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Mouse CD8$^+$ CD122$^+$ T Cells with Intermediate TCR Increasing with Age Provide a Source of Early IFN-γ Production

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Although CD8$^+$ IL-2Rβ (CD122)$^+$ T cells with intermediate TCR reportedly develop extrathymically, their functions still remain largely unknown. In the present study, we characterized the function of CD8$^+$ CD122$^+$ T cells with intermediate TCR of C57BL/6 mice. The proportion of CD8$^+$ CD122$^+$ T cells in splenocytes gradually increased with age, whereas CD8$^+$ IL-2Rβ-negative or -low (CD122$^-$) T cells conversely decreased. The IFN-γ production from splenocytes stimulated with immobilized anti-CD3 Ab in vitro increased with age, whereas the IL-4 production decreased. When sorted CD8$^+$ CD122$^+$ T cells were stimulated in vitro by the anti-CD3 Ab, they promptly produced a much larger amount of IFN-γ than did CD8$^+$ CD122$^-$ T cells or CD4$^+$ T cells, whereas only CD4$^+$ T cells produced IL-4. The depletion of CD8$^+$ CD122$^+$ T cells from whole splenocytes greatly decreased the CD3-stimulated IFN-γ production and increased the IL-4 production, whereas the addition of sorted CD8$^+$ CD122$^+$ T cells to CD8$^+$ CD122$^+$ T cell-depleted splenocytes restored the IFN-γ production and partially decreased IL-4 production. It is of interest that CD8$^+$ CD122$^+$ T cells stimulated CD4$^+$ T cells to produce IFN-γ. The CD3-stimulated IFN-γ production from each T cell subset was augmented by macrophages. Furthermore, CD3-stimulated CD8$^+$ CD122$^+$ T cells produced an even greater amount of IFN-γ than did liver NK1.1$^+$ T cells and also showed antitumor cytotoxicity. These results show that CD8$^+$ CD122$^+$ T cells may thus be an important source of early IFN-γ production and are suggested to be involved in the immunological changes with aging. ***The Journal of Immunology*****, 2000, 164: 5652–5658.**

The CD122$^+$ intermediate TCR cells in the liver and other organs are composed of an NK1.1$^+$ subset and an NK1.1$^-$ subset, and the former are CD4$^+$ or CD4$^-$8$^-$, whereas the latter are mainly CD8$^+$ (1, 2). Intermediate TCR cells that gradually increase with age in the liver and other organs belong to the NK1.1$^-$ subset, whereas the NK1.1$^+$ subset remains proportionally rare except in the liver in mice of all ages (2, 3). The NK1.1$^+$ subset with intermediate TCR (NKT) cells produces IFN-γ and/or IL-4 and seems to play an important role in polarizing either the Th1 or Th2 immune responses (4–9), which are probably dependent on the types of Ags. They can produce IFN-γ by IL-12 stimulation and can be strong antitumor and antimetastatic effectors (8–14). On the other hand, although we previously suggested that NK1.1$^-$ intermediate TCR cells could be involved in the immunological change with aging (2, 3), their function remains largely unknown. There have been substantial studies wherein the proliferation and cytokine production of T cells of aged mice stimulated with various mitogens reportedly decreased in comparison with those of young mice (15–18). For example, anti-CD3 Ab-, PHA-, or Con A-induced T cell proliferation and IL-2 production decrease with aging (3, 18). On the other hand, mitogen-induced IFN-γ production from T cells in aged hosts was also reported to increase (18, 19). However, the function of CD8$^+$ CD122$^+$ T cells in comparison with the functions of other T cell subsets and the interaction among them have not been studied. In the present study, we clearly show that CD8$^+$ CD122$^+$ T cells rapidly produce a greater amount of IFN-γ by CD3 stimulation in vitro than that of regular CD8$^+$ CD122$^-$ T cells with high TCR or of CD4$^+$ T cells with high TCR and can be antitumor effectors. In addition, CD8$^+$ CD122$^+$ T cells produced an even greater amount of IFN-γ than did NKT cells. Furthermore, CD8$^+$ CD122$^+$ T cells with intermediate TCR activated CD4$^+$ T cells to produce IFN-γ and partially inhibited IL-4 production from splenocytes. These findings suggest that CD8$^+$ CD122$^+$ T cells are thus an important cellular component in the Th1 immune response and that they are also responsible for immunological changes with aging.

Materials and Methods

Mice

C57BL/6 Ly5.2 mice were obtained from Japan SLC (Hamamatsu, Japan) and were maintained in our animal facility for 10–50 wk. C57BL/6 Ly5.1 mice were provided by Dr. H. Watanabe at Niigata University School of Medicine (Niigata, Japan).

Abs and reagents

Anti-mouse CD3ϵ mAb (145-2C11) (20) and anti-mouse CD8 mAb (53-6.72) (21) were prepared from ascites of hybridomas. FITC-conjugated anti-CD8 mAb (5H10-1), PE-conjugated anti-mouse CD122 (IL-2R β-chain) mAb (TMβ1), PE-conjugated anti-mouse CD4 mAb (GK1.5), PE-conjugated anti-mouse NK1.1 mAb (PK136), biotin-conjugated anti-mouse TCR-$\alpha\beta$ mAb (H57-597), biotin-conjugated anti-mouse Ly5.1 Ab (CD45.1, A20) and anti-Ly5.2 Ab (CD45.2, 104) were purchased from PharMingen (San Diego, CA). FITC-conjugated anti-mouse CD5 mAb (CG16) was purchased from Immunotech (Marseille, France). Magnetic bead-coupled anti-mouse CD4 mAb (RL172.4) and magnetic bead-coupled anti-mouse CD8 mAb (3.155) were purchased from Dynal (Lake Success,

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NY). RED670-conjugated streptavidin was obtained from Life Technologies (Grand Island, NY).

Flow cytometric analysis

The spleen was pressed through a stainless steel mesh, and mononuclear cells (MNC)[3] were obtained after lysing erythrocytes with an erythrocyte lysis solution (0.17 mM NH_4Cl, 0.01 mM Na_2EDTA, and 0.1 M Tris (pH 7.3)). The MNC were incubated with FITC-conjugated anti-CD8 mAb, PE-conjugated anti-CD122 mAb, and biotin-conjugated anti-mouse TCR-$\alpha\beta$ mAb for 20 min on ice and were washed twice with staining solution (PBS containing 2% FBS and 0.1% sodium azide). Subsequently, MNC were incubated with R-phycoerythrin-Cy5-conjugated streptavidin (Dako, Kyoto, Japan) for 20 min on ice and then were washed twice. Finally, MNC were resuspended in staining solution, filtrated with nylon mesh, and applied to a flow cytometric analyzer (FACSCalibur; Becton Dickinson, Cockeysville, MD) with Cell Quest software (Becton Dickinson).

Preparation of each T cell subset

Under ether anesthesia, the mice were killed by bleeding from the subclavian artery and vein, and the spleen or liver was thus obtained. Splenic MNC were incubated on 150-mm plastic tissue culture dishes (Corning Costar, Cambridge, MA) with 10% FBS RPMI 1640 and 50 μM 2-ME (complete medium) in 5% CO_2 at 37°C for 1 h, and adherent cells (most of the macrophages and a major part of the B cells) were removed. Nonadherent cells were incubated with magnetic bead-coupled anti-CD4 mAb for 30 min at 4°C, and most of the $CD4^+$ cells were removed. MNC were incubated with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD122 mAb at 4°C for 20 min, and $CD8^+$ $CD122^+$ T cells or $CD8^+$ $CD122^-$ T cells were sorted by Epics Elite (Coulter, Miami, FL). $CD4^+$ $CD122^-$ T cells were also sorted from splenic MNC. Liver MNC were obtained as previously described (9). Liver MNC were stained with FITC-conjugated anti-CD5 mAb and PE-conjugated anti-NK1.1 mAb, and $CD5^+$ $NK1.1^+$ T cells were obtained by sorting. For the depletion of $CD8^+$ $CD122^+$ T cells, whole splenocytes were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD122 mAb while the $CD8^+$ $CD122^+$ T cells were sorted out.

Cell cultures and assays for IFN-γ and IL-4 levels

Flat-bottom 96-well culture plates (Corning Costar, Cambridge, MA) were coated with 100 μl of purified anti-CD3 mAb (145-2C11, 10 μg/ml or 1 μg/ml in PBS) overnight at 4°C to immobilize mAb, and the plates were rinsed with PBS three times before use. Whole splenocytes (4×10^5 cells/well) and the sorted splenocytes (4×10^5 cells/well) were cultured with immobilized anti-CD3 Ab in complete medium for 24 or 48 h at 37°C in a 5% CO_2 incubator. In some experiments, 100 μl of purified anti-CD28 mAb (37.51; 10 μg/ml; PharMingen) was immobilized with anti-CD3 mAb in a 96-well plate and used to stimulate T cells. To obtain macrophages, whole splenocytes (4×10^5/ml) were incubated at 37°C in a 5% CO_2 incubator for 1 h, and nonadherent cells were gently removed and plastic adherent cells were regarded as macrophages. After cultivation, the IFN-γ and IL-4 levels of culture supernatants were evaluated by using cytokine-specific ELISA commercially available from Endogen (Woburn, MA), and the results were expressed as the means ± SD of triplicate cultures from three to five individual mice.

Intracellular IFN-γ staining

The splenocytes were stimulated with anti-CD3 Ab for 36 h at 37°C in a 5% CO_2 incubator, and Golgistop (0.7 μl/ml; PharMingen) was added before an additional 12-h culture. The splenocytes were collected and washed once. The splenocytes were incubated with PE-conjugated anti-CD4 Ab or PE-conjugated anti-CD8 Ab and biotin-conjugated anti-Ly5.2 or Ly5.1 Ab (PharMingen) at 4°C for 20 min and were washed twice. Biotin-conjugated Abs were developed with streptavidin-conjugated RED670. Cells were incubated with Cytofix/Cytoperm solution (PharMingen) at 4°C for 20 min and were washed twice with Perm/Wash solution (PharMingen). Thereafter, the cells were incubated with FITC-conjugated anti-mouse IFN-γ Ab (rat IgG1; XMG1.4; PharMingen) or isotype control (R3-34; PharMingen) at 4°C for 30 min, were washed twice with Perm/Wash solution, and were analyzed by a flow cytometric analyzer.

[3] Abbreviations used in this paper: MNC, mononuclear cell; $\beta_2m^{-/-}$, β_2-microglobulin-deficient; BM, bone marrow.

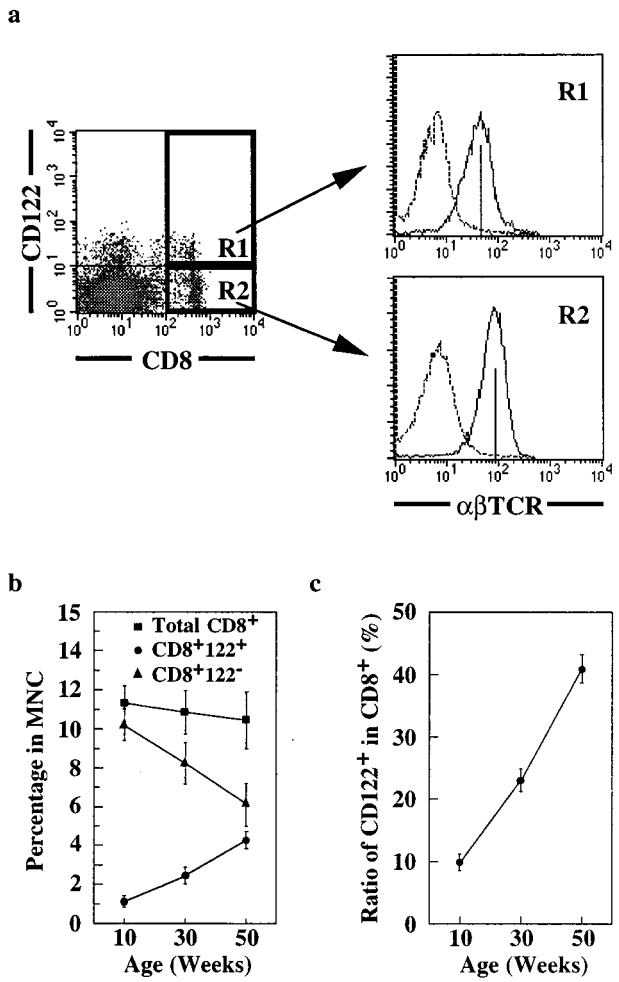

FIGURE 1. $CD8^+$ $CD122^+$ T cells expressed an intermediate TCR and increased the spleen increase with age, whereas $CD8^+$ $CD122^-$ T cells with high TCR decreased. $CD8^+$ $CD122^+$ T cells expressed an intermediate TCR, whereas $CD8^+$ $CD122^-$ T cells expressed high TCR (*a*). $CD8^+$ $CD122^+$ T cells gradually increased with age, whereas $CD8^+$ $CD122^-$ T cells conversely decreased (*b* and *c*). The ratio of $CD8^+$ $CD122^+$ T cells in total $CD8^+$ T cells also increased with age (*c*). All data represent the means ± SEs from eight mice in each group.

Assay for NK activity (cytotoxic assay)

The target cells used were NK-sensitive YAC-1 (lymphoma cells of A/S origin). The target cells were labeled with 100 μCi $Na_2(^{51}Cr)O_4$ (Amersham, Cleveland, OH) for 60 min at 37°C in an RPMI 1640 medium containing 10% FBS and were washed three times with medium. The labeled targets (10^4/well) were incubated in a total volume of 200 μl with 10^5 effector cells (E:T ratio was 10:1) in 10% FBS-RPMI 1640 in 96-well round-bottom microtiter plates. The plates were centrifuged and then incubated for 4 h in 5% CO_2 at 37°C, after which the supernatants were harvested and counted in a gamma counter. The cytotoxicity was calculated as the percentage of releasable counts after subtracting the spontaneous release. The spontaneous release was less than 15% of the maximum release.

Statistical analysis

Differences between the groups were analyzed by the Mann-Whitney *U* test or by an ANOVA analysis with Fisher's protected least significant difference using the Stat View program (Abacus Concepts, Berkeley, CA) on an Apple computer. Differences were considered to be significant when p was <0.05.

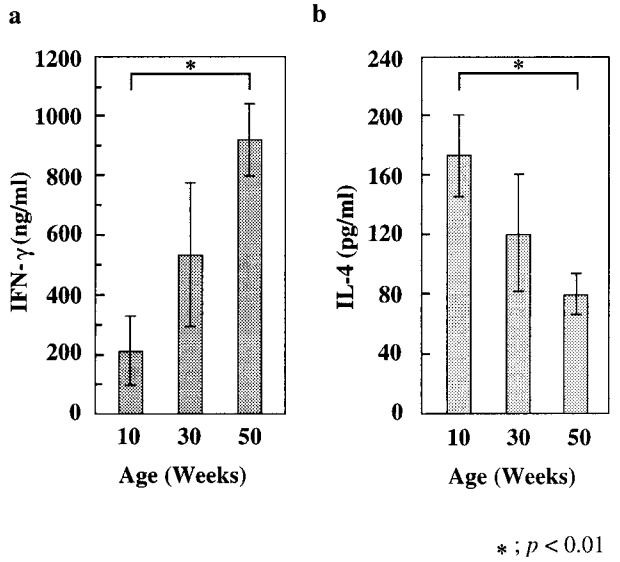

FIGURE 2. IFN-γ (*a*) and IL-4 (*b*) production from splenocytes of mice with various ages. A total of 4 × 10^5 splenocytes were stimulated with immobilized anti-CD3 Ab in 96-well flat-bottom plates for 48 h, and culture supernatants were subjected to ELISA. All data represent the means ± SEs of five to eight independent experiments from different mice of various ages.

Results

CD8$^+$ CD122$^+$ T cells express intermediate TCR and increase in the spleen with mouse age

As we previously reported (1, 2), CD8$^+$ CD122$^+$ T cells expressed intermediate TCR, whereas CD8$^+$ CD122$^-$ T cells expressed high TCR (Fig. 1*a*). The proportion of CD8$^+$ CD122$^+$ T cells in the splenocytes gradually increased with age, whereas that of CD8$^+$ CD122$^-$ T cells decreased (Fig. 1, *b* and *c*). The proportion of these cells in splenocytes of 10-wk-old mice was 1.1 ± 0.2% (mean ± SE, n = 8), whereas the proportion increased to 4.3 ± 0.5% in mice 50 wk old (n = 8; Fig. 1*b*). Nearly half of all CD8$^+$ T cells were CD8$^+$ CD122$^+$ T cells in 50-wk-old mice (Fig. 1*c*).

Production of IFN-γ and IL-4 from splenocytes of mice with various ages

The IFN-γ production from whole splenocytes stimulated with immobilized anti-CD3 Ab gradually increased with age (Fig. 2*a*), whereas the IL-4 production conversely decreased (Fig. 2*b*).

CD8$^+$ CD122$^+$ T cells produce a large amount of IFN-γ

When various T cell populations purified by cell sorting were stimulated with anti-CD3 Ab for 24 and 48 h, CD8$^+$ CD122$^+$ T cells produced a greater amount IFN-γ than those produced by CD8$^+$ CD122$^-$ T cells and regular CD4$^+$ CD122$^-$ T cells (Fig. 3*a*). It is noteworthy that CD8$^+$ CD122$^+$ T cells promptly responded to CD3 stimulation and produced IFN-γ within 24 h. Neither subset of CD8$^+$ T cells produced any detectable IL-4, whereas regular CD4$^+$ T cells did produce IL-4 (Fig. 3*b*). When these T cell subsets were cultured for longer periods (72 and 96 h), CD8$^+$ CD122$^+$ T cells also produced a 4- to 5-fold greater amount of IFN-γ than those produced by the other two subsets (not shown). CD4$^+$ CD122$^+$ T cells were NKT cells (1, 2) and are described later in this section.

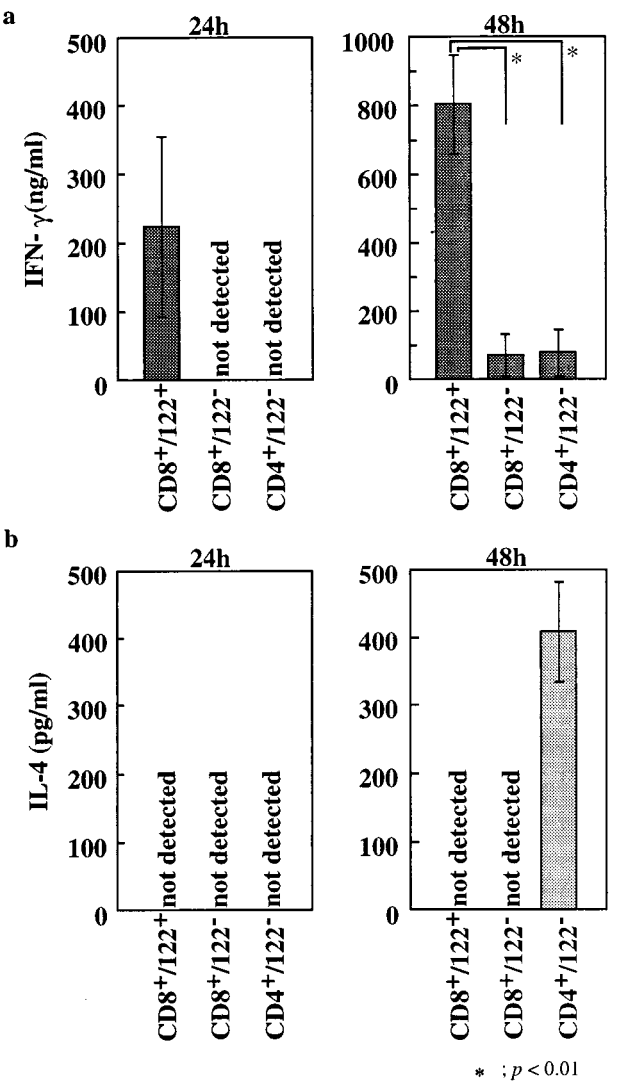

FIGURE 3. IFN-γ (*a*) and IL-4 (*b*) production from various T cell subsets of splenocytes. Spleens of 30-wk-old mice were obtained, the indicated T cell subsets were purified by cell sorting, and 4 × 10^5 cells from each subset were stimulated with immobilized anti-CD3 Ab in 96-well flat-bottom plates for 48 h, and the culture supernatants were subjected to ELISA. The data represent the means ± SEs from three to five independent experiments.

Enhancement of the IFN-γ production and the partial inhibition of IL-4 production from splenocytes by CD8$^+$ CD122$^+$ T cells

Because the sorting procedure may affect the function of T cells, in addition to the untreated (unsorted) splenocytes, splenocytes that were just passed through the sorting machine were also added as a control (sorted total). By removal of CD8$^+$ CD122$^+$ T cells from the splenocytes by cell sorting, CD3-stimulated IFN-γ production from splenocytes greatly decreased and IL-4 production conversely increased; however, the addition of sorted CD8$^+$ CD122$^+$ T cells in serial proportions into CD8$^+$ CD122$^+$ T cell-depleted splenocytes gradually reversed the cytokine production (Fig. 4).

CD8$^+$ CD122$^+$ T cells produce IFN-γ as well as activate CD4$^+$ T cells to produce IFN-γ

To further confirm which populations of splenocytes were IFN-γ producers in the presence of CD8$^+$ CD122$^+$ T cells, CD3-stimulated splenocytes with or without CD8$^+$ CD122$^+$ T cells were stained with anti-IFN-γ Ab (intracellular staining) and with either

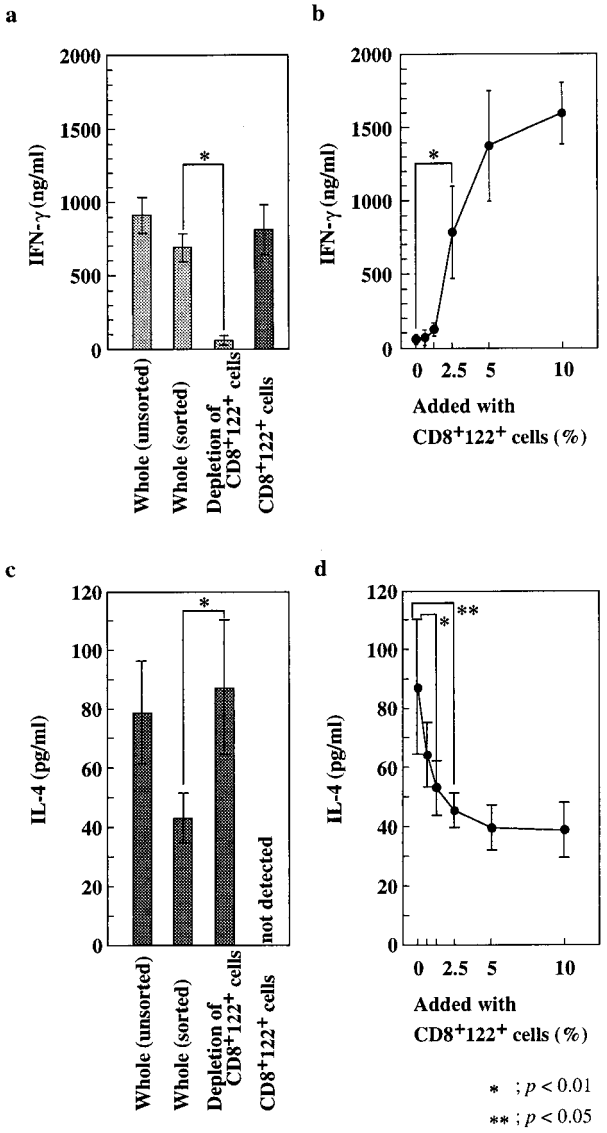

FIGURE 4. The depletion of $CD8^+$ $CD122^+$ T cells from total splenocytes abolished IFN-γ production and increased IL-4 production from splenocytes, and the addition of purified $CD8^+$ $CD122^+$ T cells into $CD8^+$ $CD122^+$ T cell-depleted splenocytes restored IFN-γ production and decreased IL-4 production. The spleens of the 50-wk-old mice were obtained, $CD8^+$ $CD122^+$ T cells were sorted out, and 4×10^5 cells were stimulated with immobilized anti-CD3 Ab in 96-well flat-bottom plates for 48 h, and the IFN-γ and IL-4 levels of culture supernatants were measured (*a* and *c*). All data represent the means ± SEs from three independent experiments. Serial proportions of $CD8^+$ $CD122^+$ T cells were added to $CD8^+$ $CD122^+$ T cell-depleted splenocytes (the sum of cell numbers was 4×10^5 cells/200 μl in each point)and were stimulated with immobilized anti-CD3 Ab in 96-well flat-bottom plates for 48 h, and the IFN-γ and IL-4 levels of culture supernatants were measured (*b* and *d*).

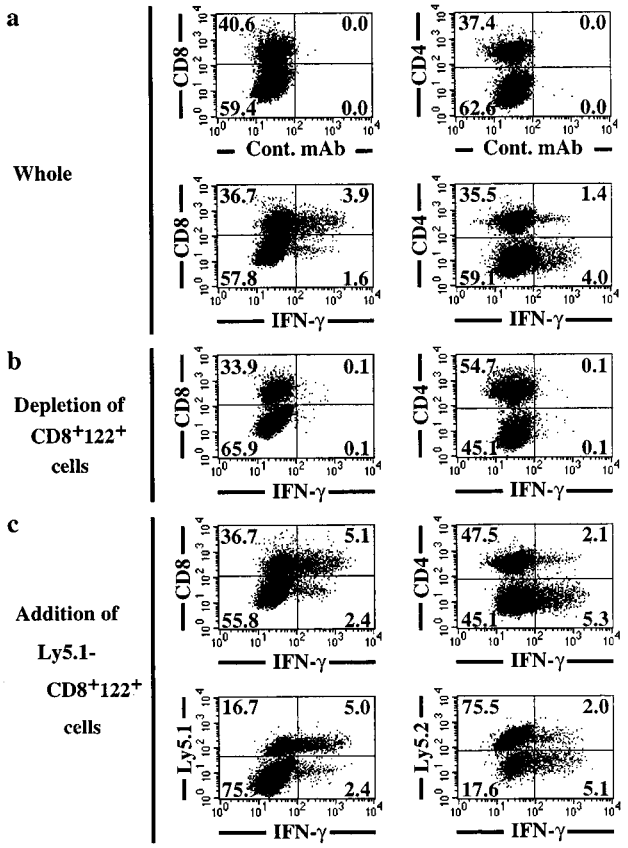

FIGURE 5. Intracellular IFN-γ production of CD3-stimulated splenocytes with or without $CD8^+$ $CD122^+$ T cells. *a*, Intracellular IFN-γ and either CD4 or CD8 expression of total splenocytes (from 40-wk-old mice) 48 h after CD3 stimulation. *b*, Intracellular IFN-γ and either CD4 or CD8 expression of $CD8^+$ $CD122^+$ T cell-depleted splenocytes 48 h after CD3 stimulation. *c*, Intracellular IFN-γ and either CD4 or CD8 expression of $CD8^+$ $CD122^+$ T cell-depleted splenocytes from Ly5.2 mice added with 10% of purified $CD8^+$ $CD122^+$ T cells from Ly5.1 splenocytes. All data shown are representative of four independent experiments with similar results.

anti-CD4 Ab or anti-CD8 Ab. The results showed that when unselected splenocytes were stimulated, intracellular IFN-γ was expressed in a significant population of $CD8^+$ cells and in a small population of $CD4^+$ cells (Fig. 5*a*). However, when $CD8^+$ $CD122^+$ T cell-depleted splenocytes were stimulated with anti-CD3 Ab, neither $CD8^+$ cells nor $CD4^+$ cells expressed intracellular IFN-γ (Fig. 5*b*). Furthermore, if sorted $CD8^+$ $CD122^+$ T cells from Ly5.1 mouse spleen were added to the $CD8^+$ $CD122^+$ T cell-depleted splenocytes (10% in proportion) of Ly5.2 mice and were stimulated with anti-CD3 Ab, $Ly5.1^+$ $CD8^+$ T cells (Fig. 5*c*) expressed intracellular IFN-γ. The proportion of IFN-γ-expressing $CD8^+$ cells (5.1%) and the proportion of IFN-γ-expressing Ly5.1 cells (5.0%) were almost the same, thus indicating that $CD8^+$ $CD122^+$ T cells but not $CD8^+$ $CD122^-$ T cells produced IFN-γ. In addition, a small number of $Ly5.2^+$ $CD4^+$ T cells also expressed intracellular IFN-γ (Fig. 5*c*).

These results were further confirmed by another experiment. The IFN-γ production from CD3-stimulated $CD4^+$ 122^- T cells was greatly augmented by the addition of 10% $CD8^+$ $CD122^+$ T cells (Fig. 6), whereas purified $CD8^+$ $CD122^-$ T cells added with 10% $CD8^+$ $CD122^+$ T cells produced only a small amount of IFN-γ (Fig. 6). These findings revealed that $CD8^+$ $CD122^+$ T cells not only produced IFN-γ by themselves but also activated $CD4^+$ 122^- T cells to produce IFN-γ, showing their synergy in IFN-γ production.

However, $CD8^+$ $CD122^+$ T cells were also suggested to be further activated by the presence of other splenocytes because $CD8^+$ $CD122^+$ T cell-depleted splenocytes supplemented with 10% $CD8^+$ $CD122^+$ T cells produced a much larger amount of IFN-γ than that estimated from the IFN-γ amount produced by purified $CD8^+$ $CD122^+$ T cells (Fig. 6), despite the fact that these cells activated $CD4^+$ T cells to produce IFN-γ. Therefore, we conducted the next experiment.

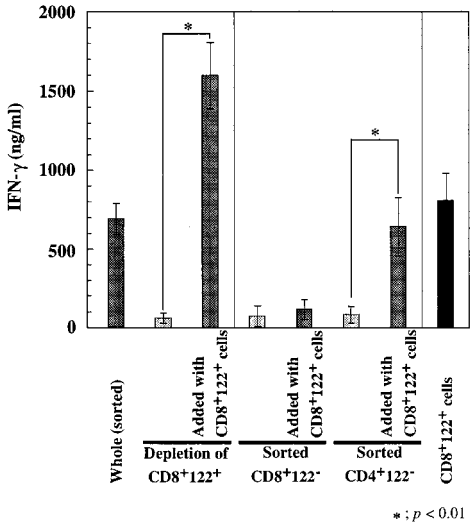

FIGURE 6. CD4$^+$ T cells added with CD8$^+$ CD122$^+$ T cells but not CD8$^+$ CD122$^-$ T cells added with CD8$^+$ CD122$^+$ T cells produce IFN-γ 48 h after CD3 stimulation. All data represent the means ± SEs from three independent experiments. A total of 4×10^5 of respective populations or 3.6×10^5 respective populations added with 0.4×10^5 (10%) CD8$^+$ CD122$^+$ T cells were stimulated with anti-CD3 Ab for 48 h, and the supernatants were subjected to ELISA. All data represent the means ± SEs from three independent experiments.

Augmentation of CD3-stimulated IFN-γ production from CD8$^+$ CD122$^+$ T cells cultured with macrophages

A total of 4×10^5 purified T cell subset was cultured with plastic adherent macrophages for 48 h. In additional experiments, anti-CD28 Ab (10 μg/ml) was precoated with anti-CD3 Ab, and the effect of anti-CD28 Ab on the IFN-γ production from the sorted T cell subsets was further examined (see *Materials and Methods*). The results showed that CD8$^+$ CD122$^-$ T cells as well as CD4$^+$ T cells cultured with macrophages produced severalfold greater amounts of IFN-γ than those produced by these cells alone (Fig. 7). CD8$^+$ CD122$^+$ T cells also produced an ~2-fold greater amount of IFN-γ (Fig. 7). In the case of CD3 and CD28 costimulation, the IFN-γ production from each T cell subset was significantly increased when these cells were cultured in a plate precoated with 1 μg/ml of anti-CD3 Ab, whereas the augmentation was not significant when each subset was cultured in a plate precoated with 10 μg/ml of anti-CD3 Ab (Fig. 7). The costimulatory molecules of macrophages and T cells (B7 of macrophages and CD28 of T cells, etc.) were thus suggested to be required for the effective IFN-γ production from T cells, especially when T cells were stimulated by a small amount of CD3 Ab. In addition, the CD3-stimulated IFN-γ production from CD8$^+$ CD122$^-$ T cells and CD4$^+$ T cells was suggested to be more dependent on either the macrophages or their costimulatory molecules than that from the CD8$^+$ CD122$^+$ T cells because the augmentation of IFN-γ production from CD8$^+$ CD122$^+$ T cells by macrophages was less prominent than that seen in the other two T cell subsets.

Comparison of IFN-γ production and the antitumor capacity between CD8$^+$ CD122$^+$ T cells and NKT cells

Because splenocytes contain only 1% NKT cells and NKT cells are abundant in the liver, NKT cells were purified from liver MNC. Because NKT cells are either CD4$^+$ CD122$^+$ or CD4$^-$ CD8$^-$ CD122$^+$ and staining with anti-CD3 Ab or anti-αβTCR Ab may activate NKT cells, NKT cells were purified by sorting after staining with anti-CD5 Ab and anti-NK1.1 Ab. The results showed that CD8$^+$ CD122$^+$ T cells produced a greater amount of IFN-γ than did NKT cells (Fig. 8*a*), whereas NKT cells also promptly produced IL-4 within 24 h (Fig. 8*b*). Both CD8$^+$ CD122$^+$ T cells and NKT cells but neither CD8$^+$ CD122$^-$ T cells nor regular CD4$^+$ T cells acquired significant antitumor cytotoxicities (Fig. 8*c*).

Discussion

CD8$^+$ CD122$^+$ T cells in the spleen gradually increased with age and IFN-γ production from CD3-stimulated splenocytes in vitro gradually increased, whereas IL-4 production gradually decreased with age. Purified CD8$^+$ CD122$^+$ T cells stimulated with anti-CD3 Ab promptly produced a large amount of IFN-γ, whereas purified CD8$^+$ CD122$^-$ T cells or CD4$^+$ T cells produced small amounts of IFN-γ. It is of interest that CD3-stimulated CD8$^+$ CD122$^+$ T cells produced IFN-γ by themselves as well as activated CD4$^+$ T cells to produce IFN-γ, indicating their synergy in

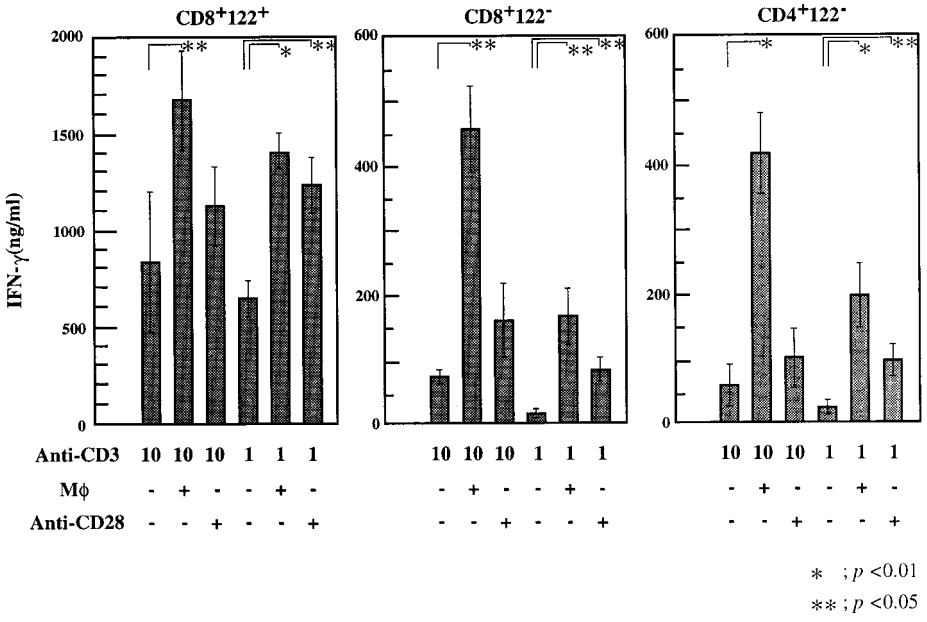

FIGURE 7. Enhancement of the CD3-stimulated IFN-γ production from each T cell subset by macrophages or CD28 costimulation. Each purified T cell subset was stimulated with a regular (10 μg/ml) or a lower amount (1 μg/ml) of immobilized anti-CD3 Ab with or without macrophages or immobilized anti-CD28 Ab. The data represent the means ± SEs from three independent experiments.

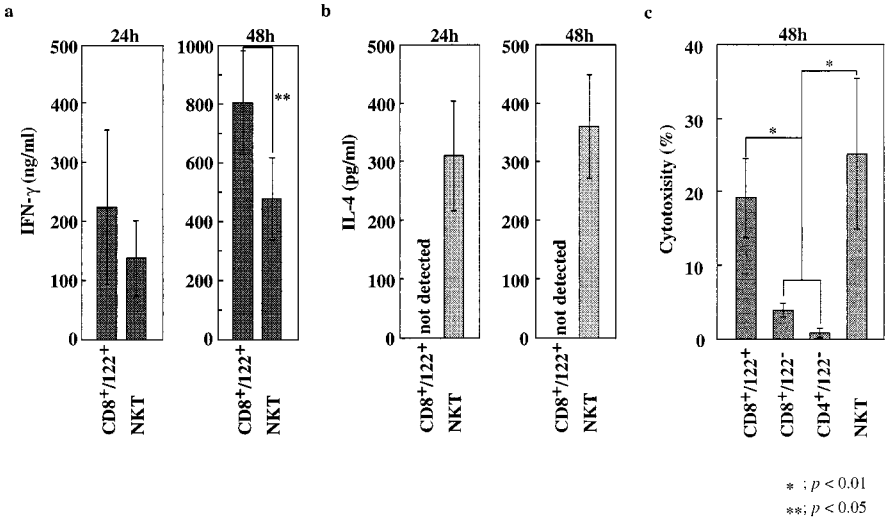

FIGURE 8. Cytokine production and the antitumor activity of $CD8^+$ $CD122^+$ T cells in comparison with those from NKT cells. Purified liver NKT cells, $CD8^+$ $CD122^+$ T cells, and other subsets were activated by anti-CD3 Ab, and their cytokine production (*a* and *b*) and cytotoxicities against YAC-1 cells (*c*) (E:T ratio was 10:1) at indicated time points were measured. All data represent the means ± SEs from three independent experiments.

IFN-γ production. In addition, $CD8^+$ $CD122^+$ T cells partially inhibited IL-4 production from CD3-stimulated splenocytes ($CD4^+$ T cells). The interaction of macrophages with each T cell subset (especially $CD8^+$ $CD122^-$ T cells and $CD4^+$ T cells) was found to enhance the CD3-stimulated IFN-γ production. Furthermore, $CD8^+$ $CD122^+$ T cells stimulated with anti-CD3 Ab produced a greater amount of IFN-γ than did NKT cells and also acquired an antitumor cytotoxicity.

$CD8^+$ $CD122^+$ T cells with intermediate TCR were previously reported to develop extrathymically (1, 22, 23) and to increase in the liver and other organs with age (2, 3). These cells were present in aged nude mice (3) and emerged thymectomized and irradiated mice that had been subjected to bone marrow transplantation (22). In addition, the adult liver contains c-*kit*$^+$ stem cells that can produce virtually all lineage leukocytes (24, 25) including $CD8^+$ $CD122^+$ T cells (25). It was suggested that these cells may have functional characteristics different from thymus-derived $CD8^+$ $CD122^-$ T cells and thus may be involved in immunological changes with aging (2, 3). Nevertheless, the function of these cells remains largely unknown.

It was previously reported by Ernst et al. (18) that the number of $CD8^+$ T cells with a memory phenotype ($CD44^{high}$) that produced a large amount of IFN-γ increased in aged mice. Because $CD8^+$ $CD122^+$ T cells in the present study are also $CD44^{high}$ as was previously reported (1–3), $CD8^+$ T cells reported by Ernst et al. are probably identical with $CD8^+$ $CD122^+$ T cells. In addition, the $CD8^+$ $CD122^+$ T cells stimulated $CD4^+$ T cells to produce IFN-γ, thus suggesting that $CD8^+$ $CD122^+$ T cells play an important role in the Th1 immune response.

It is well known that elder hosts are susceptible to infections and malignant tumors and that IFN-γ is the potent activator of T cells, NK cells, and macrophages. It was also reported in humans that $CD57^+$ $CD8^+$ T cells (presumably extrathymically developed) increased with age (26) and are a putative counterpart of mouse $CD8^+$ $CD122^+$ T cells (2). Consistently with this speculation, we recently found that CD3-stimulated human $CD57^+$ $CD8^+$ T cells produced a much larger amount of IFN-γ than that produced by regular $CD8^+$ T cells.[4] Therefore, the increases of extrathymic $CD8^+$ T cells and IFN-γ-producing potential in elder hosts seem to be reasonable changes with aging.

[4]T. Ohkawa, S. Seki, H. Dobashi, Y. Koike, Y. Habu, K. Ami, H. Hiraide, and I. Sekine. *Submitted for publication.*

NKT cells use the limited Vα (Vα14) and Vβ (Vβ7, Vβ8) chains for their TCR, whereas $CD8^+$ $CD122^+$ T cells show a more heterogeneous TCR repertoire (2, 27). Although β_2-microglobulin-deficient ($\beta_2m^{-/-}$) mice lack not only $CD8^+$ T cells but also NKT cells, it was also demonstrated that when T cell-depleted bone marrow (BM) cells from normal mice were i.v. transferred into irradiated $\beta_2m^{-/-}$ mice, NKT cells developed, whereas no regular $CD8^+$ T cells did (28–30). On the other hand, when T cell-depleted BM cells from $\beta_2m^{-/-}$ mice were injected into irradiated normal mice, regular $CD8^+$ T cells developed, whereas NKT cells did not (28–30). As a result, NKT cells appear to be selected by the β_2m-associated molecules of BM-derived cells but not by those of thymic epithelial cells. CD1 was later shown to be the β_2m-associated molecule of BM-derived cells required for NKT cell development (4, 5, 31, 32). Similarly, using the BM chimera described above, we recently found that in addition to NKT cells, a small but significant population of $CD8^+$ $CD122^+$ T cells with intermediate TCR also developed in irradiated $\beta_2m^{-/-}$ mice (which originally lack $CD122^-$ as well as $CD122^+$ $CD8^+$ T cells) after the T cell-depleted BM transplantation from normal mice (but not vice versa), whereas no regular $CD8^+$ $CD122^-$ T cells with high TCR developed (33). In addition, TAP-1 gene-mutated mice have NKT cells (34–36) but lack both $CD8^+$ $CD122^-$ and $CD8^+$ $CD122^+$ T cells (33). Because TAP-1-mutated mice do not express MHC class I molecules but do express CD1, MHC class I molecules (but not CD1) of BM-derived cells but not of thymic epithelial cells appear to be required for the development of $CD8^+$ $CD122^+$ T cells. Therefore, even though $CD8^+$ $CD122^+$ T cells and NKT cells both share the same properties of intermediate TCR and both are dependent on class I or related molecules of BM-derived cells for their development, their manners of development are different. $CD8^+$ $CD122^+$ T cells may recognize more heterogeneous Ags.

In addition, it was recently demonstrated that $CD8^+$ $CD122^+$ T cells with male H-Y Ag-specific intermediate TCR could develop in thymectomized and irradiated male mice that are subjected to BM transplantation from male H-Y Ag-specific TCR transgenic mice. In contrast, they could not develop in thymectomized and irradiated female mice (37). Because female mice lack male H-Y Ag, it was suggested that $CD8^+$ $CD122^+$ T cells with male H-Y Ag-specific TCR were positively selected by extrathymically expressed male H-Y Ag (37). Therefore, for some $CD8^+$ $CD122^+$ T cells with intermediate TCR, MHC class I expression and self-Ag

(but not foreign Ag) are both required for their extrathymic development, implying their potential autoreactivity.

Although we recently demonstrated that NKT cells are an important subset for the polarization of the Th1 immune response by the IFN-γ production (9), they rapidly produce both IL-4 and IFN-γ by the injection of anti-CD3 Ab in vivo as previously reported (4, 5, 32); this was also demonstrated in the present in vitro study. In contrast, CD3-stimulated CD8$^+$ CD122$^+$ T cells promptly produced IFN-γ but did not produce IL-4 and inhibited IL-4 production from splenocytes. Notably, NKT cells are proportionally rare in all lymphoid organs except the liver throughout the mouse life (2), whereas CD8$^+$ CD122$^+$ T cells constantly increase in the liver, spleen, and lymph nodes with aging (2, 3); up to 50% of all CD8$^+$ T cells of the spleen were CD8$^+$ CD122$^+$ T cells in 50-wk-old mice, as demonstrated in the present study. Moreover, we showed that CD8$^+$ CD122$^+$ T cells produced a greater amount of IFN-γ than did NKT cells by in vitro CD3 stimulation. As a result, the capacity of systemic CD8$^+$ CD122$^+$ T cells in aged mice for IFN-γ production is considered to be very large.

Finally, it is noteworthy that the large decrease of the CD3-stimulated IFN-γ production from CD8$^+$ CD122$^+$ T cell-depleted splenocytes and the recovery of the IFN-γ production from CD8$^+$ CD122$^+$ T cell-depleted splenocytes by the addition of the serial proportions of CD8$^+$ CD122$^+$ T cells suggest that the interaction of CD8$^+$ CD122$^+$ T cells not only with macrophages and CD4$^+$ T cells but also with other splenocytes (NK cells, etc.) may be required for a more effective IFN-γ production from CD8$^+$ CD122$^+$ T cells or from whole splenocytes, because purified CD8$^+$ CD122$^+$ T cells stimulated with anti-CD3 Ab in the presence of macrophages produced only a 2-fold larger amount of IFN-γ than that produced by purified CD8$^+$ CD122$^+$ T cells alone.

Therefore, based on the above findings, we conclude that CD8$^+$ CD122$^+$ T cells are a potent IFN-γ producer and are thus considered to be one of the important effectors in the Th1 immune response.

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