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$CD4^+$ T Cell–Depleted Lymphocyte Infusion Impairs Neither the Recovery of Recipient Thymus nor the Development of Transplanted Thymus

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Thymus transplantation, in conjunction with bone marrow transplantation (BMT), has been attracting attention for the treatment of various diseases. Recently, donor lymphocyte infusion (DLI) has been used as a helpful tool for establishing donor chimerism and preventing a relapse of leukemia/lymphoma. However, the effects of DLI on transplanted and recipient thymuses have not been explored. We therefore performed DLI in the intrabone marrow–BMT + thymus transplantation setting. We have found that DLI leads to derangements in both recipient thymuses and transplanted thymuses; by 2 wk after BMT, we saw a decrease in total cell number, a lower percentage of $CD4^+CD8^+$ cells, and the obliteration of the thymic corticomedullary junction. Four weeks later, the thymic impairment became more serious. However, when we depleted the $CD4^+$ T cells ($CD4^-$-DLI), the recipient thymic recovery and transplanted thymic development were significantly restored by the treatment. In addition, there were much greater levels of TNF-α and Fas ligand, and a lower percentage of regulatory T cells in the DLI group than in the $CD4^-$-DLI group. These findings indicate that inflammation induced by DLI, especially by $CD4^+$ T cells, plays a crucial role in the thymic impairment. *The Journal of Immunology*, 2013, 190: 2976–2983.

Allogeneic bone marrow transplantation (allo-BMT) is a potentially curative therapy for certain diseases of the hematopoietic system, immunodeficiencies, autoimmune diseases, solid malignant tumors, and so on (1–6). We have developed a new and powerful bone marrow transplantation (BMT) method: intrabone marrow–BMT (IBM-BMT) (7), in which donor bone marrow cells (BMCs) are directly injected into the recipient's bone marrow cavity. Therefore, a much greater number of donor hematopoietic stem cells and mesenchymal stromal cells (including mesenchymal stem cells) can be inoculated into the recipient bone marrow by IBM-BMT than by conventional i.v. BMT. This results in the rapid reconstitution of donor hematopoietic cells and permits a reduction in the doses of irradiation used as a conditioning regimen (8–10).

The thymus is an organ for inducing T cells and maintaining homeostasis. However, thymic functions are impaired by the conditioning regimen and the acute graft-versus-host disease (GvHD) that occurs after allo-BMT, resulting in deficient cell immunity (11, 12). In addition, there is a strong association between posttransplant autoimmune disease and the thymic dysfunction caused by chronic GvHD (13). Thymus transplantation (TT), an attractive method for improving T cell functions, has been applied clinically for patients with DiGeorge syndrome or HIV infection, which elicits the hypoplasia of the thymus (14). However, in mice, although T cell functions were restored or enhanced by TT, no concomitant GvHD was observed after TT in conjunction with allo-BMT (15). Therefore, TT can be used to treat autoimmune diseases in chimeric-resistant MRL/lpr mice and type 2 diabetes mellitus, and to suppress tumor growth (16–18).

Donor lymphocyte infusion (DLI) is often used after allo-BMT to prevent disease relapse in the setting of T cell–depleted BMT or nonmyeloablative conditioning regimens. It is also a combined method to convert from mixed chimerism to full donor chimerism (19, 20). However, DLI-induced GvHD is always associated with an increase in therapy-related morbidity because of its uncontrollable and fatal characteristics (21). It has been reported that many factors are involved in the damage to the recipient thymus after DLI (22, 23), whereas the effects of DLI on the transplanted thymus have hitherto remained unexplored.

In this study, we investigate the influence of DLI on both recipient and transplanted thymuses in the IBM-BMT + TT setting. Because we have found that TT using newborn thymus is most effective in tumor suppression (18), we used newborn thymus in this study. We show in this article that $CD4^+$ T cell–depleted lymphocyte infusion ($CD4^-$-DLI) impairs neither the recovery of recipient thymus nor the development of transplanted thymus.

Materials and Methods

Mice

C57BL/6 (B6), enhanced GFP (eGFP) transgenic (tg) B6, and BALB/c mice were purchased from Shimizu Laboratory Supplies (Shizuoka, Japan). Eight- to 12-wk-old male mice were used for BMT and DLI. For TT, 1 d after birth, B6 mice were sacrificed to obtain newborn thymuses. All the mice were maintained in a specific pathogen-free room.

Experimental protocol

As shown in Fig. 1, BALB/c mice were lethally irradiated with 7 Gy using the Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada) with two

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Abbreviations used in this article: allo-BMT, allogeneic bone marrow transplantation; B6, C57BL/6; BMC, bone marrow cell; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion; eGFP, enhanced GFP; FasL, Fas ligand; GvHD, graft-versus-host disease; IBM-BMT, intrabone marrow–BMT; tg, transgenic; Treg, regulatory T cell; TT, thymus transplantation; WSP, whole spleen.

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[^{137}Cs] sources, and the next day, these mice received IBM-BMT from B6 mice (group I). Some mice additionally received TT from B6 mice (group II). On the same day, some mice also received whole spleen (WSP-), CD4$^-$-, or CD8$^-$-DLI from B6 mice: WSP-DLI (group III), CD4$^-$-DLI (group IV), and CD8$^-$-DLI (group V). The treated mice were sacrificed 5 d, 2 wk, or 4 wk after the treatments.

Reagents and flow cytometric analysis

The Abs used in this study were as follows: purified rat anti-mouse CD4 and CD8 Ab (eBioscience, San Diego, CA); FITC-conjugated anti-mouse CD4 and H-2K^b Ab; PE-conjugated anti-mouse H-2K^d, CD4, CD8, and B220 Ab; and PerCP-Cy5.5–conjugated anti-mouse CD45 Ab (BD Pharmingen, San Diego, CA). For the thymus and peripheral blood analysis, leukocytes were first gated by CD45$^+$ cells, which were estimated as nuclear cells. To analyze the percentage of regulatory T cells (Tregs) in the spleen, we performed intracytoplasmic FoxP3 staining using an eBioscience FITC–anti-mouse/rat FoxP3 staining set in accordance with the manufacturer's instructions. Samples were analyzed using an FACSCalibur flow cytometer (BD Biosciences).

IBM-BMT and TT

One day after irradiation, the BMCs were prepared by flushing them from the medullary cavities of the femurs and tibias of B6 mice with PBS. The BMCs (1×10^7) were then injected directly into the tibial cavity of the recipient mice via the IBM route, as previously described (24). One whole newborn thymus from a B6 mouse was transplanted under the renal capsule of each recipient of IBM-BMT.

Cell preparation and DLI

Whole splenocytes from donor B6 mice were injected via the tail vein in the WSP-DLI group. CD4$^+$ or CD8$^+$ T cells were depleted from the splenocytes using purified CD4 or CD8 Ab with Dynabeads anti-rat IgG (Invitrogen Dynal AS, Oslo, Norway) for the CD4$^-$- or the CD8$^-$-DLI group, respectively. A total of 1×10^7 splenocytes were injected in the WSP-, CD4$^-$-, and CD8$^-$-DLI groups.

Assessment of GvHD

Survival was monitored daily. The severity of GvHD was assessed as previously described (25). In brief, the recipients were scored every 4 or 5 d for six clinical parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea. A severity scale of 0–2 was used for each parameter, with a maximum score of 12. Histopathological analysis was performed by scoring the changes in the skin, intestine, and liver specimens. A severity scale from 0–4 was used, with a maximum score of 12.

ELISA

Peripheral blood was collected from the recipient mice by EDTA tube (BD, Franklin Lakes, NJ) and centrifuged to get the mouse plasma within 30 min. The plasma was stored at −20°C until assay. TNF-α and Fas ligand (FasL) were measured using the commercial kit (R&D Systems, Minneapolis, MN). Samples and standards were run in duplicate.

Blockade of inflammatory cytokine

For cytokine blockade, purified rat anti-mouse TNF-α Ab and FasL Ab were purchased from BioXCell and BioLegend, respectively. After WSP-DLI, some mice were injected i.p. with 1 mg anti–TNF-α Ab and 2 mg anti-FasL Ab (WSP + Abs group) on days 0, 4, 8, and 12 as described previously (26).

Statistical analysis

The results are represented as means ± SD. The Student t test was used to determine any statistical significance. A p value <0.05 was considered to be a significant difference.

Results

In the IBM+TT setting, WSP-DLI induces serious GvHD but CD4$^-$ -DLI does not

We carried out IBM-BMT, TT, and DLI from B6 to BALB/c mice. The protocol is described in detail in Fig. 1 and in *Materials and Methods*.

The TT group (group II) showed similar results to group I (Fig. 2), although TT provides an abundance of thymocytes (immature T cells), whereas DLI contains mature T cells and attacks the target organs after priming. Serious GvHD was induced not only according to the clinical scores (Fig. 2A), but also according to the histopathological evaluation (Fig. 2B) in the WSP-DLI group (group III). However, when CD4$^+$ T cells were depleted, the severity of the GvHD decreased dramatically (group IV), whereas CD8$^+$ T cell–depleted DLI (CD8$^-$-DLI: group V) induced GvHD, as seen in the WSP-DLI group (group III). Therefore, we omitted group V from the subsequent experiments because in vivo experiments require a large number of mice. Thus, the mice with CD4$^-$-DLI exhibited only a slight and transient loss of body weight, but no serious GvHD (Fig. 2).

The recovery of recipient thymus is impaired by WSP-DLI but not CD4$^-$-DLI

The cytoreductive conditioning regimens in the context of BMT, especially irradiation, result in severe recipient thymic atrophy and defects in the immune system (13). Moreover, after BMT, the transplanted bone marrow–derived thymic progenitors migrate to the recipient's thymus where they support thymopoiesis. We examined the effects of DLI on the recovery of the recipient thymus. The recipient mice were sacrificed and analyzed 5 d, 2 wk, and 4 wk after BMT. Five days after BMT, the recipient thymuses in all groups showed severe atrophy (Fig. 3A) and the total cell number decreased. The cell numbers in the WSP- and CD4$^-$-DLI groups were much lower than in the TT group (Fig. 3B). The size and weight of the recipient thymus in the TT group gradually increased (Fig. 3A, 3C, 3D). Histologically, the recipient thymus already displayed well-defined cortical and medullary areas 2 wk after BMT (Fig. 3E). In addition, the percentage of CD4$^+$CD8$^+$ cells had also increased to the normal level (Fig. 3F).

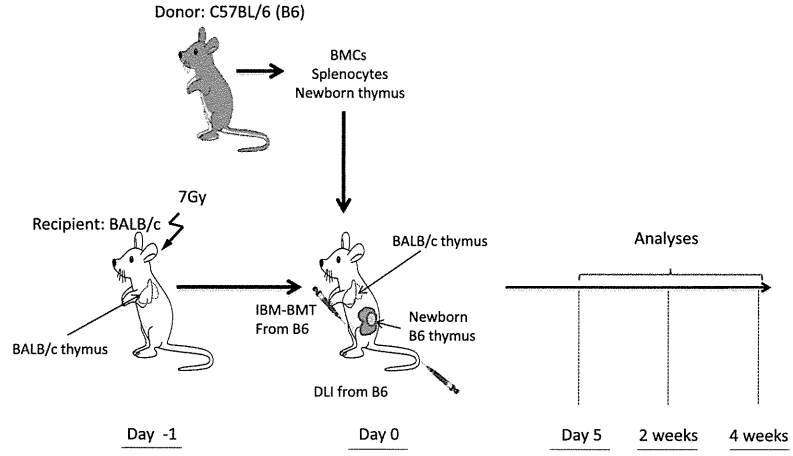

FIGURE 1. Experimental protocol. BALB/c mice were lethally irradiated (7 Gy from [^{137}Cs]). The next day, all the mice received IBM-BMT from B6 mice (group I). Some mice additionally received newborn TT from B6 mice (group II). On the same day, some mice also received WSP-, CD4$^-$-, or CD8$^-$-DLI from B6 mice: WSP-DLI (group III), CD4$^-$-DLI (group IV), and CD8$^-$-DLI (group V). The treated mice were sacrificed 5 d, 2 wk, or 4 wk after the treatments.

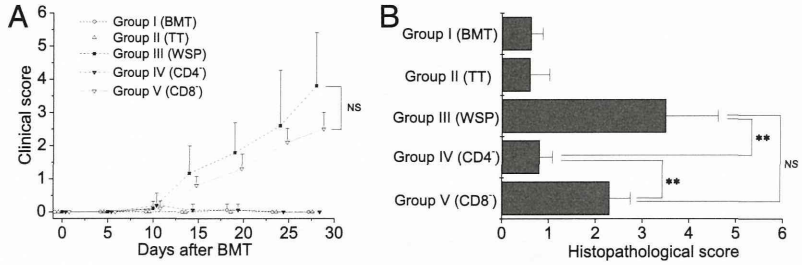

FIGURE 2. Clinical and histopathological scores of different groups. Irradiated BALB/c mice received IBM-BMT alone (BMT) or with newborn TT. Whole, $CD4^+$, or $CD8^+$ T cell–depleted splenocytes were injected in the WSP-, $CD4^-$- and $CD8^-$-DLI groups, respectively. Donor BMCs, thymuses, and splenocytes were all from B6 mice. After BMT, the body weight of the recipients was recorded and clinical signs of GvHD were assessed every 4 or 5 d (**A**). Autopsies were performed on mice that had been sacrificed 4 wk after BMT. Tissues from GvHD target organs (liver, skin, and intestine) were prepared for histopathological scoring (**B**). n = 4–8/group; experiments were performed three times. $^{**}p < 0.01$.

However, when the whole B6 splenocytes were injected, the process of recovery was disturbed: 1) the recipient thymus remained atrophic (Fig. 3A, 3C, 3D); 2) it was structurally damaged and, histologically, lost the corticomedullary junction (Fig. 3E); and 3) the percentage of $CD4^+CD8^+$ cells decreased seriously (Fig. 3F, 3G).

When $CD4^+$ T cells were depleted, the effects of DLI on the impairment of the recipient thymus appeared to be ameliorated at 2 wk after the BMT (Fig. 3C, 3E, 3F). Four weeks after the BMT, there was no significant difference in the structure or percentage of $CD4^+CD8^+$ cells between the TT and $CD4^-$-DLI groups (Fig. 3E, 3G). These findings suggested that DLI, especially with $CD4^+$ T cells, impaired the recovery of the recipient thymus.

The development of transplanted thymus is also impaired by WSP-DLI but not $CD4^-$-DLI

The transplanted thymus was also examined at the time points described earlier. In the TT group (group II), macroscopic findings indicated that the transplanted thymus developed well in all the recipient mice (Fig. 4A). Two weeks after the BMT, the transplanted thymus showed normal architecture and percentage of $CD4^+CD8^+$ cells (Fig. 4C, 4D). The thymus had also grown large by 4 wk after BMT (Fig. 4A, 4C). In the WSP-DLI group, the total cell number of the transplanted thymus was lower than that of the TT group on day 5 after BMT, but there was no significant difference between the TT and $CD4^-$-DLI groups (Fig. 4B). In the WSP-DLI group, only 37.5% of transplanted thymuses could be observed at 2 wk after the BMT, and those thymuses were smaller, had lost the thymic corticomedullary junction, and had a decreased percentage of $CD4^+CD8^+$ cells (Fig. 4A, 4C, 4D). The impairment in the transplanted thymus had become even more serious by the later time point, at which time only 16.7% of the transplanted thymuses could be observed (Fig. 4A, 4C, 4E). In contrast, 2 wk after BMT, the transplanted thymus in the $CD4^-$-DLI group was much larger than that in the WSP-DLI group. In addition, both the thymic structure and the percentage of $CD4^+CD8^+$ cells were similar to those in the TT group. Four weeks after BMT, there were no significant differences in any of the thymic parameters between the TT and $CD4^-$-DLI groups (Fig. 4A, 4C–E). These results indicated that the WSP-DLI had a remarkable impact on the development of the transplanted thymus, and that the $CD4^+$ T cells played a crucial role in the impairment.

DLI-derived $CD4^+$ T cells induce thymic impairment

Because both the recovery of the recipient's thymus and the development of the transplanted thymus had already been disturbed in the WSP-DLI group by 2 wk after BMT, we investigated the origin of the population that played a role in the impairment at an earlier time point. It has been reported that TT can also significantly increase the percentage of $CD4^+$ T cells (but not $CD8^+$ T cells) compared with BMT alone (27). Because no serious GvHD or thymus impairment was induced by TT, we hypothesized that the derivation of $CD4^+$ T cells could be the key point. We used splenocytes of eGFP tg B6 mice to distinguish the derivation of the T cells and confirm the role of the $CD4^+$ T cells. In this model, BMCs and newborn thymus were from B6 mice, whereas splenocytes from eGFP tg mice were injected for DLI. The percentages of non-DLI (donor BMCs or TT) and DLI-derived $CD4^+$ and $CD8^+$ T cells were analyzed 5 d after the BMT. The results showed that the percentage of DLI-derived alloreactive $CD4^+$ T cells was much higher in the WSP-DLI group than in the $CD4^-$-DLI group, suggesting that the DLI-derived $CD4^+$ T cells induced the impairment of both the recipient and transplanted thymus (Fig. 5A). Although the percentage of $CD8^+$ T cells in both the WSP- and $CD4^-$-DLI groups was higher than in the TT group, there was no significant difference between the WSP- and $CD4^-$-DLI groups, indicating that the $CD8^+$ T cells were likely not responsible for the thymus impairment (Fig. 5B). Moreover, there were no significant differences in the percentages of non–DLI-derived $CD4^+$ and $CD8^+$ T cells between the WSP- and $CD4^-$-DLI groups (Fig. 5A, 5B). These data showed that mature $CD4^+$ T cells from DLI played an important role in the impairment of the recovery of the recipient thymus and the development of the transplanted thymus. In addition, lymphopenia, especially B lymphopenia, was seen in the WSP-DLI group, whereas normal reconstitution was observed in the TT and $CD4^-$-DLI groups at 4 wk after BMT (Table I), proving that donor $CD4^+$ (not $CD8^+$) T cells were responsible for the induction of hematopoietic niche injury (28). The recipients in all groups showed full donor chimerism (>98%, data not shown).

Mechanism for $CD4^+$ T cell–induced thymic impairment

It has been shown that alloreactive $CD4^+$ T cells induce epithelial and organ damage in recipients, otherwise known as acute GvHD; many kinds of soluble inflammatory agents, such as TNF-α, IFN-γ, IL-1, and NO, are involved (29). The target organs in acute GvHD include the skin, liver, intestine, and thymus. Teshima and colleagues (30) have shown that the damage does not require alloantigen expression on the target epithelium, and that the neutralization of TNF-α prevents the damage. In addition, FasL-dependent donor T cell–mediated cytotoxicity has been implicated in the development of the thymus and bone marrow GvHD (22, 28). Therefore, we hypothesized that the inflammatory cytokines of TNF-α and FasL should induce the impairment of both recipient and transplanted thymuses. We thus examined the plasma levels of TNF-α and FasL 5 d after BMT, because we know that, at that

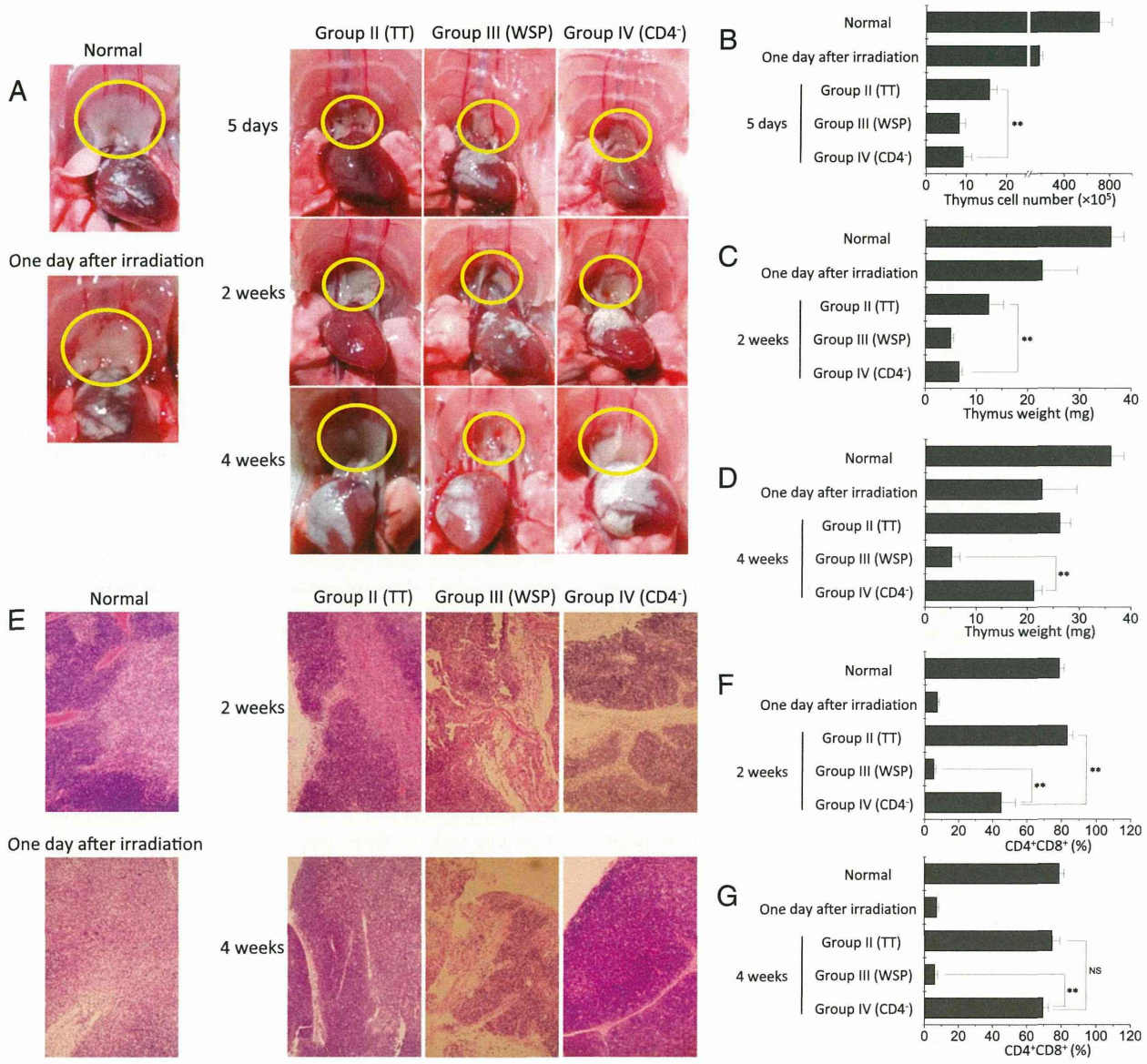

FIGURE 3. WSP-DLI, but not $CD4^-$-DLI, induces recipient thymic impairment. Recipient mice were sacrificed for analysis 5 d, 2 wk, or 4 wk after BMT. Thymuses from normal B6 mice and B6 mice 1 d after irradiation (7 Gy, no BMT) were used for control. (**A**) Representative macroscopic findings. Yellow circles indicate location of recipient thymus. (**B**) Total cell number of recipient thymus on day 5. (**C–G**) Weight, representative histological findings, and $CD4^+CD8^+$ cell percentage of recipient thymus are shown for later time points [(C, F): 2 wk; (D, G): 4 wk]. (E) H&E staining (original magnification ×100). n = 4–8/group; experiments were performed three times. $^{**}p < 0.01$.

time point, the processes of recipient thymic recovery and transplanted thymic development were disturbed by WSP-DLI. The levels of TNF-α and FasL were, indeed, significantly higher in the WSP-DLI group than in the TT and $CD4^-$-DLI groups on day 5 after BMT (Fig. 6A). To determine whether the production of inflammatory cytokines was causally related to the thymic impairment, we blocked TNF-α and FasL after WSP-DLI by Abs as described previously (26). The mice injected with Abs were sacrificed 2 or 4 wk after BMT. Both recipient and transplanted thymuses in the WSP + Abs group showed similar results to those in the $CD4^-$-DLI group 2 wk after BMT, including macroscopic (Supplemental Fig. 1A) and histological (Supplemental Fig. 1B) observations, weight of recipient thymus (Supplemental Fig. 1C), and percentages of $CD4^+CD8^+$ cells in the recipient (Supplemental Fig. 1D) and transplanted thymuses (Supplemental Fig. 1E). Blockade of TNF-α and FasL also prevented thymic impairment, according to observations made 4 wk after BMT (data not shown). These results proved that the inflammatory cytokines of TNF-α and FasL mediated the impairment of both recipient and transplanted thymuses.

We also compared the percentage of Tregs in the recipient spleen, which reflects the suppressive capability of inflammation 4 wk after BMT at the time lymphopenia occurs. The percentage of Tregs in the WSP-DLI group was much lower than in the TT and $CD4^-$-DLI groups (Fig. 6B). These data suggested that inflammation induced by DLI-derived $CD4^+$ T cells resulted in the impairment of recipient thymic recovery and transplanted thymic development.

Discussion

In this study, we examined the effects of $CD4^-$-DLI on both recipient and transplanted thymuses after allo-IBM-BMT + TT. In

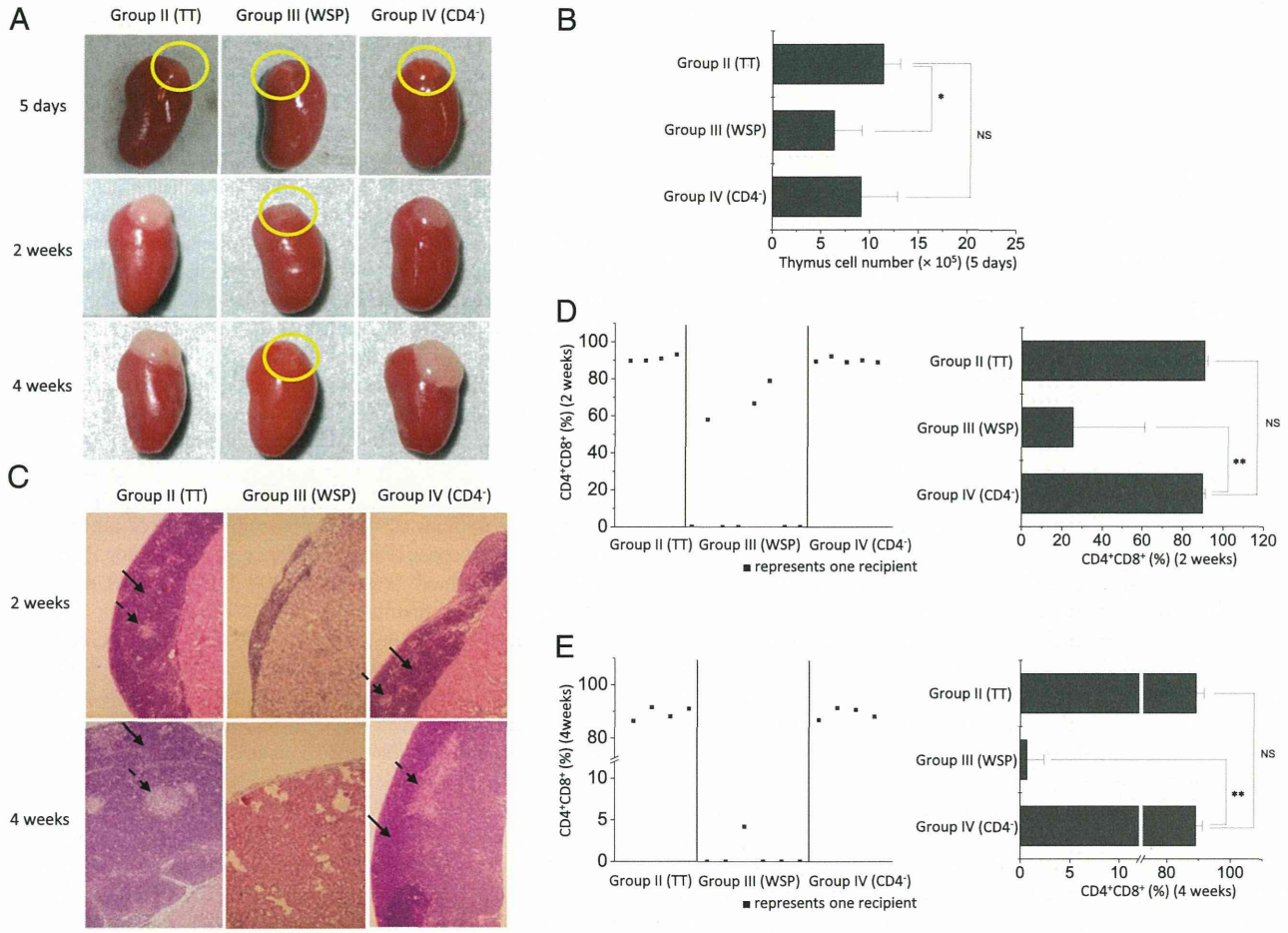

FIGURE 4. The development of transplanted thymus is impaired by WSP-DLI, but not $CD4^-$-DLI. Analysis of transplanted thymus was also performed at the same time as that in Fig. 3. (**A**) Representative macroscopic findings. Yellow circles indicate location of transplanted thymus. (**B**) Total cell number on day 5. (**C**) Representative histological findings. H&E staining (original magnification ×100). Plain arrows indicate cortex; dotted arrows indicate medulla. (**D** and **E**) Percentage of $CD4^+CD8^+$ cells is shown 2 or 4 wk after BMT. Each small square (■) represents the percentage for each recipient mouse in the *left panels*. Values of small squares on the x-axis are 0 and mean that no transplanted thymus could be observed or obtained for analysis. The *right panels* show the percentage by mean ± SD. $*p < 0.05$, $**p < 0.01$.

the BMT + TT setting, DLI induced serious damage, as evidenced in the classical GvHD target organs such as the skin, liver, and intestine. In addition, recovery of the recipient thymus was impaired, as was the development of the transplanted thymus. DLI led to decreased total cell number, lower percentages of $CD4^+$ $CD8^+$ cells, and loss of the thymic corticomedullary junction in

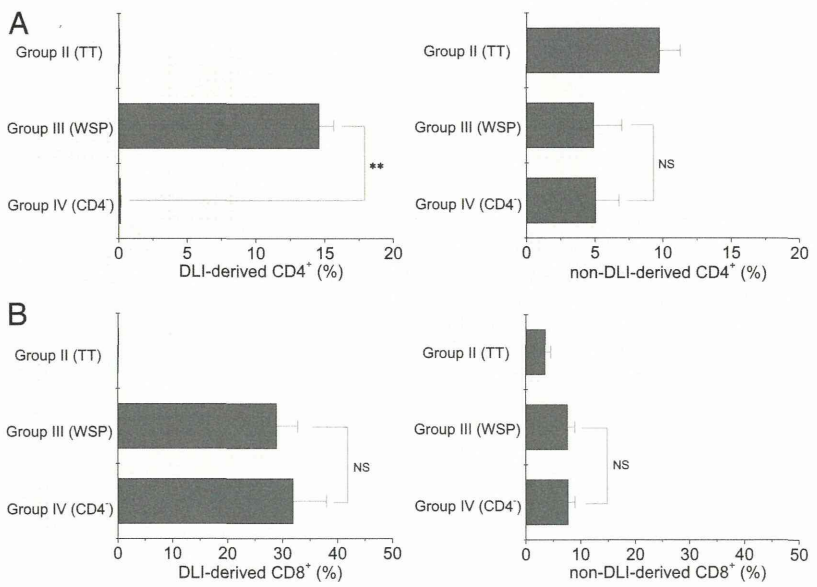

FIGURE 5. Percentages of DLI- and non–DLI-derived $CD4^+$ and $CD8^+$ T cells 5 d after BMT. BMCs and newborn thymus from B6 mice were used for BMT and TT, whereas splenocytes from eGFP tg mice were injected for DLI. Peripheral blood was analyzed 5 d after BMT. The percentages of DLI- ($eGFP^+$) and non–DLI-derived ($eGFP^-$) $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells in the leukocytes are shown. n = 3–4/group; experiments were performed three times. $**p < 0.01$.

Table I. Reconstitution of donor-derived hematopoietic cells 4 wk after BMT

Groups	Percentage of Donor Cells		
	CD4	CD8	B220
Group II (TT)	11.4 ± 2.7	7.7 ± 0.9	44.7 ± 1.0
Group III (WSP)	2.4 ± 1.1	4.7 ± 1.4	2.0 ± 1.2
Group IV ($CD4^-$)	10.7 ± 2.4**	7.0 ± 1.3*	45.5 ± 4.0**

Four weeks after BMT, the reconstitution was analyzed using the peripheral blood by flow cytometer. Full donor chimerism was observed in all the recipient mice (>98%, checked by H-$2K^b$ and H-$2K^d$ staining). The percentages of H-$2K^{b+}CD4^+$, H-$2K^{b+}CD8^+$, and H-$2K^{b+}B220^+$ cells in the peripheral blood are shown. n = 4–6/group, experiments were performed three times.

*$p < 0.05$, **$p < 0.01$, $CD4^-$-DLI group versus WSP-DLI group. There were no significant differences in the percentages of donor-derived CD4, CD8, and B220 cells between the TT and $CD4^-$-DLI groups.

both the recipient and transplanted thymuses. However, the thymic impairment was significantly ameliorated after $CD4^+$ T cell depletion, indicating that $CD4^+$ T cells play a crucial role in the impairment.

Complete reconstitution of the recipient's immune system is imperative for there to be a favorable outcome from allo-BMT. The recovery of peripheral T cells occurs via both thymus-independent and thymus-dependent pathways (31), and it seems likely that mature T cells injected by DLI expand rapidly in response to recipient APCs and provide protective immunity after allo-BMT via the thymus-independent pathway. However, they induce uncontrollable and fatal GvHD.

Donor hematopoietic progenitors enter and restore the thymus, and differentiate into T cells via the thymus-dependent pathway after BMT. Because the recipient thymus is impaired by the conditioning regimen (especially irradiation), the thymic reconstitution takes ~10 wk; T cells are the last hematopoietic lineage cells to recover after BMT in mice and humans (32). To improve this situation, we combined TT with BMT to provide a new environment for T cell induction and differentiation. TT was able to significantly enhance not only the percentage, but also the number of $CD4^+$ T cells, which helped restore early T cell functions whereas avoiding any concomitant GvHD (14, 27).

In this study, we combined IBM-BMT with TT and DLI, because IBM-BMT can efficiently recruit donor-derived stromal cells (including mesenchymal stem cells), support the donor-derived hematopoietic stem cells, and help to suppress GvHD. As shown in Fig. 2, GvHD was induced after WSP-DLI but was preventable with $CD4^-$-DLI. The process of recipient thymic recovery was disturbed, and the recipient thymus showed decreased total cell number, a lower percentage of $CD4^+CD8^+$ cells, and obliteration of the thymic corticomedullary junction at 2 or 4 wk after BMT (Fig. 3C–G). Because GvHD is considered to be systemic, the recipient thymus can also be considered to be a target for GvHD. Lower doses of DLI resulted in thymic damage without any obvious clinical signs of GvHD, suggesting that the thymus is probably exquisitely sensitive to GvHD (22, 31).

Effector mechanisms of acute GvHD are thought to include both CTL-mediated and cytokine-mediated cytotoxicity. In our model, recipient thymic impairment was significantly ameliorated in the $CD4^-$-DLI group, indicating that mature $CD4^+$ T cells play a central role in the impaired recovery of the recipient thymus. We also excluded the effects of TT-derived $CD4^+$ T cells and DLI-derived $CD8^+$ T cells by using splenocytes from eGFP tg B6 mice (Fig. 5). Our findings of the relative importance of allo-reactive $CD4^+$ T cells are consistent with recent reports showing that $CD4^+$ T cells are much more powerful (>100 times) than $CD8^+$ T cells in destroying the hematopoietic niche (28), although it has been reported that $CD8^+$ T cells play a key role in mediating GvHD (33, 34). The mature T cells from DLI are activated and proliferate in the secondary lymphoid tissues under the stimulation of APCs. The activated $CD4^+$ T cell–dependent GvHD damage is mediated primarily by soluble inflammatory agents such as TNF-α, IFN-γ, IL-1, and NO. The attack on the epithelium, induced by the soluble inflammatory agents, has been proved to not require Ag expression (30). This novel evidence explains the impairment of not only the recovery of the recipient thymus, but also the development of the transplanted thymus (Fig. 4B–E). Methods to inhibit the activated $CD4^+$ T cell–induced inflammation, such as neutralization of TNF-α and the administration of anti-CD4 Ab, ameliorated the effects of inflammatory impairment on the recipient epithelium or bone marrow niche (28, 30). Recent reports have proved that FasL, which is a T cell cytolytic molecule, is required for the thymus and bone marrow GvHD (22, 28). The results from one clinical study show a clear correlation between the level of serum-soluble FasL and liver damage during GvHD (35). Based on these findings, we examined the concentration of TNF-α and soluble FasL in the plasma, and compared the levels in

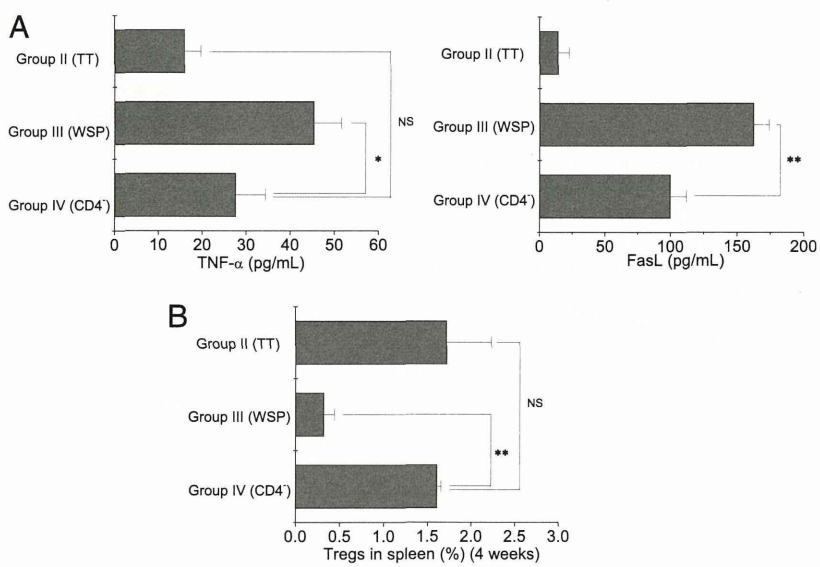

FIGURE 6. The levels of TNF-α and FasL and percentage of Tregs. (**A**) The levels of TNF-α and FasL in the recipient plasma were measured 5 d after BMT. (**B**) Percentage of Tregs in the recipient splenocytes was analyzed 4 wk after BMT. The Tregs were of donor origin as they showed H-$2K^{b+}$. n = 3–4/group; experiments were performed three times. *$p < 0.05$, **$p < 0.01$.

the TT, WSP-DLI, and CD4$^-$-DLI groups. The results showed that the TNF-α and FasL levels in the plasma in the WSP-DLI group were much higher than in the other two groups. Blockade of TNF-α and FasL by Abs prevented inflammatory cytokine-mediated impairment of both recipient and transplanted thymuses, confirming the role of TNF-α and FasL in thymic impairment. In addition, the percentage of Tregs that could suppress the inflammatory reaction was also decreased dramatically after WSP-DLI, but not CD4$^-$-DLI. It thus appears that the inflammation induced by the allo-CD4$^+$ T cells mediates the unselective impairment of both the recovery of the recipient's thymus and the development of the transplanted thymus.

There are thymic epithelial cells, endothelial cells, and fibroblasts in the thymic stroma. The destruction on the thymic stroma impairs the thymic cellularity and function. Recipient thymic GvHD is largely due to the impairment of the stromal cells of the thymus induced by allo-activated T cells. We have further proved a causative link between the activated CD4$^+$ T cells (but not CD8$^+$ T cells) and the impairment of the thymic stromal cells using an in vitro system (data not shown).

Newborn TT increased the percentage of CD4$^+$ T cells significantly in the peripheral blood after BMT (27). This population did not induce any serious recipient organ injury, suggesting that these cells experienced reconstitution via a thymus-dependent pathway. We have obtained encouraging results using thymus-independent CD8$^+$ T cells in treating solid tumors (36). This infers that the graft-versus-tumor effects of TT and CD4$^-$-DLI can be combined for tumor suppression without severe thymic impairment or GvHD.

In this study, we have shown that DLI leads to derangements in both recipient and transplanted thymuses in the IBM-BMT + TT setting. The inflammation mediated by allo-CD4$^+$ T cells plays a central role in the impairment of the recipient thymic recovery and the transplanted thymic development. The impairment would be significantly ameliorated by the depletion of CD4$^+$ T cells.

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Disclosures

The authors have no financial conflicts of interest.

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REVIEW

Novel conditioning regimens for bone marrow transplantation

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Abstract: Bone marrow transplantation (BMT) has evolved into an effective strategy for the treatment of hematological and oncological disorders. Radiotherapy and chemotherapy are used as conditioning regimens prior to BMT to suppress host immunity and reduce tumor burden. High doses of total body irradiation are conventionally administered along with alkylating agents, ie, the myeloablative regimen, to help ensure rapid engraftment of donor cells and to prevent relapse. However, the toxicity of the myeloablative conditioning regimen and unacceptable nonrelapse mortality rules out this approach for older patients by whom less intense preparative regimens are likely to be better tolerated. The reduced-intensity and nonmyeloablative conditioning regimens have been demonstrated by many investigators to be novel approaches resulting in a lower nonrelapse mortality rate and lower incidence of severe acute graft versus host disease. Here, we review the conditioning regimens employed as a pretreatment for BMT, and focus on the novel conditioning regimens and cutting edge developments.

Keywords: myeloablative conditioning regimen, reduced-intensity conditioning, nonmyeloablative conditioning regimen, relapse, nonrelapse mortality, graft versus host disease

Introduction

Bone marrow transplantation (BMT) was originally developed to treat congenital immunodeficiencies and hematologic disorders.[1,2] BMT has also become a powerful strategy for treating autoimmune and metabolic diseases.[3–7] Diseases frequently encountered in BMT are listed in Table 1. Radiotherapy and/or chemotherapy are prerequisites for recipients of BMT, because these conditioning regimens are essential for successful transplantation. Because the majority of BMT procedures are performed for the treatment of malignant disease, the conditioning regimens could be used to provide tumor cytoreduction and ideally disease eradication. The therapeutic effects of BMT on malignancies are also mediated via induction of the graft versus tumor effect by immunocompetent cells in the graft. Conditioning regimens that can minimize graft versus host disease without jeopardizing engraftment and graft versus tumor effects are being explored.[8]

The intensity of the conditioning regimens varies significantly. Based on the expected duration and reversibility of cytopenia after BMT, Bacigalupo et al classified the conditioning regimens into three categories, ie, myeloablative, reduced-intensity, and nonmyeloablative conditioning regimens.[9] Myeloablative conditioning regimens result in irreversible cytopenia, and stem cell support is required after BMT. The high-dose radiotherapy and chemotherapy used in the myeloablative conditioning regimens

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Table 1 Diseases frequently encountered in bone marrow transplantation

Autologous BMT	Allogeneic BMT
Multiple myeloma	Acute myeloid leukemia
Non-Hodgkin lymphoma	Acute lymphoblastic leukemia
Hodgkin disease	Chronic myeloid leukemia
Acute myeloid leukemia	Chronic lymphocytic leukemia
Neuroblastoma	Myeloproliferative disorders
Germ cell tumors	Myelodysplastic syndromes
Autoimmune disorders	Multiple myeloma
	Non-Hodgkin lymphoma
	Hodgkin disease
	Aplastic anemia

Note: Multiple myeloma continues to be the most common indication for autotransplantation and acute myeloid leukemia for allogeneic transplantation.

reduce immunocompetent cells in the recipient, permitting rapid engraftment of even unrelated, mismatched donor bone marrow cells. However, the myeloablative conditioning regimens are associated with considerable morbidity and mortality.[10] Therefore, these approaches have been restricted to young patients without comorbidities, and 50 years is considered an upper age limit.

In contrast with the consensus on a definition of myeloablative conditioning regimens, there are different opinions about reduced-intensity and nonmyeloablative conditioning regimens. Researchers sometimes refer to some of the conventional nonmyeloablative regimens as "reduced-intensity" conditioning regimens.[11–13] "Reduced-intensity conditioning" has also been used instead of "nonmyeloablative" directly.[14] Even though these regimens have been variably named nonmyeloablative conditioning or reduced-intensity conditioning regimens, they share one important characteristic, ie, both result in reversible myelosuppression (usually within 28 days) when given without stem cell support.[12] Above all, these methods use lower doses of cytoreductive treatments and result in low nonhematological toxicity. Some researchers use terms such as "intermediate-intensity" conditioning and semi-intensive conditioning rather than reduced-intensity conditioning.[15,16] Therefore, we have put reduced-intensity and nonmyeloablative conditioning regimens together in this review when discussing those regimens that are not myeloablative and are less toxic.[17–19]

Here, we review the conditioning regimens that are performed as pretreatments for BMT, and focus on some novel conditioning methods (reduced-intensity and nonmyeloablative conditioning regimens) with lower intensity that have expanded the use of BMT to older patients and to those with comorbidities.

Myeloablative conditioning regimens

Total body irradiation was the first conditioning method developed from research of radiation exposure and has been widely used in the conditioning regimens for its powerful immunosuppressive effects and activity against a variety of malignancies. Early myeloablative total body irradiation regimens were carried out using single large fractions of 8–10 Gy. However, such treatment was not tolerated well and was associated with serious toxicity, which resulted in interstitial pneumonitis, and severe nausea/vomiting.[20,21] To reduce the side effects of these high doses of total body irradiation while maintaining or improving efficacy, both fractionation and reductions in dose rates were developed.[22,23]

Host bone marrow can also be ablated with chemotherapy. Furthermore, chemotherapy can reduce or eradicate the tumor burden, while reducing the long-term sequelae of total body irradiation, including cataracts, sterility, and secondary malignancies. The combination of busulfan and cyclophosphamide is currently the most widely used myeloablative conditioning regimen without incorporating total body irradiation to treat malignant and nonmalignant hematological disorders with allogeneic BMT.[24–26] Yet, specific metabolites of cyclophosphamide are known to be associated with increased transplantation-induced mortality after conditioning, especially with busulfan. Therefore, fludarabine, a purine analog, has been used in an attempt to replace the cyclophosphamide in the busulfan and cyclophosphamide combination for myeloablative allogeneic BMT as well as nonmyeloablative transplantation. Fludarabine has considerable efficacy in both immunosuppression and tumor cell killing with minimal extramedullary toxicity. The regimen of busulfan and fludarabine has exhibited lower nonrelapse mortality and higher overall survival in patients with low-risk disease than busulfan and cyclophosphamide.[25,27]

Other possible alkylating agents, such as nitrosoureas (eg, carmustine), melphalan, thiotepa, and etoposide, have been included in the conditioning regimens in some trials. For example, the combination of carmustine, etoposide, cytarabine, and melphalan (BEAM) was designed to provide antilymphoma activity without the toxicity of total body irradiation. BEAM has proven to be an effective preparative regimen for its feasibility and tolerability in patients with lymphoma treated by both autologous and allogeneic BMT.[28,29]

Total body irradiation is often combined with chemotherapy in conditioning regimens and appears to provide benefit over conditioning with chemotherapy alone in many settings.[30–32] The combination of cyclophosphamide and total body irradiation (CyTBI) is considered to be one of the

standard regimens by many transplant centers.[23,26,33,34] Some studies have shown the combination of alkylating agents and total body irradiation to have a number of advantages in the conditioning for treatment of high-risk malignancies and solid tumors but with less toxicity.[35] CyTBI and busulfan and cyclophosphamide are currently the most commonly used regimens in all BMT clinical practice, including myeloablative, reduced-intensity, and nonmyeloablative conditioning regimens.[11,26,34] Comparative studies on clinical therapeutic effects of CyTBI and busulfan and cyclophosphamide have indicated that different regimens and types of malignant diseases may affect the outcome.[36] The myeloablative regimens mentioned above are summarized in Table 2.

Reduced-intensity and nonmyeloablative conditioning regimens

Pros and cons of reduced-intensity and nonmyeloablative conditioning regimens

Although dose intensification of myeloablative conditioning regimens had been shown to be effective in reducing the incidence of relapses, it resulted in unacceptable nonrelapse mortality due to regimen-related toxicity. Many studies have endeavored to identify an ideal conditioning regimen that would provide sufficient disease control to allow for sustained remission but without inducing unacceptable levels of toxicity and nonrelapse mortality. Reduced-intensity and nonmyeloablative conditioning regimens have been offered as alternatives to conventional high-dose radiotherapy and chemotherapy for older patients undergoing BMT on the basis that the less intense preparative regimens are likely to produce considerably less organ toxicity, so would be better tolerated by such patients. One report compares the outcome of myeloablative and nonmyeloablative conditioning regimens in patients older than 50 years, and suggests that the nonmyeloablative conditioning regimen led to improved overall survival at one year and 2 years. A significantly lower nonrelapse mortality rate was observed in the nonmyeloablative conditioning regimen group than in the myeloablative conditioning group (32% versus 50%).[37] For patients who were heavily pretreated and already refractory to therapy, such as in indolent lymphoma, the majority demonstrated donor engraftment and there was a high rate of complete remission.[13] One third of patients who underwent nonmyeloablative conditioning had failed prior high-dose myeloablative conditioning BMT.[38] Therefore, reduced-intensity and nonmyeloablative conditioning regimens have been used with increasing frequency, particularly in older patients with hematological malignancies and in patients considered at high risk for treatment-related toxicity and mortality associated with high-dose myeloablative conditioning regimens.[39,40]

Secondly, reduced-intensity and nonmyeloablative conditioning regimens may reduce the risk of severe acute graft versus host disease. These regimens cause only limited host damage, which may subsequently translate into less release of inflammatory cytokines which, it has been proposed, provide a proinflammatory milieu for development of graft versus host disease.[41] In addition, development of transient mixed donor-host chimerism after reduced-intensity and nonmyeloablative conditioning regimens may facilitate the establishment of mutual tolerance, which in turn downregulates the activity of graft versus host disease.[42] Residual host T cells also play a role in the suppression of graft versus host disease. Results from the Fred Hutchinson Cancer Research Center showed that the incidence of severe acute graft versus host disease was significantly lower in nonmyeloablative conditioning patients (grades III–IV acute graft versus host disease, 17% in the nonmyeloablative conditioning group versus 35% in the myeloablative conditioning group).[38] There is some controversy regarding the incidence of severe acute graft versus host disease in patients given reduced-intensity and nonmyeloablative conditioning regimens.[37] The timing of onset of acute graft versus host disease after reduced-intensity and nonmyeloablative conditioning regimens is delayed, and may develop after day 100, at a time when chronic graft versus host disease is usually diagnosed after

Table 2 Summary of frequently used myeloablative regimens

Regimen	Radiotherapy and chemotherapy	Total dose	Reference
BuCy	Busulfan	po: 16 mg/kg or	22–26,37,
	Cyclophosphamide	iv: 12.8–16 mg/kg	38
		iv: 120 mg/kg or	
		3.6 g/m^2	
BuFlu	Busulfan	po: 16 mg/kg or	25,27
	Fludarabine	iv: 12.8 mg/kg	
		iv: 180–200 mg/m^2	
CyTBI	Cyclophosphamide	iv: 120 mg/kg or	23,26,33,
		3.6 g/m^2	34,38
	TBI	8–15.75 Gy	
BEAM	Carmustine	iv: 300 mg/m^2	28,29
	Etoposide	iv: 400–800 mg/m^2	
	Cytarabine	iv: 800–1600 mg/m^2	
	Melphalan	iv: 140 mg/m^2	
CyATG	Cyclophosphamide	iv: 200 mg/kg	63
	ATG	iv: 90 mg/kg	

Abbreviations: po, per os; iv, intravenous; TBI, total body irradiation; ATG, antithymocyte globulin.

the myeloablative conditioning regimens.[42] More recently developed nonmyeloablative conditioning regimens have shifted some or all of the burden of killing tumor cells from the conditioning regimen to the graft versus tumor effects.[43] Therefore, donor lymphocyte infusion, which has been used as a helpful tool for inducing a sustained complete response of malignancies but which is always followed by serious graft versus host disease in myeloablative conditioning regimens, could replace high-dose cytotoxic therapy because of its graft versus tumor effects in reduced-intensity and nonmyeloablative conditioning regimens.[44] Donor lymphocyte infusion performed after these conditioning regimens has shown promising results, even in the treatment of solid malignancies in both animal and clinical studies.[17,45]

Thirdly, the defense provided by the host's immune system is partly protected because the reduced-intensity and nonmyeloablative conditioning regimens do not immediately and completely eliminate host-derived immunocompetent cells, and the level of host neutropenia is reduced. This is extremely important for early immunity after transplantation, and infectious complications may be reduced.[46]

Recent advances with reduced-intensity and nonmyeloablative conditioning regimens have significantly decreased early mortality and acute graft versus host disease, while enabling robust and prompt engraftment, and hence enhancing the therapeutic benefits of BMT.[47] However, there are also potential disadvantages of using these condition regimens, disease relapse being a primary cause of treatment failure for patients receiving them. In one study, a higher rate of relapse (albeit not statistically significant) was observed in patients with myelodysplastic syndrome or acute myeloid leukemia in a nonmyeloablative conditioning group than in the myeloablative conditioning group (46% versus 30%, $P = 0.052$).[37] Similar results have been reported by other groups, and greater intensity leads to less relapse, although possibly at the expense of higher nonrelapse mortality.[48,49] Chronic graft versus host disease is another disadvantage of reduced-intensity and nonmyeloablative conditioning regimens. The incidence and times of onset of chronic extensive graft versus host disease were similar between myeloablative and reduced-intensity and nonmyeloablative conditioning regimens.[38]

Candidate patients for reduced-intensity and nonmyeloablative conditioning regimens often have adverse characteristics, including advancing age, higher risk diseases, and higher pretransplantation comorbidity scores. However, despite the potential disadvantages, considering these unfavorable factors and the improvements in nonrelapse mortality, acute graft versus host disease suppression, progression-free survival, and overall survival, the overall outcome of these conditioning regimens is encouraging, and the number of BMT operations performed using them for a variety of hematological conditions is increasing dramatically.[11,37]

Examples of reduced-intensity and nonmyeloablative conditioning regimens

Low-dose (2–3 Gy) total body irradiation alone is an easy and convenient nonmyeloablative conditioning regimen. Its intensity is, to the best of our knowledge, the lowest in use today. Fludarabine is added in low doses in an attempt to reduce the risk of graft rejection. Low-dose total body irradiation, with or without fludarabine 90 mg/m^2, is a minimally toxic regimen developed for allogeneic BMT to treat patients with advanced hematological malignancies who are older or have comorbid conditions. It is one of the most widely used regimens by clinical centers.[11,38,42–44,50,51] Prospective clinical allogeneic BMT trials have shown that, in patients aged 60–75 years treated with this regimen, 5-year overall and 5-year progression-free survival rates were 35% and 32%, respectively.[52]

Other chemotherapy drugs, especially alkylating agents, are often combined with fludarabine. FAI is a regimen consisting of fludarabine, cytarabine, and idarubicin, and busulfan and fludarabine is a regimen used in myeloablative conditioning, but at much lower doses.[37,49]

The intensity of regimens increases with the doses of chemotherapy. Similar doses of fludarabine plus intermediate doses of one or two alkylating agents or low dose total body irradiation would be more powerful in host cytoreduction. Lim et al defined the intermediate doses of alkylating agents as oral busulfan (8–10 mg/kg), intravenous melphalan (80–140 mg/m^2), intravenous cyclophosphamide (600–1200 mg/m^2), or intravenous thiotepa (5–10 mg/kg).[48] The doses employed by different clinical centers may be quite different. For example, the combination of fludarabine and melphalan varies from fludarabine 100–150 mg/m^2 and melphalan 140–180 mg/m^2 to fludarabine 90–120 mg/m^2 and melphalan 90–140 mg/m^2.[49,53,54] In the regimen consisting of fludarabine and cyclophosphamide, the dose of cyclophosphamide in the research of Anderlini et al is 2.5–3 times the dose used by Lim et al.[48,54]

New drugs have been developed and added to the conditioning regimens. Treosulfan has been used as a substitute for busulfan in frail patients, because the side effects and toxicity are supposedly less severe. Treosulfan-based conditioning regimens have shown a favorable safety profile with fast

and sustained engraftment.[18,19,55] Recently, Nemecek et al have reported that a conditioning regimen consisting of treosulfan and fludarabine is well tolerated and yields encouraging survival rates and disease control with minimal nonrelapse mortality in patients with high-risk hematological malignancies.[39] Clofarabine is a second-generation purine nucleoside analog that combines the properties of fludarabine and cladribine. It is one of the most effective single agents against leukemic blast.[56] The combination of clofarabine with the reduced-intensity conditioning regimen showed good antileukemic efficacy, even in patients with high-risk acute myeloid leukemia or myelodysplastic syndrome.[57] Tyrosine kinase inhibitors can lead to cytogenetic remissions in patients with chronic myeloid leukemia and have been used before reduced-intensity or nonmyeloablative conditioning regimens. Warlick et al reported that allogeneic reduced-intensity conditioning BMT for older patients with chronic myeloid leukemia can control relapse with acceptable toxicity.[58]

Novel reduced-intensity and nonmyeloablative conditioning regimens

Total lymphoid irradiation

Efforts have been made clinically to reduce toxicity through using total lymphoid irradiation rather than total body irradiation to protect critical organs. Total lymphoid irradiation was initially used with the combination of conventional myeloablative regimens to increase immunosuppression and engraftment further.[59] Research in animals showed that total lymphoid irradiation increased the proportion of regulatory natural killer T cells. These natural killer T cells prevented acute graft versus host disease by inhibiting the proliferation and cytokine secretion of conventional T cells without affecting graft versus tumor activity.[60,61] Lowsky et al took advantage of the immune system's regulatory natural killer T cells and evaluated the total lymphoid irradiation-based reduced-intensity conditioning regimen in patients with lymphoid malignancies or acute leukemia undergoing allogeneic BMT. Eight Gy total lymphoid irradiation was delivered with fractions using fields to encompass the thymus, spleen, and lymph nodes. The results showed that 95% of patients scored as grade 0 according to standard scores for graft versus host disease, and the incidence of severe acute graft versus host disease was only 3%. The reduced-intensity conditioning regimen containing total lymphoid irradiation did not adversely affect the graft versus tumor effects of the allogeneic graft.[62]

Monoclonal antibodies

In 1994, Storb et al reported a conditioning regimen for patients with aplastic anemia using a high-dose combination of cyclophosphamide and monoclonal antibody to CD3 (antithymocyte globulin).[63] Subsequently, reduced-intensity conditioning regimens that consisted of antithymocyte globulin and alkylating agents were applied to both nonmalignant and malignant hematological disorders.[15,64] Promising outcomes were confirmed after regimens that contained antithymocyte globulin by the low incidence of acute graft versus host disease, although chronic graft versus host disease remained a major problem.[65,66] Therefore the use of other antibodies was explored, a representative being alemtuzumab (monoclonal antibody to CD52, marketed as Campath®, Genzyme Corporation, Cambridge, MA). Alemtuzumab has since been proven to be effective and safe in the reduced-intensity conditioning regimens by several goups.[67–69] Recently, a minimal-intensity conditioning regimen using alemtuzumab with fludarabine and cyclophosphamide has been developed by Marsh et al.[70] The results show that the regimen consisting of fludarabine, cyclophosphamide and alemtuzumab was associated with not only durable engraftment but also a much lower risk of chronic graft versus host disease compared with the conventional regimen containing antithymocyte globulin.[70] A retrospective study concluded that reduced-intensity conditioning consisting of fludarabine, melphalan, and alemtuzumab significantly improved survival rates over a myeloablative conditioning regimen consisting of busulfan, cyclophosphamide, and antithymocyte globulin plus or minus etoposide.[71] The anti-CD20 monoclonal antibody, rituximab, has also been used in both myeloablative and reduced-intensity conditioning regimens.[72] For patients with lymphoma who experienced disease recurrence following autologous BMT, allogeneic BMT prepared with a nonmyeloablative conditioning regimen consisting of fludarabine, cyclophosphamide and rituximab was suggested to be an effective option.[73]

Radioimmunotherapy usage

Antibodies conjugated with radionuclides, known as radioimmunotherapy, have been used for the treatment of cancer both in animal experiments and clinically.[74,75] By way of radioimmunotherapy, radiotherapy could be directly delivered to the surface of the targeted cells in continuous low-dose rate irradiation without increasing the toxicity, thereby sparing normal tissue. Therefore, radioimmunotherapy has been used in conditioning regimens to reduce the tumor burden while allowing for long-term disease control through graft versus

tumor effects in both myeloablative and reduced-intensity conditioning regimens.[76] Clinically, the radiolabeled anti-CD20 antibody (yttrium-90 ibritumomab tiuxetan) had been administered prior to reduced-intensity conditioning regimens for patients with advanced lymphoma and refractory disease or relapse after a previous autologous BMT. The treatment is associated with favorable outcomes, including no additional toxicity, enhanced cytoreduction, acceptable graft versus host disease, and absence of relapse.[77,78] Pagel et al have combined iodine-131 labeled anti-CD45 antibody with a standard reduced-intensity conditioning regimen for the treatment of older high-risk patients with acute myeloid leukemia or myelodysplastic syndrome. The results showed that CD45-targeted radiotherapy could be safely combined with a reduced-intensity conditioning regimen to yield encouraging overall survival.[79] The frequently used reduced-intensity and nonmyeloablative conditioning regimens are summarized in Table 3.

Table 3 Summary of frequently used reduced-intensity and nonmyeloablative conditioning regimens

Regimen	Radiotherapy and chemotherapy	Total dose	Reference
TBI	TBI	2–3 Gy	11,42–44, 51
FluTBI	Fludarabine	iv: 90 mg/m^2	11,38,
	TBI	2–3 Gy	42–44,50, 51
FAI	Fludarabine	iv: 120 mg/m^2	49
	Cytarabine	iv: 4 g/m^2	
	Idarubicin	iv: 36 mg/m^2	
BuFlu	Busulfan	iv: 3.2 mg/kg	37
	Fludarabine	iv: 120 mg/m^2	
FluCy	Fludarabine	iv: 125 mg/m^2	54
	Cyclophosphamide	iv: 3 g/m^2	
FluCyATG	Fludarabine	iv: 125 mg/m^2	54
	Cyclophosphamide	iv: 3 g/m^2	
	ATG	iv: 60 mg/kg	
FluMel	Fludarabine	iv: 90–150 mg/m^2	49,53,54
	Melphalan	iv: 90–180 mg/m^2	
TreoFlu	Treosulfan	iv: 30–42 g/m^2	39
	Fludarabine	iv: 150 mg/m^2	
TLIATG	TLI	8 Gy	62
	ATG	iv: 7.5 mg/kg	
TreoFluATG	Treosulfan	iv: 30–42 g/m^2	18,39
	Fludarabine	iv: 150 mg/m^2	
	ATG	iv: 30–90 mg/m^2	
BuFluATG	Busulfan	iv: 12.8 mg/kg	66
	Fludarabine	iv: 250 mg/m^2	
	ATG	iv: 4.5 mg/kg	
FCC	Fludarabine	iv: 120 mg/m^2	70
	Cyclophosphamide	iv: 1200 mg/m^2	
	Alemtuzumab	iv or sc: 40–100 mg	
FluCyRit	Fludarabine	iv: 90 mg/m^2	73
	Cyclophosphamide	iv: 2250 mg/m^2	
	Rituximab	iv: 1375 mg	

Abbreviations: TBI, total body irradiation; iv, intravenous; TLI, total lymphoid irradiation; ATG, antithymocyte globulin; sc, subcutaneous.

Conclusion

BMT remains a potentially dangerous procedure due to the many possible complications. The myeloablative conditioning regimens depending on high doses of radiotherapy and chemotherapy induce intense toxicity and have a high nonrelapse mortality rate; therefore, the conventional myeloablative conditioning regimens have been modified with the goal of reducing toxicity while maintaining or improving efficacy. Reduced-intensity and nonmyeloablative conditioning regimens have proven to be less toxic, making them suitable for older patients and those with comorbidities. These novel regimens are also associated with a lower rate of nonrelapse mortality and incidence of severe acute graft versus host disease. Host immunity is not completely destroyed in reduced-intensity and nonmyeloablative conditioning regimens and provides partial protection from infections. Reduced-intensity and nonmyeloablative conditioning regimens are appealing alternatives to myeloablative conditioning regimens and make BMT more acceptable by directly or indirectly ameliorating the complications. Efforts to improve effects and outcomes further continue to be explored by researchers, with potentially promising results.[80]

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Disclosure

The authors declare no competing financial interests in this work.

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Mismatched human leukocyte antigen class II-restricted CD8$^+$ cytotoxic T cells may mediate selective graft-versus-leukemia effects following allogeneic hematopoietic cell transplantation

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Partial human leukocyte antigen (HLA)-mismatched hematopoietic stem cell transplantation (HSCT) is often performed when an HLA-matched donor is not available. In these cases, CD8$^+$ or CD4$^+$ T cell responses are induced depending on the mismatched HLA class I or II allele(s). Herein, we report on an HLA-DRB1*08:03-restricted CD8$^+$ CTL clone, named CTL-1H8, isolated from a patient following an HLA-DR-mismatched HSCT from his brother. Lysis of a patient Epstein–Barr virus-transformed B cell line (B-LCL) by CTL-1H8 was inhibited after the addition of blocking antibodies against HLA-DR and CD8, whereas antibodies against pan-HLA class I or CD4 had no effect. The 1H8-CTL clone did not lyse the recipient dermal fibroblasts whose HLA-DRB1*08:03 expression was upregulated after 1 week cytokine treatment. Engraftment of HLA-DRB1*08:03-positive primary leukemic stem cells in non-obese diabetic/severe combined immunodeficient/γc-null (NOG) mice was completely inhibited by the *in vitro* preincubation of cells with CTL-1H8, suggesting that HLA-DRB1*08:03 is expressed on leukemic stem cells. Finally, analysis of the precursor frequency of CD8$^+$ CTL specific for recipient antigens in post-HSCT peripheral blood T cells revealed a significant fraction of the total donor CTL responses towards the individual mismatched HLA-DR antigen in two patients. These findings underscore unexpectedly significant CD8 T cell responses in the context of HLA class II. (*Cancer Sci* 2011; 102: 1281–1286)

Allogeneic hematopoietic stem cell transplantation (HSCT) has been used successfully for the treatment of hematological malignancies. Although HSCT from human leukocyte antigen (HLA)-identical siblings or unrelated donors is feasible to minimize the risk of acute graft-versus-host disease (aGVHD), HSCT from HLA-mismatched donors can be performed when a patient has advanced disease and no HLA matched donor is available.[1] It has been shown that aGVHD and survival rates are comparable between patients receiving HLA-mismatched unrelated HSCT and those receiving fully HLA-matched HSCT when the mismatch combination is not non-permissive.[2] Because the mismatched HLA molecule(s) may serve as a target for donor T cells, the immune response to these HLA in patients receiving a zero non-permissible mismatch HSCT could give rise to a favorable graft-versus-leukemia (GVL) effect with minimal risk of aGVHD. Following HLA-mismatched HSCT, it is commonly believed that CD8$^+$ or CD4$^+$ T cell responses are induced, depending on the mismatched HLA class I or II allele(s), based on the binding of cognate coreceptors to MHC molecules stabilizing weak interactions between T cell receptors (TCR) and MHC.[3]

In the present study, we characterized an HLA class II-restricted CTL clone isolated from a patient with acute myeloid leukemia who received HLA-DR/DP loci-mismatched HSCT. The CTL clone, named CTL-1H8, was CD8$^+$ and its cytotoxicity was blocked by an anti-CD8 antibody as well as by an anti-HLA-DR antibody. The CTL-1H8 clone lysed primary leukemic cells possessing the mismatched HLA-DRB1*08:03, but not cytokine-treated recipient dermal fibroblasts. Engraftment of HLA-DRB1*08:03-positive primary leukemic stem cells in immunodeficient mice[4] was completely inhibited by *in vitro* preincubation with CTL-1H8. Furthermore, we demonstrated by CTL precursor (CTLp) frequency analysis that a significant fraction of the total donor CD8$^+$ CTL response in this patient was directed against the HLA-DRB1*08:03 molecule. These findings underscore the *in vivo* immunological relevance of a CD8$^+$ T cell response against mismatched HLA class II molecule(s).

Materials and Methods

Cells, HLA transfectants, and antibodies. Peripheral blood mononuclear cells (PBMC) were collected and cryopreserved before and after HSCT from a male patient who had received his brother's bone marrow (BM) for AML (M6; French American British subtype, M6). The HLA genotype of the recipient was A*24:02/*33:03, B*52:01/*44:03, C*12:02/*14:03, DRB1*08:03/*13:02, DQB1*06:01/*06:04, DPB1*02:02/*04:01, whereas that of the donor was mismatched by DRB1*15:02 instead of DRB1*08:03 and by DPB1*05:01 instead of DPB1*02:02. The patient developed grade II aGVHD limited to the skin and extensive chronic GVHD, but has been free from disease recurrence for over 2 years. B-Lymphoblastoid cell lines (B-LCL) were established from the donor and recipient, as well as from normal volunteers. All blood, BM, and tissue samples were collected after the subjects had provided written informed consent, and the study was approved by the Institutional Review Board of Aichi Cancer Center. The B-LCL, including the HLA class I negative B-LCL line 721.221, were

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