Brief Definitive Report

Developmental Arrest of NK1.1$^+$ T Cell Antigen Receptor (TCR)-α/β^+ T Cells and Expansion of NK1.1$^+$ TCR-γ/δ^+ T Cell Development in CD3ζ-deficient Mice

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Summary

The relationship between the structure of the T cell antigen receptor (TCR)–CD3 complex and development of NK1.1$^+$ T cells was investigated. The TCR complex of freshly isolated NK1.1$^+$ TCR-α/β^+ thymocytes contained CD3ζ homodimers and CD3ζ-FcRγ heterodimers, whereas that of the majority of NK1.1$^-$ T cells did not contain FcRγ. The function of CD3ζ and FcRγ in the development of NK1.1$^+$ T cells was determined by analyzing CD3ζ- and FcRγ-deficient mice. The NK1.1$^+$ T cells from wild-type and CD3ζ-deficient mice had equal levels of CD3 expression. However, the development of NK1.1$^+$ TCR-α/β^+ T cells was almost completely disrupted in thymus and spleen in CD3ζ-deficient mice, whereas no alteration was observed in FcRγ-deficient mice. In contrast, the number of novel NK1.1$^+$ TCR-γ/δ^+ thymocytes expressing a surface phenotype similar to NK1.1$^+$ TCR-α/β^+ thymocytes increased approximately six times in CD3ζ-deficient mice. These findings establish the distinct roles of the CD3ζ chain in the development of the following different thymic T cell compartments: NK1.1$^-$ TCR$^+$, NK1.1$^+$ TCR-α/β^+, and NK1.1$^+$ TCR-γ/δ^+ thymocytes, which cannot be replaced by CD3η or FcRγ chains.

A T cell population expressing NK1.1, a marker previously thought to be specific for natural killer cells, has been found in CD4$^+$CD8$^-$ T cells and CD4$^-$CD8$^-$ T cells (1–4). The NK1.1$^+$ TCR-α/β^+ T cells express skewed TCR Vα (5) and Vβ (1, 2, 4) families. Recently, a similar T cell population with a skewed TCR repertoire was also identified in human lymphocytes (5–7). These T cells secrete several kinds of lymphokines, such as IL-2, IL-4, and IFN-γ (8, 9). Furthermore, we have shown that NK1.1$^+$ TCR-α/β^+ thymocytes are the only population within the thymus that expresses Fas ligand and is cytotoxic against immature CD4$^+$CD8$^+$ thymocytes expressing Fas antigen (10, 11). NK1.1$^+$ TCR-α/β^+ thymocytes appear to be an important population that may regulate the development of the immune system by secreting several different lymphokines (12) or by killing Fas-expressing cells (11). Although both NK1.1$^+$ CD4$^+$CD8$^-$ and NK1.1$^+$ CD4$^-$CD8$^-$ T cells were shown to be selected by β_2-microglobulin–associated MHC class I antigen (13–16), it has remained unclear what antigen the NK1.1$^+$ TCR-α/β^+ T cells recognize and how the NK1.1$^+$ TCR-α/β^+ T cells develop.

Recently, IL-2–activated NK1.1$^+$ TCR-α/β^+ thymocytes were shown to express TCR complexes associated with FcRγ homodimers (17) similarly to CD8α/α^+ TCR-γ/δ^+ T cells in intestinal intraepithelial lymphocytes (18–20). These studies raised the possibility that NK1.1$^+$ TCR-α/β^+ thymocytes may have TCR complexes and signaling functions distinct from conventional NK1.1$^-$ T cells. In the present study, to understand the signals required for the development of NK1.1$^+$ TCR-α/β^+ T cells, we analyzed these populations in CD3ζ- (21) and FcRγ-deficient mice (22). CD3ζ was required for the development of these T cells, although FcRγ deficiency did not alter their development. On the other hand, the number of NK1.1$^+$ TCR-γ/δ^+ thymocytes significantly increased in CD3ζ-deficient mice. These findings demonstrated that CD3ζ has distinct roles in the development of not only the major NK1.1$^-$ T cells (20, 21, 23) but also of NK1.1$^+$ T cells.

Materials and Methods

Mice. CD3ζ-deficient mice were made as previously described (21). FcRγ-deficient mice were kindly provided by Dr. J. V. Ravetch (The Rockefeller University, New York) (22). These mice were maintained in our animal facility and back-crossed to C57BL/6 mice six times for CD3ζ-deficient mice and three times for FcRγ-deficient mice, and NK1.1 expression was confirmed for all mice.

Cell Preparation. NK1.1$^+$ TCR-α/β^+ thymocytes were pre-

pared by FACS® sorting as described previously (11). The purity of NK1.1$^+$ TCR-α/β^+ thymocytes was >99%. LN T cells were prepared by removing surface Ig$^+$ B cells by magnetic particles coupled with goat anti-mouse IgG Ab (Advanced Magnetics, Inc., Cambridge, MA).

Immunoprecipitation and Two-dimensional SDS-PAGE Analysis. Immunoprecipitation was carried out basically as described by Kim et al. (24). Briefly, 3 × 10^6 purified NK1.1$^+$ TCR-α/β^+ thymocytes and LN T cells were solubilized in lysis buffer (1% digitonin, 50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml antipain, 2.5 μg/ml chymostatin, 10 μg/ml pepstatin A, 1 mM PMSF, and 10 mM iodoacetamide). Immunoprecipitation was performed with anti-CD3 (2C11) mAb precoupled by dimethylpimelimidate to protein A Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ). The total precipitates were biotinylated as previously described (18). After biotinylation, immunoprecipitates were resolved by two-dimensional nonreducing–reducing SDS-PAGE and transferred onto polyvinylidene fluoride membrane. The biotinylated protein on the membrane was detected by enzyme chemiluminescence as described previously (18).

FACS® Analysis. 1 × 10^6 thymocytes or red cell–depleted splenocytes were stained with FITC–anti–heat stable antigen (HSA) mAb, PE–anti-NK1.1 mAb, and biotin–anti-CD3, TCR-α/β or TCR-γ/δ mAb (all from Pharmingen, San Diego, CA), followed by Tricolor-streptavidin (Caltag Laboratories, San Francisco, CA). For analysis of various surface marker expressions, thymocytes were stained with FITC–anti-HSA mAb, PE–anti-NK1.1 mAb, and biotin–anti-CD4, -CD8, -CD44,–MEL-14 (LECAM-1) (all from Pharmingen)–IL-2Rβ (kindly provided by Dr. Miyasaka), or–IL-7R mAb (gratefully received from Dr. Nishikawa, Kyoto University, Japan), followed by Tricolor-streptavidin. The stained cells were analyzed by FACScan® (Becton Dickinson & Co., Mountain View, CA).

Results

Association of FcRγ with the TCR Complexes of Freshly Isolated NK1.1$^+$ TCR-α/β^+ Thymocytes. Recent analysis by S. Koyasu revealed that IL-2–activated NK1.1$^+$ TCR-α/β^+ T cells express unique TCR complexes associated with FcRγ homodimers (17). However, because of the limited number of these cells isolated from mice, analysis of the TCR complexes in freshly isolated NK1.1$^+$ TCR-α/β^+ T cells has been difficult. In the present study, we applied a newly developed sensitive labeling method (24) for analyzing the TCR complexes of freshly isolated NK1.1$^+$ TCR-α/β^+ thymocytes. As shown in Fig. 1, immunoprecipitation of the TCR complexes of NK1.1$^+$ TCR-α/β^+ thymocytes with anti-CD3ϵ mAb showed that the complexes contained CD3ζ homodimers and CD3ζ-FcRγ heterodimers. However, FcRγ homodimers, which were observed in the TCR complexes of IL-2–activated NK1.1$^+$ T cells (17), were not detected in the complexes of the freshly isolated NK1.1$^+$ TCR-α/β^+ thymocytes. On the other hand, only CD3ζ homodimers were detected in TCR complexes of LN T cells as a representative population of the NK1.1$^-$ conventional T cells. Therefore, NK1.1$^+$ TCR-α/β^+ thymocytes seemed to possess a unique composition of the TCR–CD3 complexes, perhaps reflecting a distinct TCR signaling capability of these cells.

Requirement of CD3ζ for Development of NK1.1$^+$ TCR-α/β^+ T Cells. We next examined the functional differences between CD3ζ and FcRγ expressed on NK1.1$^+$ TCR-α/β^+ T cells in their development by using CD3ζ- (21) and FcRγ-deficient mice (22). Because NK1.1$^+$ CD3$^+$ thymocytes do not express HSA antigen in normal mice (1, 3, 4, 9) and in CD3ζ-deficient mice (data not shown), we analyzed the expression of NK1.1 and TCR on HSA$^-$ thymocytes. As shown in Fig. 2 *A*, NK1.1$^+$ thymocytes from $\zeta^{+/-}$ and $\zeta^{-/-}$ mice expressed the same level of CD3. However, the number of NK1.1$^+$ TCR-α/β^+ cells was found to be severely reduced in $\zeta^{-/-}$ mice. Staining the cells with anti-Vβ8 mAb showed a similar result (data not shown). The proportion of NK1.1$^+$ TCR-α/β^+ thymocytes to NK1.1$^-$ TCR-α/β^+ thymocytes in CD3ζ-deficient mice decreased to one-third of that in wild-type mice. This indicates that the reduction of the cell number of NK1.1$^+$ TCR-α/β^+ cells in CD3ζ-deficient mice was more drastic than that of NK1.1$^-$ TCR-α/β^+ cells. When NK1.1$^+$ CD3$^+$ cells in $\zeta^{-/-}$ mice were stained with anti-TCR-γ/δ mAb, it was found that most of these cells were TCR-γ/δ^+. To understand the composition of the thymocytes in $\zeta^{-/-}$ mice, the total cell numbers of the NK1.1$^+$ TCR-α/β^+ and NK1.1$^+$

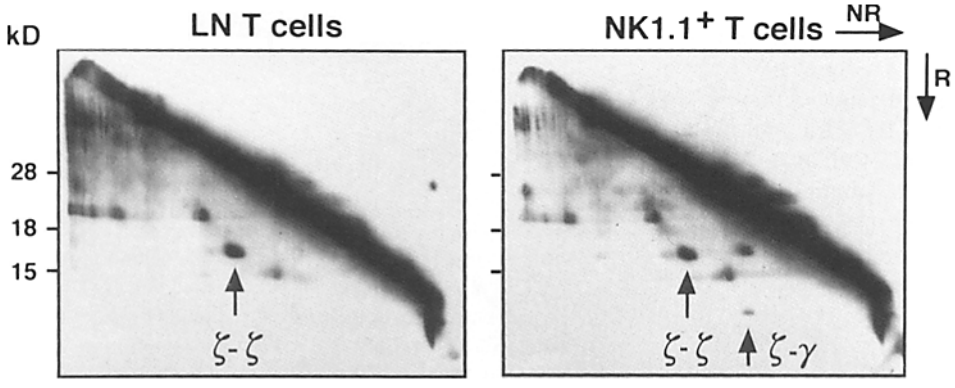

Figure 1. Involvement of the FcRγ chain in the TCR complex of freshly isolated NK1.1$^+$ TCR-α/β^+ thymocytes. Total cell lysate from 3–5 × 10^6 NK1.1$^+$ TCR-α/β^+ thymocytes and LN T cells was precipitated with anti-CD3ϵ mAb. The immunoprecipitates were biotinylated on beads and analyzed by two-dimensional nonreducing (*NR*)–reducing (*R*) SDS-PAGE, and total protein was visualized. Positions of CD3ζ homodimers (ζ-ζ) and CD3ζ-FcRγ heterodimers (ζ-γ) as well as molecular size markers are indicated in the figure. An off-diagonal spot with a molecular mass of 14 kD seems to be an unknown molecule detected by the biotinylation method used.

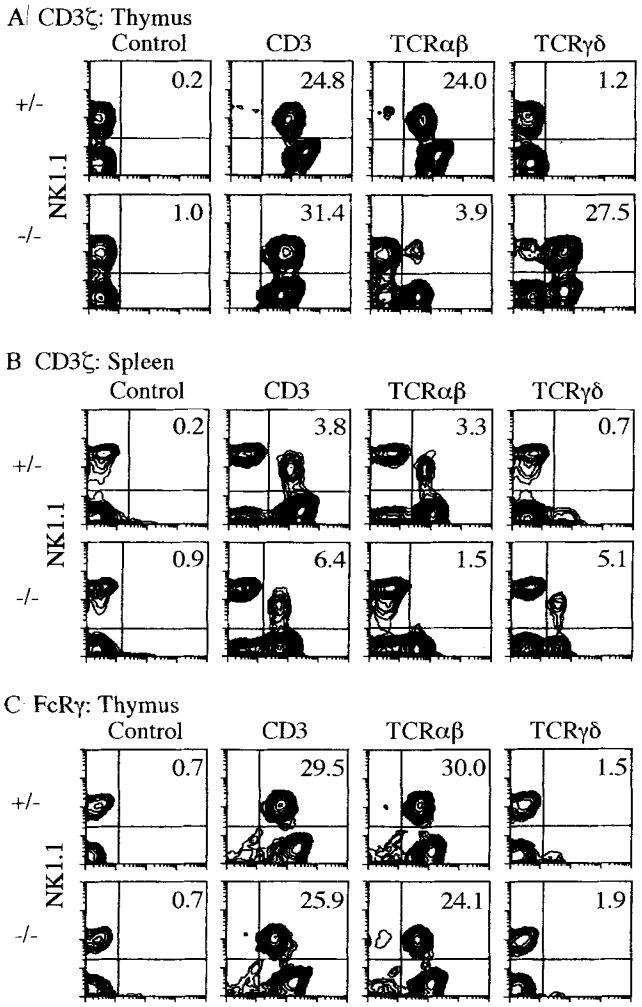

Figure 2. Analysis of NK1.1$^+$ T cells in mice lacking CD3ζ (*A*, *B*) or FcRγ (*C*). Thymocytes (*A*, *C*) and splenocytes (*B*) from each line of mice were stained with FITC–anti-HSA mAb, PE–anti-NK1.1 mAb, and biotin–anti-CD3ε, -TCR-β, or -TCR-δ mAb followed by Tricolor-streptavidin. Expressions of NK1.1 (*vertical*) and CD3, TCR-α/β, or TCR-γ/δ (*horizontal*) on HSA$^-$ thymocytes from each line of mice were illustrated. Proportions of upper-right regions were indicated in the figure. The proportion of NK1.1$^+$ TCR-α/β$^+$ cells to NK1.1$^-$ TCR-α/β$^+$ cells in the thymus is 0.15 in ζ$^{+/-}$ and 0.05 in ζ$^{-/-}$ mice, and that in the spleen is 0.041 in ζ$^{+/-}$ and 0.010 in ζ$^{-/-}$ mice. Data represent five independent experiments.

TCR-γ/δ$^+$ thymocytes were calculated (Fig. 3). While the number of NK1.1$^+$ TCR-α/β$^+$ thymocytes was reduced dramatically, the number of NK1.1$^+$ TCR-γ/δ$^+$ thymocytes increased about six times in ζ$^{-/-}$ mice as compared with those in ζ$^{+/-}$ mice. This expansion of the NK1.1$^+$ TCR-γ/δ$^+$ thymocyte population was not simply a reflection of a general increase of TCR-γ/δ$^+$ T cells in ζ$^{-/-}$ mice, as the number of NK1.1$^-$ TCR-γ/δ$^+$ thymocytes did not significantly increase in ζ$^{-/-}$ mice, although their relative proportion within thymocytes increased (data not shown). Similar to thymocytes, the development of NK1.1$^+$ TCR-α/β$^+$ T cells was also arrested, and NK1.1$^+$ TCR-γ/δ$^+$ T

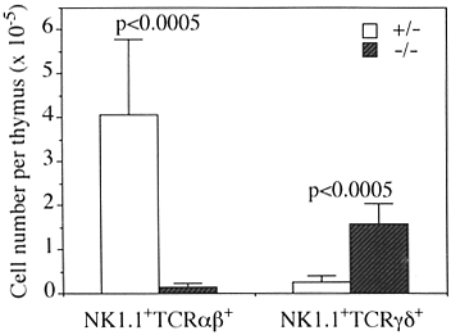

Figure 3. Total cell numbers of NK1.1$^+$ TCR-α/β$^+$ cells and NK1.1$^+$ TCRγδ$^+$ cells in the thymus of CD3ζ-deficient mice. The total numbers of NK1.1$^+$ TCR-α/β$^+$ thymocytes and NK1.1$^+$ TCR-γ/δ$^+$ thymocytes from ζ$^{+/-}$ (n = 7) and ζ$^{-/-}$ (n = 6) mice were calculated. Data were presented as mean ± SD. Significance was evaluated by Student's t test.

cells were increased in the spleen of CD3ζ-deficient mice (Fig. 2 *B*).

When FcRγ-deficient mice were analyzed, no obvious difference was observed in the development of NK1.1$^+$ TCR-α/β$^+$ thymocytes as compared with wild-type mice (Fig. 2 *C*). In addition, the development of NK1.1$^+$ TCR-γ/δ$^+$ thymocytes, though small in number, was not affected by the lack of FcRγ expression (Fig. 2 *C*). These findings indicate that the expression of CD3ζ is essential for the development of NK1.1$^+$ TCR-α/β$^+$ T cells but not of NK1.1$^+$ TCR-γ/δ$^+$ thymocytes. On the other hand, FcRγ, though involved in the TCR complexes of NK1.1$^+$ TCR-α/β$^+$ T cells, was not crucial for the development of NK1.1$^+$ TCR-α/β$^+$ T cells.

Surface Phenotype of NK1.1$^+$ TCR-γ/δ$^+$ Thymocytes in CD3ζ-deficient Mice. To characterize the novel NK1.1$^+$ TCR-γ/δ$^+$ thymocytes that expanded in CD3ζ-deficient mice, we analyzed the expression of several surface markers on NK1.1$^+$ TCR-γ/δ$^+$ thymocytes. As previously reported (3, 4, 10, 25), the surface phenotype of NK1.1$^+$ TCR-α/β$^+$ thymocytes is homogeneously CD8$^-$ CD44$^+$ MEL-14$^-$ IL-2Rβ$^+$ IL-7R$^+$, and almost half of them express CD4 (Fig. 4). When the surface phenotype of NK1.1$^+$ thymocytes in ζ$^{-/-}$ mice (most were TCR-γ/δ$^+$ cells) was compared with that in ζ$^{+/-}$ mice (most were TCR-α/β$^+$ cells), almost all of the NK1.1$^+$ TCR-γ/δ$^+$ thymocytes were CD8$^-$ CD44$^+$ MEL-14$^-$ IL-2Rβ$^+$ IL-7R$^+$, which was the same phenotype as NK1.1$^+$ TCR-α/β$^+$ thymocytes, except that most of the NK1.1$^+$ TCR-γ/δ$^+$ thymocytes were CD4$^-$ (Fig. 4).

Discussion

In the present study, we found that freshly isolated NK1.1$^+$ TCR-α/β$^+$ thymocytes express TCR complexes associated with CD3ζ-FcRγ heterodimers as well as CD3ζ homodimers. Considering that TCR complexes of most NK1.1$^-$ T cells do not contain FcRγ, NK1.1$^+$ TCR-α/β$^+$ thymocytes may possess a unique TCR signaling pathway via FcRγ. However, the development of NK1.1$^+$ TCR-

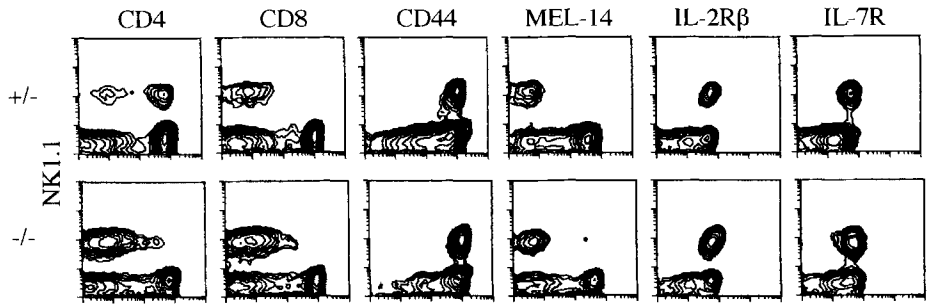

Figure 4. Surface phenotype analysis of NK1.1$^+$ thymocytes from CD3ζ-deficient mice. Thymocytes from $\zeta^{+/-}$ and $\zeta^{-/-}$ mice were stained with FITC-anti-HSA mAb, PE-anti-NK1.1 mAb, and biotin-anti-CD4, -CD8, -CD44,-MEL-14, -IL2Rβ, or -IL7R mAb. Expressions of NK1.1 (*vertical*) and indicated markers (*horizontal*) on HSA$^-$ thymocytes were illustrated.

α/β^+ T cells was almost completely blocked in CD3ζ-deficient mice, but not in FcRγ-deficient mice. These observations demonstrated that the expression of CD3ζ but not of FcRγ was a primary requisite for the development of NK1.1$^+$ TCR-α/β^+ T cells. Since the proportion of thymic and splenic NK1.1$^+$ TCR-α/β^+ T cells to NK1.1$^-$ TCR-α/β^+ T cells was severely reduced in CD3ζ-deficient mice (Fig. 2, *A* and *B*), the development of NK1.1$^+$ TCR-α/β^+ T cells was more dependent on the expression of CD3ζ than that of NK1.1$^-$ TCR-α/β^+ T cells.

In parallel with the developmental arrest of NK1.1$^+$ TCR-α/β^+ T cells, novel NK1.1$^+$ TCR-γ/δ^+ T cells were expanded in CD3ζ-deficient mice. The NK1.1$^+$ TCR-γ/δ^+ T cells showed almost the same phenotype as NK1.1$^+$ TCR-α/β^+ T cells, suggesting that they both belong to the same lineage. The exact mechanism of the expansion of NK1.1$^+$ TCR-γ/δ^+ thymocytes in CD3ζ-deficient mice is not clear at present. Considering that the number of TCR-γ/δ^+ T cells did not increase in the mice lacking TCR-α/β T cells (26, 27), and the number of NK1.1$^-$ TCR-γ/δ^+ thymocytes did not increase in our CD3ζ-deficient mice, the expansion of NK1.1$^+$ TCR-γ/δ^+ T cells does not seem to be merely a compensation for the reduction of TCR-α/β^+ T cells in CD3ζ-deficient mice. Therefore, we assume that the expansion of NK1.1$^+$ TCR-γ/δ^+ T cells may result from the lack of signals through CD3ζ. It is possible that signals through CD3ζ on TCR-γ/δ^+ T cells may inhibit the development of NK1.1$^+$ TCR-γ/δ^+ T cells in normal mice. Indeed, recent observations that the overexpression of CD3ζ suppressed the recombination-activating gene expression and blocked the development of TCR-α/β^+ T cells (28) are consistent with this idea. Therefore, signals from CD3ζ may regulate the development of thymocytes and T cells positively or negatively, depending on the developmental stages and specific properties of T cell subsets.

The development of NK1.1$^+$ TCR-α/β^+ thymocytes was not altered in FcRγ-deficient mice (Fig. 2 *B*). Furthermore, NK1.1$^+$ TCR-α/β^+ thymocytes from FcRγ-deficient mice responded to the stimulation by immobilized anti-TCR mAb and had grown in the presence of IL-2 as in normal mice (Arase, H., and T. Saito, unpublished observation). Therefore, FcRγ does not seem to be critical for the development and function of NK1.1$^+$ TCR-α/β^+ T cells. However, considering that FcRγ has an antigen recognition activation motif and transduces signals distinct from that through CD3ζ in vitro (29), it may contribute partly to the TCR signaling in NK1.1$^+$ TCR-α/β^+ thymocytes. Further studies will be required to clarify the function of FcRγ expressed in NK1.1$^+$ TCR-α/β^+ thymocytes.

Collectively, it is concluded that at least three populations, NK1.1$^-$ TCR-α/β^+ T cells, NK1.1$^+$ TCR-α/β^+ T cells, and NK1.1$^+$ TCR-γ/δ^+ T cells, require different TCR signals for their development. Although the precise manner and location of the differentiation of NK1.1$^+$ TCR-α/β^+ T cells and NK1.1$^+$ TCR-γ/δ^+ T cells are still unclear, our present findings that CD3ζ signals control the development of TCR-α/β^+ and TCR-γ/δ^+ T cells provide an important insight into the biology of NK1.1$^+$ T cells.

We would like to thank Dr. J. V. Ravetch for providing FcRγ-deficient mice, Drs. S. Sato and S. Masaki for maintaining CD3ζ-deficient mice, and Ms. C. Ogata for technical assistance.

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture and from the Agency for Science and Technology. H. Arase received a grant-in-aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research and from The Tokyo Biochemical Research Foundation.

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Received for publication 3 January 1995 and in revised form 25 April 1995.

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