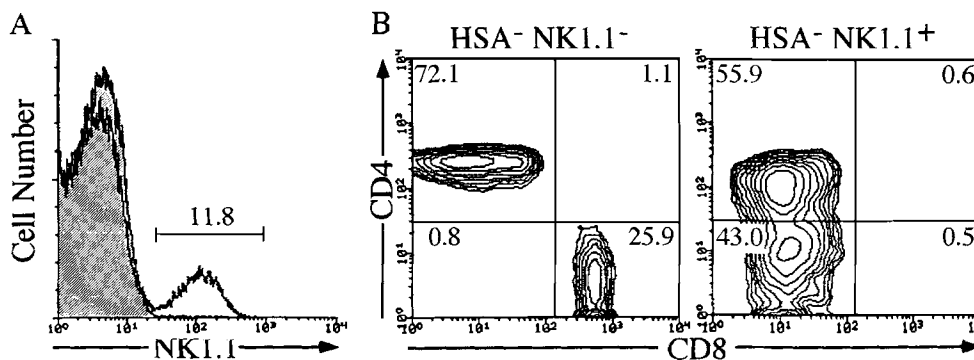
**Mice.** C57BL/10 (B10), B10.BR/SgSnSlc (B10.BR) mice were obtained from the Shizuoka Laboratory Animal Cooperation (Hamamatsu, Shizuoka Prefecture, Japan). C57BL/6J Jcl were obtained from the Japan Clea Animal Cooperation (Tokyo, Japan). B6 *lpr/lpr*

and B6 *gld/gld* were obtained from stock maintained in our animal facility at Hokkaido University.

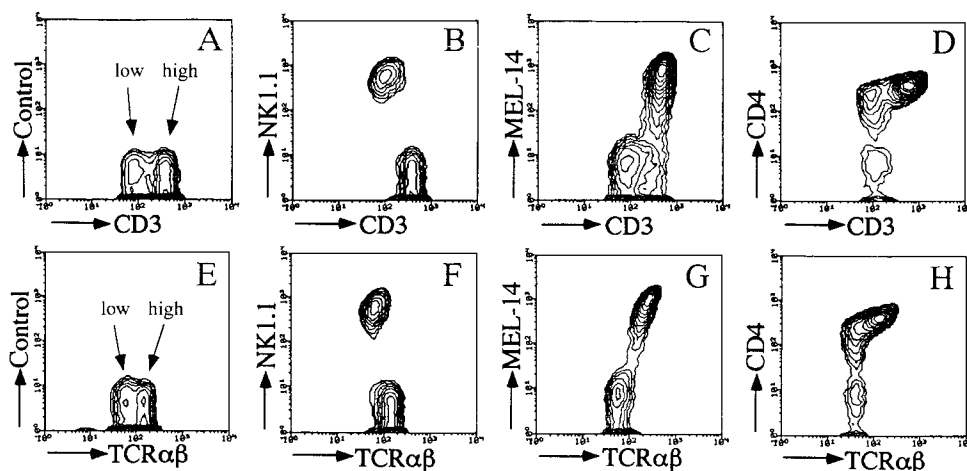
**Antibodies and Reagents.** Primary mAbs used for immunofluorescence staining and flow cytometry in the present experiments were 2C11 (anti-CD3- $\epsilon$ , a kind gift from Dr. J. A. Bluestone, University of Chicago, Chicago, IL) (17), H57.597 (anti-TCR- $\alpha/\beta$ , a kind gift from Dr. R. T. Kubo, National Jewish Center for Immunology, Denver, CO) (18), MEL-14 (anti-gp90<sup>MEL-14</sup>) (19), J11d (anti-HSA) (20), PK-136 (anti-NK1.1) (purchased from American Type Culture Collection, Rockville, MD) (21), and 020-400 (anti-Lyt2.2) (Meiji Institute of Health, Kanagawa, Japan). PE anti-CD4 and FITC anti-CD8 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). Fluorescence-labeled secondary antibodies and avidin used herein were FITC anti-mouse IgG, FITC anti-rat IgG, FITC anti-hamster IgG, biotin anti-hamster IgG (Cappel Organon Teknika Co., West Chester, PA), PE anti-mouse IgG, PE anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL), PE-streptavidin, and TANDEM-streptavidin (Southern Biotechnology Associates). Calcium ionophor (A23187) was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

**Surface Marker Analysis.** Thymocytes were treated with anti-HSA mAb or mixture of anti-HSA and anti-CD8 mAbs followed by C treatment. The resultant cells were treated with Dynabeads coupled with anti-mouse or anti-rat IgG (Dynal, Oslo, Norway) to completely remove CD8<sup>+</sup> or HSA<sup>+</sup> cells. Thereafter, the purified HSA<sup>-</sup> cells were stained with anti-NK1.1 mAb or medium followed by biotin anti-mouse IgG and TANDEM-streptavidin. After blocking the nonspecific binding site of anti-mouse IgG by purified mouse Ig, the cells were stained with PE anti-CD4 and FITC anti-CD8 mAbs (Fig. 1). On the other hand, the purified HSA<sup>-</sup> CD8<sup>-</sup> cells were mixed with anti-NK1.1, MEL-14, anti-CD4 mAb, or medium (control) followed by PE anti-mouse IgG or PE anti-rat IgG. After blocking the nonspecific binding site of anti-mouse IgG or anti-rat IgG by purified mouse or rat Ig, the cells were mixed with anti-CD3 mAb or anti-TCR- $\alpha/\beta$  mAb followed by FITC anti-hamster IgG that had been preabsorbed with mouse and rat Ig (Fig. 2). The stained cells were analyzed by FACScan<sup>®</sup> (Becton Dickinson & Co.).

**Cell Sorting.** To prepare effector cells, HSA<sup>-</sup> MEL-14<sup>-</sup> CD4<sup>+</sup>CD8<sup>-</sup>, HSA<sup>-</sup> MEL-14<sup>-</sup> CD4<sup>+</sup>CD8<sup>+</sup>, HSA<sup>-</sup> MEL-14<sup>-</sup> NK1.1<sup>+</sup> CD8<sup>-</sup>, HSA<sup>-</sup> NK1.1<sup>+</sup> CD8<sup>-</sup>, HSA<sup>-</sup> TCR- $\alpha/\beta$ <sup>high</sup> CD4<sup>+</sup>CD8<sup>-</sup>, and HSA<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> CD4<sup>+</sup>CD8<sup>+</sup> cells were purified by sorting. After purification of CD8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> or CD8<sup>-</sup> HSA<sup>-</sup> cells from thymocytes of 6–10-wk-old mice, cells were stained with FITC anti-rat IgG/PE anti-CD4, FITC anti-rat IgG/anti-NK1.1/PE anti-mouse IgG or FITC anti-rat IgG/anti-TCR- $\alpha/\beta$ /biotin anti-hamster IgG/PE-streptavidin. Thereafter, CD4<sup>-</sup> MEL-14<sup>-</sup>, CD4<sup>+</sup> MEL-14<sup>-</sup>, NK1.1<sup>+</sup>, NK1.1<sup>-</sup>, TCR-



**Figure 1.** FACS<sup>®</sup> analysis of HSA<sup>-</sup> thymocytes from B10 mice. HSA<sup>-</sup> thymocytes were stained with anti-CD4, CD8, and NK1.1 mAbs. (A) NK1.1 expression of HSA<sup>-</sup> thymocytes (hatched histogram, control; open histogram, NK1.1). (B) CD4 and CD8 expression on HSA<sup>-</sup> NK1.1<sup>-</sup> or HSA<sup>-</sup> NK1.1<sup>+</sup> thymocytes from B10 mice. Proportions of each population are indicated in the figure.



**Figure 2.** Surface marker of CD8<sup>-</sup> HSA<sup>-</sup> thymocytes. CD8<sup>-</sup> HSA<sup>-</sup> thymocytes from 8-wk-old B10 mice were stained with anti-CD3 (A-D) or anti-TCR- $\alpha/\beta$  (E-H) mAb and anti-NK1.1 (B and F), MEL14 (C and G), or anti-CD4 (D and H) mAbs.

$\alpha/\beta^{\text{low}}$  or TCR- $\alpha/\beta^{\text{high}}$  cells were sorted by FACStar<sup>®</sup> (Becton Dickinson & Co.). The purity of the cells was always >99%.

**Cytotoxic Assay.** Target thymocytes were prepared from syngeneic 1-4-wk-old mice unless otherwise indicated. CD4<sup>+</sup>8<sup>+</sup> target thymocytes were prepared by sorting. HSA<sup>-</sup> NK1.1<sup>-</sup> target thymocytes were prepared by treatment with anti-HSA + anti-NK1.1 mAb plus C followed by Dynabead treatment. Cytotoxic assay was carried out as previously reported (3, 22). Briefly, target cells were labeled with PKH-2 green fluorescence dye (Zynaxis Cell Science, Inc., Malvern, PA) and were mixed with various numbers of effector cells for 16 or 10 h in a V-bottomed 96-well microplate (Flow Laboratories, Inc., McLean, VA) in 200  $\mu$ l of RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and  $5 \times 10^{-5}$  M 2-ME after centrifuge. Thereafter, the proportion of dead cells among PKH-2-stained cells were analyzed by staining the cultured cells with propidium iodide red fluorescence dye (Sigma Chemical Co., St. Louis, MO) using FACScan<sup>®</sup>. In our preliminary experiments with tumor cell targets, no difference was observed between this PKH-2 staining method and the <sup>51</sup>Cr release assay. Data are presented as mean  $\pm$  SD from triplicate cultures.

**Induction of Anti-H-2<sup>d</sup> CTL.** Anti-H-2<sup>d</sup> cytotoxic T cells were generated by stimulating purified B10.BR lymph node T cells with 20-Gy irradiated B10.D2 splenocytes for 4 d (23). Cytotoxicity was determined by a 5-h assay as described above.

**Fas Antigen Transfectants.** The cDNA encoding the truncated mouse Fas antigen (90 amino acids had been removed from the COOH-terminal end) was obtained by reverse transcription PCR using an oligonucleotide corresponding to nucleotides 27-46 and 740-760 of the published sequence (24). L5178Y cells were cotransfected with EcoRI-digested pMAMneo (Clontech Laboratories, Inc., Palo Alto, CA), and PvuI-digested pME18S (25) containing mouse cDNA encoding the whole Fas molecule (24) or the truncated mouse Fas molecule by electroporation at 290 V with a capacitance of 960  $\mu$ F using Gene Pulser (Bio-Rad Laboratories, Richmond, CA). A-1 transfectant expressing intact mouse Fas antigen or F-10 transfectant expressing truncated Fas antigen was obtained after selection by culturing with 400  $\mu$ g/ml G418 and cloning by limiting dilution.

**Anti-Fas mAbs.** B cell hybridomas secreting anti-Fas mAb were produced by fusing NS-1 mouse myeloma cells with splenocytes from rat immunizing with recombinant fusion soluble mouse Fas molecule (Nishimura, Y. and S. Yonehara, manuscript submitted for publication). Thereafter, a hybridoma cell line secreting anti-

Fas antibody (RMF6, rat IgG2a), that bound to A-1 cells but not to parental L5178Y cells, was cloned.

## Results

**Surface Marker Analysis of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{+}}$  Thymocytes.** NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes have been reported to be present in HSA<sup>-</sup> CD4<sup>-</sup>8<sup>-</sup> (1, 2, 4, 9) or HSA<sup>-</sup> CD4<sup>+</sup>8<sup>-</sup> population (12, 13). However, direct comparison of CD4 and CD8 expressions on the NK1.1<sup>+</sup> cells has not been performed. To clarify the surface characteristics of NK1.1<sup>+</sup> T cells, we first analyzed expressions of NK1.1 antigen on HSA<sup>-</sup> thymocytes. As shown in Fig. 1 A, ~12% of HSA<sup>-</sup> thymocytes expressed NK1.1 antigens. When CD4 and CD8 expressions on HSA<sup>-</sup> NK1.1<sup>-</sup> or HSA<sup>-</sup> NK1.1<sup>+</sup> cells were analyzed by three-color flow cytometry, only CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> populations were seen among the HSA<sup>-</sup> NK1.1<sup>-</sup> cells (Fig. 1 B). By contrast, HSA<sup>-</sup> NK1.1<sup>+</sup> cells were revealed to consist of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> populations. Actually no NK1.1<sup>+</sup> cells were detected in CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>-</sup>8<sup>+</sup> thymocyte populations.

We then analyzed expressions of NK1.1, MEL-14, or CD4 antigens on CD8<sup>-</sup> HSA<sup>-</sup> thymocytes in combination with CD3 or TCR- $\alpha/\beta$  expression. The CD8<sup>-</sup> HSA<sup>-</sup> thymocytes consisted of CD3/TCR- $\alpha/\beta^{\text{low}}$  and CD3/TCR- $\alpha/\beta^{\text{high}}$  populations (Fig. 2, A and E). More than 99% and more than 98% of the NK1.1<sup>+</sup> CD8<sup>-</sup> HSA<sup>-</sup> cells expressed low levels of CD3 and TCR- $\alpha/\beta$ , respectively (Fig. 2, B and F). On the other hand, NK1.1<sup>+</sup> cells expressing distinguishably low amounts of CD3/TCR- $\alpha/\beta$  were MEL-14<sup>-</sup> (Fig. 2, C and G). Approximately half of the NK1.1<sup>+</sup> cells expressed CD4 antigens (Fig. 2, D and H). In contrast, almost all NK1.1<sup>-</sup> cells expressing high amounts of CD3/TCR- $\alpha/\beta$  were MEL-14<sup>+</sup> and CD4<sup>+</sup>. These analyses on surface characteristics of the HSA<sup>-</sup> thymocytes revealed a distinct and unique NK1.1<sup>+</sup> CD3/TCR- $\alpha/\beta^{\text{low}}$ , MEL-14<sup>-</sup> subpopulation in both CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>-</sup> populations.

**Cytotoxicity of Freshly Isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  Thymocytes against Thymocyte Targets.** In our previous study (3), we found that NK1.1<sup>+</sup> CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes exhibited considerable LAK activity against thymocytes in

a 4-h cytotoxic assay. However, we could not detect cytotoxicity in freshly isolated NK1.1<sup>+</sup> CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes in the 4-h cytotoxic assay. Since we observed in this study (3) that the NK1.1<sup>+</sup> CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes showed significant redirected cytotoxicity against tumor cells bearing Fc receptors before these cells acquired LAK activity, we attempted to detect cytotoxic activity of freshly isolated thymocyte subpopulations using different experimental conditions. When cytotoxicity of purified CD4<sup>-</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (double negative, DN) and CD4<sup>+</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (CD4) thymocytes, most of which were NK1.1<sup>+</sup> cells, was evaluated against thymocyte targets in a 16-h cytotoxic assay, both of these two populations exhibited considerable cytotoxicity toward syngeneic thymocytes (Fig. 3 A). The cytotoxicity, however, was low when evaluated in a 10-h cytotoxic assay (Fig. 3 B). On the other hand, neither unfractionated (whole) thymocytes nor lymph node T cells (LN T) showed any killing activity toward thymocytes (Fig. 3 A). In addition, lymph node T cell blasts induced by stimulation with immobilized anti-TCR mAbs showed no cytotoxicity against thymocyte targets (data not shown).

We then sorted CD8<sup>-</sup> HSA<sup>-</sup> thymocytes into NK1.1<sup>+</sup> lymph node T cells (LN/T) and NK1.1<sup>-</sup> or into TCR- $\alpha/\beta$ <sup>low</sup> and TCR- $\alpha/\beta$ <sup>high</sup> populations to confirm the population that is responsible for the cytotoxicity. Fig. 3 C shows that NK1.1<sup>+</sup> or TCR- $\alpha/\beta$ <sup>low</sup> population but neither NK1.1<sup>-</sup> nor TCR- $\alpha/\beta$ <sup>high</sup> population possesses significant cytotoxic activity. From these observations, we concluded that only NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> cells among CD8<sup>-</sup> HSA<sup>-</sup> thymocytes exhibit substantial cytotoxicity against syngeneic thymocytes.

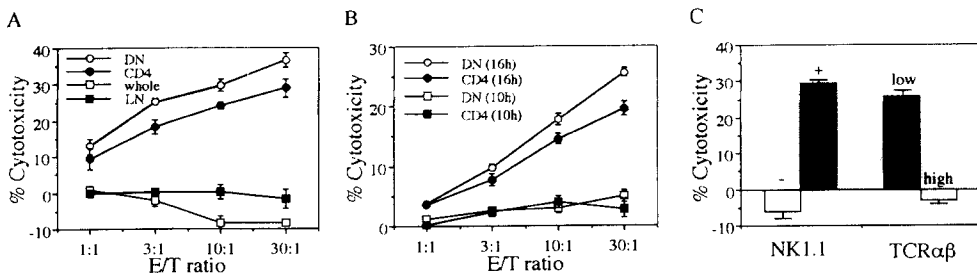
**Thymocyte Subpopulation Susceptible to the Cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> Thymocytes.** Next, to determine the population that is susceptible to the cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes, we prepared CD4<sup>+</sup>8<sup>+</sup> (double positive, DP) cells as a representative of immature thymocyte targets, HSA<sup>-</sup> NK1.1<sup>-</sup> (HSA<sup>-</sup>) cells as a representative of mature thymocyte targets and LN T as a representative of mature peripheral T cell targets. NK1.1<sup>+</sup> CD4<sup>-</sup>8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes killed CD4<sup>+</sup>8<sup>+</sup> thymocytes (Fig. 4 A). By contrast, these NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> cells killed neither mature HSA<sup>-</sup> NK1.1<sup>-</sup> thymocytes nor LN T. Similarly, the

NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes showed no cytotoxicity against NK1.1<sup>-</sup> CD4<sup>-</sup>8<sup>-</sup> thymocytes, although almost half of the CD4<sup>-</sup>8<sup>-</sup> population was dead spontaneously in the 16-h culture either in the absence or presence of effector cells (data not shown). These observations may permit us to conclude that cells killed by the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes are CD4<sup>+</sup>8<sup>+</sup> thymocytes.

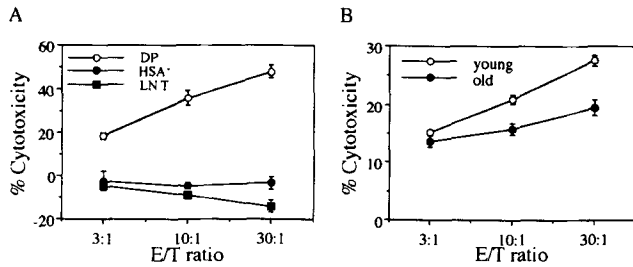
When the susceptibility of target thymocytes to NK1.1<sup>+</sup> effector cells was compared between young mice (1 wk old) and adult mice (12 wk old), thymocytes from young mice constantly showed higher susceptibility to the NK1.1<sup>+</sup> thymocytes than those from adult mice did (Fig. 4 B). On the other hand, no significant difference was observed in proportion of CD4<sup>+</sup>8<sup>+</sup> thymocytes between young and adult mice (data not shown). This finding suggests that proportion of the CD4<sup>+</sup>8<sup>+</sup> subpopulation susceptible to the cytotoxicity by NK1.1<sup>+</sup> killer cells is high in the thymus of young mice as compared with adult mice. Therefore, we used thymocytes from young mice as targets throughout the present study unless otherwise indicated.

**Requirement of Fas Expression on the Targets for Susceptibility to the Cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> Thymocytes.** We then analyzed influence of genetic differences on the susceptibility of thymocyte targets to NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes. CD8<sup>-</sup> HSA<sup>-</sup> NK1.1<sup>+</sup> thymocytes, in which >98% of the cells were TCR- $\alpha/\beta$ <sup>low</sup>, from B10.BR (H-2<sup>k</sup>) mice killed both H-2 compatible (B10.BR) and H-2 incompatible (B10) (H-2<sup>b</sup>) thymocytes (Fig. 5 A). Thus, it appeared that H-2 differences between killer and target cells did not influence the cytotoxicity. However, it should be noted in Fig. 5 B that when NK1.1<sup>+</sup> thymocytes from B10.BR mice were analyzed for cytotoxicity toward B6 *lpr/lpr* (H-2<sup>b</sup>) or B6 *+/+* thymocytes, the NK1.1<sup>+</sup> cells exhibited only negligible cytotoxicity against thymocyte targets from 3-wk-old B6 *lpr/lpr* mice. By contrast, the NK1.1<sup>+</sup> thymocytes showed substantial cytotoxicity against B6 *+/+* thymocytes. Similarly, the NK1.1<sup>+</sup> thymocytes from B10.BR mice killed thymocytes from MRL *+/+* (H-2<sup>k</sup>) mice but not those from MRL *lpr/lpr* mice. On the other hand, NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes from B6 *lpr/lpr* mice showed substantial cytotoxicity against thymocytes from *+/+* mice (data not shown).

When susceptibility of the target thymocytes to calcium



**Figure 3.** Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes against syngeneic thymocytes. (A) Cytotoxicity of CD4<sup>-</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (DN), CD4<sup>+</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (CD4), unfractionated thymocytes (whole) or LN T (LN) from B10.BR mice against syngeneic thymocytes. (B) Cytotoxicity of CD4<sup>-</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (DN) or CD4<sup>+</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> (CD4) thymocytes against target thymocytes determined by a 16- (circle) or 10-h (square) cytotoxic assay. (C) Cytotoxicity of CD8<sup>-</sup> HSA<sup>-</sup> NK1.1<sup>+</sup> (open histogram, left column), CD8<sup>-</sup> HSA<sup>-</sup> NK1.1<sup>-</sup> (solid histogram, left column), CD8<sup>-</sup> HSA<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> (solid histogram, right column) or CD8<sup>-</sup> HSA<sup>-</sup> TCR- $\alpha/\beta$ <sup>high</sup> (open histogram, right column) thymocytes against syngeneic thymocytes. (E/T ratio = 30:1). These data are representative from at least three independent experiments.

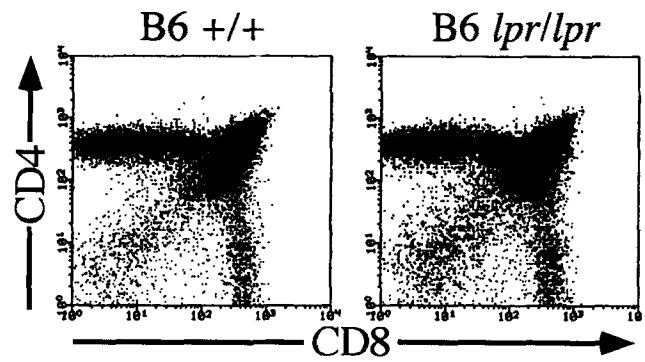


**Figure 4.** Susceptibility of CD4<sup>+</sup>8<sup>+</sup> (DP) thymocytes to NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes. (A) Cytotoxicity of CD4<sup>-</sup>8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> thymocytes against CD4<sup>+</sup>8<sup>+</sup> thymocytes (DP), HSA<sup>-</sup> NK1.1<sup>-</sup> thymocytes (HSA<sup>-</sup>) or lymph node T cells (LN T). (B) Cytotoxicity of CD8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> NK1.1<sup>+</sup> thymocytes against thymocyte from young (1 wk) or adult (12 wk) mice. These data are representative of at least three independent experiments.

ionophor (26, 27) was compared between B6 *+/+* and B6 *lpr/lpr* mice, both B6 *+/+* and B6 *lpr/lpr* thymocytes showed the same levels of susceptibility to the calcium ionophor (Fig. 5 C). Furthermore, no difference in the proportion of major thymocyte subpopulations was noted between young normal (*+/+*) and young *lpr* mice (Fig. 6). Expression patterns of MHC class I and LFA-1 molecules on thymocytes were essentially identical between these mice (data not shown).

Recently, the *lpr* gene has been identified as a mutation in *fas* gene that encodes Fas antigen that transduce signals to induce apoptosis (14–16). Indeed, it has been reported that the Fas antigen is not expressed on the surface of thymocytes from *lpr* mice, although most of thymocytes from normal counterpart mice (congenic at the *lpr* locus) express the Fas antigen at high levels (15, 28, 29). Thus, the present findings that NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes exhibited substantial cytotoxicity only toward thymocyte targets of normal mice but not those of *lpr* mice suggest that the cytotoxicity is associated with the Fas expression on the targets.

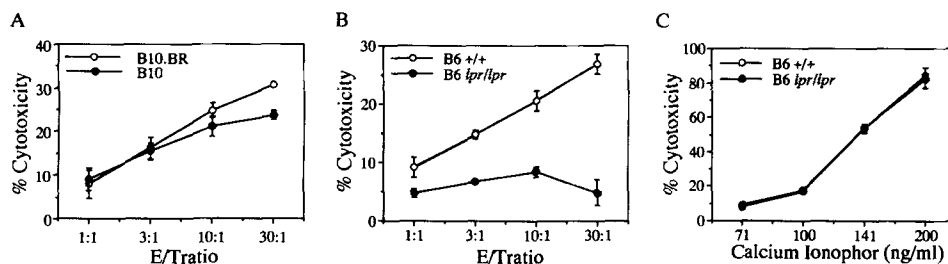
**Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  Thymocytes toward Target Cells Transfected with *fas* Genes.** Next, to examine directly the involvement of Fas antigens on the cytotoxicity, we analyzed cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes against target cells transfected with *fas* gene. L5178Y (a T lymphoma line derived from DBA/2) cells were transfected with either whole *fas* cDNA or mutated *fas* cDNA in which cytoplasmic domain is truncated (90 amino acids had been



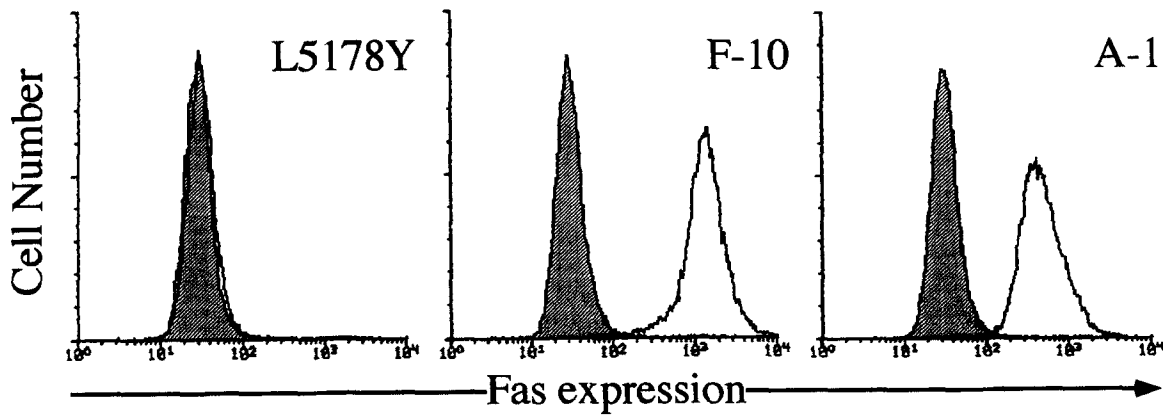
**Figure 6.** CD4 and CD8 expressions on thymocytes from 3-wk-old B6 *+/+* or B6 *lpr/lpr* mice.

removed from the COOH-terminal end). Fig. 7 shows that the parental L5178Y cells do not express Fas antigen. By contrast, *fas* gene transfectants (A-1) or mutant *fas* gene transfectants (F-10) express significant amounts of Fas antigens on their surface. When these cells were used as targets, NK1.1<sup>+</sup> thymocytes from B10.BR mice killed A-1 transfectants more extensively than parental L5178Y cells or F-10 transfectants (Fig. 8 A). On the other hand, these transfectants and parental cells showed the same level of susceptibility to the cytotoxicity by anti-H-2<sup>d</sup> CTL (Fig. 8 B). Thus, the difference in susceptibility to NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes seen among three target cell lines appears not to be attributable to their different sensitivities to the cytotoxic agents. It seems that intact Fas expression on the targets is directly associated with the susceptibility to NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes.

**Cytotoxic Activity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  Thymocytes from *gld* Mice.** *gld* mice are known to have lymphoid disorders similar to *lpr* mice (30–32). Accumulations of B220<sup>+</sup> T cells are seen in lymphoid tissues that are accompanied by a large amount of autoantibody production in the *gld* mice. However, unlike *lpr* mice, Fas antigen is expressed and functions normally in *gld* mice (reference 16 and Yonehara, S., unpublished observation). It has been suggested that a mutation in Fas ligand molecule may occur and be the basis of disorders observed in the *gld* mice (33, 34) and recently Takahashi et al. (35) identified the point mutation in the COOH-terminal region of Fas ligand.



**Figure 5.** Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes against target thymocytes from different mouse strains. (A) Cytotoxicity of CD8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> NK1.1<sup>+</sup> thymocytes (B10.BR, H-2<sup>k</sup>) against syngeneic (B10.BR, H-2<sup>k</sup>, open circle) or allogeneic (B10, H-2<sup>b</sup>, solid circle) thymocytes. (B) Cytotoxicity of CD8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> NK1.1<sup>+</sup> thymocytes against thymocytes from 3-wk-old B6 *+/+* (open circle) or B6 *lpr/lpr* mice of the same age (solid circle). (C) Viability of thymocytes cultured in the presence of calcium ionophor (A23187) to induce apoptosis (B6 *+/+*, open circle, B6 *lpr/lpr*, solid circle). These data are representative from three independent experiments.



**Figure 7.** Fas antigen expression on transfectants. Parental L5178Y (T cell lymphoma derived from DBA/2 mice [H-2<sup>d</sup>]), truncated Fas antigen expressing cells (F-10) or normal Fas antigen expressing cells (A-1) were stained with anti-Fas mAb (RMF6) plus FITC-anti-rat IgG (open histogram) or only with FITC anti-rat IgG (hatched histogram).

We then, purified NK1.1<sup>+</sup> cells from the thymus of *gld* mice and analyzed their cytotoxicity against thymocyte targets. Fig. 9 A shows that the NK1.1<sup>+</sup> thymocytes from *gld* mice do not kill thymocyte targets from normal mice. On the other hand, when susceptibility of target thymocytes to the NK1.1<sup>+</sup> effector cells from normal (B6 +/+) mice was compared between *gld* mice and normal mice, thymocytes from *gld* mice showed slightly but consistently higher susceptibility than those from normal mice (Fig. 9 B). These results support interpretation for our present experimental results that NK1.1<sup>+</sup> TCR<sup>low</sup> thymocytes kill targets at least in some part via Fas antigen-Fas ligand interaction.

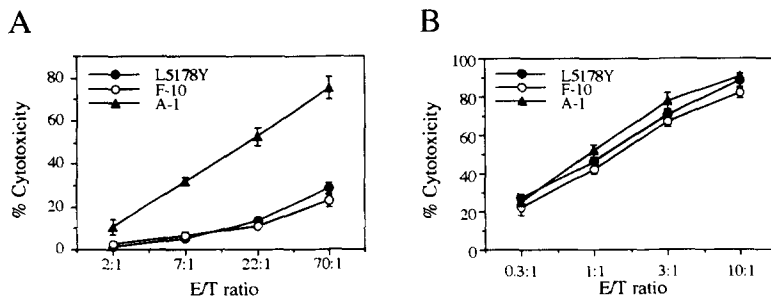
## Discussion

NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes are detected in both CD4<sup>-</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>-</sup> thymocyte populations (4, 9, 12). In spite of extensive analysis of this population, physiological role of the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes has been unclear. In the present study, using a 16-h cytotoxic assay we demonstrated that freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes exhibited significant cytotoxicity against thymocyte targets from either syngeneic or allogeneic mice. Furthermore, the target population susceptible to the killing activity by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes was revealed to be immature CD4<sup>+</sup>8<sup>+</sup> thymocytes. Although it took a long time (16 h) for the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes to ex-

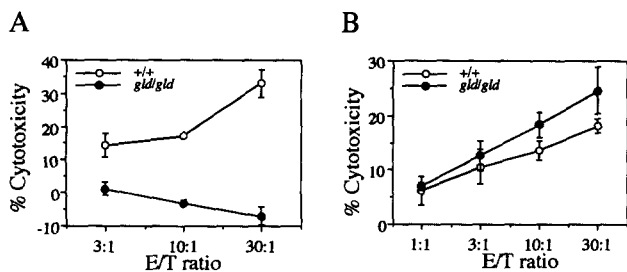
press detectable levels of cytotoxicity against CD4<sup>+</sup>8<sup>+</sup> thymocytes in vitro as compared with conventional CTL, NK, or LAK cells, it seems interesting and somewhat surprising that effector (killer) cells and their target cells are coexisting within the same organ: the thymus.

Furthermore, we found that NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes did not exhibit significant cytotoxicity against CD4<sup>+</sup>8<sup>+</sup> thymocytes from *lpr* mice. The *lpr* mice have recently been reported to carry mutated *fas* gene in which early transposon is inserted (16, 36–38). In fact extensively low levels of Fas expression are noted on thymocytes in *lpr* mice (15, 29). Thus, the present finding that thymocytes from *lpr* mice but not from +/+ normal mice were resistant to cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes suggests the involvement of Fas antigen in the cytotoxicity. Furthermore, NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> cells killed the T lymphoma line transfected with *fas* genes but killed neither the parental T lymphoma line nor the T lymphoma line transfected with *fas* genes in which the cytoplasmic domain is truncated. On the other hand, these T lymphoma lines showed the same levels of susceptibility to CTLs specific for H-2<sup>d</sup> alloantigens. Thus, these experimental results may permit us to conclude that Fas antigen and subsequent signaling from Fas are directly involved in the killing by NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes.

This postulate is supported by the finding of the last experiment that NK1.1<sup>+</sup> thymocytes from *gld* mice could not kill CD4<sup>+</sup>8<sup>+</sup> thymocytes from normal mice. It has been



**Figure 8.** Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes against Fas antigen transfectants. (A) Cytotoxicity of CD8<sup>-</sup> HSA<sup>-</sup> MEL-14<sup>-</sup> NK1.1<sup>+</sup> thymocytes from B10.BR mice against L5178Y (solid circle), F-10 (open circle) or A-1 (solid triangle) cells. Cytotoxicity was determined by a 16-h assay. (B) Cytotoxicity of anti-H-2<sup>d</sup> cytotoxic T cells against L5178Y (solid circle), F-10 (open circle) or A-1 (solid triangle) cells. These data are representative from three independent experiments.



**Figure 9.** Cytotoxicity and susceptibility of thymocytes from *gld* mice. (A) Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes from B6 +/+ mice (open circle) or B6 *gld/gld* mice (solid circle) against thymocyte targets from B6 +/+ mice were analyzed. (B) Cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes from B6 +/+ mice against thymocyte targets from 6-wk-old B6 +/+ (open circle) or B6 *gld/gld* mice (solid circle) were analyzed. These data are representative from three independent experiments.

suggested that *lpr* and *gld* autoimmune prone mice carry mutations of genes encoding an interacting pair of molecules involved in T cell development (33) and very recently a point mutation in the Fas ligand was determined in the *gld* mice (35). In *gld* mice, Fas antigen is normally expressed (16) and can deliver signals for apoptosis into thymocytes (Yonehara, S., unpublished observation). Thus, it seems that NK1.1<sup>+</sup> thymocytes of *gld* mice carrying defective Fas ligand can not kill the normal CD4<sup>+</sup>8<sup>+</sup> thymocytes. This interpretation is consistent with the next finding that CD4<sup>+</sup>8<sup>+</sup> target thymocytes from *gld* mice showed consistently higher sensitivity than those from +/+ mice of the same age to NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  killer population of normal mice. We reasoned that the CD4<sup>+</sup>8<sup>+</sup> thymocytes from *gld* mice which might not have been killed in vivo exhibited high sensitivity to the NK1.1<sup>+</sup> killer cells of normal mice. These results suggest strongly the contribution of the Fas antigen-Fas ligand interaction on the cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes and may interpret, at least in part, the immunological disorders seen in *lpr* and *gld* mice.

A similar killing system via Fas molecule was reported by Rouvier et al. (39). These authors reported that certain kinds of CTLs which had been stimulated with target antigens in vivo showed both Fas- and non-Fas-mediated cytotoxicity. Rat Fas ligand was cloned from a novel (rat  $\times$  mouse) T cell hybridoma and demonstrated to be a molecule of TNF family (40). Although we attempted to detect Fas ligand expression on NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes using a soluble form of recombinant Fas molecules, we could not detect the expression of the Fas ligand on the effector cells (data not shown). Suda et al. (40) reported that stimulation of rat spleen cells with Con A significantly increased mRNA for the Fas ligand. Therefore, activation of T cells seems to elevate levels of the Fas ligand expression, which eventually results in induction of Fas-mediated CTL. Thus, the expression of Fas ligand on freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes might not reach a detectable level when we analyzed them in the present study. Alternatively, NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes may deliver signals to Fas molecules using unknown molecules different from the integral membrane Fas ligand. These points

should be pursued in further studies in which more efficient detection system of murine Fas ligand is introduced.

At present, it has remained unclear whether recognition of antigens by TCR is involved in the cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes against CD4<sup>+</sup>8<sup>+</sup> thymocytes. The NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes express skewed TCR V $\beta$  family and express self Mls-1<sup>+</sup> reactive V $\beta$ 8.1 TCR in Mls-1<sup>+</sup> mice (1, 2, 5, 12). Recent analysis of  $\beta_2$ -microglobulin deficient mice revealed that the predominant V $\beta$ 8.2 expression in CD4<sup>+</sup>8<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes results from positive selection by self-antigen presented on MHC class I (41). Similarly, we have found that the proportion of NK1.1<sup>+</sup> CD4<sup>+</sup>8<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes is significantly reduced in  $\beta_2$ -microglobulin-deficient mice (Arase, H., N. Arase, and K. Onoé, unpublished observation). It seems that the NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes may recognize certain antigens associated with class I antigen in the absence of CD8 coreceptors and in this way acquire Fas-mediated cytotoxicity against CD4<sup>+</sup>8<sup>+</sup> thymocytes. However, it should be taken into consideration that the cytotoxicity by NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes was shown to cross the H-2 barrier and that CD4<sup>+</sup>8<sup>+</sup> target thymocytes express low amounts of classical class I antigen.

Thymocytes are known to be positively or negatively selected and most of thymocytes die by apoptosis before they mature into CD4<sup>+</sup>8<sup>+</sup> or CD4<sup>+</sup>8<sup>-</sup> thymocytes from CD4<sup>+</sup>8<sup>+</sup> thymocytes. We demonstrated herein that a subpopulation of CD4<sup>+</sup>8<sup>+</sup> thymocytes appeared to be targets for NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  killer cells in vitro. Thus, it seemed that NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes might contribute to the negative selection in the thymus. However, T cells with endogenous superantigen reactive TCR repertoire are almost normally eliminated in *lpr* mice (42-44). Similarly, the T cells with self-reactive TCR have been already eliminated in newborn mice in which NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes are hardly detected. Thus, it seems to us that the cytotoxicity of the NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes toward immature thymocytes is not directly related to the clonal elimination of self-reactive T cells.

In *lpr* and *gld* mice, abnormal B220<sup>+</sup> CD4<sup>+</sup>8<sup>-</sup> TCR- $\alpha/\beta^{\text{low}}$  cells accumulate in lymphoid tissues of adult mice. Although the exact origin of the B220<sup>+</sup> CD4<sup>+</sup>8<sup>-</sup> T cells is unclear, several reports have suggested that these cells are derived from CD4<sup>+</sup>8<sup>+</sup> thymocytes (42, 45-47). Thus, the defect of cytotoxicity of NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes against CD4<sup>+</sup>8<sup>+</sup> thymocytes may be correlated with abnormal T cell development in *lpr* and *gld* mice. In addition, a high production rate of whole CD4<sup>+</sup>8<sup>+</sup> thymocyte population as compared with normal mice has been reported in *lpr* mice (45).

Both CD4<sup>+</sup>8<sup>-</sup> NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  and CD4<sup>+</sup>8<sup>-</sup> NK1.1<sup>+</sup> TCR- $\alpha/\beta^{\text{low}}$  thymocytes appear late in life (1, 5, 12). On the other hand, it was shown that culture of TCR- $\alpha/\beta^{\text{low}}$  cells in bone marrow generated in CD4<sup>+</sup>8<sup>-</sup> TCR- $\alpha/\beta^{\text{low}}$  cells (48, 49). Furthermore, Kikly and Dennert (50) reported that NK1.1<sup>+</sup> CD4<sup>+</sup>8<sup>-</sup> TCR- $\alpha/\beta^{\text{low}}$  T cells in bone marrow could differentiate in extrathymic tissues. Thus,

it seems that the NK1.1<sup>+</sup> CD4<sup>-</sup> 8<sup>-</sup> TCR- $\alpha/\beta$ <sup>+</sup> thymocytes are generated not only in the thymus (51) but also in extrathymic tissues. The latter population may enter the thymus after full differentiation. Indeed, it has been reported that activated T cells can enter the thymus (52, 53). Taken together, these cells seem to have differentiated in a way different from major CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Furthermore, proportions of NK1.1<sup>+</sup> T cells are relatively high in the thymus (4, 12), bone marrow (12, 54), and liver (reference 55 and Arase, H., unpublished observation), although the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> cells are detectable in almost all lymphoid organs. Thus, the target cells killed by the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> T cells may not be restricted to CD4<sup>+</sup> 8<sup>+</sup> thymocytes. Further analysis of the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> cells distributed to a variety of tissues will be needed to clarify the total functions of the cells.

A T cell population similar to that shown in the murine system has been reported in human. In the thymus (56) and peripheral blood (57), T cells with natural killer cell marker such as CD56 (NKH-1) have been detected. In addition, human CD4<sup>-</sup> 8<sup>-</sup> TCR- $\alpha/\beta$ <sup>+</sup> cells express unique TCR

repertoire like murine CD4<sup>-</sup> 8<sup>-</sup> TCR- $\alpha/\beta$ <sup>low</sup> T cells (58, 59) and a certain repertoire among these CD4<sup>-</sup> 8<sup>-</sup> TCR- $\alpha/\beta$ <sup>+</sup> cells has been shown to be specific for class I-like CD1 molecules (60). On the other hand, Fas antigen is also expressed on most of the human thymocytes (Yonehara, S., Y. Kobayashi, and Y. Nishimura, manuscript submitted for publication). Therefore, the similar regulatory mechanism mediated by NK-like T cells may play a role even in human system.

In conclusion, we have demonstrated herein that freshly isolated NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes kill directly CD4<sup>+</sup> 8<sup>+</sup> thymocytes expressing Fas antigens. Although we have not elucidated the recognition system of the NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> cells, these findings appear to be important when the physiological role of NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> thymocytes and the Fas antigen expressed on most thymocytes are considered. The defective interaction between NK1.1<sup>+</sup> TCR- $\alpha/\beta$ <sup>low</sup> killer thymocytes and target CD4<sup>+</sup> 8<sup>+</sup> thymocytes seems to be related to lymphoid disorders seen in both *lpr* and *gld* mice (33, 35).

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We thank Dr. S. Kobayashi and Dr. T. Saito for helpful comments, and Drs. K. Mori, M. Murakami, M. Inobe, and T. Uede for providing B6 *lpr/lpr* and B6 *gld/gld* mice. We would also like to thank Ms. Michiyo Konishi for her kind assistance in preparing this manuscript.

This work was supported by The Special Grant-in-Aid for Promotion of Education and Science in Hokkaido University provided by the Ministry of Education, Science, and Culture, Japan; the Uehara Memorial Foundation; and the Nishimura Aging Fund. H. Arase and N. Arase were supported by a grant-in-aid for Japan Society for Promotion of Science.

Address correspondence to Dr. Kazunori Onoé, Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-17, Kita-ku, Sapporo 060, Japan. The present address of Hisashi Arase and Noriko Arase is the Division of Molecular Genetics Center for Biological Research Science, Chiba University School of Medicine, 1-8-1 Inohara, Chuo-ku, Chiba 260, Japan.

Received for publication 15 February 1994 and in revised form 18 April 1994.

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