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Expansion of NK1.1$^-$ intermediate TCR cells and granulocytes in mice transplanted with TAP-1-deficient cells

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ABSTRACT

Missing self which lacked the expression of MHC class I antigens was prepared in irradiated B6.Ly5.1 mice (H-2^b) which had undergone bone marrow transplantation (BMT) (depleted of T cells) of TAP-1 (-/-) (Ly5.2, H-2^b) mice. Donor cells (Ly5.2$^+$) and recipient cells (Ly5.1$^+$) were identified by Ly5 markers. For purposes of comparison, syngeneic (B6.Ly5.2 mice) BMT and allogeneic (BALB/c mice, H-2^d) BMT were also conducted. In the case of missing self cells, the ratio of expanding donor cells increased in the liver, spleen and bone marrow on days 14 and 21 after BMT. Such donor cells were mainly NK cells and NKT cells, especially in the liver. The interacting recipient lymphocytes were NKT cells at the early stage (day 7). However, the major lymphocytes became IL-2Rβ^+CD3^{int} cells which lacking NK1.1 at the fulminant stage (days 14 and 21). At this time, granulocytes expanded prominently. Since IL-2Rβ^+CD3^{int} cells (NK1.1$^-$) lacked cytotoxicity, the suppression of expanding donor cells might be mediated by granulocytes. Granulocytes were activated by inflammatory cytokines. These results suggest that in addition to NK1.1-expressing cells (e.g., NK and NKT cells), IL-2Rβ^+CD3^{int} cells lacking NK1.1 may be also the lymphocyte subset which recognizes MHC class I-deficient self.

Many investigators have believed that missing self (i.e., MHC-negative self cells) is recognized by NK cells and that such abnormal cells generated in the body might be eliminated by NK cell-mediated cytotoxicity (3, 4, 8, 12, 23, 28). At such time, the function of killer inhibitory receptor (KIR) on NK cells is important for recognition of the deficiency of MHC molecules on abnormal self (16, 24, 25). At this time, 9 Gy-irradiation of lethal dose was applied. However, this lethal dose is somewhat dangerous as a clinical dose of irradiation before bone marrow transplantation (BMT). In this regard, we applied 6 Gy-irradiation for BMT in the present study.

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It is speculated that NK cells are the most primordial lymphocytes in phylogeny (1). In addition to NK cells, other primordial lymphocytes have been characterized as components of innate immunity. Such cells include natural killer T cells (NKT cells) (13, 21, 22) and NK1.1$^-$ intermediate TCR cells (NK1.1$^-$TCR^{int} cells) (18, 19, 32). NKT cells are generated through an alternative pathway in the thymus and home to the liver (5–7, 10, 11), whereas NK1.1$^-$TCR^{int} cells are generated extrathymically in the liver. All T cells present in athymic nude mice belong to NK1.1$^-$TCR^{int} cells (9). It is of interest that these NK1.1$^-$TCR^{int} cells express Ly49 inhibitory receptors (Kawamura T, unpublished observation).

In the present study, we investigated how these primordial T cells are associated with the recognition of missing self. C57BL/6 (B6) Ly5.1 (B6.Ly5.1) mice were used as recipients after 6 Gy-irradiation. On the other hand, bone marrow cells isolated from

TAP-1 (-/-) mice (Ly5.2$^+$, lack of MHC class I expression) were intravenously injected as a source of missing self. Contrary to our initial expectation, in the present study, NK1.1$^-$TCR^{int} cells (extrathymic T cells) were found to be major lymphocytes which interact with abnormally missing self, at least under present applied conditions.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) (H-2^b Ly5.2$^+$), B6.Ly5.1 (H-2^b Ly5.1$^+$), TAP-1 (-/-) (H-2^b Ly5.2$^+$) (29), and BALB/c (H-2^d) mice at the age of 8 to 12 weeks were used. These mice were originally obtained from Charles River Japan (Kanagawa, Japan) and were maintained in the animal facility of Niigata University. All mice were fed under specific pathogen-free conditions.

Bone marrow transplantation into 6 Gy-irradiated B6 mice. Recipient B6 mice were irradiated with 6 Gy, and 1×10^7 bone marrow cells of various mice were immediately injected. Bone marrow cells were depleted of mature T cells by in vitro treatment with anti-Thy1.2 (5a-8) mAb and complement (rabbit sera which had absorbed mouse lymphocytes). Donor mice were syngeneic B6.Ly5.2 mice, TAP-1 (-/-) mice, and allogeneic BALB/c mice.

Cell preparation. Mice anesthetized with ether were sacrificed by complete exsanguination through axillary arteries and veins (31). Specimens from the liver and spleen were removed and kept in PBS (pH 7.2) on ice until cell preparation. To obtain liver leukocytes, the liver obtained from one mouse was cut into small pieces with scissors, pressed through 200-gauge stainless-steel mesh, and then suspended in 40 ml of MEM supplemented with 5 mM Hepes (Nissui Pharmaceutical Co., Tokyo, Japan) and 2% heat-inactivated newborn calf serum. After being washed once with the medium, the cells were fractionated by centrifugation in 15 ml of 35% Percoll solution containing 100 IU/ml heparin for 15 min at 2000 rpm. The pellet of cells containing RBC was resuspended and washed with the medium. To deplete RBC, ammonium chloride (155 mM NH_4Cl + 10 mM $KHCO_3$ + 1 mM EDTA-Na)/Tris-HCl buffer (0.17 M, pH 7.6) solution was added, and the solution was kept on ice for 5 min. After being washed twice with the medium, liver leukocytes were suspended in 1 ml of the medium and the number of cells was counted.

Spleen cells were obtained by forcing each organ through 200-gauge stainless-steel mesh. To deplete RBC, spleen cells were lysed with the ammonium chloride-Tris buffer and washed twice with the medium.

Bone marrow cells were obtained by flushing femurs with the medium. To deplete RBC, bone marrow cells were lysed.

Immunofluorescence tests. The surface phenotype of cells was analyzed using mAbs in conjunction with a Tri-color immunofluorescence test (27). The mAbs used here included FITC-, phycoerythrin (PE)-, or biotin-conjugated reagents of anti-CD3 (145-2C11), anti-H-2K^b (AF6-88.5), anti-IL-2Rβ (TM-β1), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-NK1.1 (PK136), anti-Ly5.1 (A20, 1.7), anti-Gr-1 (RB6-8C5) mAbs (PharMingen Co., San Diego, CA), and anti-Mac-1 (M1/70.15) mAb (Caltag Lab., San Francisco, CA). Biotin-conjugated reagents were developed with Tri-color conjugated streptavidin (Caltag Lab., San Francisco, CA). The fluorescence-positive cells were analyzed with FACScan using Lysis II software (Becton-Dickinson Co., Mountain View, CA).

Cytotoxicity assay. Target cells were YAC-1 cells for NK cytotoxicity (20). Con A blasts of splenic lymphocytes obtained from various mice were also used as target cells for the assay of auto- and allo-reactivity. Splenic lymphocytes were cultured *in vitro* with Con A (2 μg/ml) for 2 days and then used as a target. Cytotoxicity activity was measured by a specific ^{51}Cr release assay (14). Labeled targets (2×10^4/well) were incubated in a total volume of 200 μl with effectors in RPMI 1640 medium supplemented with 10% FCS in a 96-well round-bottomed microculture plate. Incubation for 4 h was performed.

Reverse transcription (RT)-PCR for IL-1β, IL-6, TNFα, and GM-CSF. Total RNA was extracted from tissues (30). To detect mRNAs of IL-1β, IL-6, TNFα, and GM-CSF, RNA was subjected to reverse transcription using the primers of these genes and such cDNA was further amplified by the PCR method (30). Briefly, total RNA was prepared from tissues of various organs by using Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 5 μg RNA with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) and Oligo (dT) 15 Primer (Promega, Madison, WI). PCR amplification of synthesized cDNA was conducted as previously described (30). PCR products as well as markers were estimated by staining with ethidium bromide. Primers for G3PDH were used to assess

the integrity of the RNA preparation.

Histology. Tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections 4 μm in thickness were stained with hematoxylin and eosin.

ELISA assay for the detection of IL-2 and IFNγ. Pooled sera were used to detect the concentrations of IL-2 and IFNγ by ELISA assay using Opt EIA mouse IL-2 and IFNγ sets (PharMingen).

Luminol-dependent chemiluminescence. Luminol-dependent chemiluminescence was used to determine the superoxide production in granulocytes, by use of a lumiphotometer (TD-4000: Labo Science, Tokyo, Japan) as reported previously (2). Liver and spleen cells were suspended to a concentration of 2×10^6/ml in 50 μl of buffer II, which consisted of 10 mM Hepes, 5 mM KCl, 145 mM NaCl, and 5.5 mM glucose (pH 7.4). To the cells were added 100 μl of 200 μM luminol solution (Wako, Osaka, Japan), 50 μl of buffer I, which consisted of buffer II supplemented with 1 mM $CaCl_2$ and 100 μl of phorbol mirystate acetate (PMA) (10 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA). Chemiluminescence was monitored by use of the lumiphotometer for 20 min and expressed in relative light units (rlu).

Depletion of NK and NKT cells. To deplete NK and NKT cells, mice were intraperitoneally injected with 0.5 mg of anti-NK1.1 mAb (PK136) every 2 days, starting on day 3 before BMT.

RESULTS

Expansion of missing self cells in recipient mice

Six Gy-irradiated B6 mice (H-2^b, Ly5.1^+) were injected with bone marrow cells from various sources (depleted of T cells), including syngeneic B6 mice (Ly5.2^+), TAP-1 (-/-) mice (B6 background, Ly5.2^+), and allogeneic BALB/c mice (H-2^d). Using a marker of Ly5.1 or Ly5.2, we distinguished the lymphocyte origin of either recipient or donor. In the case of allogeneic combination, the lymphocyte origin of a recipient or a donor was identified by the expression of H-2^b or H-2^d.

The lymphocyte origin was identified in the liver, spleen, and BM of recipient mice on days 7, 14, and 21 after BMT (Fig. 1a). In the liver, total lymphocytes were recovered almost equally in all mice. In the spleen, total lymphocytes increased rapidly in mice which had undergone BMT of TAP-1 (-/-) origin and allogeneic origin. On the other hand, the recovery of bone marrow cells tended to be retarded in mice which had undergone BMT of allogeneic origin (especially on day 14).

Identification of donor cells among total lymphocytes was then conducted (Fig. 1b). The highest rate of donor cells was found in the liver, spleen, and BM of recipient mice which had undergone syngeneic BMT. On the other hand, the rate of donor cells was somewhat lower in mice which had undergone BMT of TAP-1 (-/-) origin than in mice which had undergone BMT of syngeneic origin. In sharp contrast, the lowest rate of donor cells was seen in mice which had undergone allogeneic BMT. These results suggested that the expansion of missing self cells, in spite of syngeneic combination (H-2^b), i.e., MHC class I⁻ of TAP-1 (-/-) cells, were somewhat suppressed in the recipient mice. The magnitude of this suppression was lower than that of the allogeneic combination. Namely, missing self cells significantly expanded in recipient mice.

Histological study

In an earlier experiment, it was suggested that many recipient lymphocytes (approximately 60%) interact-

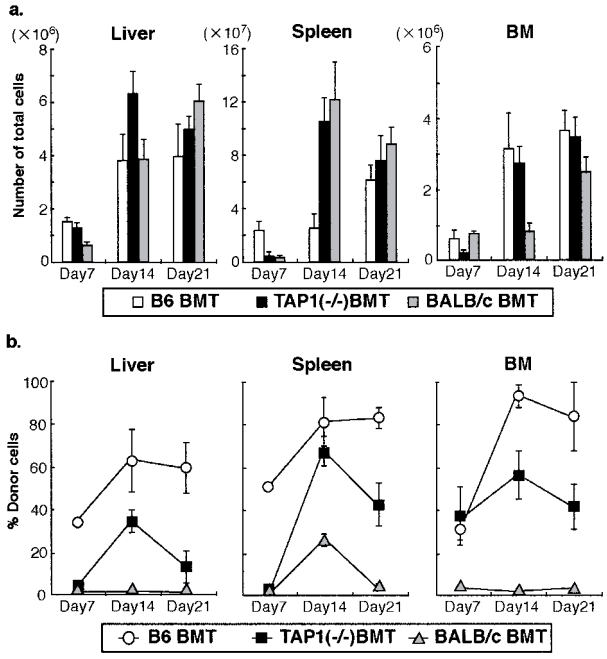

Fig. 1 Recovery of lymphocytes after BMT. a. total lymphocytes including both donor and recipient cells, b. recovery of donor cells. Recipient mice which had undergone BMT were B6.Ly5.1 mice (H-2^b), whereas donor mice were B6 mice (Ly5.2), TAP-1 (-/-) mice or BALB/c mice (H-2^d). The mean and one SD were produced from 4 experiments.

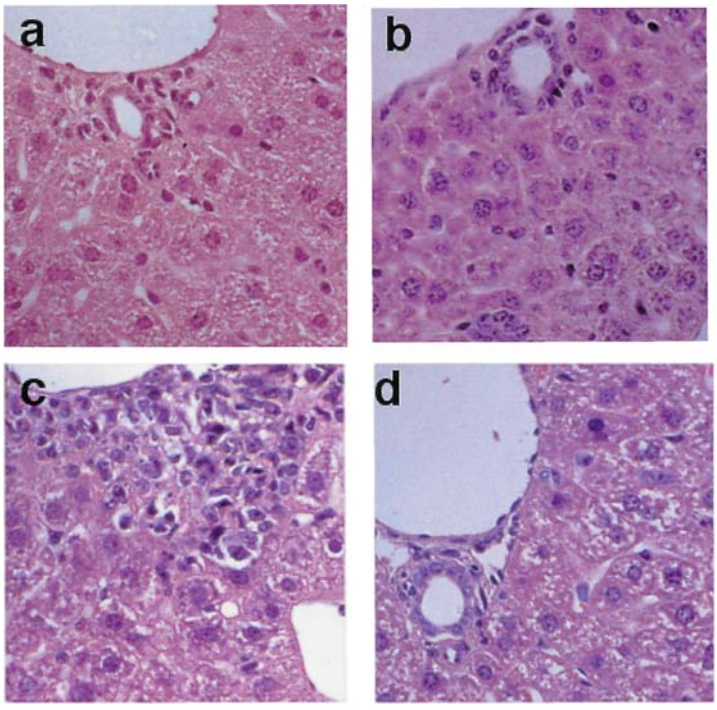

Fig. 2 Histology of the liver on day 21 after BMT. a. normal liver, b. liver of mice which had undergone syngeneic BMT, c. liver of mice which had undergone BMT from TAP-1 (-/-) mice, d. liver of mice which had undergone allogeneic BMT. Histology of the liver was produced by hematoxylin and eosin.

ed with missing self cells (approximately 40%), which were generated by BMT of TAP-1 (-/-) origin, in the liver on day 14. This liver histology (Fig. 2) was compared with others (a. normal liver, b. syngeneic BMT on day 14, and d. allogeneic BMT on day 14). The most prominent accumulation of lymphocytes was seen in the liver of mice which had undergone BMT of TAP-1 (-/-) mice (Fig. 2c).

Identification of lymphocyte subsets among expanding cells

To identify lymphocyte subsets among expanding donor ($Ly5.1^-$) cells, three-color staining for Ly5.1, CD3, and NK1.1 was conducted (Fig. 3). In the case of syngeneic BMT, all lymphocytes expanding in the liver, spleen, and BM were NK cells or $NK1.1^-$ $CD3^-$ cells (undetermined subsets). On the other hand, not only NK cells but also NKT cells expanded in the liver, spleen, and BM of recipient mice with BMT of TAP-1 (-/-) origin. In contrast, $CD3^+$T cells (including $CD3^{int}$ and $CD3^{high}$ cells), but neither NK nor NKT cells, expanded in mice with allogeneic BMT.

Experiments were then focused on the identification of lymphocyte subsets among expanding recipient ($Ly5.1^+$) cells. In these experiments, day 7 (Fig. 4a) and day 21 (Fig. 4b) were examined. All NK, NKT and T cells expanded in the liver and spleen of recipient mice which had undergone syngeneic BMT (Fig. 4a). The results from BMT of TAP-1 (-/-) origin were of also interest. The major expanding lymphocyte subset was estimated to be NKT cells, especially in the liver and BM. To some extent, this

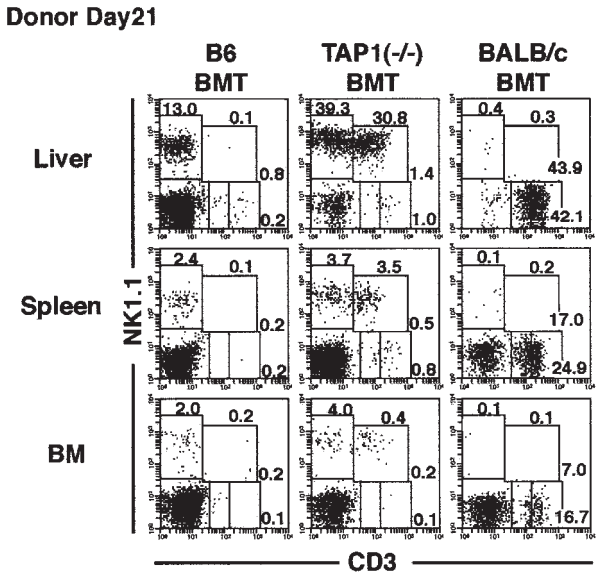

Fig. 3 Phenotype of donor cells in various organs. Lymphocytes were isolated on day 21 after BMT. Three-color stainings for Ly5.1 (or H-2^d), NK1.1 and CD3 were conducted. By gated analysis ($Ly5.1^-$ or H-2^{d+}), the expression of NK1.1 and that of CD3 were determined. Representative findings of three experiments are depicted.

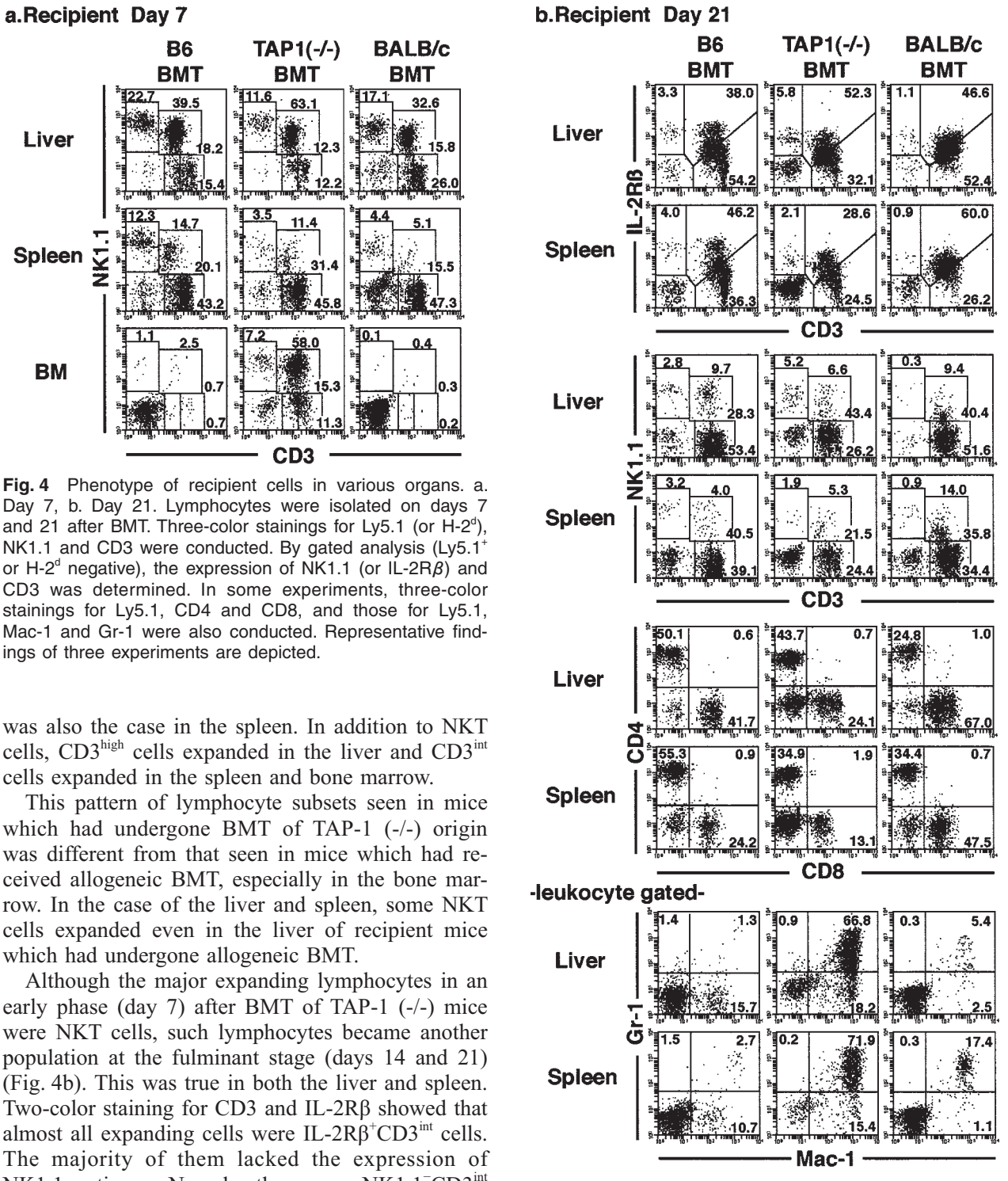

Fig. 4 Phenotype of recipient cells in various organs. a. Day 7, b. Day 21. Lymphocytes were isolated on days 7 and 21 after BMT. Three-color stainings for Ly5.1 (or H-2^d), NK1.1 and CD3 were conducted. By gated analysis (Ly5.1^+ or H-2^d negative), the expression of NK1.1 (or IL-2Rβ) and CD3 was determined. In some experiments, three-color stainings for Ly5.1, CD4 and CD8, and those for Ly5.1, Mac-1 and Gr-1 were also conducted. Representative findings of three experiments are depicted.

was also the case in the spleen. In addition to NKT cells, $CD3^{high}$ cells expanded in the liver and $CD3^{int}$ cells expanded in the spleen and bone marrow.

This pattern of lymphocyte subsets seen in mice which had undergone BMT of TAP-1 (-/-) origin was different from that seen in mice which had received allogeneic BMT, especially in the bone marrow. In the case of the liver and spleen, some NKT cells expanded even in the liver of recipient mice which had undergone allogeneic BMT.

Although the major expanding lymphocytes in an early phase (day 7) after BMT of TAP-1 (-/-) mice were NKT cells, such lymphocytes became another population at the fulminant stage (days 14 and 21) (Fig. 4b). This was true in both the liver and spleen. Two-color staining for CD3 and IL-2Rβ showed that almost all expanding cells were IL-2Rβ^+CD3^{int} cells. The majority of them lacked the expression of NK1.1 antigens. Namely, they were $NK1.1^-CD3^{int}$ cells and a mixture of $CD4^+$ and $CD8^+$ cells. A similar result was observed in mice which had undergone syngeneic and allogeneic BMT. In the case of allogeneic BMT, $CD8^+$ cells were much more predominant than $CD4^+$ cells.

Another evidence was that granulocytes (i.e., Mac-1^+Gr-1^+) became abundant in mice which had undergone BMT of TAP-1 (-/-) origin (see Fig. 4b, bottom). In other words, missing self cells were eliminated (or suppressed) by the expansion of both $NK1.1^-CD3^{int}$ cells and granulocytes.

Functional assays for cytotoxicity of lymphocytes

It was speculated that either lymphocytes ($NK1.1^-$ $CD3^{int}$ cells) or granulocytes might act as suppressor cells against missing self. In this regard, cytotoxicity against various targets was examined using effector cells (i.e., hepatic lymphocytes) isolated from mice which had undergone BMT of TAP-1 (-/-) origin (Fig. 5). Effector cells were also isolated from normal B6 mice and mice which received allogeneic BMT. NK cytotoxicity against YAC-1 cells was examined in parallel. NK activity was only seen in effector cells of normal B6 mice. More interestingly, effector cells isolated from normal B6 mice and mice which had undergone BMT of TAP-1 (-/-) origin did not have any cytotoxicity against Con A blasts of B6 origin, including those of TAP-1 (-/-) mice. This was also true in effector cells of mice which had undergone allogeneic BMT. On the other hand, allogeneic cytotoxicity against Con A blasts of BALB/c origin was induced in effector cells of mice which had undergone allogeneic BMT.

Superoxide production of granulocytes

We previously reported that $NK1.1^-CD3^{int}$ cells sometimes induced the activation of granulocytes via their production of IFNγ, TNFα, or GM-CSF (i.e., inflammatory cytokines) (15, 19). This possibility was also examined in these experiments (Fig. 6). The activation of granulocytes was examined as an indicator of superoxide production by chemiluminescence. Granulocytes were isolated from both the liver and spleen and stimulated with PMA. It was found that granulocytes isolated on days 14 and 21 after BMT of TAP-1 (-/-) origin produced a high titer of superoxides upon stimulation, especially those isolated from the liver.

Cytokine profile

We then examined the cytokine profile of lymphocytes isolated from mice which had undergone various BMT. First, the production of IFNγ and IL-2 was examined in sera of various mice (Fig. 7a). Only mice which had undergone allogeneic BMT produced IFNγ and IL-2 (i.e., Type 1 cytokine profile).

Other cytokines were analyzed by the RT-PCR method (Fig. 7b). In this case, total mRNA was isolated from the tissue itself, because it is known that

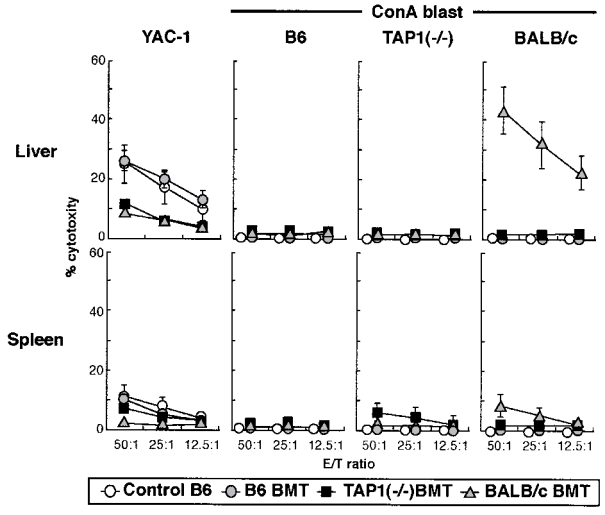

Fig. 5 Cytotoxicity activity against YAC-1 cells and Con A blasts. Effector lymphocytes were isolated on day 21 after BMT. Target cells were YAC-1 cells for NK cytotoxicity and Con A blasts were of various origins for specific cytotoxicity. Cytotoxicity was determined by 4-h incubation at the indicated E/T ratios. The mean and one SD were produced in triplicate cultures. Representative findings of three experiments are depicted.

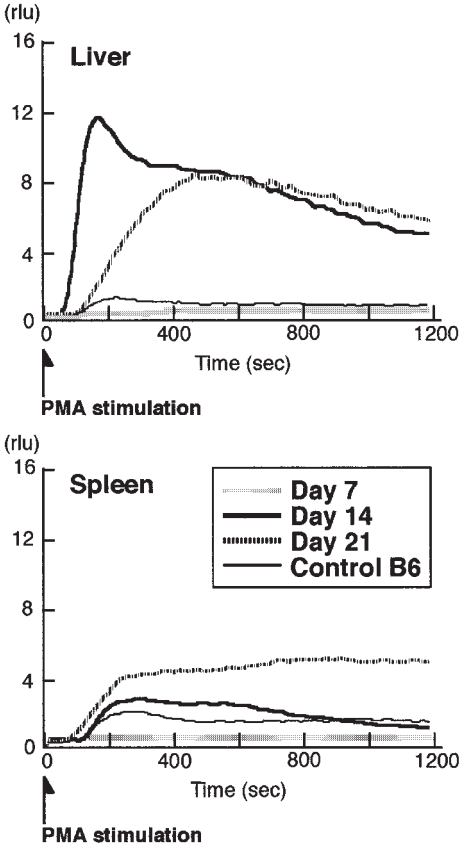

Fig. 6 Superoxide production by granulocytes in the liver and spleen. Whole leukocytes were isolated from the liver and spleen at the indicated days after BMT from TAP-1 (-/-) mice. PMA stimulation was conducted at the chemiluminescence assay.

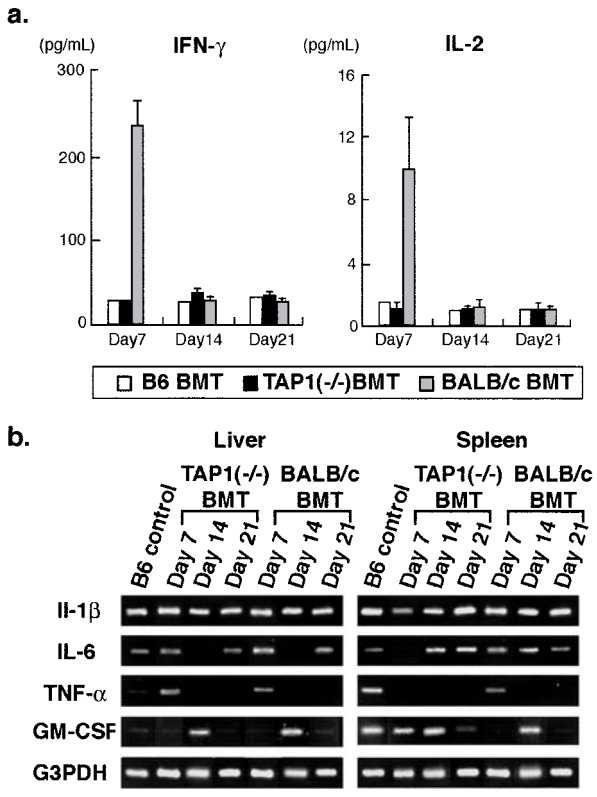

Fig. 7 Cytokine profile after BMT. a. serum level of IFNγ and IL-2, b. inflammatory cytokines identified by RT-PCR. Serum was obtained at the indicated days after BMT, and IFNγ and IL-2 were detected by ELISA assay. Total RNA was isolated from the whole liver and spleen on day 7, 14, and 21 after BMT. Message of inflammatory cytokines was examined by RT-PCR method.

hepatocytes or Kupffer cells are important producers of inflammatory cytokines. The spleen was also used for mRNA isolation. Although the peak time varied, IL-1β, IL-6, TNFα, and GM-CSF were found to be produced by both the liver and spleen.

Effect of the elimination of NK and NKT cells on the recognition of missing self

To directly examine the function of NK and NKT cells in the present phenomenon, experiments in which these cells were eliminated by anti-NK1.1 mAb (PK136) were conducted (Fig. 8). Irradiated mice with or without the treatment were subjected to BMT of TAP-1 (-/-) origin. The number of lymphocytes yielded by the liver and bone marrow remained almost unchanged (Fig. 8a). However, the ratio of donor cells increased prominently in both the liver and bone marrow (Fig. 8b).

At an early stage (day 7), NK and NKT cells expanded and the treatment of PK136 was effective (Fig. 8c). Even in mice treated with PK136, the major expanding lymphocytes were IL-2Rβ$^+$CD3^{int} cells expressing the phenotype of NK1.1$^-$, CD4$^+$ (Fig. 8d). In this case, the expansion of granulocytes became prominent (Fig. 8d) and resulted in an increase in leukocytes accumulating in the liver (Fig. 8e). These results indicated that NK and NKT cells first recognized missing self and then suppressed the expansion of such missing self (i.e., donor cells). When NK and NKT cells were absent, NK1.1$^-$ CD3^{int} cells and granulocytes interacted with the missing self from the early stage.

DISCUSSION

In the present study, we demonstrated that NK1.1$^-$ TCR^{int} cells were the major lymphocytes which interacted with missing self. Missing self cells were produced in the present study by BMT from TAP-1 (-/-) mice into 6 Gy-irradiated B6 mice. In an early stage after BMT (day 7), NK cells and NKT cells were rather predominant lymphocytes in various immune organs of the recipient mice. This was due to their radioresistance to recipient cells and to their early recovery property of donor cells. However, in the subsequent fulminant stage (days 14 and 21), NK1.1−TCR^{int} cells became the most predominant lymphocytes (of recipient origin) in all tested organs. Since donor MHC class I$^-$ cells began to expand at that stage, these recipient cells might interact with such missing self cells. Another evidence was that granulocytes parallelly expanded in these mice which had undergone BMT of TAP-1 (-/-) origin. On the other hand, expanding donor cells tended to become NK and NKT cells. It is speculated that conventional T cells (and B cells) might be sensitive to the suppression by recipient effector cells.

The origins of donor cells and recipient cells were identified by markers of Ly5.1, Ly5.2, and alloantigen in the present study. The suppression of donor cells was most prominent in allogeneic BMT. It is easily speculated that allorecognition was the greatest in this combination. On the other hand, the suppression of expanding donor cells was greater in missing self BMT than in syngeneic BMT. In the other words, missing self cells were positively recognized by recipient lymphocytes and such lymphocytes were found to be IL-2Rβ$^+$TCR^{int} cells (NK1.1$^-$). They were a mixture of CD4$^+$ and CD8$^+$ cells. As we have previously reported (13, 21), these lymphocytes are present in all lymphoid organs, including the liver, and are estimated to be of truly extrathy-

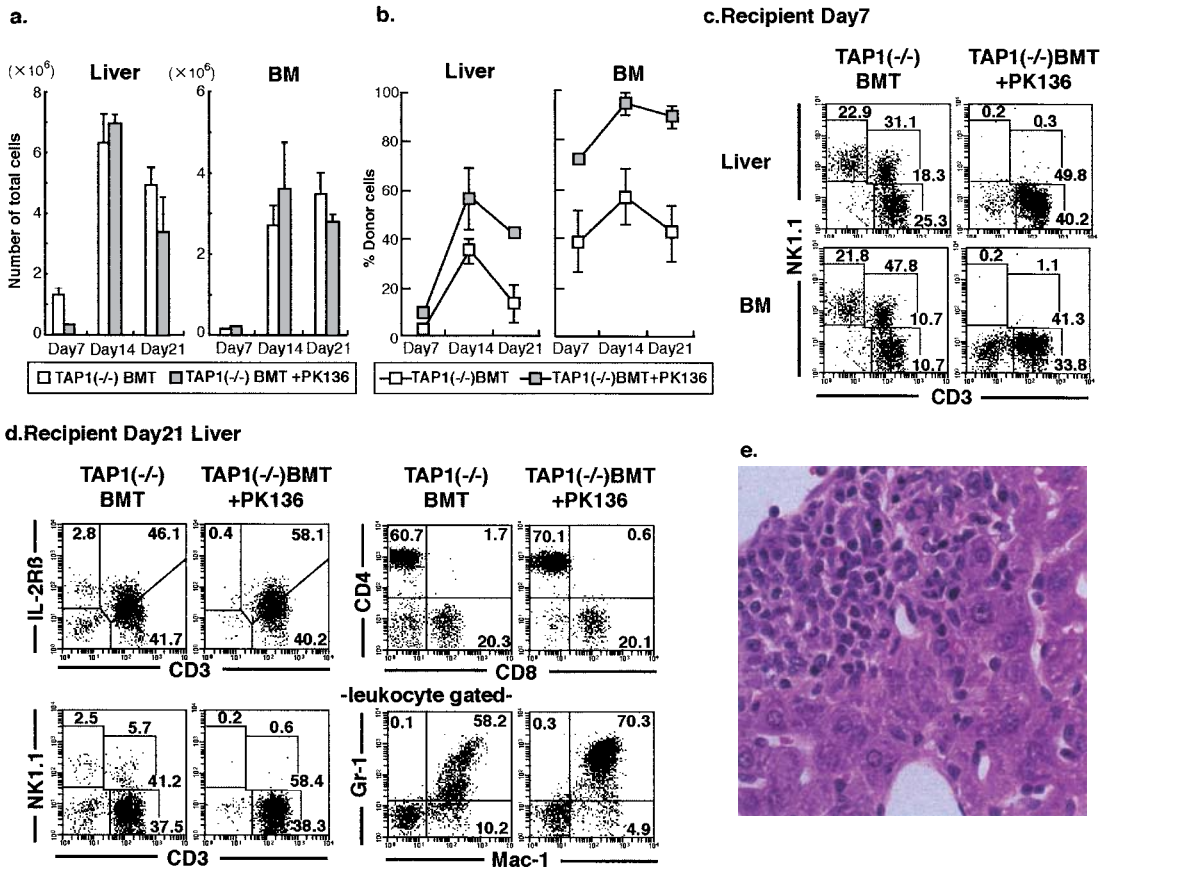

Fig. 8 Effect of the elimination of NK1.1$^+$ cells on the expansion of donor and recipient lymphocytes after BMT from TAP-1 (-/-) mice. a. Number of lymphocytes in the liver and bone marrow (BM), b. Ratio of donor cells in the liver and BM, c. Phenotype of recipient lymphocytes on day 7, d. Phenotype of recipient cells on day 21, e. Histology of the liver on day 21 after BMT, i.e., TAP-1 (-/-) +PK136. Phenotype of recipient lymphocytes was identified by three-color staining for Ly5.1, NK1.1, and CD3. (or combinations of Ly5.1 and others).

mic origin (1, 9).

We could not detect a direct cytotoxicity against missing self cells of TAP-1 (-/-) origin in recipient mice (days 14 and 21). In sharp contrast, allogeneic cytotoxicity was induced in the liver of mice which had undergone allogeneic BMT. At this time, no augmentation of NK cytotoxicity against YAC-1 cells was evident. In other words, IL-2Rβ^+CD3^{int} cells (NK1.1$^-$) primarily lacked cytotoxicity in other cases. Since the expansion of IL-2Rβ^+CD3^{int} cells (NK1.1$^-$) is known to accompany the activation of granulocytes (15, 19), parameters of granulocytes were examined in parallel. It was found that there was a prominent expansion of granulocytes in both the liver and spleen at the fulminant stage. These granulocytes were eventually at the activated state in terms of superoxide production. Taken together, these findings indicate that granulocytes, rather than IL-2Rβ^+TCR^{int} cells lacking cytotoxic function, might be actual effector cells which suppress the expansion of missing self cells (Fig. 9).

We then examined how TCR^{int} cells activated granulocytes after BMT from TAP-1 (-/-) mice. Various signs of the production of inflammatory cytokine were demonstrated at the late fulminant phase. Such cytokines included IL-1β, IL-6, TNFα, and GM-CSF. It is speculated that these inflammatory cytokines mediate the activation of granulocytes as shown by Fig. 9.

There have been many studies of NK cytotoxicity against missing self cells (TAP-1 (-/-) Con A blasts) *in vitro* (17, 26). In those experiments, NK cells activated by rIL-2, or Tilorone (IFNγ inducer) were used as effector cells. In the present study, we confirmed that naive lymphocytes isolated from the liver and spleen of normal mice did not have cytotoxicity against TAP-1 (-/-) blasts *in vitro*. This result suggests that NK cytotoxicity for missing self

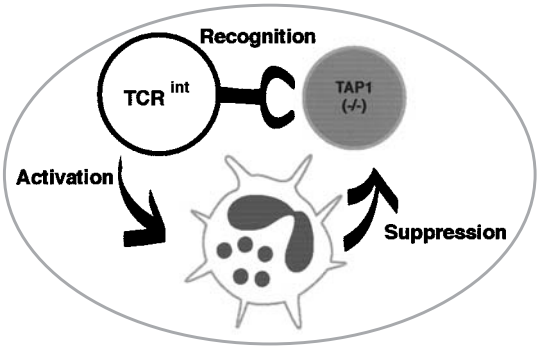

Fig. 9 Schematic representative of the recognition of missing self cells by TCR^{int} cells. Since TCR^{int} cells lack cytotoxicity, granulocytes activated by TCR^{int} cells may directly suppress the expansion of missing self cells.

cells requires activation by IL-2, INFγ, or some other cytokines. We did not detect IL-2 and IFNγ in sera of mice which had undergone TAP-1 (-/-) BMT, but only in those which had undergone allogeneic BMT at the early phase. These results can be understood as follows: the function of KIR on NK cells is important for protection of self cells expressing MHC class I from attack by activated-NK cells in cases of exposure to allo-antigen and such abnormal cells. However, in the case of TAP-1 (-/-) BMT, NK cells cytotoxicity did not have cytotoxicity against TAP-1 (-/-) blasts, but were suppressed. Therefore, the function of NK cells is indicated to be incomplete, which explains missing self rejection *in vivo*.

As one of the control experiments, allogeneic BMT was conducted in this study. The results were of interest, because the most prominent suppression of donor cells (i.e., allogeneic suppression) was seen in this experiment. In this situation, $NK1.1^{-}$T cells were the major donor cells which expanded in various organs. This was also true in recipient cells. Allogeneic killer activity was induced in these $NK1.1^{-}$ T cells. Granulocytes did not expand in this case. These results confirmed our previous observation that extrathymic T cells recognize allogeneic lymphocytes with both MHC class I disparity and MHC class II disparity in athymic nude mice (9).

In a final portion of experiments, we examined the actual function of NK and NKT cells in the recognition process of missing self. It was demonstrated that the expansion of missing self cells became prominent by the *in vivo* treatment of anti-NK1.1 mAb (PK136) in both the liver and bone marrow, especially at the early phase. However, such expansion of missing self cells was finally suppressed at the fulminant phase in recipient mice. At that time, $IL\text{-}2R\beta^{+}CD3^{int}$ cells expressing the phenotype of $NK1.1^{-}$, $CD4^{+}$ became prominent and granulocytes also expanded in the liver. These results suggest that the major lymphocyte subset which interacted with missing self was $NK1.1^{-}TCR^{int}$ cells and that direct effector cells which suppressed missing self were activated granulocytes.

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