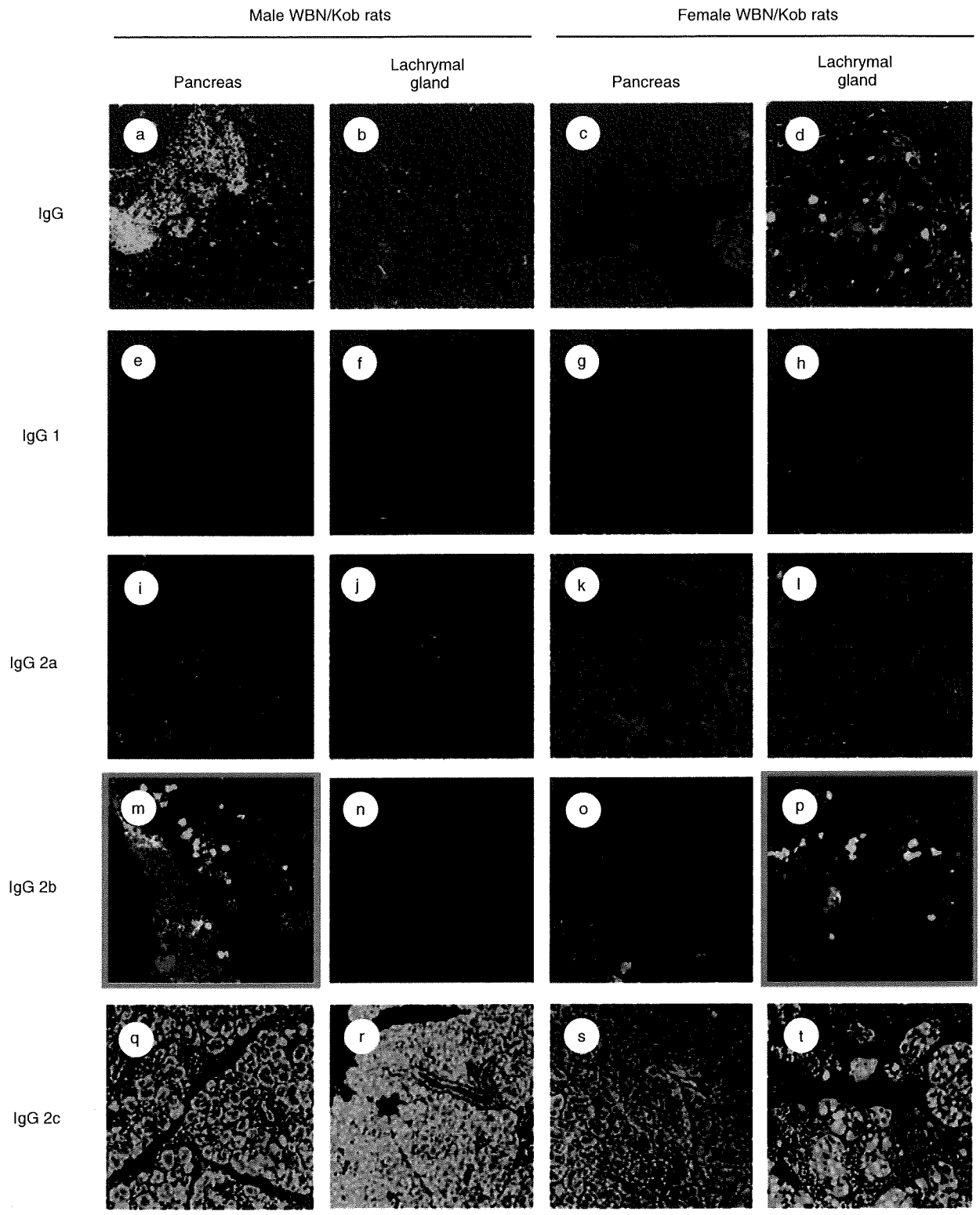

Fig. 5. Detection of tissue-specific antibody. Paraffin sections of pancreas and lachrymal gland were prepared from male and female Wistar Bonn/Kobori (WBN/Kob) rats (4 months of age) and stained with biotinylated anti-IgG (a–d), anti-IgG1 (e–h), anti-IgG2a (i–l), anti-IgG2b (m–p) or IgG2c (q–t). They were visualized by additional staining with fluorescein isothiocyanate–streptavidin.

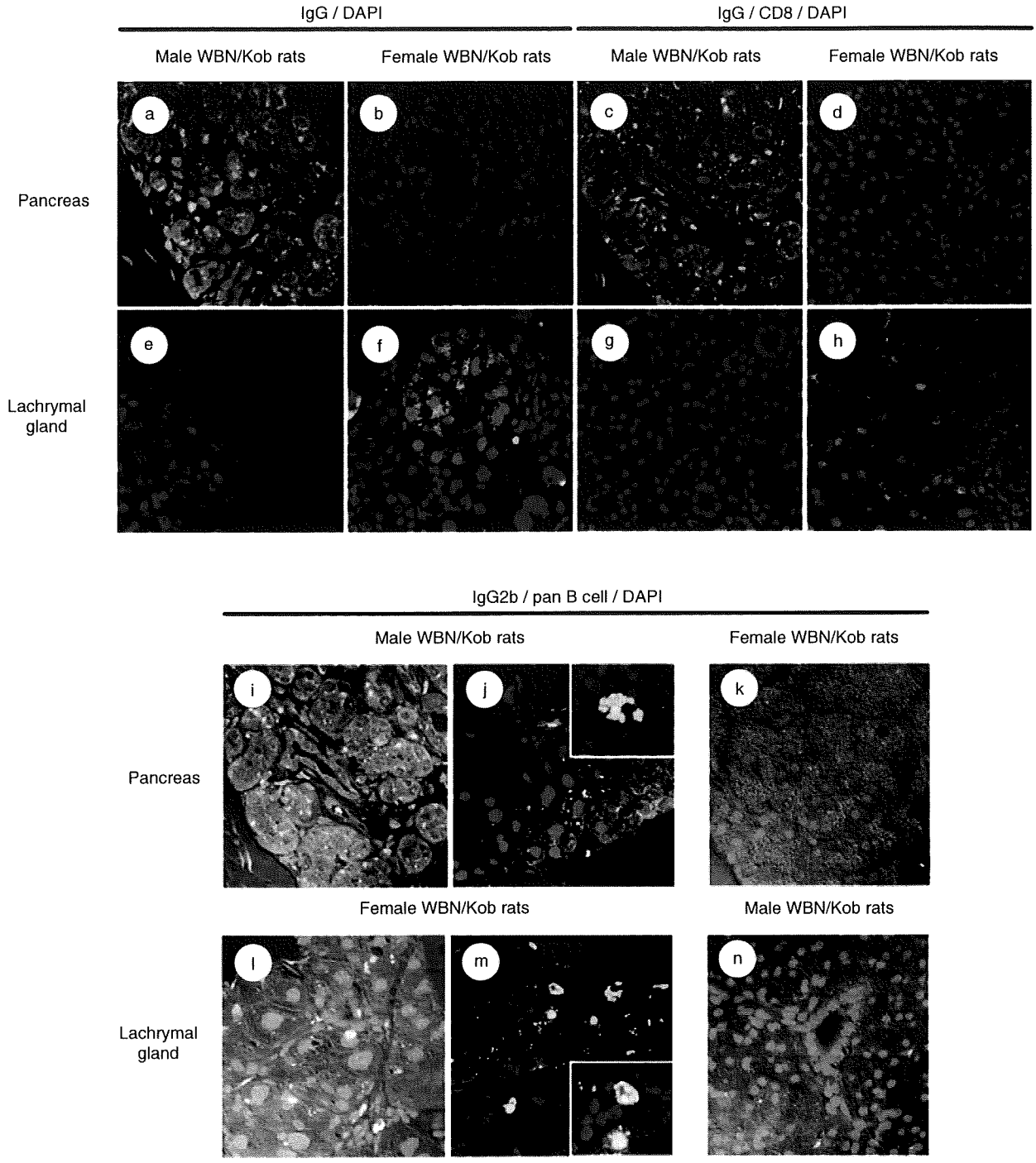

Fig. 6. Detection of infiltrated IgG-producing cells and $CD8^+$ T cells. Paraffin sections of pancreas and lachrymal gland were prepared from male and female Wistar Bonn/Kobori (WBN/Kob) rats (4 months of age) and stained with biotinylated anti-IgG monoclonal antibodies (mAb). (a,b) Pancreas; (e,f) lachrymal gland) followed by fluorescein isothiocyanate (FITC)–streptavidin. Nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI). Paraffin sections were double-stained with anti-IgG mAb plus phycoerythrin (PE)-anti-CD8 mAb. (c,d) Pancreas; (g,h) lachrymal gland. Paraffin sections of the pancreas and lachrymal gland were stained with anti-IgG2b followed by FITC–streptavidin, PE-anti-pan B cell mAb and DAPI. (i–k) Pancreas; (l–n) lachrymal gland).

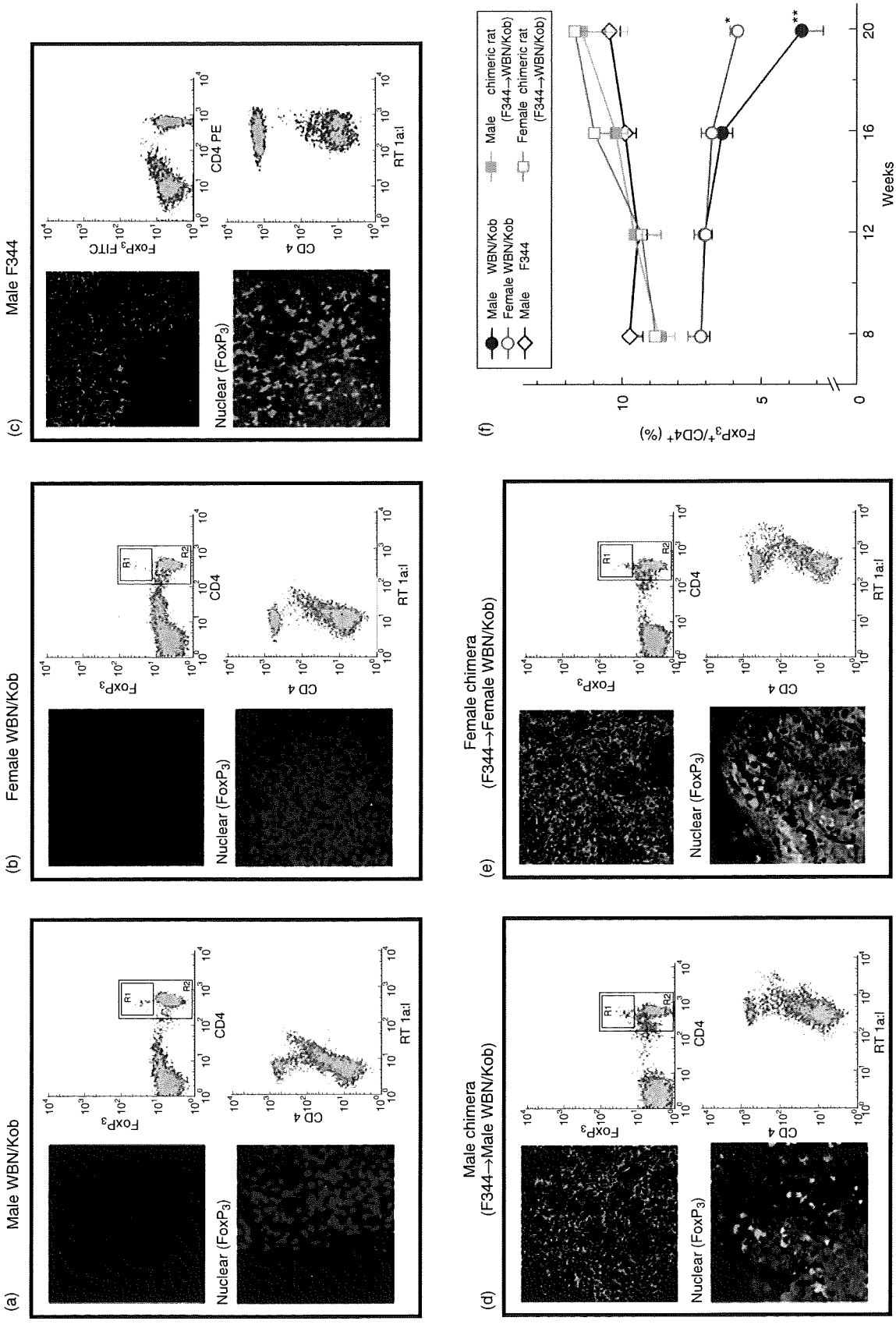

Fig. 7. Detection of Forkhead box P3 (FoxP3)$^+$ cells in the spleen. Paraffin sections of the spleen were prepared (4 months of age) and stained with fluorescein isothiocyanate (FITC)-anti-FoxP3 monoclonal antibody (mAb) and 4,6-diamidino-2-phenylindole. Spleen cells were prepared and stained with FITC-anti-CD4 mAb followed by the intracytoplasmic staining with phycoerythrin (PE)-anti-FoxP3 mAb to detect T regulatory cells. The stained cells were analysed by a fluorescence activated cell sorter scan. (a) Male Wistar Bonn/Kobori (WBN/Kob) rat; (b) female WBN/Kob rat; (c) male F344 rat as a normal control; (d) male WBN/Kob rat treated with intrabone marrow–bone marrow transplantation (IBM–BMT) from F344; (e) female WBN/Kob rat treated with IBM–BMT from F344). Spleen cells were also stained with FITC-anti-RT1A^l [donor type rat major histocompatibility complex (MHC)] and PE-anti-CD4 mAbs to detect donor-derived cells. (a) Male WBN/Kob rat; (b) female WBN/Kob rat; (c) male F344 rat as a normal control; (d) male WBN/Kob rat treated with IBM–BMT from F344; (e) female WBN/Kob rat treated with IBM–BMT from F344). The frequency of FoxP3$^+$/CD4$^+$ cells was measured kinetically and summarized in Fig. 7F. Symbols and bars represent means ± standard deviations of seven rats. $^*P < 0{\cdot}05$.

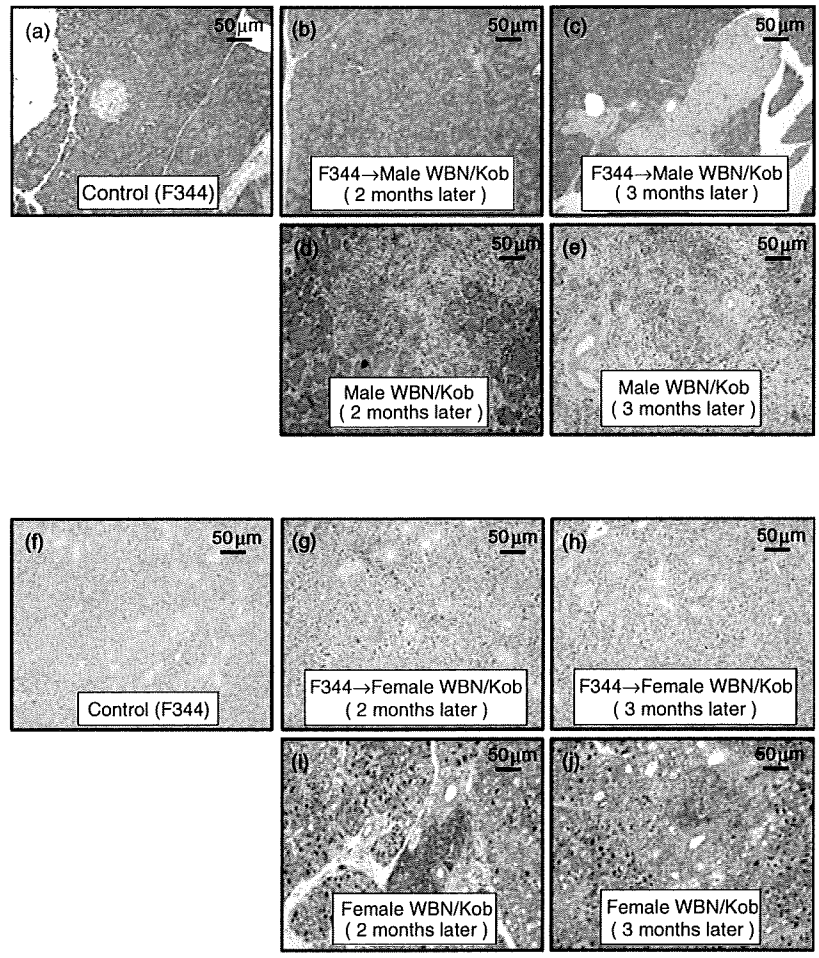

Fig. 8. Pathological findings of pancreas and lachrymal gland after intrabone marrow–bone marrow transplantation (IBM–BMT). Male and female Wistar Bonn/Kobori (WBN/Kob) rat treated with IBM–BMT from F344 (F344→WBN/Kob) were examined histologically after staining with haematoxylin and eosin. Paraffin sections of pancreas and lachrymal grand were prepared. (a–e) Pancreas; (f–j) lachrymal gland. (a) Male F344 rat as a normal control; (b) male WBN/Kob rat treated with IBM–BMT from F344 (F344→WBN/Kob, 3 months of age, 2 months after IBM–BMT); (c) male F344→WBN/Kob, 4 months of age, 3 months after IBM–BMT; (d) male WBN/Kob rat 3 months of age; (e) male WBN/Kob rat 4 months of age; (f) female F344 rat as a normal control; (g) female F344→WBN/Kob, 3 months of age, 2 months after IBM–BMT; (h) female F344→WBN/Kob, 4 months of age, 3 months after IBM–BMT; (i) female WBN/Kob rat 3 months of age; (j) female WBN/Kob rat 4 months of age.

in WBN/Kob rats, which allowed us to understand more clearly the pathogenic mechanism of AIP. Recently, AIP has been divided histologically into two distinctive types, LPSP and granulocyte epithelial lesion (GEL). In Japan and Korea, most patients with AIP show the LPSP type associated with systemic exocrinopathy in the aged male. By contrast, in Caucasians, younger patients with the GEL type of AIP associated with ulcerative colitis are often observed without gender difference as well as LPSP. The LPSP type of AIP has been thought to be a multi-focal fibrosclerosing disease with abundant infiltration of IgG4-positive plasmacytes, because extrapancreatic lesions, such as sclerosing cholangitis, sialoadenitis or dacryoadenitis (similar to Mikulicz's disease or Kuttner's tumour), retroperitoneal fibrosis, interstitial nephritis or thyroiditis, are often associated with AIP. It is noted that these lesions can be treated by administration of steroid hormone [27], suggesting that sclerosing cholangitis or sialoadenitis with AIP is different from primary sclerosing cholangitis or typical Sjøgren's syndrome respectively [28].

In the present study, we found that WBN/Kob rats showed different manifestations, depending on gender. In the pancreas of the male rats, many lymphocytes infiltrated around the pancreatic ducts, which seemed to destroy the pancreatic ducts and acinar cells. On the other hand, the lachrymal and salivary glands of the female rats showed massive infiltration of lymphocytes similar to the male pancreas (Fig. 1). Moreover, aged WBN/Kob rats (>18 months) showed thyroiditis, sclerotic cholangitis and even tubulointerstitial nephritis in both genders (Fig. 2). These findings suggest that WBN/Kob rats are an animal model for the LPSP type of AIP, showing extra-pancreatic sclerosing lesions without inflammatory bowel disease, but not the GEL type. Subsequently, analyses of the lymphocyte surface markers (Fig. 3), serum γ-globulin levels (Fig. 4), specific autoantibodies (Fig. 5) and the deposits of immune globulin (Fig. 6) showed that these lesions met the characteristics of autoimmune diseases defined by Witebsky and Mackay [29,30]. In addition to immunohistological findings, the prevention of pancreatitis and dacryoadenitis by the reconstitution of immune tolerance using BMT supports the correspondence as an autoimmune disease model (Fig. 7).

Human AIP involves both humoral immunity (autoantibody) and cell-mediated immunity (cytotoxic lymphocyte). We have reported previously that autoantibodies against

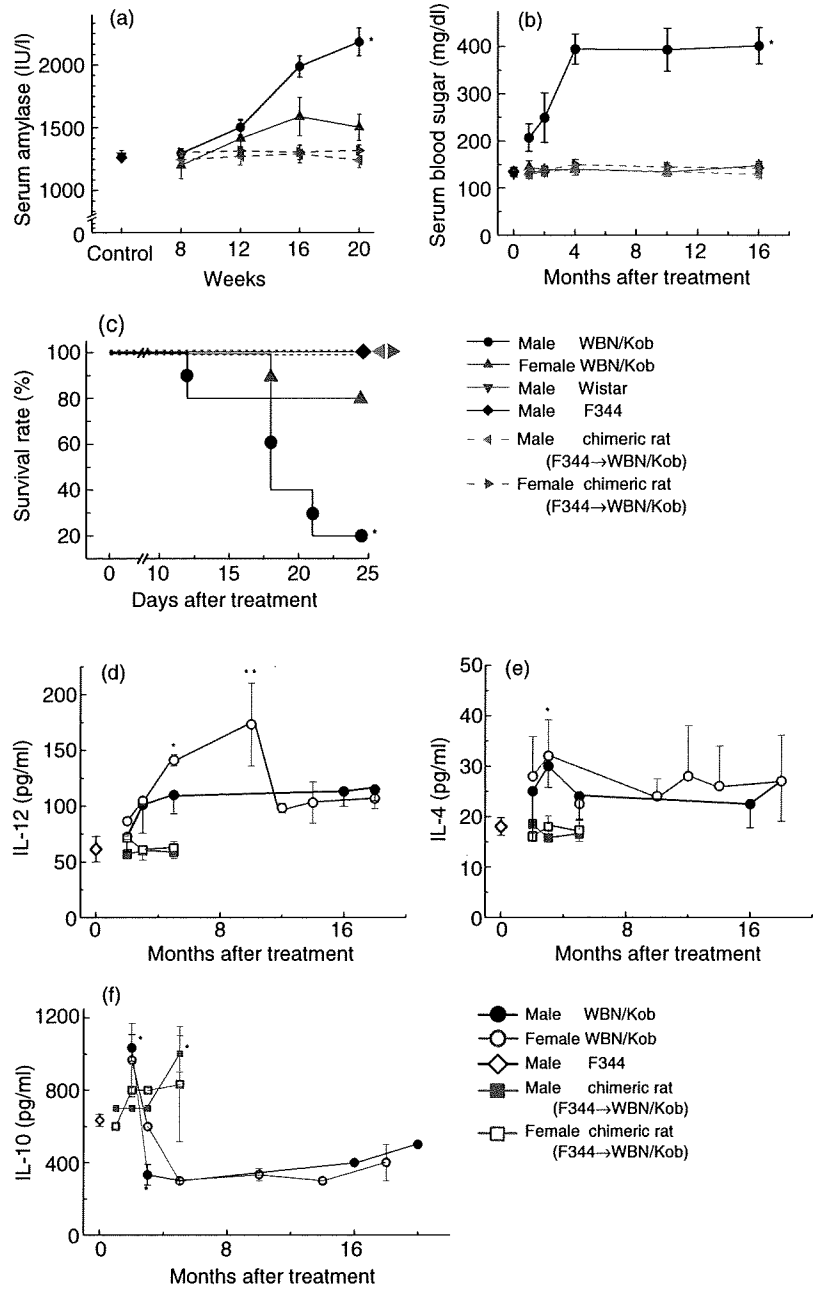

Fig. 9. Measurement of serum amylase, glucose, survival rate and cytokine levels. Serum amylase (a), glucose (b), survival rate (c), interleukin (IL)-12 (d), IL-4 (e) and IL-10 (f) were measured kinetically. Symbols and bars represent means ± standard deviations of seven rats. $^*P < 0{\cdot}05$.

CA-II and LF are identified frequently in patients with AIP, and that the prevalence of these two antibodies is independent [31]. Although the stage-dependent immunity in human AIP still remains unclear, T helper type 1/T helper type 2 (Th1/Th2) imbalance in the microenvironment is thought to reflect exacerbation progress. In fact, cytokine balance in the WBN/Kob rats suggested that Th2 cells are involved mainly in early development and Th1 cells are involved in progression, in comparison with the immunity of other AIP models that shifted into Th1 balance only because of artificial alloimmunization [18,19,32]. We also detected the specific autoantibodies of IgG2b type in WBN/Kob rats. Although details of the IgG subclass in rats remain unclear, rat IgG2b, a minor subclass of IgG, is separated in a similar position to human IgG4 by electrophoresis [33]. IgG2b subclass antibodies in the rats reflect the Th1/Th2 immune balance [34]. Up-regulation of the Th1 cytokine (interferon-γ) and down-regulation of IL-4 alter the Th1/Th2 balance to up-regulate IgG2b in the memory response, expand polyclonal activation and also expand the capacity of specific T helper cells. As reported previously, $CD4^+$ T cells react to CA-II or LF, which allows them to escape from negative selection in the thymus and depletion of T_{regs} such as $CD4^+$ $CD25^+$ T cells in the periphery. Although we identified

the specific antibody belonging to the IgG2b subclass in WBN/Kob rats in the present study, the details of other targeting antigens still remain unclear. Further studies on target antigens are necessary.

Recently, great attention has been paid to relations between various autoimmune diseases and T_{regs}. T_{regs} control immunological self-tolerance in the periphery [35,36]. The regulatory function is not mediated via CTLA-4 and cannot be blocked by antibodies to IL-4, IL-10 or transforming growth factor-β [6,13]. It has been clarified that their transcriptional factor, FoxP3, is the master gene for differentiation and function of T_{regs} [36]. In mice, neonatal thymectomy (NTx) induces the escape of T cells from negative selection in the thymus and depletes T_{regs} in the periphery, which results in the failure of both humoral and cellular immunity. Therefore, the NTx BALB/c mice, which had been immunized with CA-II or lactoferrin, nude mice (in which spleen cells of NTx mice had been transferred) developed immune-mediated pancreatitis and exocrinopathy [37]. Similarly, the WBN/Kob rats showed decreased numbers of peripheral $CD4^+$ $CD25^+$ T cells and $FoxP3^+$ splenic cells as reported in other autoimmune disease patients or animal models [38,39]. Our results showed that reconstitution of immune tolerance using BMT recovered the numbers of peripheral $CD4^+$ $CD25^+$ T cells and $FoxP3^+$ splenic cells, which resulted in the prevention of exocrinopathy in WBN/Kob rats. However, it still remains unclear as to whether or not all autoimmune diseases can originate from the abnormalities of T_{regs}. In WBN/Kob rats, cytokine alteration indicates that Th2 cells are involved mainly in early development and Th1 cells are involved in later development. Although it still remains unclear as to why organ-specific inflammation in human AIP and WBN/Kob rats depends on gender difference, factors such as sex hormones and genetic diathesis might be involved.

New immune therapies, such as suppressive, supportive and anti-cytokine therapies, can help to maintain and induce the remission of various autoimmune diseases [40]. However, as serious problems such as side effects of immune suppression often occur, these are radical treatments. Recently, haematopoietic stem cell transplantation has shown therapeutic efficacy in various kinds of autoimmune diseases [41]. In the WBN/Kob rats, we succeeded finally in preventing the development of inflammation by the achievement of complete chimerism; direct injection of the donor BMCs into the bone marrow cavity (by IBM–BMT) reduced the donor bone marrow trapping in the lung or liver [42]. The increased size of the $CD4^+CD25^+$ population and the up-regulation of FoxP3 after IBM–BMT as a treatment for ongoing pancreatitis and dacryoadenitis provide important evidence for the involvement of T_{regs} in the process of tolerance observed after induced relapses. The efficacy of IBM–BMT is not only a transient immunosuppression but, as we demonstrated, multiple interconnected mechanisms of immunomodulation. These findings suggest that IBM–BMT therapy may be a promising treatment of AIP in humans, although some complications such as rejection and infection should be carefully monitored. Again, taken together the WBN/Kob rats represent a novel model for the study of AIP with autoimmune extrapancreatic exocrinopathy.

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Prevention of graft-*versus*-host disease by intrabone marrow injection of donor T cells: involvement of bone marrow stromal cells

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Summary

We have developed a new and effective method for bone marrow transplantation (BMT): bone marrow cells (BMCs) are injected directly into the bone marrow (BM) cavity of recipient mice. The intrabone marrow injection of BMCs (IBM-BMT) greatly facilitates the engraftment of donor-derived cells, and IBM-BMT can attenuate graft-*versus*-host reaction (GVHR), in contrast to conventional intravenous BMT (i.v.-BMT). Here, we examine the mechanisms underlying the inhibitory effects of IBM-BMT on GVHR using animal models where GVHR is elicited. Recipient mice (C57BL/6) were irradiated and splenic T cells (as donor lymphocyte infusion: DLI) from major histocompatibility complex-disparate donors (BALB/c) were injected directly into the BM cavity (IBM-DLI) or injected intravenously (i.v.-DLI) along with IBM-BMT. The BM stromal cells (BMSCs) from these recipients were collected and related cytokines were examined. The recipient mice that had been treated with IBM-BMT + i.v.-DLI showed severe graft-*versus*-host disease (GVHD), in contrast to those treated with IBM-BMT + IBM-DLI. The suppressive activity of BMSCs in this GVHD model was determined. The cultured BMSCs from the recipients treated with IBM-BMT + IBM-DLI suppressed the proliferation of responder T cells remarkably when compared with those from the recipients of IBM-BMT + i.v.-DLI in mixed leucocyte reaction. Furthermore, the level of transforming growth factor-β and hepatocyte growth factor in cultured BMSCs from IBM-BMT + IBM-DLI increased significantly when compared with those from the recipients of IBM-BMT + i.v.-DLI. Thus, the prevention of GVHD observed in the recipients of IBM-BMT + IBM-DLI was attributable to the increased production of immunosuppressive cytokines from BMSCs after interaction with host reactive T cells (in DLI).

Keywords: bone marrow stromal cells, donor lymphocyte infusion, graft-*versus*-host disease, intrabone marrow-bone marrow transplantation

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Introduction

Allogeneic bone marrow transplantation (BMT) has been used as a potentially curative therapy for patients with a wide variety of diseases, including haematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases and solid tumours [1–6]. However, there are several problems to be resolved in allogeneic BMT. One of the important issues is how to control graft-*versus*-host disease (GVHD), which remains a major cause of post-transplantation morbidity and mortality.

We have recently developed intrabone marrow (IBM)-BMT, in which bone marrow cells (BMCs) are injected directly into the bone marrow (BM) cavity [7]. We have found that IBM-BMT allows us not only to use low-dose irradiation as a preconditioning regimen [7,8] but also to suppress GVHD [9], as IBM-BMT can efficiently recruit donor-derived stromal cells [including mesenchymal stem cells (MSCs)] that can support donor-derived haemopoietic stem cells [1,9–12].

It is noted that IBM-BMT can be used to prevent GVHD, even when intensive donor lymphocyte infusion (DLI) is

carried out [9]. We attempted to inject allogeneic T cells as DLI into the BM cavity (IBM-DLI) or intravenously (i.v.-DLI) with IBM-BMT. The prolongation of survival rate and reduction of GVHD were observed clearly in the recipients treated with IBM-BMT + IBM-DLI, but not in those with IBM-BMT + i.v.-DLI [13]. These findings prompted us to examine the regulatory function of BM stromal cells (BMSCs) after interaction with T cells that had been injected into the BM cavity. Evidence has been accumulated that BMSCs play a critical role in the regulation of haemopoiesis by promoting cell-to-cell interactions and constitutively secreting immunoregulatory soluble factors [14–23]. In fact, BMSCs suppress the proliferation of allogeneic T cells in a major histocompatibility complex (MHC)-independent manner [24–31].

In the present study, we examine the suppressive activity of BMSCs that had been in contact with T cells *in vivo*, and evaluate the effect of T cell polarization and several factors produced by BMSCs.

Materials and methods

Mice

C57BL/6 (B6, H-2^b), BALB/c (H-2^d) mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). C57BL/6 mice at the age of 7–9 weeks were used as recipients, and BALB/c mice at the age of 7–9 weeks were used as donors. All mice were kept in our animal facilities under specific pathogen-free conditions. All animal procedures were performed in accordance with protocols approved by the Animal Experimentation Committee, Kansai Medical University.

Irradiation

C57BL/6 mice were irradiated at 8·5 Gy (1·0 Gy/min) from a ^{137}Cs source (Gammacell 40 Exactor, Nordion, International Inc., Ottawa, Ontario, Canada) 1 day before the BMT.

Bone marrow transplantation and donor lymphocyte infusion

Bone marrow cells were flushed from the femoral and tibial bones of the BALB/c mice, and then suspended in RPMI-1640. The BMCs were then filtered through a 70-μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove debris, washed and adjusted to $1{\cdot}5 \times 10^9$ cells/ml in RPMI-1640. The BMCs, thus prepared, were injected directly into the BM cavity as described previously [7]. Briefly, the region from the inguen to the knee joint was shaved and a 5-mm incision was made on the thigh. The knee was flexed to 90 degrees and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the BM cavity. Using a microsyringe (10 μl; Hamilton Co., Reno, NV, USA) containing the donor BMCs ($1{\cdot}5 \times 10^9$ cells/ml), the donor BMCs were injected from the said bone holes into the BM cavity of the left tibia (10^7 cells/7 μl/tibia) (IBM-BMT). In some groups, BMCs were injected intravenously.

T cells were purified from the spleens by positive selection by a MACS® system using CD4 and CD8α microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) after depletion of red blood cells, or by an EPICS ALTRA flow cytometer (Coulter, Hialeah, FL, USA) after staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD4/CD8 monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA, USA).

Splenic T cells were injected into the BM cavity of the right tibia (10^7 cells/7 μl/tibia: intrabone marrow T cell injection as DLI; IBM-DLI) or injected intravenously (i.v.-DLI; 10^7 cells/0·5 ml) into the recipient mice along with the IBM-BMT. Recipients treated with IBM-BMT alone (without DLI) served as negative controls (termed NO-DLI) [13].

Preparation of freshly isolated BMSCs

Three days after the DLI, BMCs were flushed from the right tibial bones of the recipient mice, and non-haemopoietic MSC-enriched cells (defined as $CD45^-/CD106^+$ cells) were sorted immediately by an EPICS ALTRA flow cytometer (Coulter, Hialeah, FL, USA) after staining with FITC- or PE-conjugated anti-CD45/CD106 mAbs (BD Pharmingen, San Diego, CA, USA). Freshly isolated non-haemopoietic BMSCs-enriched populations, sorted as $CD45^-/CD106^+$ cells, were prepared from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI, or IBM-BMT alone (NO-DLI). Haemopoietic BMC-enriched populations, sorted as $CD45^+/CD106^-$ cells, were also prepared from the recipients and used as controls.

Preparation of cultured BMSCs

Bone marrow cells from the right tibia, into which T cells had been injected as DLI, were collected from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) 3 days after treatment, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Two days later, non-adherent cells were removed. Adherent cells were detached using trypsin-ethylenediamine tetraacetic acid, and passaged when 80% confluence was reached and then replated. After 2 weeks (short-term culture) or 3 months (long-term culture) the cultures were discontinued, and BMSCs were harvested and used for further experiments, including mixed leucocyte reaction (MLR) and real-time reverse transcription–polymerase chain reaction (RT–PCR) assay. The culture-expanded BMSCs from the recipients of IBM-BMT +

IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were stained with FITC-anti-CD45 and PE-anti-CD106 mAbs and analysed by a fluorescence activated cell sorter (FACScan) (BD Pharmingen).

Mixed leucocyte reaction

Various numbers of freshly prepared (defined as $CD45^-/CD106^+$ BM cells) or cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.V-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR (4-day culture) where 2×10^5 responder $CD4^+$ T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml. $CD45^+/CD106^-$ haemopoietic cells or whole BMCs served as controls for BMSCs added to the culture. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period.

Activation of T cells with concanavalin A

Splenic T cells (2×10^6 cells) from BALB/c mice were cultured with 2·5 μg/ml of concanavalin A (ConA) for 4 days. Activated T cells, thus prepared, were used as a positive control in real-time RT–PCR assay and enzyme-linked immunosorbent assay (ELISA) to detect cytokines.

Flow cytometric analyses of intracellular cytokines

CD4-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice with cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) in round-bottomed plates (RPMI-1640) with 10% FCS. Cells were harvested 6 days later and stained with biotin-conjugated anti-H-$2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and FITC-anti-CD4 mAb (BD Pharmingen) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized with Cutofix/Cytoperm solution™ (BD Pharmingen). Intracellular cytokines were detected after the staining of cells with PE-anti-interleukin (IL)-2, -interferon (IFN)-γ or -IL-4 using an Intracellular Cytokine Staining Kit® (BD Pharmingen). Cells stained with isotype control cocktail (BD Pharmingen) served as a control. The stained cells were analysed by a FACScan® (Becton Dickinson Co., Mountain View, CA, USA).

Real-time RT–PCR assay

Cytokine messages of BMSCs were determined by real-time RT–PCR. We prepared some primers for transforming growth factor (TGF)-β (forward: TTTCGATTCAGCGCT CACTGCTCTTGTGAC, reverse: ATGTTGGACAACTGCT CCACCTTGGGCTTGC), hepatocyte growth factor (HGF) (forward: AAGAGTGGCATCAAGTGCCAG, reverse: CTG GATTGCTTGTGAAACACC), IL-2 (forward: TGGAGCA GCTGTTGATGGAC, reverse: CAATTCTGTGGCCTGCTT GG), IL-4 (forward: ACAGGAGAAGGGACGCCAT, reverse: GAAGCCCTACAGACGAGCTCA), IL-10 (forward: GGTT GCCAAGCCTTATCGGA, reverse: ACCTGCTCCACTGC CTTGCT) and IL-15 (forward: CATCCATCTCGTGCTAC TTGTGTT, reverse: CATCTATCCAGTTGGCCTCTGTTT) (Nisshinbo, Chiba, Japan).

Real-time RT–PCR was conducted on a DNA engine Opticon2 System (MJ Japan Ltd, Tokyo, Japan) by using SYBR Green I as a double-stranded DNA-specific binding dye and continuous fluorescence monitoring. The cycling conditions consisted of a denaturation step for 10 min at 95°C, 40 cycles of denaturation (94°C for 15 s), annealing (60°C for 30 s) and extension (72°C for 30 s). After amplification, melting curve analysis was performed with denaturation at 95°C, then continuous fluorescence measurement from 65°C to 95°C at 0·1°C/s. All reactions were run at least in duplicate, and included control wells without cDNA.

Detection of cytokines in MSC culture supernatant

Mesenchymal stem cell culture supernatants were collected 2 weeks later, and the amounts of IL-2, IL-4, IFN-γ and TGF-β were determined by ELISA kits.

Statistical analyses

Non-parametric analyses (Mann–Whitney *U*-test and log-rank test) were performed using StatView software (Abacus Concepts, Berkley, CA, USA). Values of $P < 0·05$ were considered statistically significant.

Results

In vitro immunosuppressive effects of BMSCs on T cell proliferation

Three days after DLI, BMCs were collected from the recipients, and non-haemopoietic BMCs (defined as $CD45^-/CD106^+$ cells) were isolated immediately as shown in Fig. 1a. The average number of these sorted cells per mouse were as follows. $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI: 31 033 ± 2450 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + i.v.-DLI: 29 850 ± 2728 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT alone (without DLI): 36 630 ± 5244 cells (four mice). There were no statistical differences among these groups regarding the yields of $CD45^-/CD106^+$ cells. The sorted $CD45^-/CD106^+$ cells from these recipients were added to the culture of one-way MLR. As shown in Fig. 2, all the $CD45^-/CD106^+$ cells isolated from

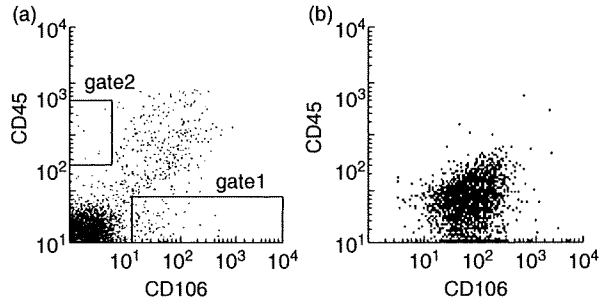

Fig. 1. Flow cytometric profiles of freshly isolated and cultured bone marrow stromal cells (BMSCs). (a) Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI) after the staining of cells with fluorescein isothiocyanate (FITC)-anti-CD45 and phycoerythrin (PE)-anti-106 monoclonal antibodies (mAbs). The dot-plot profile of $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) was similar to (a). Haemopoietic bone marrow cell-enriched populations, sorted as $CD45^+/CD106^-$ cells (gate 2), were also prepared from the recipients, and used as controls. (b) Cultured BMSCs (for 2 weeks) obtained originally from the right tibia of the recipients of IBM-BMT + IBM-DLI were stained with FITC-anti-CD45 and PE-anti-106 mAbs, and analysed by a fluoresence activated cell sorter scan. The dot-plot profile of cultured BMSCs from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) was similar to (b).

the BM of IBM-DLI, i.v.-DLI and IBM-BMT alone (without DLI) suppressed MLR only slightly, but not significantly (not statistically significant among three groups). This is the case when haemopoietic $CD45^+/CD106^-$ cells or whole BMCs were added to the culture. Thus, non-haemopoietic BMCs freshly isolated from the site of IBM-DLI could not significantly suppress T cell proliferation in MLR. This might be due to the heterogeneity of non-haemopoietic BMCs. Therefore, we next examined the inhibitory effect of cultured BMSCs after IBM-DLI.

Three days after DLI, BMCs were collected from the recipients, and cultured in DMEM with 10% FCS for 2 weeks, as shown in *Materials and methods*. The phenotypes of BMSCs, thus prepared, were negative for CD45 and CD34, but positive for CD90 and CD106 (Fig. 1b). These BMSCs were added to the culture of MLR to examine their suppressive effects.

As shown in Fig. 3a and b, the BMSCs prepared from the recipients treated with IBM-BMT + IBM-DLI significantly suppressed MLR in a dose-dependent fashion when compared with those from the recipients treated with IBM-BMT + i.v.-DLI. It is surprising that the BMSCs from the recipients of IBM-BMT + IBM-DLI still showed a suppressive effect on T cell proliferation even after long-term culture (3 months) when compared with those prepared from the recipients of IBM-BMT + i.v.-DLI (Fig. 3c), suggesting that the suppressive effects of BMSCs on the BM (IBM-DLI) are long-lasting.

The frequency of IFN-γ- and IL-4-producing T cells after coculture with BMSCs

To examine the effects of BMSCs on T cell polarization, CD4-enriched T cells from donor BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice and BMSCs cultured from the recipients of IBM-BMT + IBM-

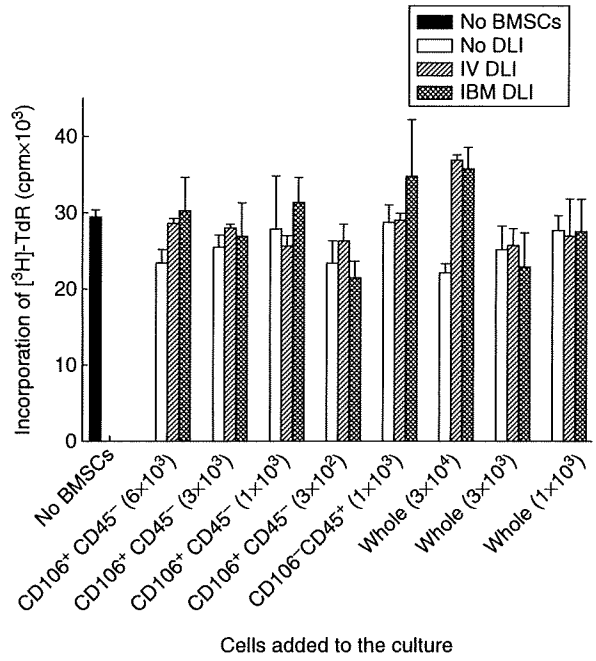

Fig. 2. Effect of freshly isolated bone marrow stromal cells (BMSCs) on T cell proliferation. Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) after the staining of cells with fluorescein isothiocyanate-anti-CD45 and phycoerythrin-anti-10^6 monoclonal antibodies (mAbs). Haemopoietic cells in the bone marrow (BM), defined as $CD45^+/CD106^-$ cells, were also obtained by a cell sort (gate 2). Graded numbers of $CD45^-/CD106^+$ BMSCs (3×10^2–6×10^3), $CD45^+/CD106^-$ haemopoietic cells (1×10^3) or whole BM cells (1×10^3–3×10^4) were added to the culture of one-way mixed leucocyte reaction where 2×10^5 responder $CD4^+$T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml and cultured for 96 h. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period. This figure shows the representative result of three experiments. The data are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient).

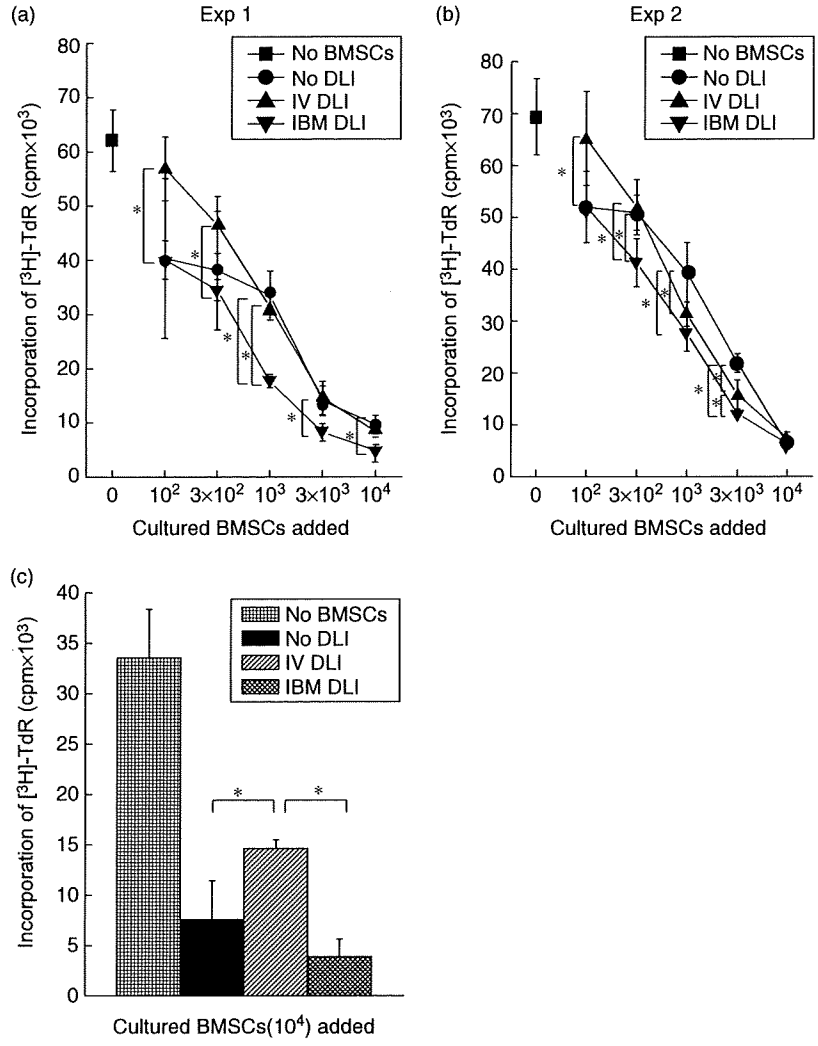

Fig. 3. Inhibitory effect of cultured bone marrow stromal cells (BMSCs) on T cell proliferation. Bone marrow cells from the right tibia were collected from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) and cultured for 2 weeks. Graded numbers of cultured BMSCs (10^2–10^4 cells) were added to the culture of one-way mixed leucocyte reaction (MLR) (a, b). BMSCs were obtained after the long-term culture (cultured for 3 months), and were added to the culture of one-way MLR (c). The data in figures are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with MLRs performed in the groups ($P < 0{\cdot}05$).

DLI, IBM-BMT + i.v.-DLI, or IBM-BMT alone (without DLI). The development of T helper 1 (Th1) or Th2 cells was defined by intracellular staining of IFN-γ or IL-4. The frequency of IL-4-producing cells was slightly but significantly higher in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4a and b *versus* 4c and summarized in 4g). Conversely, the percentage of IFN-γ-producing cells was lower in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4d and e *versus* 4f, and summarized in 4g). Furthermore, this is the case when intracellular IL-2 was examined (data not shown). Thus, the polarization of Th2 cells is facilitated strongly after co-culture with the BMSCs from the recipients of IBM-BMT + IBM-DLI, while Th1 cells are induced dominantly by co-culture with the BMSCs from the recipients of IBM-BMT + i.v.-DLI. These findings suggest strongly that T cells injected into the BM cavity can modulate the function of BMSCs after their interaction.

Bone marrow stromal cells produce immunoregulatory cytokines: TGF-β and HGF

Previous reports have shown that BMSCs can modify T cell functions by soluble factors [18,19]. Therefore, we attempted to identify molecules involved in the immune modulation by BMSCs. First, we determined the levels for IL-2, IL-10, IFN-γ and TGF-β in the culture supernatant of BMSCs using an ELISA. The culture supernatants of enriched T cells stimulated with ConA served as a control. As shown in Table 1, IL-2, IL-10 or IFN-γ were not detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI), while a significant amount of TGF-β was detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, but not in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). These results indicate that TGF-β secreted from the BMSCs obtained from the recipients of IBM-BMT + IBM-DLI

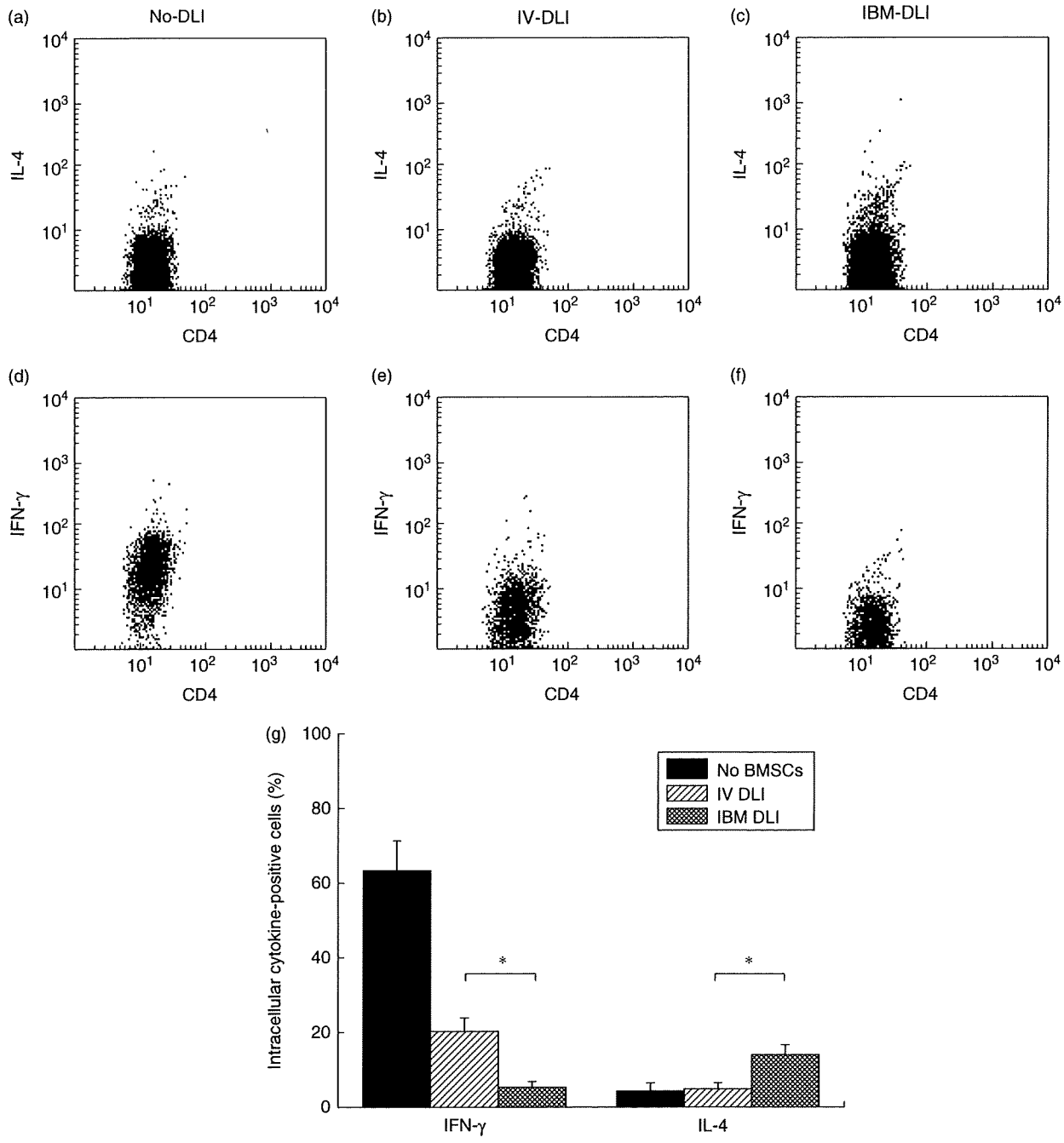

Fig. 4. Interaction of bone marrow stromal cells (BMSCs) with T cells and induction of T helper 2 cells. $CD4^+$ cell-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 and BMSCs cultured from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) in a round-bottomed plate (RPMI-1640) with 10% fetal calf serum. Cells were harvested 6 days later and stained with biotin-conjugated anti-H-$2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and fluorescein isothiocyanate-anti-CD4 monoclonal antibody (mAb) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized and intracellular cytokines were detected after the staining of cells with phycoerythrin-anti-interleukin (IL)-4 and -interferon (IFN)-γ mAbs. Representative dot-plot profiles of $CD4^+$/IL-4^+ cells (a, b, c) or $CD4^+$/IFN-γ^+ cells (d, e, f) are shown, co-cultured with BMSCs from the recipients of IBM-BMT alone (without DLI) (a, d), IBM-BMT + i.v.-DLI (b, e), or IBM-BMT + IBM-DLI (c, f). Cells in dot-plot profiles were gated positively as H-$2K^{d+}$ responder cells. Cells stained with isotype control cocktail served as a control. (g) Representative result of three experiments. Columns represent mean percentage of IFN-γ or IL-4 bearing cells ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with intracellular cytokines performed in the groups ($P < 0{\cdot}05$).

Table 1. Measurement of cytokines.

	No DLI†	i.v.-DLI	IBM-DLI	T cells with ConA‡
IL-2 (pg/ml)	0	0	0	87·3 ± 15·5
IFN-γ (pg/ml)	0	0	0	1418·2 ± 369·4
IL-10 (pg/ml)	0	0	0	1114·6 ± 103·1
TGF-β (ng/ml)	0	0·27 ± 0·4	14·2 ± 2·4	0·6 ± 0·5

†Bone marrow stromal cell (BMSC) culture supernatants from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were collected 2 weeks later. The cell supernatants were analysed for the amount of interleukin (IL)-2, IL-4, interferon (IFN)-γ and trandforming growth factor (TGF)-β by enzyme-linked immunosorbent assay. ‡Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

might be one of the candidates for attenuation of GVHD in our model system.

It has been reported that HGF also inhibits T cell proliferation or activation [18,19]. Therefore, we next determined in the culture supernatants of BMSCs whether the levels of HGF in BMSCs increased after IBM-BMT + IBM-DLI. We measured HGF (and also TGF-β) in the message level by a quantitative real-time RT–PCR because no ELISA kit is available to detect murine HGF. As shown in Fig. 5a (HGF) and 5b (TGF-β), the relative levels of both HGF and TGF-β were significantly higher in the BMSCs from the recipients of IBM-BMT + IBM-DLI than in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Furthermore, as summarized in Table 2, we did not detect substantial levels of IL-2, IL-4 or IL-15 mRNA in BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). However, it is noted that a slight but significant level of IL-10 message was detected only in the BMSCs from recipients of IBM-BMT + IBM-DLI, but not in those from recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Therefore, T cells injected directly into the BM cavity can induce the production of suppressive cytokines from BMSCs, and BMSCs might exert their inhibitory effect on T cell activation or proliferation via HGF and/or TGF-β.

Discussion

Transplantation biology has been one of the major advances in medicine during the last few decades. BMT, in particular, can cure a variety of malignancies by exploiting graft-*versus*-tumour effects exerted by the lymphocytes. In this proce-

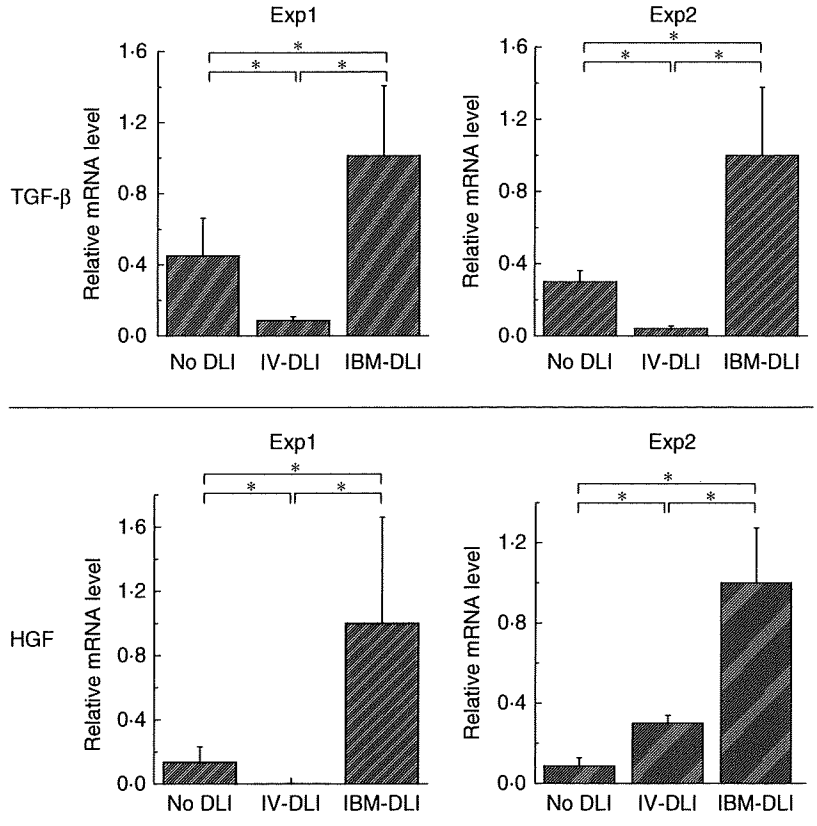

Fig. 5. Production of transforming growth factor (TGF)-β and hepatocyte growth factor (HGF) in bone marrow stromal cells (BMSCs). Culture expanded BMSCs from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time PCR. After DNase I treatment, cDNA was synthesized, amplified using HGF or TGF-β primer, and visualized with SYBR Green by real-time reverse transcription–polymerase chain reaction. Relative intensity of HGF or TGF-β mRNA was calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intensity. Columns represent relative cytokine message levels of TGF-β and HGF. Each column shows mean ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient), and we performed two separate experiments. Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with cytokine message performed in the groups ($P < 0·05$).

Table 2. Analyses of cytokine messages by real-time reverse transcription–polymerase chain reaction (RT–PCR).

Cytokines examined	No DLI†	i.v.-DLI	IBM-DLI	T cells with ConA‡
IL-2	0·47 ± 0·3§	0·31 ± 0·2	0·47 ± 0·3	8·51 ± 6·1
IL-4	0	0	0·025 ± 0·03	1277·2 ± 357·4
IL-10	0	0	2·7 ± 2·3	95 000 ± 16 000
IL-15	0	0	0	n.d.

†Culture expanded bone marrow stromal cells (BMSCs) from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time RT–PCR. After DNase I treatment, cDNA was synthesized, amplified using interleukin (IL)-2, IL-4, IL-10 or IL-15 primer, and visualized with SYBR Green by real-time RT–PCR. ‡Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

§Relative intensities of soluble factors were calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Numbers in the table represent mean intensities of cytokines ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). We performed two separate experiments. n.d., not done.

dure, one of the major problems to be solved is GVHD. We have developed recently a new protocol for BMT: IBM-BMT can induce persistent allogeneic donor-specific tolerance without the use of immunosuppressants after the treatment, even when the radiation doses are reduced to sublethal levels. Therefore, we have aimed to develop a new strategy for the successful engraftment of donor-derived haematolymphoid cells without developing GVHD even in the presence of T cells in the donor inoculum. We have found that GVHD could be alleviated when BMCs containing T cells were inoculated into the BM cavity [9]. We compared the severity of GVHD induced by the intravenous injection of T cells (i.v.-DLI) with that induced by the IBM injection of T cells (IBM-DLI). Acute GVHD was observed in recipients treated with IBM-BMT + i.v.-DLI, while reduced GVHD was seen in those treated with IBM-BMT + IBM-DLI. However, the mechanisms underlying this inhibition still remain unresolved and therefore we focused on the function of BMSCs, because T cells can interact with BMSCs in the BM cavity after the IBM-DLI. The ability of MSCs to interact with immune cells and to modulate their response has important implications in the transplantation biology. We have carried out experiments in which the sorted $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR. The inhibitory ability of non-haemopoietic BMCs to activated T cells was insufficient (Fig. 2). However, cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) showed an immunosuppressive effect in MLR in a dose-dependent fashion (Fig. 3). Furthermore, of interest and of importance is that the cultured BMSCs from the recipients of IBM-BMT + IBM-DLI suppressed MLR strongly even in small numbers (10^2–3×10^3) when compared with BMSCs from the recipients of IBM-BMT + i.v.-DLI.

Furthermore, the conversion of Th1 cells (defined by intracellular staining of IFN-γ) was clearly inhibited while the polarization of Th2 cells (defined by intracellular staining of IL-4) was facilitated by BMSCs from the recipients treated with IBM-DLI. In contrast to this, BMSCs from the recipients of i.v.-DLI prompted the polarization of Th1 cells (Fig. 4). These data suggest that BMSCs from the recipients of IBM-BMT + IBM-DLI interact with naive T cells to convert Th2 cells, which might be beneficial for GVHD management.

Several recent reports have described how BMSCs produce soluble factors, including TGF-β and HGF, which regulate T cell proliferation [18,21,22,32]. In our present study, BMSCs from the recipients of IBM-BMT + IBM-DLI produced significantly higher amounts of HGF and TGF-β than those from the recipients of IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) (Fig. 5 and Table 1).

Collectively, our findings indicate clearly that BMSCs can interact with T cells that have been injected into the BM cavity as IBM-DLI, and that the function(s) of BMSCs might somehow be modulated by this interaction to produce inhibitory cytokines and to possess the ability to convert Th0 cells to Th2 cells, but not to Th1 cells. It should be noted that the modulated features of BMSCs were maintained for at least 6 weeks, thus leading to the reduction of GvH responses. We have shown, in our GVHD model, that IBM-DLI (*in vivo* injection of donor T cells into the BM cavity) (but not i.v.-DLI) can attenuate GVHD. Therefore, our present study provides the basic information that IBM-BMT is an excellent strategy to engraft donor cells efficiently along with attenuation of GVHD, even when some quantities of T cells are contaminated in BMC preparations. Thus, IBM-BMT can control GVHD easily.

T cells can recognize MHC determinants on BMSCs *in vivo*, and the BMSC recognized by T cells can modulate their functions. Therefore, we are now investigating subcellular processes after the T–BMSC interaction and identifying molecules, other than MHC, to be essential for this interaction.

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ORIGINAL ARTICLE

Transplantation of newborn thymus plus hematopoietic stem cells can rescue supralethally irradiated mice

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We attempted to rescue supralethally irradiated (SLI) mice by transplantation of hematopoietic stem cells (HSCs) plus thymus from variously aged donors (fetus, newborn and adult). Although the transplantations of these kinds of HSCs alone showed a very short survival, newborn liver cells (NLCs) (as the source of HSCs) plus newborn thymus (NT) transplantation markedly improved the survival rate. The transplantation attenuated severe damage in the small intestine, which is one of the major causes of death by SLI. In addition, the donor-derived CD4$^+$ T cells significantly increased with additional NT transplantation. The production of interleukin (IL)-7 and keratinocyte growth factor, which plays a crucial role in protection against radiation injury in the intestine, was the highest in NT. Finally, SLI mice that had received NLC plus IL-7$^{-/-}$ NT transplantation plus IL-7 injection showed improved survival, weight recovery and an elevated number of CD4$^+$ T cells compared with the mice that had received NLC plus IL-7$^{-/-}$ NT or plus IL-7 injection alone. These findings suggest that NLCs plus NT transplantation can rescue SLI mice most effectively, and that high production of IL-7 in NT plays a crucial role with induction of CD4$^+$ T cells.

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Introduction

In recent years, bone marrow transplantation (BMT) has become a powerful strategy for the treatment of intractable diseases, such as hematological disorders (leukemia, lymphoma and aplastic anemia), congenital immunodeficiencies, metabolic disorders, autoimmune diseases and malignant tumors.[1] Using various animal models, we have found that allogeneic BMT can be used for the treatment of such diseases.[2–9] The basic theory is to replace pathogenic hematopoietic cells of hosts with normal hematopoietic stem cells (HSCs) of donors following lethal irradiation.

Exposure to supralethal irradiation (SLI) can occur, for example, in criticality accidents or in the treatment of malignant tumors.[10–13] High doses of irradiation induce severe damage not only in hematopoietic cells but also in other organs such as the gastrointestinal tract and brain,[14] leading to early death. Conventional BMT is thus ineffective for SLI recipients, because the organ damage is overwhelming. Indeed, HSC transplantation was unable to rescue a recent case of criticality accident, even though donor-derived cells were detected.[15,16] Rescue from SLI is thus extremely difficult.

The thymus is the central organ of T-cell development. We have previously reported that BMT plus thymus transplantation can accelerate hematopoietic recovery and improve survival rate, and can be used to treat autoimmune diseases in recipients such as aged or chimeric resistant hosts,[7,17] in which conventional BMT is difficult.

Interleukin (IL)-7 is produced by thymic epithelial cells, marrow stromal cells, fibroblasts and intestinal epithelia, and plays a crucial role in the early T-cell development and the functions in the thymus.[18–24] In addition, IL-7 engages in mucosal immunity, including the development of γδ T cells.[25–27] Notably, IL-7 signals have also been reported as an important factor in the regeneration of the gastrointestinal cells after irradiation.[28] Keratinocyte growth factor (KGF) is the significant cytokine for generating epithelial cells.[29] In embryogenesis, both KGF produced by thymocytes and IL-7 by thymic epithelial cells play a part in the development of the thymus.[30] Additionally, KGF is effective in treating intestine injured by irradiation and chemotherapy.[31]

In the present study, we attempted to rescue SLI mice using HSC transplantation plus thymus transplantation from variously aged donors, since the functions of the thymus greatly differ with age.[32,33] We here show that the transplantation of newborn liver cell (NLCs) plus newborn thymus (NT) can most effectively rescue SLI mice. It is likely that the high production of IL-7 by NT transplantation plays an important role in the induction of CD4$^+$ T cells.

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Materials and methods

Mice
Female 6- to 8-week-old, newborn (≤48 h after birth) and 16-day fetus C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were obtained from Shimizu Laboratory Supplies (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. IL-7 gene null (IL-$7^{-/-}$) mice with B6 background were kindly provided by Professor Ikuta from Kyoto University (Kyoto, Japan).[27]

HSCs and thymus transplantation
The 6- to 8-week-old female BALB/c mice received lethal irradiation (7 Gy) or SLI (9.5 Gy) 1 day before HSC transplantation. The next day, 1×10^7 B6 HSCs were injected intravenously into these mice. Bone marrow cells were collected from the femurs and tibias of 6- to 8-week-old B6 mice. Newborn and fetal livers were obtained and single-cell suspensions were created for the use of NLCs and fetal liver cells as the source of HSCs.[34,35] Adult thymus (AT), NT and fetal thymus (FT) tissues were removed from the aged mice. For thymus transplantation, one-quarter of the AT, or one NT or one FT was simultaneously transplanted under the renal capsule in some recipients with HSC transplantation. Thymus transplantation alone was also performed in other mice.

IL-7 treatment in vivo
Recombinant mouse IL-7 (Perpro Tech EC, London, UK) in PBS was injected intraperitoneally into chimeric mice for 7 days after HSC transplantation (1 μg per mice per day). Control mice were injected with PBS alone.

Reverse transcription-PCR
Reverse transcription-PCR analysis was employed for the determination of IL-7 mRNA. In brief, total RNA was extracted from each isolated thymus using RNagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Reverse transcription of 1 μg of RNA to cDNA was performed using oligo(dT) (Perkin Elmer Cetus, Norwork, CT, USA). Primer sequences of IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PCR condition were as follows: IL-7 (forward), 5′-ACAT CATCTGAGTGCCACA-3′; IL-7 (reverse), 5′-CTCTCA GTAGTCTCTTTAG-3′ (355 bp); KGF (forward), 5′-ATC CTGCCAACTCTGCTACAGA-3′; KGF (reverse), 5′-CT TCCCTTTGACAGGAATCCCCTT3′; GAPDH (forward), 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH (reverse), 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp). Each reaction was performed at 94 °C for 30 s for denaturation, then optimal annealing temperature (IL-7, 45 °C; KGF, 55 °C; GAPDH, 55 °C) for 30 s and 72 °C for 30 s for elongation (35 cycles). PCR products were analyzed by electrophoresis in 2% agarose gels and made visible by staining with ethidium bromide.

Western blotting
Each thymus tissue sample (1 mg per sample) was lysed on ice for 40 min in 20 μl of cell lysis buffer (0.5% Nonidet P-40 (Sigma, St Louis, MO, USA), 0.15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.2) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Following centrifugation at 8000 r.p.m. for 10 min, the lysate supernatants were normalized for protein concentration using the Bradford reagents (Pierce Chemical, Rockford, IL, USA). Samples were boiled for 5 min in SDS-reducing buffer, separately treated by SDS–PAGE (12% acryl-amide, wt/vol), and then electrophoretically transferred onto nitrocellulose membranes. Membranes were probed for invariant chain with the IN-1 Moabs. Anti-human KGF antibody (goat anti-mouse affinity-purified IgG) (R&D Systems, Minneapolis, MN, USA) and anti-mouse IL-7 antibody (goat anti-mouse affinity-purified IgG) were applied at 1:100 dilution. Binding was detected using a horseradish peroxidase-conjugated anti-goat IgG (American Pharmacia Biotech, Piscataway, NJ, USA) diluted at 1:1500 and visualized by chemiluminescence.

Analysis of surface markers and the numbers of lymphocytes by flow cytometry
Surface markers on lymphocytes from peripheral blood and spleen cells were analyzed by three-color fluorescence staining using a FACScan system (Becton Dickinson, Franklin Lakes, NJ, USA). FITC-conjugated anti-H-$2K^b$ MoAbs (Pharmingen, San Diego, CA, USA) were used to determine chimerism. FITC-, phycoerythrin- or biotin-conjugated CD4, CD8 or B220 (Becton Dickinson or Pharmingen) was used for analyses of lymphocyte subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used for the third color in the avidin/biotin system. The numbers of lymphocyte subsets in peripheral blood or in spleen cells were calculated as the total lymphocyte numbers of WBCs measured by SF-3000 with SFVU-1 unit (Sysmex, Kobe, Japan), or as the total lymphocyte numbers of spleen cells multiplied by the percentage of the lymphocyte cells.

Pathological findings
The small intestine, grafted thymus under the renal capsule and other organs from chimeric mice were fixed in 10% formaldehyde solution and embedded in paraffin. Sections 4-μm thick were prepared and stained using hematoxylin and eosin. Histology was examined under microscopy.

Statistical analysis
Nonparametric analyses (paired or unpaired Mann–Whitney *U*- and log-rank tests) were performed using StatView software (Abacus Concepts, Berkeley, CA, USA). Values of $P<0.05$ were considered statistically significant.

Results

Survival rates and chimerism in SLI mice receiving HSCs with or without thymus transplantation from variously aged donors
We first examined the effects on survival rates in SLI (9.5 Gy) mice that had received HSCs with or without thymus transplantation from variously aged (fetus,

newborn and adult) donors (Figure 1). In total, 80% of BALB/c mice that had been irradiated with a conventional low dose (7 Gy) survived >100 days after the transplantation of 1×10^7 bone marrow cells of B6 mice. In contrast, most of the 9.5-Gy-irradiated BALB/c mice died within 14 days after the transplantation of 1×10^7 HSCs from variously aged B6 mice (Figure 1a), since BALB/c mice are radio-sensitive and 9.5 Gy is an SLI dose. Next, we performed additional thymus transplantation in SLI mice (Figure 1b). Interestingly, NLCs with NT transplantation significantly improved the survival rate (70% survival at 100 days after transplantation), in comparison with NLC transplantation alone and all the other combinations. NT transplantation alone did not improve the survival rate. The engrafted thymus showed a normal structure under the renal capsule, and normal T-cell differentiation was observed in the thymus 8 weeks after transplantation (Figure 2).

Histology and body weight in SLI mice receiving NLC plus NT transplantation

Next, we investigated the causes of death in SLI mice. Histologically, the most damaged organ was the small intestine in the mice that had received NLC transplantation alone. In contrast to normal small intestine (Figure 3a; i), the mucosa displayed marked necrosis, and only a few cryptae were left 7 days after transplantation (Figure 3a; ii). However, with NT transplantation, severity was attenuated (Figure 3a; iii) and the mucosa with cryptae displayed good regeneration 14 days after the transplantation (Figure 3a; iv). The body weight of SLI mice that had received NLC transplantation alone was significantly reduced compared with conventional dose (7 Gy)-irradiated mice at 7 days (Figure 3b). However, it was significantly recovered with additional NT transplantation. SLI mice that had received HSCs alone or HSCs with AT or FT transplantation showed short survival rates (data not shown).

Analyses of chimerism and lymphocyte subsets from SLI mice receiving NLCs with or without NT transplantation

Supralethal irradiation mice that had received NLCs plus NT transplantation showed full donor-type chimerism (H-2K^{b+}) at 2 weeks after transplantation, and it continued for more than 12 weeks (Figure 4a). However,

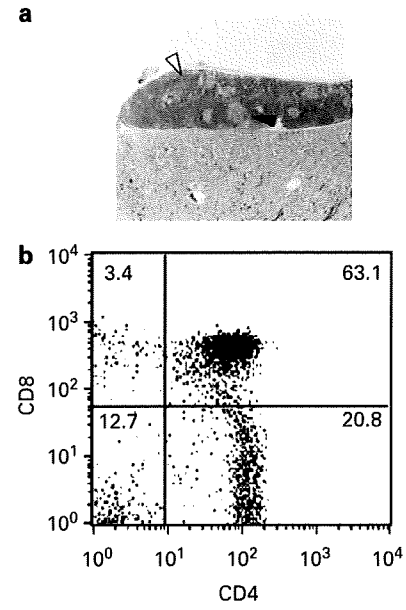

Figure 2 Histology and CD4/CD8 expression in transplanted thymus from SLI mice receiving NLCs plus NT transplantation. Histology (hematoxylin and eosin, ×200) (**a**) and percentages of CD4$^+$ and CD8$^+$ thymocytes (**b**) in engrafted NT from SLI BALB/c mice that had received 1×10^7 NLCs plus NT transplantation from B6 mice at 8 weeks after transplantation. The engrafted thymus is seen under the renal capsule, and cortical (open arrow) and medullary areas (closed arrow) were well demarcated (**a**). Cells were stained with anti-mouse CD4 and CD8 MoAbs and analyzed by flow cytometry (**b**). Representative data are shown from five independent experiments. NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

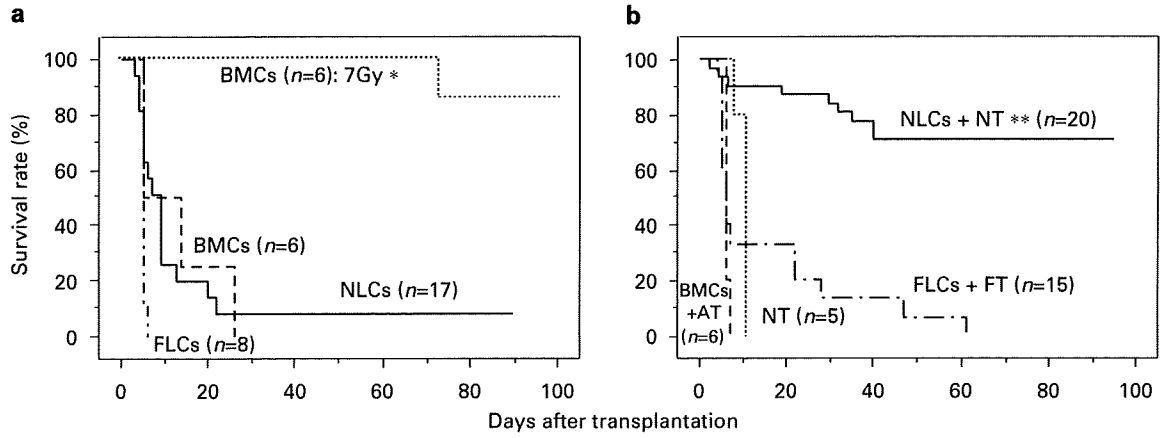

Figure 1 Survival rate in lethally irradiated or SLI mice receiving HSCs with or without thymus transplantation from variously aged donors. Survival rate for lethally irradiated mice (7 Gy) that had received 1×10^7 BMCs alone and SLI BALB/c mice (9.5 Gy) that had received 1×10^7 FLCs, NLCs or BMCs alone (**a**). Survival rate for SLI BALB/c mice (9.5 Gy) that had received 1×10^7 FLCs plus FT transplantation, NLCs plus NT transplantation, BMCs plus AT transplantation or NT transplantation alone (**b**). *$P<0.005$ compared with BMCs, NLCs or FLCs. **$P<0.005$ compared with NLCs, NT, BMCs plus AT or FLCs plus FT. AT = adult thymus; BMCs = bone marrow cells; FT = fetal thymus; HSCs = hematopoietic stem cells; FLCs = fetal liver cells; NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.