

Intensive generation of NK1.1⁻ extrathymic T cells in the liver by injection of bone marrow cells isolated from mice with a mutation of polymorphic major histocompatibility complex antigens

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SUMMARY

Whether intermediate TCR (TCR^{int}) cells and natural killer T (NKT or NK1.1⁺TCR^{int}) cells are extrathymically generated remains controversial. This arises from the fact that there are few of these T cells in athymic nude mice and neonatally thymectomized mice. However, when athymic mice were provided with appropriate microenvironments or stimulation, many TCR^{int} cells (mainly NK1.1⁻) were found to arise in the liver. NKT cells are known to be positively selected by monomorphic major histocompatibility complex (MHC) -like antigens (e.g. CD1d). This is true even if they are CD4⁺. In other words, a MHC class I-like antigen is restricted to CD4 antigen. This rule is somewhat different from that seen in conventional T cells (i.e. the restriction of class II with CD4 and that of class I and CD8). In the case of NK1.1⁻TCR^{int} cells, they were selected by polymorphic MHC antigens, but their MHC restriction to CD4 or CD8 antigen was incomplete. This was revealed by experiments of bone marrow transfer with class I (bm 1) or II (bm 12) disparity. Depending on the disparity, a unique cytokine profile in sera was detected. These results suggest that the development of T lineage lymphocytes and MHC restriction to CD4 and CD8 might have occurred in parallel as a phylogenetic event, and that NK1.1⁻ extrathymic T cells (i.e. NK1.1⁻TCR^{int}) are at an intermediate position between NKT cells and conventional T cells in phylogeny.

INTRODUCTION

Unusual T cells, such as double-negative (DN) CD4⁻CD8⁻ T cells or T cells coexpressing a natural killer (NK) marker (NK1.1), i.e. natural killer T (NKT) cells, have been found in the thymus of mice,^{1–3} although these T cells are also present in other immune organs. In contrast to conventional T cells, which are generated through the mainstream of T-cell differentiation in the thymus, these DN (and CD4⁺) NKT cells are generated by an alternative intrathymic pathway. Subsequent studies have revealed that NKT cells recognize some antigens in the context of monomorphic major histocompatibility complex (MHC) -like (or MHC) antigens (e.g. CD1d and

TL)^{4,5} and use a restricted T-cell receptor (TCR) repertoire of V α 14J α 281 and V β 8.2 (or V β 7 or V β 2).⁶ On the other hand, we have independently characterized intermediate TCR (TCR^{int}) cells which, similarly to NK cells, consistently express interleukin-2 receptor β (IL-2R β).⁷ These T cells are abundant in the liver and are considered to be of extrathymic origin.

A subsequent study has shown that IL-2R β ⁺ TCR^{int} cells in the liver comprise both NK1.1⁺ and NK1.1⁻ subsets at a ratio of approximately 1:1.⁸ Moreover, similar NK1.1⁺ TCR^{int} (i.e. NKT cells) and NK1.1⁻ TCR^{int} populations are found even in the thymus. This raises two questions as follows: whether all NK1.1⁺ TCR^{int} and NK1.1⁻ TCR^{int} cells in the liver are supplied by the thymus and how NK1.1⁺ TCR^{int} and NK1.1⁻ TCR^{int} cells are regulated by MHC antigens in their differentiation. The first question arises from the fact that there are few of these unusual T cells in the liver of athymic nude mice⁹ and neonatally thymectomized mice.¹⁰ The second question stems from the fact that there is little information about the regulation of NK1.1⁻ TCR^{int} cells by MHC antigens. On the other hand, NKT cells are characterized as being regulated by non-classical MHC-like antigens.^{4,5} In detail, NKT cells recognize some peptide antigens¹¹ or glycolipid antigens (e.g. α -galactosylceramide) in the context of CD1d.¹²

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Abbreviations: BMT, bone marrow cell transfer; DN, double-negative; IL-2R β , interleukin-2 receptor β -chain; NKT cells, natural killer T cells; TCR^{high} cells, high T cells; TCR^{int} cells, intermediate T cells.

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However, some NKT cells appear to recognize some antigens in the context of non-CD1d molecules.¹³

In this study, we examined what conditions are appropriate for the induction of extrathymic T cells in athymic nude mice. We then examined what types of MHC antigens regulate the differentiation of such extrathymic T cells. For this purpose, we investigated whether IL-2R β^+ TCR^{int} cells (including the NK1.1⁺ and NK1.1⁻ subsets) recognize the point mutations on polymorphic MHC antigens (i.e. bm 1 and bm 12 mutations). In bm 1 mice, there are three point mutations on the polymorphic MHC class I antigen (i.e. H-2K^{bm1}),¹⁴ while in bm 12 mice there are three such mutations on the polymorphic MHC class II antigen (i.e. I-A^{bm12}).¹⁵ Intensive studies have thus far indicated that the disparity of bm 1 is recognized by CD8⁺ conventional T cells and results in acute graft-versus-host disease (GVHD) after bone marrow transplantation (BMT; bm 1 \rightarrow B6 and *vice versa*).¹⁶ The disparity of bm 12 is recognized by CD4⁺ conventional T cells and results in chronic GVHD after BMT (bm12 \rightleftharpoons B6). We can thus expect to determine whether extrathymic T cells recognize the MHC antigen disparity of bm 1 or bm 12.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) and B6-nu/nu mice were used at the age of 8–40 weeks. These mice were originally obtained from Charles River Japan, Tokyo, Japan and were maintained in the animal facility of Niigata University under specific pathogen-free conditions. In some experiments, B6-C-H-2^{bm1} (bm 1) mice (i.e. H-2K^{bm1}) and B6-C-H-2^{bm12} (bm 12) mice (i.e. I-A^{bm12}) were used at the age of 10–12 weeks. These mice were Ly5.2⁺. F₁ mice were also prepared from bm 1 or bm 12 mice in conjunction with B6.Ly5.1 mice. These (bm 1 \times B6.Ly5.1) F₁ or (bm 12 \times B6.Ly5.1) F₁ mice were used at the age of 10–12 weeks.

Administration of interleukin-12

At the age of 40 weeks, the *nu/nu* mice were intraperitoneally injected with murine interleukin-12 (IL-12; 10³ U/mouse) (Genzyme Corporation, Cambridge, MA)

Immunofluorescence tests

The phenotypes of cells were determined by two- or three-colour immunofluorescence tests.⁸ For flow cytometric analysis, anti-CD3 (145-2C11), anti-IL-2R β (TM- β 1), anti-NK1.1 (PK136), anti-CD4 (RM4-5), anti-CD8 α (53-6.7), anti-CD8 β (53-5.8), anti-TCR $\alpha\beta$ (H57-597), and anti-TCR $\gamma\delta$ (GL3) monoclonal antibodies (mAb) were purchased from PharMingen (San Diego, CA). Mouse anti-Ly5.1 (A20.1.7) mAb was provided by Dr T. Kina (Kyoto University, Japan). All mAbs were used in a fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), or biotin-conjugated form. Biotinylated reagents were developed with TRI-COLOR-conjugated streptavidin (Caltag Lab., San Francisco, CA). Cells were analysed by fluorescence-activated cell sorter (FACScan; Becton Dickinson Co., Mountain View, CA). To prevent non-specific binding of mAb, CD32/16 (2-4G2) was added before staining with labelled mAb.

Cell transfer experiments

B6-*nu/nu* mice were irradiated with a sublethal dose (4 Gy), and 10⁷ bone marrow cells, obtained from mutant mice, were injected 1 day after the irradiation. Bone marrow cells were isolated from B6, bm 1, bm 12 (bm 1 \times Ly5.1) F₁, and (bm 12 \times Ly5.1) F₁ mice and were depleted of Thy-1⁺ cells by the complement-mediated cell lysis. These bone marrow cells were injected intravenously.

Identification of serum levels of interferon- γ and IL-4 by ELISA

Sera were harvested and levels of interferon- γ (IFN- γ) and IL-4 were determined by enzyme-linked immunosorbent assay (ELISA) as described previously.^{17,18}

RESULTS

Induction of NK1.1⁻ CD3^{int} cells in the liver of athymic nude mice, with aging or by the injection of IL-12

To determine whether TCR^{int} or CD3^{int} cells are generated in athymic nude mice, young (12-week-old) and old (40-week-old) B6-*nu/nu* mice, and old *nu/nu* mice injected with IL-12 (10³ U/mouse), were examined in parallel with young B6 mice (Fig. 1). Two-colour staining for CD3 and IL-2R β , for CD3 and NK1.1 and for CD4 and CD8 was conducted.⁸ Although the proportion of IL-2R β^+ CD3^{int} cells was small (1.3%) in the liver of young *nu/nu* mice, it increased (38.7%) in the liver of old *nu/nu* mice and such mice injected with IL-12 (71.1%). Two-colour staining for CD3 and NK1.1 and for CD4 and CD8(α) showed that the majority of CD3^{int} cells in old *nu/nu* mice were CD8⁺ NK1.1⁻. A few NK1.1⁺ subsets of CD3^{int} cells appeared in the expanding CD3^{int} cells in old *nu/nu* mice injected with IL-12. They were CD8⁺ (data not shown). On the other hand, NK1.1⁻ CD3^{int} cells in these mice were a mixture of CD8⁺ (30%) and DN CD4⁻ CD8⁻ (40%).

We further examined the phenotypes of these cells, in terms of TCR and CD8, in the liver of adult (25-week-old) *nu/nu* mice (Fig. 2). The majority were CD8 $\alpha^+\beta^+$ $\alpha\beta$ TCR⁺ cells, while there were few $\gamma\delta$ TCR⁺ cells.

Prominent expansion of NK1.1⁻ CD3^{int} cells in the liver, when athymic mice were sublethally irradiated and were subjected to BMT with MHC disparity

We conducted BMT from B6, bm 1, or bm 12 mice (depleted of Thy-1⁺ cells) into sublethally irradiated (4 Gy) *nu/nu* mice (8-week-old). Under these experimental conditions, either donor or recipient cells were recovered and gave rise to lymphocytes. Two weeks after BMT, lymphocytes were obtained from the liver, spleen and bone marrow and their phenotype was identified (Fig. 3). Two-colour staining for CD3 and IL-2R β showed that the expansion of NK cells and CD3^{int} cells had begun in the liver of these BMT mice (Fig. 3a). Although the expansion of NK cells was prominent, that of CD3^{int} cells was not so prominent in B6 \rightarrow B6 *nu/nu* mice. In sharp contrast, the expansion of CD3^{int} cells was prominent in bm 1 \rightarrow B6 *nu/nu* mice and in bm 12 \rightarrow B6 *nu/nu* mice. Similar to the case of control nude mice, these BMT mice lacked IL-2R β^+ TCR^{high} conventional T cells which were seen in control euthymic B6 mice.

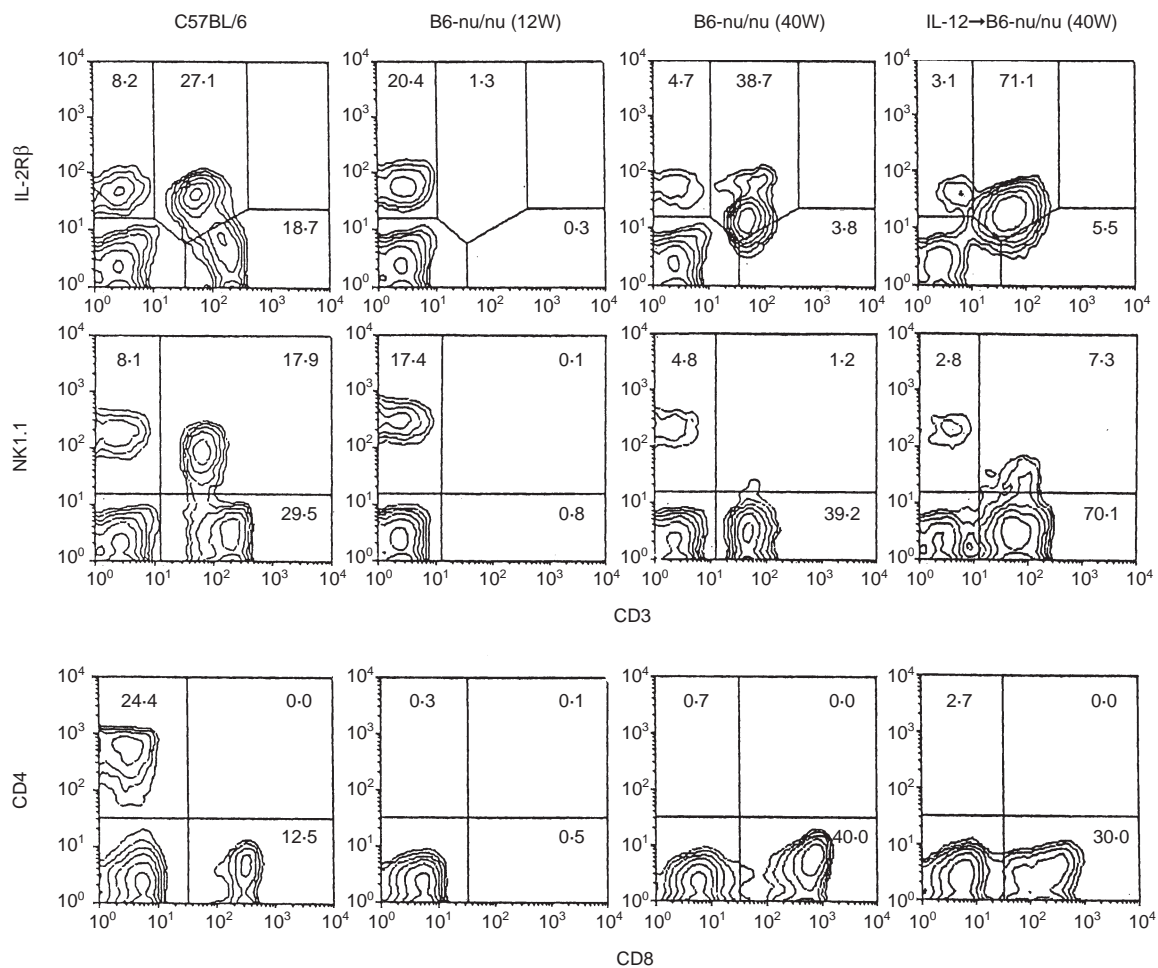


Figure 1. Athymic B6-*nu/nu* mice have the ability to produce IL-2R β^+ CD3^{int} cells. B6 and B6-*nu/nu* mice were used at the age of 12 weeks. The *nu/nu* mice at the age of 40 weeks were intraperitoneally injected with murine IL-12 (10^3 U/mouse) and were examined on day 3. Two-colour staining for CD3 and IL-2R β , for CD3 and NK1.1 and for CD4 and CD8 were conducted. Numbers in the figure represent the percentages of fluorescence-positive cells. The data shown here are representative of three experiments.

We examined which subset of CD3^{int} cells expanded in these BMT (bm 1 and bm 12) *nu/nu* mice (Fig. 3b). In both groups of mice, the majority of CD3^{int} cells were NK1.1⁻. In the case of control B6 mice, 67% (12.3/18.3) of CD3^{int} cells were NK1.1⁺.

Further characterization of expanding T cells in the liver was conducted by two-colour staining for the indicated combinations (Fig. 4). In the case of bm 1 BMT mice, the major expanding cells were CD8⁺ (20.7%) and DN (7.6%). On the other hand, a mixture of CD4⁺ (4.2%) and CD8⁺ (7.9%) cells expanded in bm 12 BMT mice. Both $\alpha\beta$ T (21.1%) and $\gamma\delta$ T (7.7%) cells expanded in bm 1 BMT mice, whereas only $\alpha\beta$ T cells expanded in bm 12 BMT mice.

In a final set of experiments, we determined the distribution of CD4⁺, DN CD4⁻ CD8⁻ and CD8⁺ cells among expanding T cells in the liver (Table 1). Normal B6 mice and B6 nude mice were examined in parallel. It was found that the number of CD3⁺ cells (0.09×10^6 /mouse) in the liver of nude mice was fewer than that (0.66×10^6 /mouse) in the liver of control B6 mice. However, bm 1 BMT nude (1.68×10^6 /mouse) and bm 12 BMT (0.75×10^6 /mouse) nude mice showed a high level of the number of CD3⁺ cells (all CD3^{int} cells) in the liver.

It was also confirmed that CD8⁺ and DN cells mainly expanded in the liver of bm 1 BMT nude mice while CD4⁺ and CD8⁺ cells mainly expanded in the liver of bm 12 BMT nude mice. In this experiment, the proportion of DN CD4⁻ CD8⁻ cells was evaluated by calculation, using the values of CD3⁺ cells, CD4⁺ cells and CD8⁺ cells which were produced by two-colour staining. Since the absolute number of NK1.1⁻ CD3^{int} cells yielded by the liver of bm 1 and bm 12 BMT mice was apparently greater than that by the liver of control B6 nude mice, the numbers of CD8⁺ and DN cells in bm 1 BMT nude mice and of CD4⁺ and CD8⁺ cells in bm 12 BMT nude mice were substantial.

Determination of the recipient or donor origin in the expanding T cells

To determine the interaction of lymphocytes between donor and recipient origins, we prepared (bm 1 \times Ly5.1) F_1 and (bm 12 \times Ly5.1) F_1 mice.¹⁹ When bone marrow cells from these F_1 mice were injected into irradiated *nu/nu* mice, we were able to distinguish the origins of recovering lymphocytes by the expression of Ly5.1 or Ly5.2 alloantigen. We conducted

Ly5.1 staining in the liver and spleen of control and BMT mice (Fig. 5). Lymphocytes of *nu/nu* mice (Ly5.2⁺) lacked Ly5.1 while all those of (bm 12 × Ly5.1) *F*₁ and (bm 1 × Ly5.1) *F*₁ mice (data not shown here) expressed Ly5.1. There was a big difference in the expression of Ly5.1 between (bm 1 × Ly5.1) *F*₁ → *nu/nu* mice and (bm 12 × Ly5.1) *F*₁ → *nu/nu* mice. These stainings were done on day 21 after BMT. The majority of recovering cells were of recipient origin in (bm 1 × Ly5.1) *F*₁ → *nu/nu* mice. In contrast, the recovering cells were a half-and-half mixture of donor and recipient origins in (bm 12 × Ly5.1) *F*₁ → *nu/nu* mice.

We also performed a time-kinetic study by repeated experiments as indicated in Fig. 5 (Fig. 6). In the liver and spleen, a similar pattern was observed. In the case of (bm 1 × Ly5.1) *F*₁ → *nu/nu* mice, the recovery of recipient cells was rapid but that of donor cells was suppressed. In the case of (bm 12 × Ly5.1) *F*₁ → *nu/nu* mice, the recovery of recipient cells was rather suppressed and that of donor cells was substantial.

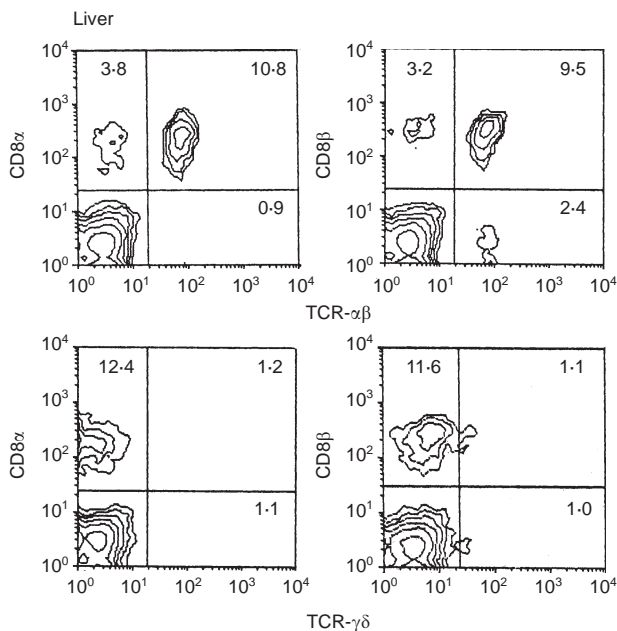


Figure 2. Further phenotypic characterization of CD3^{int} cells. Two-colour staining for αβTCR and CD8α (or CD8β) and for γδTCR and CD8α (or CD8β) were conducted. In this experiment, *nu/nu* mice at the age of 25 weeks were used and lymphocytes in the liver were examined.

Serum levels of IFN-γ and IL-4 in athymic nude mice after the subjection to BMT of bm 1 or bm 12 origin

In a final element of the present experiments, we examined what types of cytokines were released into the sera by the interaction of NK1.1⁻ CD3^{int} cells with class I or class II disparity cells (Fig. 7). In the case of bm 1 BMT (bm 1 → B6-*nu/nu*), high titres of both IFN-γ and IL-4 were detected on days 14 and 21 in the sera of nude mice. On the other hand, a high titre of only IL-4 was induced in the sera of nude mice subjected to bm 12 BMT (bm 12 → B6-*nu/nu*). Since control mice (B6 → B6-*nu/nu*) did not show any positive levels of IFN-γ and IL-4 in the sera, the positive results of bm 1 → B6-*nu/nu* and bm 12 → B6-*nu/nu* mice were suggested to be derived from the interaction of bm 1 and bm 12 mutations with the recipient mice.

DISCUSSION

In the present study, new evidence regarding several points emerged. Euthymic mice carried a large number of IL-2Rβ⁺ TCR^{int} and NK1.1⁺ TCR^{int} cells (i.e. NKT cells) in the liver. On the other hand, athymic nude mice, especially in youth, produced neither IL-2Rβ⁺ TCR^{int} cells nor NK1.1⁺ TCR^{int} cells even in the liver. However, with aging, athymic mice produced IL-2Rβ⁺ TCR^{int} cells but still did not produce NK1.1⁺ TCR^{int} cells in the liver. Namely, the majority of TCR^{int} cells were the NK1.1⁻ subset (CD8α⁺β⁺ or DN). The microenvironments in the liver or a lack of thymic influence might be associated with this unique profile seen in athymic nude mice. The absence of IL-2Rβ⁺ TCR^{high} conventional T cells was confirmed in athymic mice under all tested conditions. In the case of NKT cells, a few CD8⁺ NKT cells emerged, but CD4⁺ (or DN) NKT cells did not emerge in athymic mice. In all tested conditions, the usage of an invariant chain of Vα14Jα281 was not detected in these athymic mice by reverse transcription-polymerase chain reaction (data not shown).

We then investigated whether the precursors of extrathymic T cells could recognize polymorphic MHC class I or II antigens. Even in young athymic mice, many IL-2Rβ⁺ TCR^{int} cells, especially the NK1.1⁻ subset, expanded in the liver but not in the bone marrow or the spleen. There was proof of the recognition of polymorphic MHC class I and II antigens by extrathymic T cells during differentiation. Firstly, the disparity of class I antigens induced the expansion of CD8⁺

Table 1. Distribution of CD3⁺ cells with CD4⁺, DN CD4⁻ CD8⁻ and CD8⁺ phenotypes in the liver

Mouse strain	Absolute no. of CD3 ^{int} cells (× 10 ⁶)	% Positive cells among CD3 ⁺ cells		
		CD4 ⁺	CD4 ⁻ CD8 ⁻	CD8 ⁺
B6 ^a	0.66 ± 0.07	64.2 ± 2.3	5.9 ± 0.8	30.2 ± 1.1
B6- <i>nu/nu</i> ^b	0.09 ± 0.06	27.4 ± 2.4	20.4 ± 9.4	52.1 ± 7.5
bm1 → B6- <i>nu/nu</i>	1.68 ± 1.0	6.9 ± 1.9	26.6 ± 5.6	67.7 ± 4.6
bm12 → B6- <i>nu/nu</i>	0.75 ± 0.67	30.5 ± 4.3	13.9 ± 9.9	55.6 ± 5.7

^aLiver lymphocytes in B6 mice were a mixture of CD3^{int} and CD3^{high} cells.

^bLiver lymphocytes in nude mice were all CD3^{int} cells.

Four mice were used to produce the mean and one SD in each experiment.

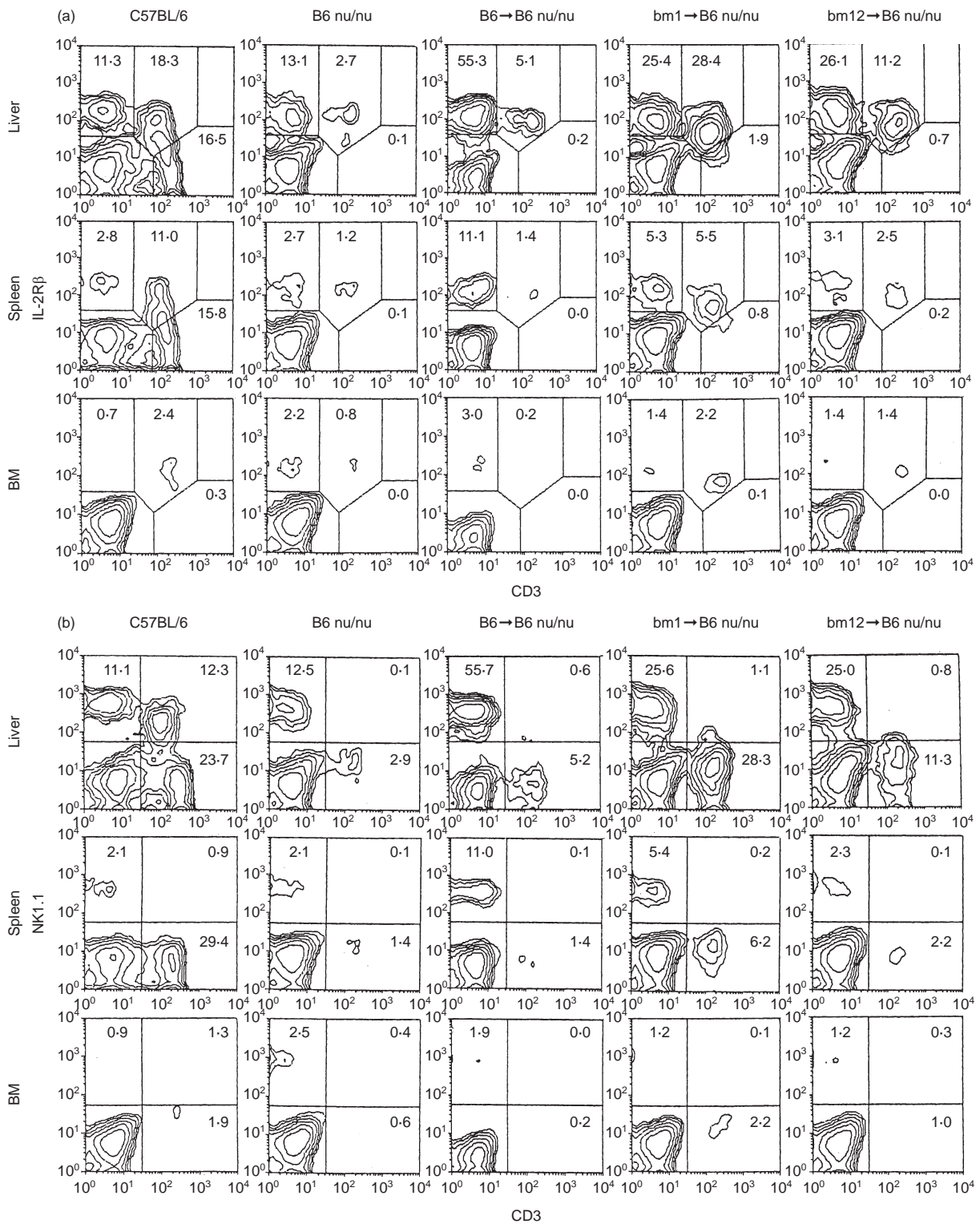


Figure 3. Generation of NK1.1⁻ CD3^{int} cells by positive selection of the mutation on polymorphic MHC antigens. (a) Two-colour staining for CD3 and IL-2R β . (b) Two-colour staining for CD3 and NK1.1. B6 and B6-*nu/nu* mice were used at the age of 10 weeks as controls. B6-*nu/nu* mice at the age of 8 weeks were irradiated (4 Gy) and were injected with 10^7 bone marrow cells of bm1 or bm12 origin. Immunofluorescence tests of lymphocytes in the liver, spleen and bone marrow (BM) were performed on day 14 after BMT. The data shown here are representative of five experiments.

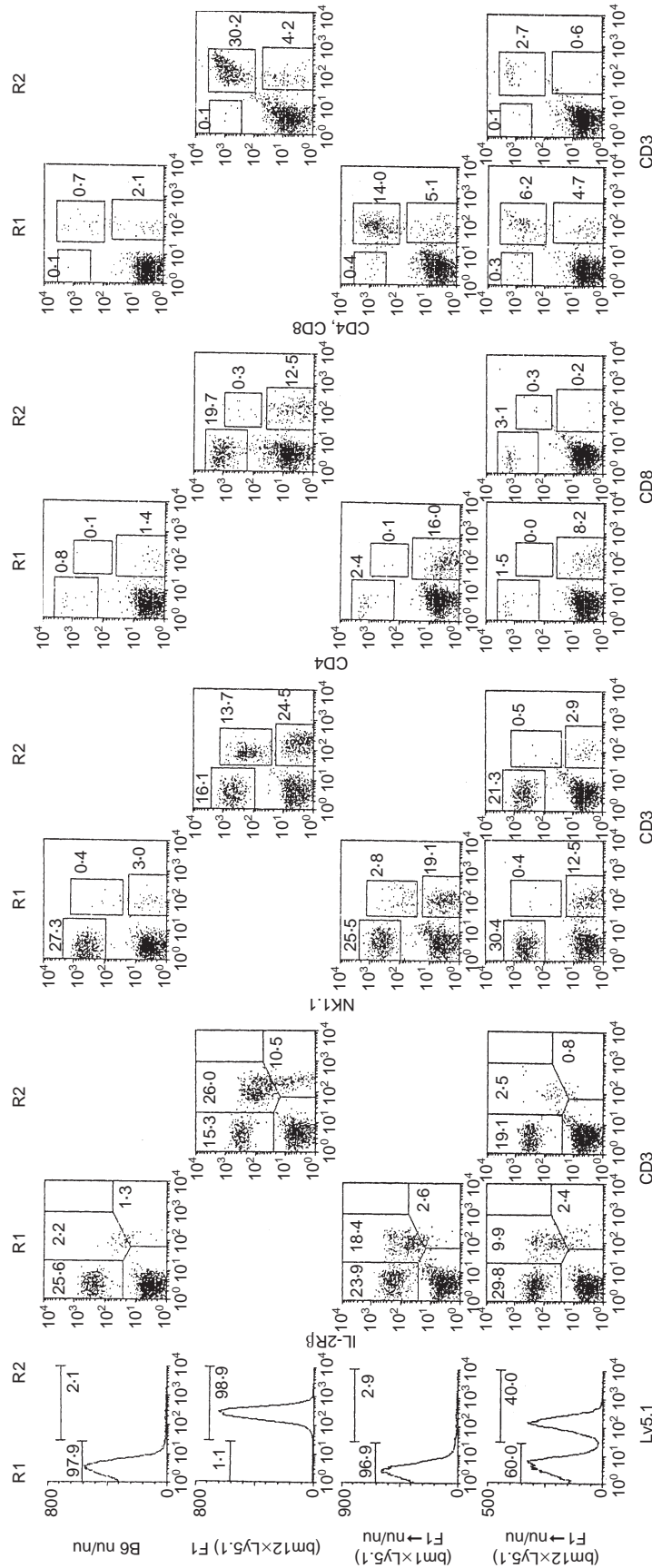


Figure 5. Three-colour staining of Ly5.1, CD3 and IL-2Rβ and those of the other indicated combinations in the liver of mice. B6 nu/nu, (bm 12 × Ly5.1) F₁ mice, (bm 1 × Ly5.1) F₁ → B6 nu/nu mice, and (bm 12 × Ly5.1) F₁ → B6 nu/nu mice were used.

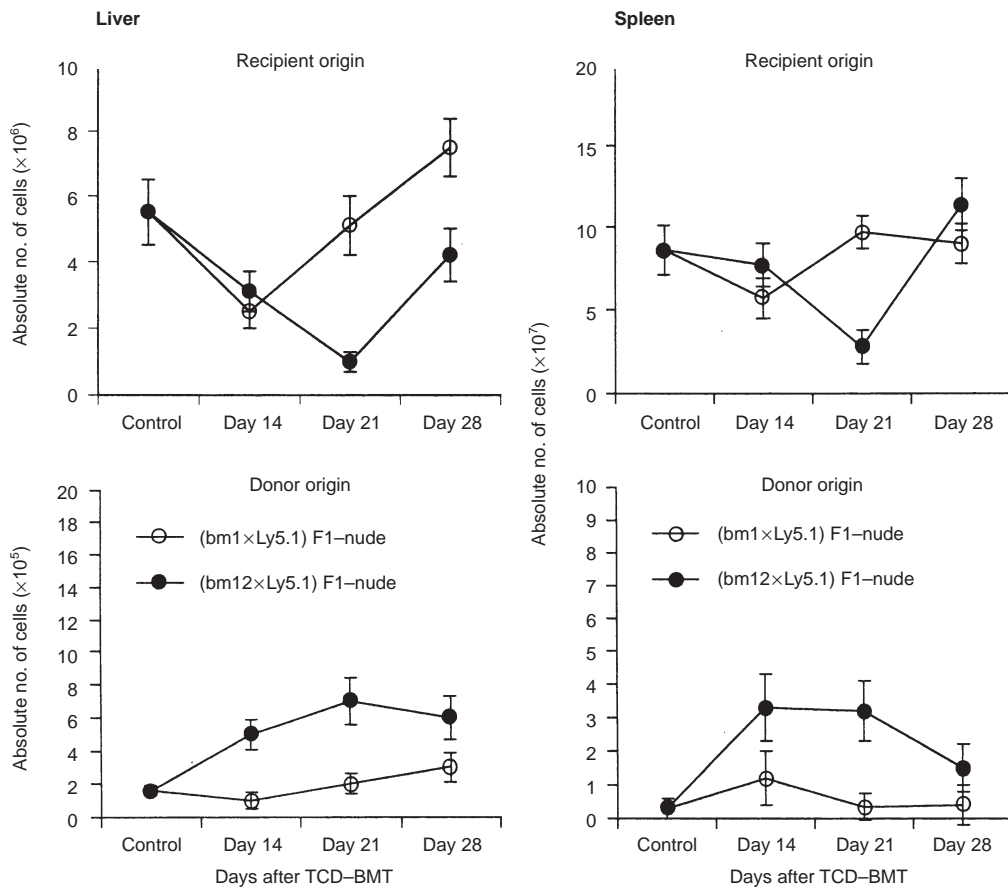


Figure 6. A time-kinetic study on the recovery of recipient or donor cells after BMT. Bone marrow cells (depleted of Thy-1⁺ cells, 10⁷/mouse) from either (bm 1 × Ly5.1) F₁ or (bm 12 × Ly5.1) F₁ mice were injected into 4 Gy-irradiated *nu/nu* mice at the age of 8 weeks. The origin of lymphocytes was determined by the gated analysis of Ly5.1. This result was representative of day 21. The repeated experiments of Fig. 5. were conducted on days 14, 21 and 28. The mean and one SD at each time-point are produced from four mice.

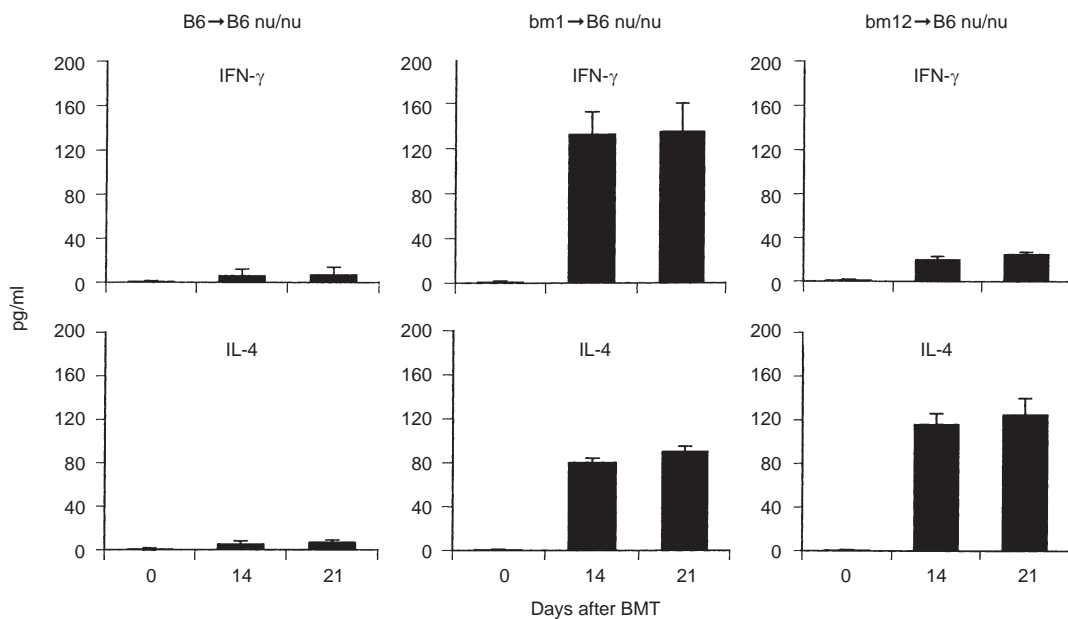


Figure 7. Serum levels of IFN- γ and IL-4 in B6-*nu/nu* mice with BMT of bm 1 origin (bm 1 → B6-*nu/nu*) and of bm 12 origin (bm 12 → B6-*nu/nu*). Sera were obtained from four mice at the indicated time-points. The mean and one SD are represented.

MHC antigens might be an event mainly involving NK1.1⁻ TCR^{int} cells among non-conventional T cells. However, the restriction of polymorphic class I and CD8⁺ cells and that of polymorphic class II and CD4⁺ cells were incomplete in the case of NK1.1⁻ TCR^{int} cells. Namely, the disparity of polymorphic class I accompanied DN cells while that of polymorphic class II accompanied CD8⁺ cells. This phenomenon is unique when compared with their complete restriction in conventional T cells.¹⁶ It should also be mentioned that the mutations on polymorphic class I antigens were recognized by $\gamma\delta$ T cells as well as by $\alpha\beta$ T cells, while those on polymorphic class II antigens were recognized by only $\alpha\beta$ T cells.

The present results may suggest the possible nature of NK1.1⁻ TCR^{int} cells seen in autoimmune-prone mice such as MRL-*lpr/lpr* mice,²⁵ NOD mice^{26,27} and neonatally thymectomized mice.^{28,29} It is speculated that NK1.1⁻ TCR^{int} cells might be generated extrathymically (or by an alternative intrathymic pathway) and have the ability to induce certain autoimmune diseases when the regulatory function of NKT cells decreases.^{26,27,30} SJL mice, which lack NKT cells, also carry a large proportion of NK1.1⁻ TCR^{int} cells.^{31,32}

To determine the physiological significance of the cell interaction *in vivo*, we examined the cytokine profile of the sera in nude mice subjected to BMT. The production of both IFN- γ and IL-4 in the case of class I disparity (bm 1 BMT \rightarrow B6-*nulnu*) resembled the T helper 0 (Th0) type of cytokine profile. In contrast, only the production of IL-4 in the case of class II disparity (bm 12 BMT \rightarrow B6-*nulnu*) resembled the Th2 type of cytokine profile. An ability to produce IFN- γ and/or IL-4 by NK1.1⁻ TCR^{int} cells depending on stimulation resembles such ability ascribed to NKT cells.^{33,34}

In any case, we report herein that NK1.1⁻ extrathymic T cells have the ability to recognize polymorphic MHC antigens. Since the strongest recognition of polymorphic MHC antigens is that of conventional T cells, a population of extrathymic T cells might acquire a similar recognition system during phylogenetic development. In other words, NK1.1⁻ TCR^{int} cells stand at an intermediate position between NKT cells and conventional T cells in phylogeny.

We could ignore neither the presence of CD8⁺ NK1.1⁻ TCR^{int} cells (of extrathymic origin) nor their function. In an earlier study, it was reported that some unusual T-cell populations recognize alloantigens and simultaneously induce self-reactive cytotoxicity.³⁵ In this paper, the expression of NK1.1 antigen was not determined. The function of CD8⁺ NK1.1⁻ TCR^{int} cells might be intimately related to this phenomenon. In future studies, we aim to ascertain whether such CD8⁺ NK1.1⁻ TCR^{int} cells in the liver are responsible for the induction of the autoimmune-like disorder seen in (C57BL/6 \times DBA/2) F₁ mice injected with T cells of DBA/2 mice. These F₁ mice fall victim to autoimmune liver disease by CD8⁺ NK1.1⁻ TCR^{int} cells which are activated by injected allogeneic T cells (manuscript in preparation).

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