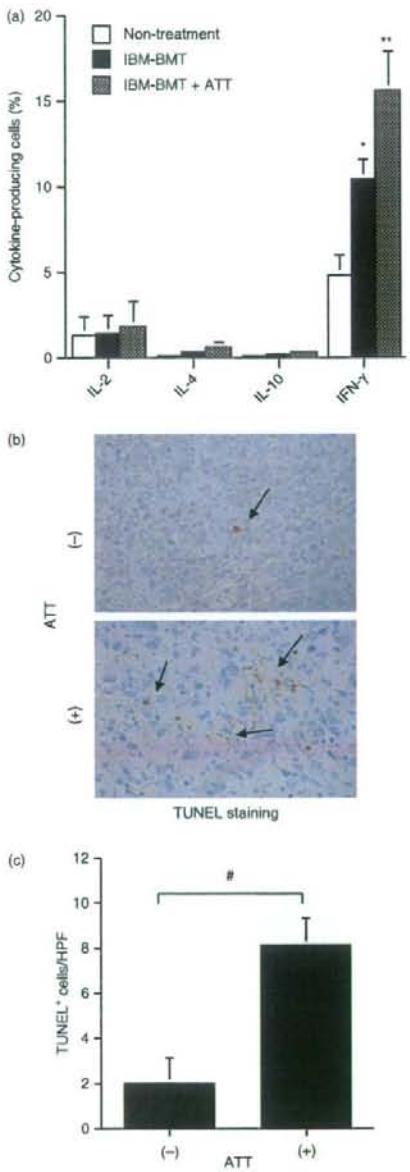

Figure 6. Analysis of cytokine production and apoptotic cells in tumours following intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). (a) Spleen cells were intracytoplasmically stained with phycoerythrin-anti-interleukin (IL)-2, IL-4, IL-10 or interferon (IFN)-γ monoclonal antibodies (mAbs) to determine the per cent of IL-2-, IL-4- or IL-10-producing cells. Non-treatment, $n = 4$; IBM-BMT alone, $n = 4$; IBM-BMT + ATT, $n = 4$. $^{*}P < 0{\cdot}05$ compared with non-treatment; $^{**}P < 0{\cdot}05$ compared with non-treatment and IBM-BMT alone. (b) Representative findings for tumour cells with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) staining (×400). TUNEL-positive tumour cells were observed (arrows). Numbers of TUNEL^{+} cells after treatment with IBM-BMT with and without ATT were compared. Numbers of TUNEL-positive tumour cells were significantly elevated in the mice treated with IBM-BMT + ATT in comparison with those treated with IBM-BMT. IBM-BMT + ATT, $n = 5$; IBM-BMT alone, $n = 5$. Data are shown as mean ± standard deviation (SD). $^{\#}P < 0{\cdot}01$.

Discussion

In the present study, we have demonstrated that allogeneic IBM-BMT + ATT successfully induces high thymopoiesis. Although this treatment induced mild GVHR, survival rate, weight and donor-derived chimerism did not differ from those in mice that received allogeneic IBM-BMT alone. Interestingly, when IBM-BMT + ATT was performed in tumour-bearing mice, tumour growth was significantly inhibited, compared with non-treatment or IBM-BMT alone. IBM-BMT + DLI did not produce a longer survival time than IBM-BMT + ATT. The donor-derived CD8 T cells markedly infiltrated the tumour after IBM-BMT + ATT, and the tumour cells underwent apoptosis as a result of lymphocyte infiltration with elevation of IFN-γ. These findings strongly indicate that IBM-BMT + ATT induces high thymopoiesis, thereby eliciting strong GVT effects with mild GVHR.

We first examined the effects of IBM-BMT + ATT on normal mice. The transplanted thymus showed a normal structure under the renal capsule and normal thymocyte differentiation. Although the number of spleen cells did not differ between the mice treated with IBM-BMT alone and the mice treated with IBM-BMT + ATT, the numbers of both CD4 and CD8 T-cell subsets were significantly higher with ATT than without ATT. In addition, the number of TRECs in the mice treated with IBM-BMT + ATT was significantly higher than in the mice treated with IBM-BMT alone, with the number increasing over time. These results indicate the successful induction of high and continuous thymopoiesis by IBM-BMT + ATT, and that most T cells are derived from TT.

As some T cells were produced by the transplanted allogeneic thymus in the mice treated with IBM-BMT + ATT, the cells should display anti-host activity, as seen in GVHR. However, in contrast to IBM-BMT + DLI, which induces severe GVHD with rapid mortality,[41] IBM-BMT + ATT elicits only mild GVHD.

We next attempted to explain the present data by the frequency of Tregs, detected as FoxP3^{+} cells in CD4^{+} T cells; this frequency was slightly lower in the recipients of IBM-BMT + ATT than in the recipients of IBM-BMT

alone or in control B6 mice, but significantly higher than in the mice treated with IBM-BMT + DLI. The low number of CD4 T cells in mice treated with IBM-BMT + DLI may result from the progression of GVHD. Much evidence has recently accumulated that Tregs are involved in the regulation of GVHD in mice,[42,43] and that, in humans, the reduced frequency of Tregs is also observed in patients with chronic GVHD and is negatively correlated with its severity.[44] This was the case in the present study, in which severe GVHD was observed in recipients with few Tregs after IBM-BMT + DLI, while only mild GVHD was observed in recipients possessing relatively high numbers of Tregs in the spleen after IBM-BMT + ATT. IBM-BMT alone produced no GVHD and a high proportion of Tregs, comparable to that in non-treated mice. These findings strongly suggest the participation of Tregs in the inhibition or negative regulation of GVHD.

Next, we investigated GVT effects associated with IBM-BMT + ATT. Interestingly, the growth of Meth-A tumours was most significantly inhibited by IBM-BMT + ATT, compared with non-treatment. IBM-BMT alone also produced significant regression, compared with non-treatment. This may be partially attributable to the effects of irradiation. In addition, IBM-BMT plus a high dose of DLI also induced strong tumour regression, but death from severe GVHD, whereas IBM-BMT plus a low dose of DLI elicited less tumour regression but mild GVHD with a long survival time. In histological analysis, IBM-BMT + ATT produced marked lymphocyte infiltration inside the tumour involving significant high numbers of donor-derived CD8 T cells compared with IBM-BMT alone. In addition, IFN-γ, which is a T helper type 1 (Th1) cytokine, also showed significantly high production in the mice with IBM-BMT + ATT, compared with the non-treated mice and the mice treated with IBM-BMT alone. Because IFN-γ itself has a GVT effect, it may not only promote an immune response with GVH effects, but also facilitate tumour regression. The mice treated with IBM-BMT alone also showed higher IFN-γ production than did the non-treated mice, suggesting that not only irradiation but also IFN-γ may play a role in tumour regression in these mice, too. As a result, the tumour cells also displayed significantly increased apoptosis. Although other tumour cell lines should be examined, these findings indicate that IBM-BMT + ATT induces strong GVT effects with donor cytotoxic $CD8^+$ T lymphocytes against the tumour.

In the MLR analyses, T cells showed a low anti-host response in the mice treated with IBM-BMT + ATT. As the mice treated with IBM-BMT alone showed no response to host cells, the difference is probably derived from ATT. Notably, the anti-host response was lower than in the mice treated with IBM-BMT + DLI. In addition, there was a positive correlation with the degree of GVHD and a negative correlation with the percentage of Tregs.

We could thus induce GVT effects without inducing severe GVHD by treatment with allogeneic IBM-BMT + ATT. Although the details of the mechanisms are still unknown, there are a number of possibilities. One is that Tregs also function to create the GVT effect in recipients treated with IBM-BMT + ATT, as it has been reported that Tregs suppress GVHR induced by CD4 T cells, but do not reduce GVT effects induced by CD8 T cells;[29,45] Tregs suppress the peripheral proliferation of CD8 T cells but do not inhibit cytotoxic T lymphocyte (CTL) activity. Thus, the intermediate proportion of $CD4^+$ $FoxP3^+$ Tregs induced by IBM-BMT + ATT can only achieve incomplete – but continuous – inhibition of GVHR but can maintain CTL activity, which leads to strong GVT effects. The other possibility, from the viewpoint of effector T-cell development, is that, in the case of IBM-BMT + DLI, the mature T cells with allo-MHC reactivity that were present easily induced severe GVHD. In the case of IBM-BMT + ATT, the reactivity of allo-specific T cells derived from the grafted thymus might be insufficient as a result of incomplete repertoire formation, as the thymic dendritic cells in the engrafted B6 thymus may present BALB/c-derived molecules (including their MHC) in negative selection during thymopoiesis. However, tumour-specific antigens are difficult to detect in the transplanted thymus because the tumour itself is located far from the thymus. As a result, the small number of allo-specific T cells (but not Tregs) may have proliferated peripherally and led to the low ratio of Tregs in CD4 T cells. To confirm these findings, further studies are required of the expression of CD4, CD8 and Foxp3 staining in GVHD and GVT sites. In addition, direct transfer and/or deletion experiments using Tregs should be carried out.

The continuous supplementation of T cells from the allogeneic ATT may induce mild GVHD and strong GVT effects with well-balanced effector and regulatory T cells. Alternatively, the transplanted thymus itself may regulate homeostasis of the cells. Thymus transplantation thus initially appears to be a simple method, but may prove to be an effective approach in that it supplies the organ in which T cells are differentiated, produced and functionally regulated. The method may be adequate to cure slow progressive diseases such as cancers, whereas the direct transfer of T cells, including Tregs, may be adequate for acute diseases such as infection and in the case of acute rejection.

Overall, we have found that allogeneic IBM-BMT + ATT induces high thymopoiesis with a mild GVH reaction and elicits strong GVT effects. Although it may clinically be difficult to obtain adequate thymus ethically and technically (with problems including donor age), grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as previously utilized.[33] In this respect, we have recently found that, even if the thymus

donor is different from the donor of BMCs, the effect is comparable to that seen with transplantation from the same donor using triple chimeric mice.[46] In addition, a method of regenerating the thymus has also been developed dramatically.[47] We thus believe that IBM-BMT + ATT could become a viable strategy for the treatment of malignant tumours in humans.

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ORIGINAL ARTICLE

Allogeneic intra-BM-BMT plus adult thymus transplantation from same donor has benefits for long-term survival even after sublethal irradiation or low-dose BM cell injection

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We examined the effects of intra-BM-BMT (IBM-BMT) plus adult thymus transplantation (ATT) from the same donor after 5.5 Gy sublethal irradiation (SubLI) or low-dose (3×10^6) BM cell injection (LDBMCI). With SubLI, BALB/c mice that had received 1×10^7 bone marrow cells by IBM-BMT plus ATT from B6 mice showed 73% donor chimerism, whereas those treated with IBM-BMT alone showed 45% chimerism. In the LDBMCI with 7Gy irradiation, IBM-BMT plus ATT resulted in a 90% survival rate with 90% chimerism, whereas IBM-BMT alone resulted in a 55% survival rate with 44% chimerism. Although the number of CD4 T cells was higher in IBM-BMT plus ATT than in IBM-BMT alone, the percentages of FoxP3$^+$/CD4$^+$ T cells and lymphocyte functions in the former were almost identical to those in the latter. When treated with IBM-BMT plus donor lymphocyte infusion (DLI), the mice showed a reduced survival time as a result of GVHD, with low numbers of FoxP3$^+$CD4 T cells under either condition, although 100% chimerism was induced. These results suggest that IBM-BMT plus ATT is effective in reconstituting the recipients with donor-derived cells even after SubLI or LDBMCI.

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Keywords: thymus transplantation; T cells; nonmyeloablative regimen; injection of a low number of BMCs; T_{reg} cells

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Introduction

BMT has been used as a potentially curative therapy for patients with a wide variety of diseases, including hematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases and solid tumors.[1–6]

However, there are some factors to take into account to ensure that BMT is performed safely and effectively. It is, for example, important to reduce the dose of irradiation to prevent side effects such as radiation-induced organ injury.[7,8] To resolve these problems, attempts have been made to transplant high doses of hemopoietic stem cells (HSCs), and/or carry out donor lymphocyte infusion (DLI).[9,10] However, there may be difficulties in obtaining sufficient numbers of HSCs, or lethal GVHD may be induced by DLI. The development of a new cell-based method is therefore required.

We have developed various new allogeneic BMT methods in mice. To supply recipients with MHC -matched donor BM stromal cells, we previously performed BMT plus bone grafts from the same donor.[5] To induce extramedullary hematopoiesis in the liver, we injected whole BM cells (BMCs; HSCs and stromal cells) from the portal vein.[11] Finally, we have recently developed intra-BM (IBM)-BMT, in which BMCs are directly injected into the BM cavity.[12]

We have also developed concurrent thymus transplantation (TT) with BMT; BMT plus TT was found to be effective for recipients showing thymic involution caused by aging, supralethal irradiation, chimeric resistance or malignant tumor.[13–16] However, it is unknown whether BMT plus TT is effective when using a nonmyeloablative regimen and/or when transplanting insufficient numbers of BMCs. In addition, TT has only been clinically applied to patients with DiGeorge syndrome or HIV infection who show hypoplasia of the thymus;[17,18] the effects of TT on BMT have not been extensively examined.

In this study, we, therefore, examined the effects of adult TT (ATT) with IBM-BMT from the same donor under the condition of sublethal irradiation (SubLI) or low-dose BMC injection (LDBMCI) in an attempt to resolve the abovementioned limitations.

Materials and methods

Mice

Six- to eight-week-old female BALB/c mice (H-2^d) and age-matched female C57BL/6(B6) (H-2^b) mice were obtained from Shimizu Laboratory Supplies (Shizuoka, Japan). All mice were kept in our animal facilities under specific pathogen-free conditions. B6 mice were used as donors and BALB/c mice were used as recipients.

BMT, ATT and DLI

For SubLI, the recipient BALB/c mice were 5.5 Gy-irradiated using a ^{137}Cs irradiator (0.9 Gy/min) 1 day before BMT. BMCs were flushed from the shafts of the femora and tibias of donor mice (B6) and single-cell suspensions were prepared. BMCs (1×10^7) were injected directly into the BM cavity of the tibia, as described earlier for the IBM-BMT method.[12] To ensure the LDBMCI, the recipient BALB/c mice were lethally irradiated (7.0 Gy) 1 day before BMT; BALB/c mice are radiosensitive and 7.0 Gy is the lethal dose for BALB/c mice. BMCs (3×10^6) from the B6 mice were injected directly into the BM cavity by the IBM-BMT method. A quarter of the removed thymic lobe from the donor B6 mice was grafted simultaneously under the renal capsule of the left kidney as ATT, or 1×10^7 of spleen cells from the donor B6 mice were i.v. transplanted as a DLI. The dose of the DLI was determined in our earlier study.[16]

Pathologic findings

The transplanted thymus, liver and small intestine from the chimeric mice were fixed in 10% formaldehyde solution and embedded in paraffin. Serial tissue sections 4 μm thick were prepared and stained with hematoxylin–eosin (HE). The histology was examined under the microscope. The degree of GVHD was evaluated using a semiquantitative scoring system for abnormalities known to be associated with GVHD, as previously described.[16] The scoring system for each parameter denotes 0 as normal; 0.5 as focal and rare, 1 as focal and mild, 2 as diffuse and mild; 3 as diffuse and moderate, and 4 as diffuse and severe in GVHD. The maximum score for the liver was thus 40, and for the small intestine it was 28.

Flow cytometry

Surface markers on lymphocytes from peripheral blood and the spleen were analyzed under three-color fluorescence staining using a FACScan system (Becton Dickinson, Franklin Lakes, NJ, USA). FITC-conjugated anti-H-2K^b (Pharmingen, San Diego, CA, USA) was used to determine chimerism. Phycoerythrin or biotin-conjugated CD4 or CD8 (Pharmingen) was used to analyze lymphocyte subsets. Avidin-PE-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed in spleen cells using an FITC-antimouse/rat FoxP3 staining set (eBioscience, San Diego, CA, USA). The procedure was performed in accordance with the instructions of the manufacturer. Donor-derived chimerism was determined from the presence of more than 90% H-2K^{b+} cells by flow cytometry in the peripheral blood of the chimeric mice 2–4 weeks after BMT.

Mitogen response and MLR

To analyze lymphocyte function and tolerance, mitogen response and MLR were performed in chimeric mice 2 months after transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and untreated B6 and BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY, USA) containing 200 μl of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 μl of glutamine (Wako Pure Chemicals, Tokyo, Japan), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated FCS. For mitogen responses, responder cells were incubated with 2.5 μg/ml of ConA (Calbiochem, San Diego, CA, USA) or 25 μg/ml of lipopolysaccharide (Difco Laboratories, Franklin Lakes, NJ, USA) for 72 h. For MLR, T cells enriched by magnetic beads were used for responders as follows. Splenocytes from chimeric mice were incubated with rat antimouse CD45R, CD11b and Gr-1 (Pharmingen) for 30 min, washed and incubated for an additional 30 min with Dynabeads sheep antirat IgG (Dynal Biotech ASA, Oslo, Norway). The negative fraction was used as T-cell enriched splenocytes (purity >98%). The enriched T cells were incubated with 2×10^5 splenocytes irradiated at 15 Gy from various strains of mice including donor (B6) mice, recipient (BALB/c) mice and third-party (C3H) mice as stimulators for 96 h. Next, 20 μl of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA, USA) was introduced during the last 18 h of the culture period. Incorporation of ^{3}H-TdR was measured using Microbeta TriLux (PerkinElmer, Wellesley, MA, USA). The stimulator index for MLR was calculated as the average of ^{3}H-TdR incorporation (stimulator in medium)/^{3}H-TdR incorporation (medium) in triplicate wells.

Statistical analysis

Nonparametric analyses (Mann–Whitney *U*-test and log-rank test) were performed with StatView software (Abacus Concepts, Berkeley, CA, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Survival rates and body weight changes in mice treated with IBM-BMT alone or IBM-BMT plus ATT under condition of SubLI or LDBMCI

In our preliminary experiments, we carried out conventional BMT (i.v. injection of BMCs: i.v. BMT) under the condition of SubLI (5.5Gy) or LDBMCI (3×10^6 cells). Although all the (B6→BALB/c) mice which had been treated with i.v. BMT under these conditions showed a 100% survival rate, no donor-derived cells were detected due to rejection. Therefore, we carried out all the subsequent experiments using IBM-BMT.

First, we examined the effects of IBM-BMT plus ATT on survival rates under the condition of SubLI or LDBMCI.

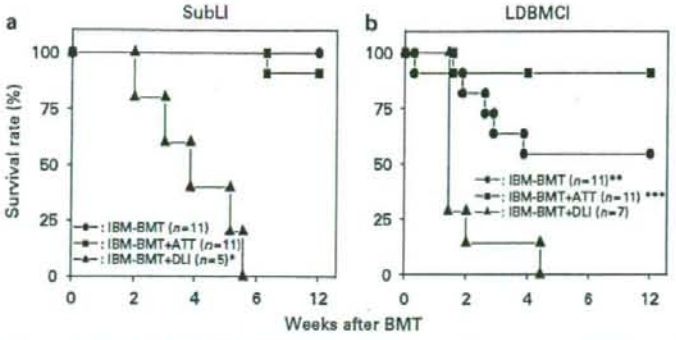

Figure 1 Survival rates of mice treated with IBM-BMT or IBM-BMT plus ATT or DLI under the condition of SubLI or LDBMCI. The survival rates of sublethally irradiated (5.5 Gy) BALB/c mice that had received 1×10^7 BM cells from B6 mice by IBM-BMT alone, or IBM-BMT plus ATT or DLI under the condition of SubLI (a). The survival rates of lethally irradiated (7.0 Gy) BALB/c mice that had received the low number of 3×10^6 BMCs from B6 mice by IBM-BMT alone or IBM-BMT plus ATT or DLI under the condition of LDBMCI (b). $^*P<0.01$ compared with the mice that received IBM-BMT alone or IBM-BMT plus ATT. $^{**}P<0.05$ or $P<0.01$ compared with the mice that received IBM-BMT plus ATT or DLI. $^{***}P<0.01$ compared with the mice that received IBM-BMT plus DLI. ATT, adult thymus transplantation; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; SubLI, sublethal irradiation.

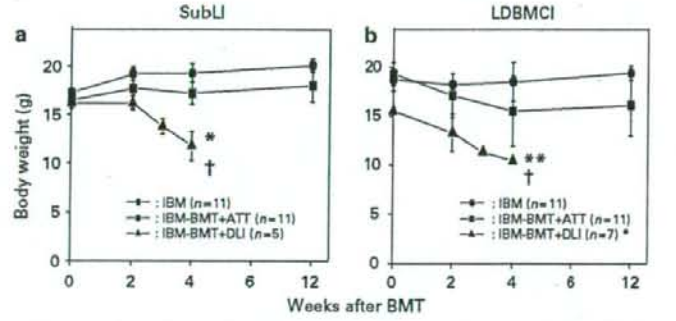

Figure 2 Body weight changes in mice treated with IBM-BMT or IBM-BMT plus ATT or IBM-BMT plus DLI under the condition of SubLI or LDBMCI. The body weight change of sublethally irradiated (5.5 Gy) BALB/c mice that had received 1×10^7 BM cells from B6 mice by IBM-BMT alone or IBM-BMT plus ATT under the condition of SubLI (a). The weight change for lethally irradiated (7.0 Gy) BALB/c mice that had received a low number (3×10^6) of BMCs from B6 mice by IBM-BMT plus ATT or IBM-BMT alone under the condition of LDBMCI (b). $^*P<0.01$ compared with the mice that received IBM-BMT alone or IBM-BMT plus ATT at 4 weeks after transplantation. $^{**}P<0.01$ compared with the mice that received IBM-BMT alone or $P<0.05$ compared with IBM-BMT plus ATT at 2–4 weeks after transplantation because of early death. †, all mice died. ATT, adult thymus transplantation; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; SubLI, sublethal irradiation.

With SubLI, most of the BALB/c (H-2^d) mice that had received a high dose (1×10^7) of BMCs from B6 mice (H-2^b) by IBM-BMT alone or IBM-BMT plus ATT survived for a long time (Figure 1a). With LDBMCI, the BALB/c mice that had received IBM-BMT plus ATT showed a significantly higher survival rate than did the mice treated with IBM-BMT alone (Figure 1b). With either SubLI or LDBMCI, the mice treated with IBM-BMT plus ATT survived more than 6 months (data not shown). In contrast, the mice that had received BMCs by IBM-BMT plus DLI (1×10^7 spleen cells) as a GVHD model showed the shortest survival under either condition, most of them having died within 5 weeks of the transplantation (described later). In the analyses of body weight, although the BALB/c mice treated with IBM-BMT plus ATT showed a slight decrease in body weight compared with the mice that were treated with IBM-BMT alone, there was no significant difference between them under either condition (Figure 2). In contrast, the mice treated with IBM-BMT plus DLI showed a significant weight loss because of GVHD under both conditions.

Effects of chimerism in mice treated with IBM-BMT alone or IBM-BMT plus ATT under condition of SubLI or LDBMCI

Next, we investigated donor-type chimerism in mice treated with IBM-BMT alone or IBM-BMT plus ATT under the condition of SubLI or LDBMCI 4 weeks after transplantation. Under SubLI, the mice treated with IBM-BMT alone showed 45.5% donor chimerism, whereas the mice treated with IBM-BMT plus ATT showed 72.7% donor chimerism (Table 1). Under the condition of LDBMCI, in the case of lethal irradiation (7 Gy), the mice treated with IBM-BMT alone showed 44.4% donor chimerism, whereas the mice that had received IBM-BMT plus ATT showed 80% donor

Table 1 Donor chimerism in mice treated with IBM-BMT alone or IBM-BMT plus adult ATT or DLI under condition of SubLI or LDBMCI

Group	Treatment	Donor type/ total[a] (%)	% of H-2K^{b+} cells in donor chimerism	
			Donor type	Host type
Experiment 1: SubLI				
1	IBM-BMT	5/11 (45.5)	99.7 ± 0.5	0.3 ± 0.4
2	IBM-BMT + ATT	8/11 (72.7)	99.2 ± 1.4	2.3 ± 3.5
3	IBM-BMT + DLI	5/5 (100)	99.6 ± 0.9	—
Experiment 2: LDBMCI				
1	IBM-BMT	4/9 (44.4)	98.2 ± 0.5	0.2 ± 0.1
2	IBM-BMT + ATT	8/10 (80.0)	94.2 ± 2.4	3.3 ± 1.4
3	IBM-BMT + DLI	7/7 (100)	99.1 ± 0.3	—

Abbreviations: ATT = adult thymus transplantation; DLI = donor lymphocyte infusion; IBM-BMT = intra-BM-BMT; LDBMCI = low-dose BM cell injection; SubLI = sublethal irradiation.
[a]Chimeric mice with more than 90% of H-2K^{b+} cells in the peripheral blood were regarded as showing chimerism by flow cytometry. Analyses were performed 4 weeks after transplantation in mice that had received IBM-BMT or IBM-BMT plus ATT and 2–4 weeks after transplantation in mice that had received IBM-BMT plus DLI because of early death. These results were obtained from three pooled experiments.

chimerism (Table 1). As the mice treated with IBM-BMT plus DLI died early, we analyzed the mice 2–4 weeks after transplantation. The mice that had received IBM-BMT plus DLI showed 100% donor chimerism under both conditions.

Histology of transplanted thymus from mice treated with IBM-BMT plus ATT

In histological analyses, the engrafted thymus showed a normal structure under the renal capsule, and normal T-cell differentiation was observed in the thymus of the mice treated with IBM-BMT plus ATT, under the condition of SubLI, 8 weeks after transplantation (Figure 3a). The transplanted thymus from the mice that had received IBM-BMT plus ATT under the condition of LDBMCI also showed the same results (data not shown).

Liver and small intestine histology in mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI

In the liver, the mice treated with IBM-BMT plus ATT showed mild lymphocyte infiltration and the GVHD score in liver was as low as that of the mice treated with IBM-BMT alone. In contrast, severe lymphocyte infiltration was observed with bile duct destruction in the mice treated with IBM-BMT plus DLI under either condition, comparable to a finding of GVHD (Figure 3b). The GVHD score was significantly higher in the mice treated with IBM-BMT plus DLI than in the mice treated with IBM-BMT alone; all these mice died of GVHD within 5 weeks after BMT. In addition, although a number of lymphocytes also infiltrated the mucosa of the small intestine with some fibrosis in the mice treated with IBM-BMT plus DLI, such findings were minimal or nonexistent in the mice treated with either IBM-BMT plus ATT or IBM-BMT alone. The GVHD score in the small intestine of the mice treated with IBM-BMT plus ATT was also as low as that of the mice treated with IBM-BMT alone.

Effects of lymphocyte subsets for mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI

We further analyzed lymphocyte subsets in mice treated with IBM-BMT alone or IBM-BMT plus ATT under the condition of SubLI or LDBMCI 4 weeks after transplantation. As the mice treated with IBM-BMT plus DLI died early, we analyzed the mice 2–4 weeks after transplantation. In the spleen, the number of CD4T cells was highest in the mice treated with IBM-BMT plus ATT, second in the mice treated with IBM-BMT alone, and lowest in the mice treated with IBM-BMT plus DLI (Figure 4). In contrast, the number of CD8T cells in IBM-BMT plus ATT was almost the same as that in IBM-BMT alone, and significantly higher than that in IBM-BMT plus DLI. The number of B220$^+$ B cells was highest in the mice treated with IBM-BMT alone, second in the mice treated with IBM-BMT plus ATT and lowest in the mice treated with IBM-BMT plus DLI. We also examined the CD4$^+$ FoxP3$^+$ regulatory T (T_{reg}) cells (Figure 5), which suppress immune responses.[19] Interestingly, although the number of T_{reg} cells in the mice treated with IBM-BMT plus ATT was significantly higher than that in the mice treated with IBM-BMT alone, the percentages of T_{reg} cells in the CD4T cells from the former were comparable to the latter under either condition (Figure 5b). However, although the percentages of CD4T cells were also high in the spleens from mice treated with IBM-BMT plus DLI (Figure 5a), the numbers and the percentages in CD4T cells of the FoxP3$^+$ T_{reg} cells were the lowest under either condition (Figure 5b).

Lymphocyte function in the spleen of mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI

Finally, we examined the lymphocyte functions in the spleen from mice treated with IBM-BMT alone or IBM-BMT plus ATT under the condition of SubLI or LDBMCI. Spleen cells in both groups showed sufficient mitogen responses to both Con A and lipopolysaccharide , the levels being comparable with those of normal B6 and BALB/c mice (Figure 6a). However, the responses were remarkably reduced in the mice treated with IBM-BMT plus DLI under the condition of SubLI or LDBMCI. The assay for MLR revealed that T cells from the mice treated with IBM-BMT alone or IBM-BMTplus ATT under the condition of SubLI or LDBMCI showed tolerance to both BALB/c (host type) and B6 (donor type) MHC determinants but normal responsiveness to third-party (C3H type) MHC determinants (Figure 6b). As expected, T cells from the mice treated with IBM-BMT plus DLI under either set of conditions showed responsiveness not only to a third-party (C3H-type) but also to BALB/c (host-type) MHC determinants; it should be noted that T cells from mice treated with IBM-BMT plus DLI showed marked responsiveness to BALB/c (host-type) MHC determinants resulting from GVHD.

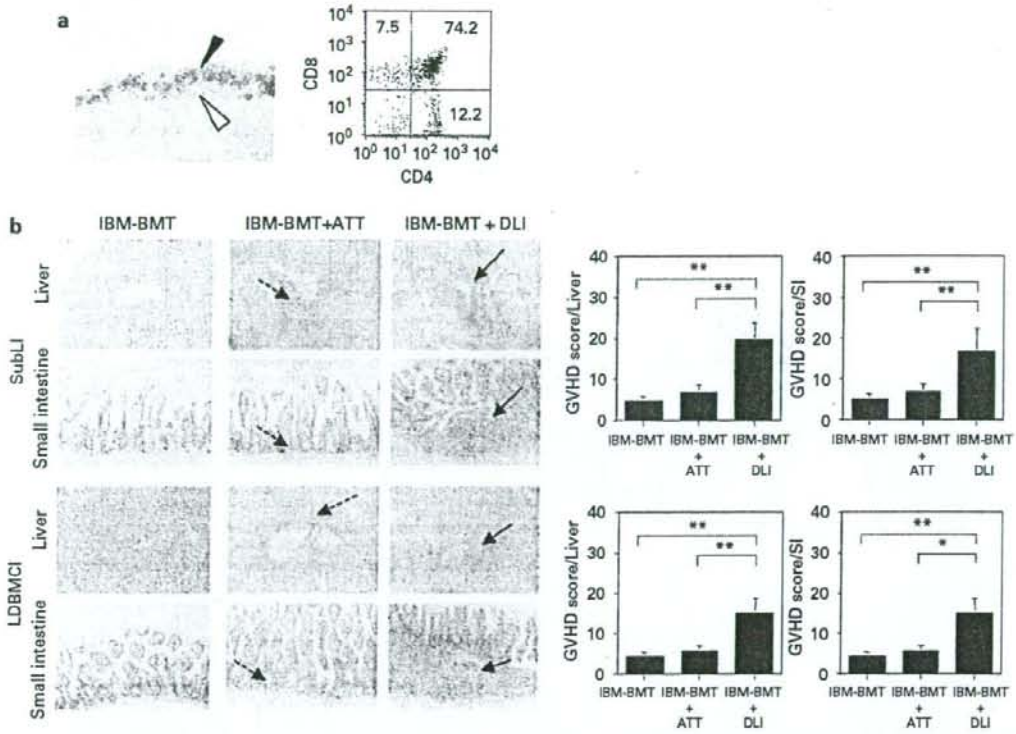

Figure 3 Histology in mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under the condition of SubLI or LDBMCI. Microscopic findings in the transplanted thymus of BALB/c mice that had been irradiated (5.5 Gy) and reconstituted with 1×10^7 BMCs from B6 mice by IBM-BMT plus ATT 8 weeks after BMT under the condition of SubLI (HE, 100×; **a**). The thymus tissue was engrafted under the renal capsule. The cortical (closed arrowhead) and medullary (open arrowhead) areas display fine construction (left panel). Representative FACS profiles for $CD4^+$ and/or $CD8^+$ thymocyte subsets are shown in the transplanted thymus (right panel). The histology of the liver and small intestine and the GVHD scores of the mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under the condition of SubLI or LDBMCI (HE staining, ×200) are shown in Figure (**b**). Lymphocytes infiltrate the portal area of the liver and the mucosa of the small intestine with fibrosis as GVHD (arrows) in mice with IBM-BMT + DLI under the condition of SubLI or LDBMCI. In contrast, only a small number of infiltrating lymphocytes were found in the mice treated with IBM-BMT plus ATT (dotted arrows) and almost no lymphocytes were found in the mice treated with IBM-BMT alone in these areas (representative results are shown). GVHD scores ± s.d. are shown (lower panels). $N=5$ in the mice treated with IBM-BMT alone, $N=5$ in mice treated with IBM-BMT plus ATT, and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of SubLI; $N=4$ in the mice that had received IBM-BMT alone, $N=5$ in the mice that had received IBM-BMT plus ATT, and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of LDBMCI. Analyses were performed 4 weeks after transplantation in the mice treated with IBM-BMT alone and the mice treated with IBM-BMT plus ATT and 2–4 weeks after transplantation in the mice treated with IBM-BMT plus DLI because of early death. $^*P<0.05$, $^{**}P<0.01$. ATT, adult thymus transplantation; BMCs, BM cells; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; SubLI, sublethal irradiation.

Discussion

In this study, we have examined the effects of ATT on allogeneic IBM-BMT under the condition of SubLI or LDBMCI. Mice treated with IBM-BMT plus ATT showed a significantly long survival rate and high donor chimerism in comparison with the mice treated with IBM-BMT alone or IBM-BMT plus ATT under either condition. Although the numbers of CD4T cells in the mice treated with IBM-BMT plus ATT were significantly higher than in the mice treated with IBM-BMT alone, the percentages of T_{reg} cells in CD4T cells from the mice treated with IBM-BMT plus ATT were similar to those in the mice treated with IBM-BMT alone; the percentages were significantly higher than those in the mice treated with IBM-BMT plus DLI, which show lethal GVHD. Finally, the mice treated with IBM-BMT plus ATT showed sufficient lymphocyte functions but tolerance to both donor-type and recipient-type MHC determinants. These findings indicate that under the condition of SubLI or LDBMCI, IBM-BMT plus ATT is superior to IBM-BMT alone.

First, we examined the effects of ATT on the survival rate and chimerism under the condition of SubLI or LDBMCI. Under the condition of SubLI, the mice treated with IBM-BMT alone and the mice treated with IBM-BMT plus ATT survived for a long time (Figure 1). However, the chimerism in the latter was much greater than in the former (Table 1). Under the condition of LDBMCI, the mice treated with IBM-BMT plus ATT showed a significantly longer survival rate with a higher percentage of donor

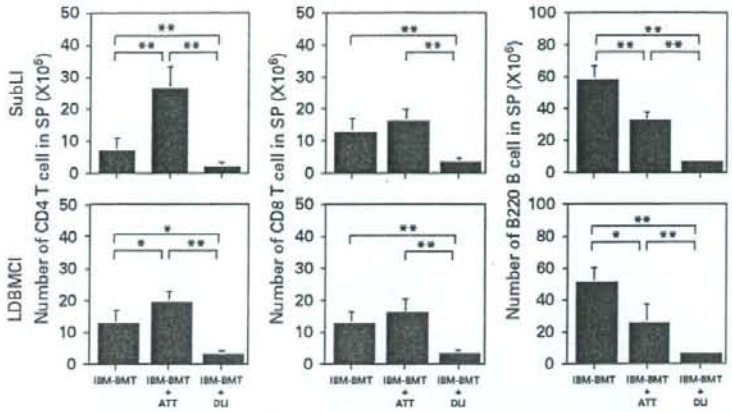

Figure 4 Analyses of numbers of lymphocyte subsets in spleen from mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI. The numbers of donor-derived H-2K^{b+} CD4$^+$ T, CD8$^+$ T or B220$^+$ B cells in the spleen from mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under the condition of SubLI or LDBMCI were analyzed (mean ± s.d.). $N=5$ in the mice treated with IBM-BMT alone, $N=5$ in the mice treated with IBM-BMT plus ATT, and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of SubLI; and $N=4$ in the mice treated with IBM-BMT alone, $N=5$ in the mice treated with IBM-BMT plus ATT, and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of LDBMCI. Analyses were performed 4 weeks after transplantation in the mice treated with IBM-BMT alone and the mice treated with IBM-BMT plus ATT and 2–4 weeks after transplantation in the mice treated with IBM-BMT plus DLI because of early death. $^*P<0.05$, $^{**}P<0.01$. ATT, adult thymus transplantation; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; SubLI, sublethal irradiation.

chimerism than did the mice treated with IBM-BMT alone. The cause of death in the mice treated with IBM-BMT alone was graft failure because of the low percentage of chimerism. In contrast, under either condition, the mice treated with IBM-BMT plus DLI showed a significantly short survival with GVHD (Figures 2 and 3), even when they showed 100% donor chimerism. Although the mice treated with either IBM-BMT plus ATT or IBM-BMT alone showed little weight gain, they survived a long time and showed no apparent histological findings of GVHD, suggesting the nondevelopment of severe or lethal GVHD, as we reported earlier in MRL/lpr[12] and normal mice[20] or tumor-bearing mice.[21] These results indicate that IBM-BMT plus ATT is superior to the other strategies for survival and/or chimerism.

Second, we studied the donor-derived lymphocyte subsets under the condition of SubLI or LDBMCI. The numbers of CD4T cells in the mice treated with IBM-BMT plus ATT were significantly higher than in the mice treated with IBM-BMT alone (Figure 4). In contrast, the numbers of CD8T cells were no different between the mice treated with IBM-BMT plus ATT or not. Although the reason for this discrepancy is unclear, we also observed the same in newborn TT with BMT in supralethally irradiated mice.[15] Transplanted thymus may predominantly supply CD4T cells or suppress CD8T cells. The low number of all lymphocyte subsets in the mice treated with IBM-BMT plus DLI may be the result of the GVHD.[22]

From the above results, there is a clear correlation between the involvement of mature T cells in transplanted cells and donor chimerism, suggesting that the alloresponse of the T cells plays an important role in reconstituting the recipients with donor cells. However, severe GVHD was not evident in the mice treated with IBM-BMT plus ATT. One explanation is that the percentages of FoxP3$^+$ T_{reg} cells in CD4T cells in the mice treated with IBM-BMT plus ATT were as high as in the mice treated with IBM-BMT alone (Figure 5), although it was lowest in the mice that had received IBM-BMT plus DLI. As FoxP3$^+$ T_{reg} cells have suppressive effects on GVHD,[19] the elevated proportion of FoxP3$^+$ T_{reg} cells may be related to the low incidence of GVHD in the mice treated with IBM-BMT plus ATT.[23]

There are two types of T_{reg} cells: one is naturally occurring T_{reg} (nT_{reg}) cells, which are derived from the thymus and the other is induced T_{reg} (iT_{reg}) cells, which are induced in the periphery from FoxP3$^-$ CD4$^+$ T cells.[24] It is likely that the former works predominantly in the mice treated with IBM-BMT plus ATT. However, it is still unknown how T_{reg} cells are supplied from ATT and functionally suppress GVHD. It has been shown that nT_{reg} cells are induced by dendritic cells, which can present autoantigens in the thymic medulla and suppress autoreactive T cells as converted negative selection.[25–27] Therefore, nT_{reg} cells induced from ATT may be responsible for the induction of specific tolerance to host antigens. It is also conceivable that the development of nT_{reg} cells from the transplanted thymus in allogeneic host plays a crucial role in the suppression of the antihost response. Further study is required to identify the exact mechanism involving the transfer and/or deletion of T_{reg} cells.

Finally, we analyzed *in vitro* the immune functions of the mice treated with IBM-BMT plus ATT under the condition of SubLI or LDBMCI. Significant immune responsiveness to both T-cell and B-cell mitogens was observed under either condition to be comparable to those in IBM-BMT alone (Figure 6a). In addition, the newly developed T cells showed a low response to the host but a normal response to

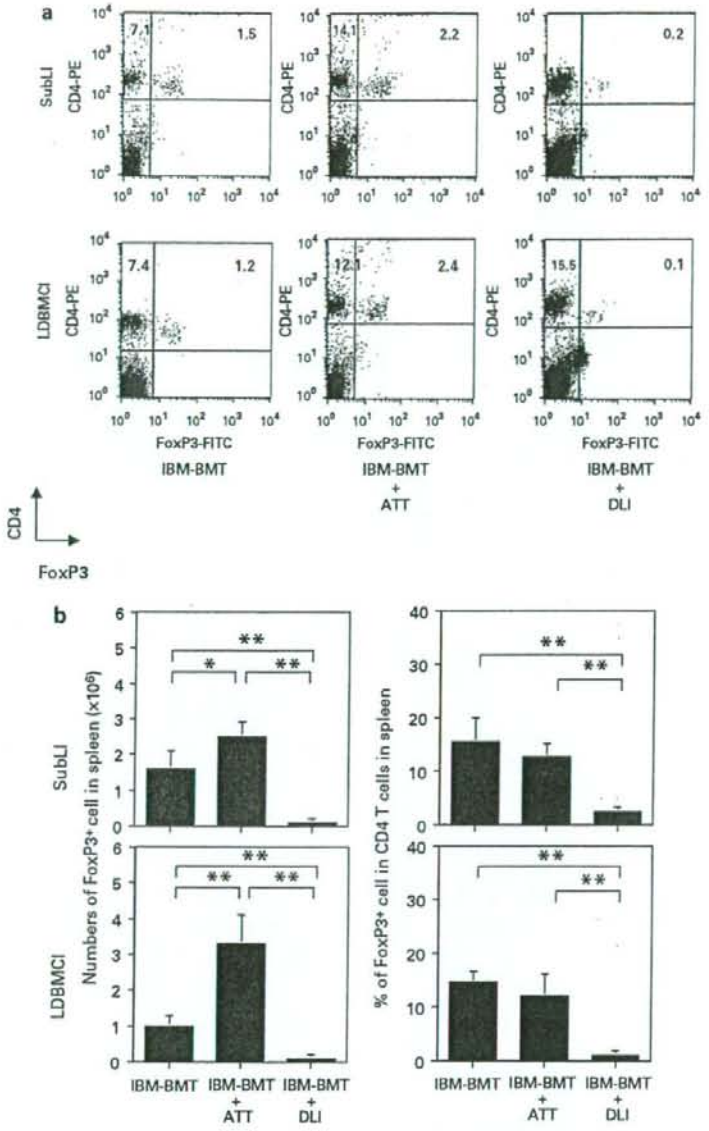

Figure 5 Analyses of the number and percentage of FoxP3$^+$/CD4$^+$ T (T_{reg}) cells in spleen from mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI. The numbers and percentage of FoxP3$^+$/CD4$^+$ T cells in the spleen from mice treated with IBM-BMT alone or IBM-BMT plus ATT or DLI under the condition of SubLI or LDBMCI were analyzed. Representative FACS profiles for FoxP3$^+$ (**a**) and the percentage of FoxP3$^+$/CD4$^+$ cells (mean ± s.d.) are shown in the spleen (**b**). $N=5$ in the mice treated with IBM-BMT alone, $N=5$ in the mice treated with IBM-BMT plus ATT and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of SubLI; and $N=4$ in the mice treated with IBM-BMT alone, $N=5$ in the mice treated with IBM-BMT plus ATT and $N=4$ in the mice treated with IBM-BMT plus DLI under the condition of LDBMCI. Analyses were performed 4 weeks after transplantation in the mice treated with IBM-BMT alone and the mice treated with IBM-BMT plus ATT and 2–4 weeks after transplantation in the mice treated with IBM-BMT plus DLI because of early death. *$P<0.05$, **$P<0.01$. ATT, adult thymus transplantation; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; SubLI, sublethal irradiation; T_{reg}, T regulatory cells.

a third party (C3H), confirming a host-specific tolerance (Figure 6b). Although we did not perform cytokine analyses in this study, we observed earlier that IFN-γ production was elevated in the mice treated with IBM-BMT plus ATT.[16] The cytokine may facilitate the engraftment of donor cells rather than promote an allo-response.

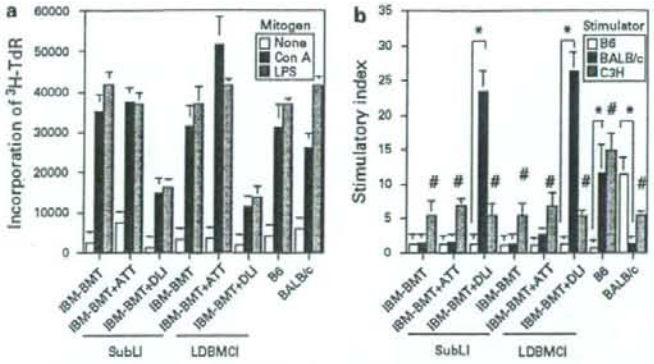

Figure 6 Mitogen responses and MLR in mice treated with IBM-BMT alone, or IBM-BMT plus ATT or DLI under condition of SubLI or LDBMCI. Mitogen responses to Con A and LPS in spleen cells (**a**) and MLR in T cells (**b**) from mice treated with IBM-BMT alone, or IBM-BMTplus ATT or DLI under the condition of SubLI or LDBMCI 12 weeks after BMT, and from the mice treated with IBM-BMT plus DLI 3 weeks after BMT. Normal B6 and BALB/c mice were also used as controls. The SI in the MLR was calculated as the average of ^{3}H-thymidineTdR incorporation (stimulator in medium)/^{3}H-thymidineTdR incorporation (medium) in triplicate wells. $^*P<0.05$. $^\#P<0.05$ compared with B6 and BALB/c stimulators in SubLI and ILNBMC in mice treated with IBM-BMT alone, IBM-BMT plus ATT and IBM-BMT plus DLI, with B6 stimulator in B6 mice, and with B6 and BALB/c stimulators in BALB/c mice. ATT, adult thymus transplantation; DLI, donor lymphocyte infusion; IBM-BMT, intra-BM-BMT; LDBMCI, low-dose BM cell injection; LPS, lipopolysaccharide; SI, stimulation index; SubLI, sublethal irradiation.

The low immune response to both mitogens, but a significantly high response to the host, were observed in the mice treated with IBM-BMT plus DLI under either condition, suggesting the presence of GVHD with immunodeficiency. Thus, successful BMT could be achieved in the mice that had received IBM-BMT plus ATT under either condition.

Overall, we have shown that IBM-BMT plus ATT is superior to IBM-BMT alone under the condition of SubLI or LDBMCI. TT alone has been clinically used for the treatment of DiGeorge syndrome and HIV infection.[17,18] From the ethical and clinical viewpoints, it would seem to be difficult to obtain the thymus and BMCs from the same young donors. However, we have recently shown that even MHC-mismatched thymus grafts can support thymopoiesis, which is comparable to a matched thymus graft.[28] In addition, there has recently been dramatic progress in research areas concerning thymus regeneration.[29,30] Thymus grafts in conjunction with IBM-BMT may become a valuable strategy for the treatment of aged patients with various intractable diseases.

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ORIGINAL ARTICLE

Aberrant expression of BCL2A1-restricted minor histocompatibility antigens in melanoma cells: application for allogeneic transplantation

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Abstract It has been shown that allogeneic hematopoietic stem cell transplantation (HSCT) can be one of the therapeutic options for patients with metastatic solid tumors, such as renal cancer. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the current setting. Thus, it is crucial to identify minor histocompatibility antigens (mHags) only expressed in tumor cells but not GVHD target organs. In this study, we examined whether three mHags: ACC-1 and ACC-2 encoded by *BCL2A1*, and HA-1 encoded by *HMHA1*, could serve as such targets for melanoma. Real-time PCR and immunohistochemical analysis revealed that the expression of both *BCL2A1* and *HMHA1* in melanoma cell lines and primary melanoma cells was comparable to that of hematopoietic cells. Indeed, melanoma cell lines were efficiently lysed by cytotoxic T lymphocytes specific for ACC-1, ACC-2, and HA-1. Our data suggest that targeting mHags encoded not only by *HMHA1*, whose aberrant expression in solid tumors has been reported, but also *BCL2A1* may bring about beneficial selective graft-versus-tumor effects in a population of melanoma patients for whom these mHags are applicable.

Keywords Minor histocompatibility antigen · Allogeneic hematopoietic stem cell transplantation · Melanoma

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure hematopoietic malignancies. The success of donor leukocyte infusion or a non-myeloablative conditioning regimen demonstrated that the therapeutic effects of allogeneic HSCT mostly rely on the allogeneic immune responses. In an HLA-matched setting, allogeneic immune responses are mediated by donor-derived cytotoxic T lymphocytes (CTLs) against minor histocompatibility antigens (mHags). Ubiquitously expressed mHags are responsible for both life-threatening graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect, whereas hematopoietic cell-restricted mHags, such as HA-1 [1] or ACC-6 [2], may be optimal target antigens which can potentially separate the GVL effect from GVHD development [3].

In some solid tumors, such as melanoma or renal cell carcinoma, there has been accumulating evidence that immunological manipulation, e.g., IL-2 [4, 5] or interferon treatment [6], can lead to clinical responses in some patients with refractory disease, although responses have been limited. Since the late 1990s, it has been reported that nonmyeloablative allogeneic HSCT leads to apparent tumor regression in these immunogenic solid tumors [7, 8]. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the

current setting. Thus, it is crucial to identify antigens that may serve as therapeutic targets for post-transplant vaccination or adoptive T-cell therapy to selectively augment the graft-versus-tumor (GVT) effects following allogeneic HSCT with modification to reduce fatal GVHD. Recently, we and others showed that the hematopoietic cell-restricted mHag HA-1^H, encoded by *HMHA1*, can be one of the potential targets for the GVT effect due to its aberrant expression in some solid tumors [9–11]. In addition, *ECGF1*-encoded mHag has been shown to be expressed in some solid tumors [12]. Thus, we sought to examine whether other mHags we had identified previously could also be expressed in any solid tumors and serve as potential targets for GVT effects.

The ACC-1 and ACC-2 mHags encoded by *BCL2A1* have been shown as hematopoietic cell lineage-restricted mHags [13]. Amino acid substitutions, ^{19}Cysteine→Tyrosine, and ^{82}Glycine→Aspartic acid, of BCL2A1 lead to donor-derived HLA-A*2402 and HLA-B*4403/4402-restricted $CD8^+$ CTL responses against the recipient's hematopoietic cells [13]. In this study, we demonstrate that *BCL2A1* is also highly expressed in melanoma cells and that they are effectively lysed by cognate CTLs. Although it has been reported that allogeneic HSCT was not promising against advanced melanoma in a small cohort of patients [14], our findings imply that targeting *BCL2A1*-encoded mHags may bring about beneficial GVT effects in a fraction of melanoma patients for whom these mHags are applicable.

2 Materials and methods

2.1 Cell lines and cell culture

$CD8^+$CTL clones recognizing $ACC1^Y$ (1B3-CTL) and ACC-2^D (3B5-CTL) were generated from post-HSCT recipients peripheral blood mononuclear cells (PBMCs) and HA-1^H (EH6-CTL) were from healthy volunteer's PBMCs, as previously described [11, 13]. These CTL clones were expanded using allogeneic PBMCs and Epstein Barr virus-transformed B cell lines (B-LCLs) as feeder cells and frozen until use.

Table 1 indicates the melanoma cell lines used in this study, kindly provided by Dr. Kawakami (Keio University, Tokyo, Japan). Their genotypes for the HLA class I and mHag allele were typed at the HLA Laboratory (Kyoto, Japan). All melanoma cell lines were cultured in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin/streptomycin. B-LCLs established by infecting PBMCs with B95-8 (ATCC, Rockville, MD, USA) supernatant and HLA class I-deficient mutant 721.221 B-LCL were cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Primary melanocytes, NHE-Ma(L) and HEMa-LP, were purchased from KURABO (Osaka, Japan) and cultured in specified medium according to the manufacturer's protocol. All blood and tissue samples were collected after obtaining written informed consent, and the study was approved by the Institutional Review Board of Aichi Cancer Center.

Table 1 HLA and mHags typing of melanoma cell lines

Cell line	HLA		mHag [a]		
	A loci	B loci	ACC-1	ACC-2	HA-1
888Mel	0101/2402	5201/5501	Y/C	D/G	R/R
HT144	0101/2402	1501/5701	Y/C	D/G	R/R
G361	2301/2601	3801/4403	Y/C	D/G	R/R
WM266	0201/2902	1302/4403	C/C	G/G	H/R
C32Mel	0201/2501	1801/4402	C/C	G/G	H/H
HS294T	0101/2501	0702/0801	C/C	G/G	R/R

[a] The phenotypes of mHags of individual melanoma cell lines are shown using a single-lettered amino acid code. ACC-1 mHag is considered to be positive when carrying a Y (tyrosine) residue (referred to as ACC-1^Y) at its polymorphic site, while it is negative when carrying a C (cysteine) residue (referred to as ACC-1^C). Similarly, ACC-2^D carrying D (asparaginic acid) is positive, while ACC-2^G carry G (glycine) is negative; HA-1^H carrying H (histidine) is positive, while HA-1^R carrying R (arginine) is negative

2.2 Messenger RNA expression of BCL2A1 and HMHA1 in melanoma cell lines

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Messenger RNA was magnetically isolated from total RNA using the μMACS mRNA Isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Complementary DNA was synthesized in the presence of oligo $(dT)_{15}$ primer (Roche) and M-MLV-reverse transcriptase (Invitrogen) according to the manufacturers' instructions.

PCR amplification and real-time quantification analysis were performed using the TaqMan assay according to the manufacturer's instructions. The following sequences were used as primers with the TaqMan probe to detect the mRNA region of each gene:

BCL2A1-sense: 5′-TGAATAACACAGGAGAATGGATAAGG-3′,
BCL2A1-antisense: 5′-TTCAGGAGAGATAGCATTTCACAGAT-3′,
BCL2A1-probe: 5′-(FAM)-CTGGCTGGATGACTTT-(MGB)-3′
HMHA1-sense: 5′-GAGGGCCTTGAGAAACTTAAGGA-3′

HMHA1-antisense: 5′-CAGCGGGTACTTGGAGATG ATC-3′,
HMHA1-probe; 5′-(FAM)-CTGCGTGTCATGCAT-(MGB)-3′

For an internal control, a primer and probe set for human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used. PCR was performed in a 1 × TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer, and 2 pmol of probe in a total volume of 25 μL in the ABI PRISM 7900HT Sequence Detector System (Applied Biosystems). The temperature profile was: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min for 40 cycles. Relative expressions were calculated by the $_{\Delta\Delta}C_T$ method after validation test described in the manufacturer's brochure (User Bulletin #2; Applied Biosystems 11 December 1997 (updated October 2001).

2.3 Immunohistochemical analysis of BCL2A1 expression in primary melanoma

To analyze the BCL2A1 protein expression in primary melanoma cells, we used frozen skin sections obtained from six patients with metastatic melanoma. The expression status was examined immunohistochemically with the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 [Santa Cruz, A1 (N-20): sc-6066].

2.4 Flow cytometric analysis of HLA Class I and BCL2A1 expression in melanoma cell lines

The cell surface HLA-class I expression of melanoma cell lines before and after treatment with interferon-γ (IFN-γ) and TNF-α was evaluated using W6/32 mAb (10 μg/mL) and FITC-conjugated anti-mouse IgG antibodies (Beckman Coulter). For the intracellular staining of BCL2A1 protein, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed once with PBS, and incubated with 40 μg/mL of goat polyclonal antibodies against BCL2A1 [Santa Cruz, A1 (N-20): sc-6066] for 15 min. After washing, bound antibodies were detected by incubation with FITC-conjugated donkey anti-goat IgG antibody (8 μg/mL, Santa Cruz, CA, USA) for 15 min. Cells were analyzed with a FACS Calibur flow cytometer and CellQuest software (Becton-Dickinson).

2.5 Cytotoxicity assay

Target cells were labeled with 0.1 mCi of ^{51}Cr for 2 h or overnight at 37°C, and 1 × 10^3 target cells/well were mixed with CTLs at an E/T ratio indicated in a standard 4-h cytotoxicity assay using 96 well, round-bottomed plates. All assays were performed at least in duplicate. Cells were treated either with IFN-γ (500 U/mL, R&D Systems) or TNF-α (10 ng/mL, Genzyme) for 48 h as indicated. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

3 Results

3.1 Melanoma cell lines express high levels of BCL2A1 gene

We previously demonstrated that *BCL2A1* is preferentially expressed in hematopoietic lineage cells but not other normal cells. By accessing a gene expression database, GNF (Genomic Institute of the Novartis Research Foundation, http://symatlas.gnf.org/SymAtlas/) [15], we found that *BCL2A1* is highly expressed in melanoma cell lines. Thus, we first tried to confirm the expression levels of *BCL2A1* in melanoma cell lines using real-time PCR. As shown in the lower part of Fig. 1a, most melanoma cell lines expressed the *BCL2A1* transcript at levels as high as B-LCLs, with the exception of the cell line HS294T, which eventually expressed a comparable level of the transcript after cytokine treatment (IFN-γ and TNF-α). Some cell lines also expressed *HMHA1* transcripts, but their levels were relatively low (Fig. 1b).

3.2 BCL2A1 expression in primary melanoma specimens

We subsequently tested whether primary melanoma cells expressed BCL2A1 protein. Skin sections from six patients (MM-1 to MM-6) with metastatic melanoma were stained with anti-BCL2A1 antibody (Fig. 2). Three specimens were positive for BCL2A1 (MM-1, 2, 3), while one was negative (MM-4). Another two samples were difficult to evaluate because of marked melanin pigmentation (MM-5, 6). Overall, 3/4 primary metastatic melanoma cells were positive for BCL2A1 protein. Along with the results of real-time PCR analysis, BCL2A1 was strongly and frequently expressed in melanoma cells.

3.3 Melanoma cell lines are susceptible to lysis by BCL2A1-specific CTL clones

To determine whether melanoma cell lines can indeed present BCL2A1-derived mHags on their cell surface HLA molecules, and are thus susceptible to lysis by CTLs specific for these mHags, we performed a standard

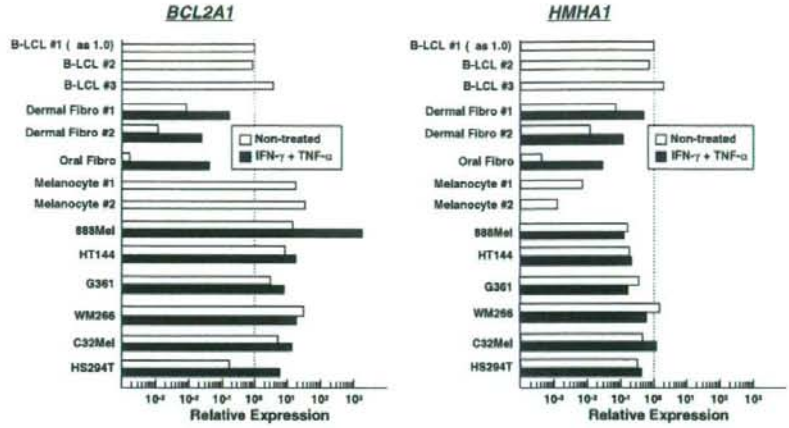

Fig. 1 Relative expression of *BCL2A1* (*left panel*) and *HMHA1* (*right panel*) in melanoma cell lines. Real-time reverse transcription PCR to quantify the mRNA expression of *BCL2A1* and *HMHA1* was performed using cDNA samples prepared from melanoma cell lines (kind gift from Dr. Kawakami, Keio University, Tokyo) and primary melanocytes, together with EBV-transformed B lymphoblasts (B-LCLs) and fibroblasts (Fibro) from skin and oral mucosa. IFN-γ + TNF-α (*solid bars*) denotes 48-h cytokine treatment with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α. *GAPDH* was used as an internal control. mRNA expression in B-LCLs is set as 1.0

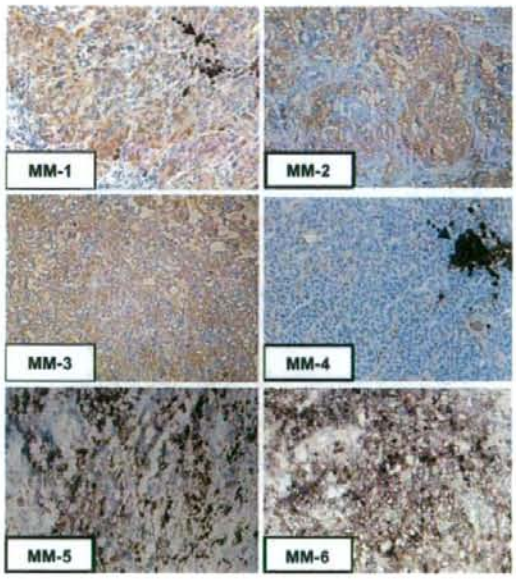

Fig. 2 Expression of BCL2A1 protein in primary metastatic melanoma (MM) cells. Frozen skin sections from six patients with metastatic melanoma were examined for BCL2A1 expression immunohistochemically by the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 (Santa Cruz, A1 (N-20): sc-6066). MM-1 to -3 were found to be positive; MM-4 negative; MM-5 and -6 showed marked melanin pigmentation. *Red arrows* in MM-1 and MM-4 indicate melanin spots, showing that the specimens were of melanoma origin

^{51}Cr-release assay. As shown in Fig. 3a, melanoma cell lines positive for respective mHags and restriction HLA alleles were lysed effectively by cognate CTL clones: 888Mel and HT144 by 18B3-CTL (HLA-A24-restricted, ACC-1^{Y}-specific), and G361 by 3B5-CTL (HLA-B44-restricted, ACC-2^{D}-specific). In contrast, HLA-class I-deficient K562 cell lines or melanoma cell lines lacking either the restriction HLA allele or cognate mHag allele that were used as control targets were not lysed at all, indicating that the observed cytotoxicity against melanoma cell lines by these CTL clones was antigen-specific. We also examined the expression of HLA-class I and intracellular BCL2A1 in these cell lines to evaluate the effect of cytokine treatment. All melanoma cell lines tested were positive for HLA-class I and BCL2A1, similarly to B-LCLs, with the one exception of HT144, whose BCL2A1 expression was 1-log lower than that of B-LCLs (Fig. 3B). Cytokine treatment upregulated HLA-class I expression in all melanoma cell lines, with one exception of 888 MEL, which might account for the increased lysis of G361 by 3B5-CTL and WM266 by EH6-CTL, respectively. The lower BCL2A1 expression in HT144 was also upregulated after treatment. However, cytokine treatment did not necessarily result in increased, specific lysis in cell lines other than G361 and WM266. Therefore, another mechanism might also be involved in the susceptibility to lysis of each cell line. In addition, two melanoma cell lines (WM266 and C32Mel) positive for the HLA-A*0201 and HA-1^{H} alleles could be recognized by EH6-CTL despite the relatively low expression of *HMHA1* compared to *BCL2A1*.

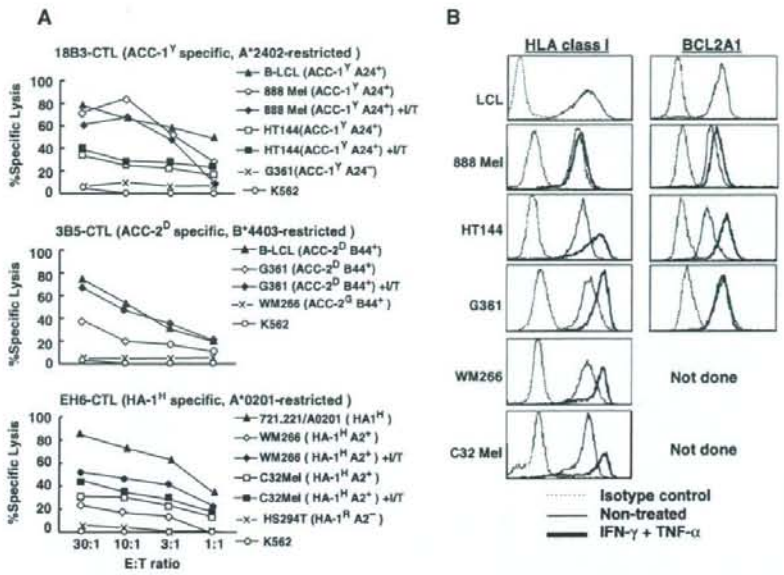

Fig. 3 Susceptibility of melanoma cell lines to mHag-specific CTL clones and the impact of cytokine treatment. **a** ^{51}Cr-release assay against melanoma cell lines. Standard 4-h ^{51}Cr-release assays were performed against various melanoma cell lines at the indicated E/T ratios and at least in duplicate. B-LCLs positive for the restriction HLA allele and mHag allele were used as positive controls for individual CTL clones. The 721.221 cell line comprised HLA-A*0201-transduced B-LCLs positive for the HA-1^{H} allele. Non-specific lysis of the individual CTL clones was examined and verified by testing against NK cell-sensitive K562 or melanoma cell lines that lacked either the cognate mHag or restriction HLA allele. I/T denotes the treatment of indicated cell lines with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α for 48 hours prior to assays; **b** HLA class I and BCL2A1 expression of melanoma cell lines. Cell surface expression of HLA class I and intracellular staining of BCL2A1 was evaluated by flow cytometry before and after treatment with the above cytokines

3.4 HMHA1 in dermal fibroblasts is also upregulated by inflammatory cytokines

It has been reported that *HMHA1* encoding HA-1 mHag is not detected in normal nonhematopoietic cells such as dermal fibroblasts [9], while *BCL2A1* is upregulated in bone marrow-derived mesenchymal stem cells by inflammatory cytokines [16]. Thus, we examined whether dermal fibroblasts upregulated these mHag genes and became susceptible to cognate CTL clones. We found that the expression of both *BCL2A1* and *HMHA1* is upregulated in the dermal fibroblasts after cytokine treatment (Fig. 1a, b, upper part), indicating that these hematopoietic cell-specific mHags might be induced in a strong inflammatory cytokine milieu such as active GVHD after HSCT. Hematopoietic cell contamination was excluded by real-time PCR or flow cytometric analysis of the expression of CD45 in these fibroblasts (data not shown). Coincident with expression, the HLA-A*0201-restricted HA-1^{H}-specific CTL clone, EH6-CTL, and A*2402 restricted ACC-1^{Y} specific CTL clone, 18B3-CTL, could lyse these cytokine-treated mHag-positive dermal fibroblasts, although their level of lysis was relatively lower than that of hematopoietic cells (Fig. 4a, b).

4 Discussion

In this study, we demonstrated that HLA-A24-restricted ACC-1^{Y} and HLA-B44-restricted ACC-2^{D} mHAgs, whose expressions were shown to be limited to hematopoietic cells including leukemia cells, were also expressed in melanoma cell lines by real-time PCR and cytotoxicity assays. Melanoma is known as one of the representative immunogenic tumors. Previously, IL-2 administration [4] or the infusion of ex vivo expanded TILs [17] was tested, but resulted in a limited clinical response. In 1990s, many antigens of melanoma origin recognized by autologous T lymphocytes were identified [18], and these antigens were subsequently tested in clinical trials by peptide vaccination or adoptive CTL infusion. To date, peptide vaccination has resulted in a limited or marginal efficacy [19] while adoptive T lymphocyte infusion including Ag-specific CTL clones or TILs, especially after a lymphodepleted conditioning regimen,

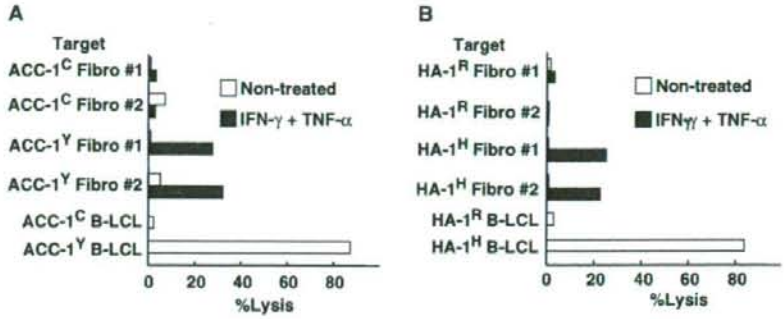

Fig. 4 Cytotoxicity of mHag-specific CTL clones against dermal fibroblasts. A standard 4-h ^{51}Cr-release assay was performed, as described above. **a** Cytotoxic activity of the HLA-A*2402-restricted, ACC-1^{Y} mHag-specific 1B3-CTL clone against HLA-A*2402-positive dermal fibroblasts (Fibro) and B-LCLs that were either ACC-1^{Y} mHag allele positive or negative (indicated as ACC-1^{C}); **b** cytotoxic activity of the HLA-A*0201-restricted, HA-1^{H}-specific EH6-CTL clone against HLA-A*0201-positive dermal fibroblasts and B-LCLs that were either HA-1^{H} mHag allele positive or negative (indicated as HA-1^{R}). IFN-γ + TNF-α treatment was performed as described in Fig. 3. The effector target ratio was fixed at 30:1

demonstrated promising results [20–22]. After allogeneic HSCT for patients with melanoma, there have been some reports indicating that CTLs against melanoma cells do exist and that these melanoma-reactive CTLs can be expanded in vitro [23, 24]. These observations suggest that allogeneic HSCT after a nonmyeloablative conditioning regimen might be a promising therapeutic strategy for patients with refractory metastatic melanoma.

Childs et al., however, reported relatively disappointing results in which 5 out of 11 metastatic melanoma patients receiving allogeneic HSCT died from rapid tumor growth, while the rest of the patients showed variable results [14]. As in the case of hematological malignancies, a high tumor burden should be one of the most unfavorable factors regarding treatment failure with allogeneic HSCT. Therefore, a treatment strategy combining the selection of patients with a lower tumor burden or slower growth kinetics and allogeneic HSCT may be explored for this poor-prognosis disease if the given donor and recipient are eligible for immunotherapy using ACC-1 and ACC-2 mHags, or other hematopoiesis-specific mHags are also highly expressed in melanoma cells. Since HSCT recipients eligible for ACC-1, ACC-2, and HA-1 mHags exist at a frequency of 11, 3, and 9%, respectively, in Japanese [25], it would be possible to apply these mHags to nearly a quarter of such patients.

BCL2A1 is a member of the B-cell lymphoma-2 (BCL2) family. *BCL2* is highly expressed in melanoma, which was shown to contribute to a chemoresistant phenotype [26]. The reduction of BCL2 by siRNA caused melanoma cells to become susceptible to chemotherapeutic agents. BCL2A1, although regulated differently from BCL2, also exerts antiapoptotic activity and is expressed even in normal melanocytes like other melanocyte differentiating antigens, such as Melan-A/MART-1 or tyrosinase. In this regard, BCL2A1 would be essential for melanoma cells and melanocyte survival, suggesting that it may be a good candidate antigen for immunotherapy against melanoma, although autoimmune depigmentation may also develop, as seen in adoptive immunotherapy targeting melanoma-associated antigens mentioned above [20, 21].

In addition, we unexpectedly found that, after cytokine treatment, dermal fibroblasts upregulated both *BCL2A1* and *HMHA1* expression and become susceptible to cognate CTL clones, respectively (Fig. 4). This suggests that, after allogeneic HSCT, they would also be upregulated under a "cytokine-storm", and may contribute in some way to the pathophysiology of skin GVHD. In the clinical setting, HA-1 was originally reported as an mHag associated with GVHD [27], and additional studies brought about mixed results, making it still too early to draw any conclusion [28, 29], while ACC-1^{Y} disparity did not seem to be associated with an increased incidence of acute GVHD [30]. In skin explant assays, it was shown that skin sections from HLA-A2^{+} HA-1^{+} individuals incubated with HA-1 CTLs developed only background grade I or low grade II GVH reactions, while male HLA-A2^{+} skin sections incubated with Y antigen-specific CTLs displayed severe GVH reactions of grade III–IV [31]. It is assumed that stronger GVH reactions might be observed if skin sections are pretreated with cytokines before incubation with HA-1-specific CTLs. IFN-γ, which is known to induce various transcription factors specific for hematopoiesis and immunity, might be a key in this upregulation of hematopoietic cell-restricted mHags in dermal fibroblasts. Since IFN-γ is strongly produced by CTLs and type 1 helper T cells, the IFN-γ secreted from mHag-specific CTLs could lead to the upregulation of target hematopoiesis-specific

mHags, resulting in GVHD or GVT effects in tumors sensitive to the IFN-γ-induced upregulation of such mHags. Therefore, it is crucial to develop a new treatment strategy to induce selective GVT effects while avoiding life-threatening GVHD using preconditioning and GVHD prophylaxis regimens to minimize GVHD, followed by selective immunotherapy targeting mHags mainly expressed in tumors and hematopoietic cells, such as ACC-1, -2, and HA-1, after the "cytokine storm" period is over.

In summary, *BCL2A1*-encoded mHags, ACC-1 and ACC-2, may be potential targets of immunological interventions for a fraction of patients with refractory, but not bulky melanoma following allogeneic HSCT.

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