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ORIGINAL ARTICLE

Roles of Host Nonhematopoietic Cells in Autoimmunity and Donor Cell Engraftment in Graft-versus-host Disease

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Background: Graft-versus-host disease (GVHD) is initiated when alloreactive donor T cells are primed by host APCs to undergo clonal expansion and maturation. Since there is a controversy regarding the role of nonhematopoietic cells in GVHD, we wanted to investigate the influence of MHC disparity on nonhematopoietic cells on the pathogenesis of GVHD in the MHC-haplomismatched C57BL/6 (H-2^b) or DBA/2 (H-2^d)→unirradiated (C57BL/6×DBA/2) F_1(BDF_1; H-$2^{b/d}$) murine model of acute GVHD (aGVHD) or chronic GVHD (cGVHD). **Methods:** We generated (BDF_1→C57BL/6), (BDF_1→DBA/2), and (BDF_1→BDF_1) chimeras and examined GVHD-related parameters and donor cell engraftment in those chimeras. **Results:** Using this experimental system, we found that 1) severe aGVHD across MHC Ag barrier depends on the expression of nonhematopoietically rather than hematopoietically derived alloAgs for maximal GVHD manifestations; 2) host APCs were sufficient to break B cell tolerance to self molecules in cGVHD, whereas host APCs were insufficient to induce autoimmunity in aGVHD; 3) donor cell engraftment was greatly enhanced in the host with MHC-matched nonhematopoietic cells. **Conclusion:** Taken together, our results provide an insight into how MHC disparity on GVHD target organs contribute to the pathogenesis of GVHD.
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INTRODUCTION

In the parent-into-F_1 graft-versus host disease (GVHD) model, the genetic background of donor strains is critical in determining the outcome of GVHD (1,2). For example, the infusion of donor T cells from the DBA/2 strain into an unirradiated (C57BL/6×DBA/2)F_1 (BDF_1) mouse induces chronic GVHD (cGVHD), whereas the infusion of T cells of the other parent, C57BL/6 (B6), induces acute GVHD (aGVHD) (3). $CD4^+$ T cells of the DBA/2 strain activate and expand host B cells with an autoreactive potential, resulting in systemic lupus erythematosus (SLE)-like symptoms such as autoAb production and glomerulonephritis (4). In aGVHD, not only do donor $CD8^+$ T cells eliminate host hematopoeietic cells, particularly host B cells, to induce massive engraftment of donor cells, but also donor $CD8^+$ T cells attack solid organs together with immune cells regulated by donor $CD4^+$ T cells, resulting in loss of body weight (1). It seems that donor $CD8^+$ T cell anergy is a restriction factor for the development of cGVHD (5,6). Even though donor $CD4^+$ T cells have the ability to break host B cell tolerance in both cGVHD and aGVHD, donor $CD8^+$ T cells have a different fate after transfer into the host; donor $CD8^+$ T cells are rapidly eliminated from the host and the remaining cells fall into anergy in cGVHD (5,6), whereas donor $CD8^+$ T cells are differentiated into effector T cells that have the ability to delete host B cells including potential autoreactive B cells in aGVHD, thus depriving the host of the opportunity to produce autoAb (7-9).

It is controversial whether cognate interactions between TCRs and MHC on nonhematopoietic target tissues are required for GVHD (10-13). In MHC-mismatched GVHD, TCRs

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can directly recognize recipient MHC (10-12). Teshima *et al.* (13) have shown that CD4-mediated GVHD, and to a lesser extent CD8-mediated GVHD do not require such direct interactions, suggesting that the direct allorecognition of donor T cells on host APCs is critical in GVHD. It is not known how donor B6 T cells induce GVHD in the B6→BDF_1 aGVHD model. In this study, F_1→parent chimeras were used to elucidate the involvement of alloAg expression by nonhematopoietic tissues in the B6→BDF_1 aGVHD model. Our results indicate that aGVHD induced solely by alloreactive donor T cells depends on the expression of nonhematopoietically rather than hematopoietically derived alloAgs.

MATERIALS AND METHODS

Mice

Female DBA/2 (H-2^d) and BDF_1 (H-$2^{b/d}$) mice, 7∼8 wk of age, were purchased from Orient (Seoul, Korea). All mice were maintained in pathogen-free conditions. These studies were approved by institutional animal care committee.

Abs and reagents

The following FITC-, PE-, PerCP- or biotin-conjugated mAbs to mouse proteins were purchased from BD Biosciences (San Diego, CA): CD4, CD8, B220, H-$2K^b$, H-$2K^d$, CD62L. HRP-conjugated rat anti-mouse IgG_1 Abs also were purchased from BD Biosciences.

Bone marrow (BM) reconstitution

BM cells were collected by flushing femurs and tibias from BDF_1 mice into MACS buffer (1×PBS containing 5 mM EDTA and 3% BSA). After erythrocyte lysis in hemolysis buffer (144 mM NH_4Cl and 17 mM Tris-HCl, pH 7.2), BM cell were incubated with biotinylated anti-CD3 mAb for 20 min on ice, washed once, then incubated with streptavidin-conjugated microbeads (Miltenyi Biotech, Auburn, CA) for 20 min at 4°C. Cells were depleted of $CD3^+$ cells using MACS buffer. Remaining $CD3^+$ cells routinely comprised less than 1% of BM cells. Recipient BDF_1 mice received 12 gray (Gy) irradiation from a cesium irradiator and were reconstituted with 5×10^6 T-cell-depleted BM from BDF_1 mice.

Induction of GVHD

GVHD was induced as described previously (14-16). In brief, single-cell suspensions in PBS were prepared from spleens and lymph nodes of normal B6 or DBA2 donors, filtered through a sterile mesh (BD Biosciences), and washed. After the erythrocytes were lysed in hemolysis buffer, the remaining cells were resuspended at 8×10^7 cells/0.2 ml in PBS. GVHD was induced by transfer of 8×10^7 of spleens/lymph node cells into the tail vein of unirradiated BDF_1 recipient mice at 12 wk after BM transplantation.

ELISA

Mice were bled from the tail vein, and serum titers of anti-DNA IgG_1 were assessed by ELISA. Plates (96-well) were incubated overnight at 4°C with 100 μl salmon sperm DNA (Sigma-Aldrich, St. Lois, MO) at a concentration of 10 μg/ml. After blocking with 2% BSA, the plates were incubated with 100 μl serially diluted serum samples for 1 h at room temperature. They were washed three times with PBS containing 0.1% Tween 20, and HRP-conjugated anti-mouse IgG_1 was added to each well and the plates were kept at room temperature for 1 h. They were washed again with the same solution and color was developed in 100 μl 3,5,3',5'-tetramethylbenzene substrate (Pierce, Rockford, IL) for 10∼15 min and stopped by adding 100 μl of 1 N HCl. The plates were then read at 450 nm with a Wallac Vector 1420 Multilabel Counter (PerkinElmer, Waltham, MA). OD values at a 1/10 dilution of sera were presented.

Flow cytometry

The spleens of GVHD mice were harvested on the indicated days after parental cell transfer. After lysis of the erythrocytes, the splenocytes were preincubated in a blocking buffer (PBS containing 2.4G2 mAb/0.2% BSA/0.1% sodium azide), and then incubated with the relevant mAbs for 30 min at 4°C. Finally, they were washed twice with staining buffer (PBS containing 0.2% BSA/0.1% sodium azide) and analyzed by FACscan (BD Biosciences).

Histopathology

Formalin-fixed kidney, liver, and large intestine were embedded in paraffin, and 5 μm thick sections were stained with H&E and evaluated by light microscopy. Slides for liver and large intestine were coded and further examined in a blinded fashion by one individual (H.J.C.), using a semi-quantitative system for abnormalities known to be associated with aGVHD (17,18). In brief, samples of liver tissue was performed by scoring 14 pathologic features, including inflammatory infiltrates in bile ducts and portal tracts, vascular endothelialitis, and hepatocellular damage as previously

reported. A severity scale from one to four was used where 0=normal, 0.5=rare scattered, 1=minimal or focal, 2=mild and more diffuse, 3=moderate damage, and 4=severe damage. Scores for each individual feature were added to yield a composite score of liver pathology. Colon abnormalities were assessed by examining crypt regeneration, surface coloncytes, colonocyte vacuolization, surface colonocyte attenuation, crypt cell apoptosis, outright crypt destruction, and lamina propria lymphocytic infiltrate. The scoring system denoted 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were added to provide a total score for each specimen. Only after scoring was performed were codes broken and data compiled.

Statistical analysis

The Student's *t* test was used to determine the statistical significance of differences between experimental groups. Error bars represent SD of the mean. Log-rank Mantel-Cox test was used for survival curves.

RESULTS

The parent-into-unirradiated GVHD model is unique among GVHD models in that the development of GVHD is initiated only by alloreactivity without inflammation induced by preconditioning. This property provides a less complication in interpreting experimental data and finds its usefulness in an experimental tool to dissect a variety of issues related to immune tolerance to alloAgs. In this study, we wanted to investigate the roles of nonhematopoietic cells in GVHD in the MHC-haplomismatched B6 or DBA/2→unirradiated BDF_1 murine model of aGVHD or cGVHD. We created (BDF_1→B6), (BDF_1→DBA/2), or (BDF_1→BDF_1) chimeras. In this experiment system, allogeneic host APCs could stimulate donor T cells, regardless of MHC mismatch in nonhematopoietic cells. PBMCs were >90% donor-derived in BDF_1→B6 or DBA/2 mice by 8 wk after BM transfer (Fig. 1). We induced aGVHD in three sets of chimeras by transferring B6 spleen/lymph node cells. BDF_1→DBA/2 chimeras had most severe aGVHD as measured by loss of body weight and histologic analysis of aGVHD target organs (Fig. 2). Except for earlier death in a small portion of mice, BDF_1→B6 chimeras barely showed clinical and pathologic symptoms of aGVHD. The earlier death could be due to a large quantity of cytokines produced when B6 donor T cells killed host BDF_1 hematopoietic cells

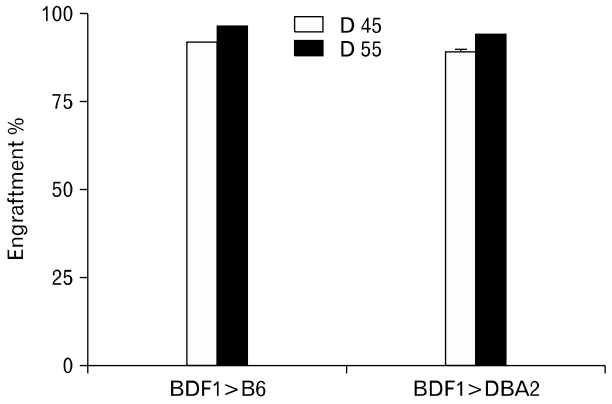

Figure 1. Confirmation of BM reconstitution. B6, DBA/2 and BDF_1 mice received 12 Gy irradiation and were reconstituted with 5×10^6 T cell-depleted BDF_1 BM cells. PBMCs were analyzed by flow cytometry at days 45 and 55 after cell transfer. Percent of donor cells were counted by staining PBMCs with anti-H-$2K^b$ or H-$2K^d$ mAbs (n=10~20 per group).

(also see Fig. 2; Ref 13). BDF_1→BDF_1 control mice had milder aGVHD than BDF_1→DBA/2 chimeras but more severe hepatic GVHD than BDF_1→B6 chimeras. Taken together, our results indicate that severe aGVHD mediated by donor T cells depends on the expression of alloAgs in nonhematopoietic cells. A similar conclusion has been reached in $CD8^+$ T cell- or $CD4^+$ T cell-mediated aGVGD across minor histocompatibility Ag (miHA) barrier (19,20).

In our experimental system, TCRs of donor B6 T cells could recognize intact BDF_1 MHC on host hematopoietic cells. Alloreactive donor T cells generated from these interactions could remove host hematopoietic cells and make a niche for donor hematopoietic stem cells. As shown in Fig. 3, MHC disparity on nonhematopoietic cells had a reverse correlation with donor cell engraftment. Even though aGVHD was not evident in BDF_1→B6 chimeras, more than 90% of splenocytes were of donor origin. In contrast, despite of severe aGVHD, DBF_1→DBA/2 chimeras had the least extent of donor cell engraftment among the three sets of chimeras. Our data indicate that MHC disparity on nonhematopoietic cells is a greater hurdle to donor cell engraftment.

It is thought that alloreactive donor T cells exhibit an activation status in MHC-mismatched GVHD, because they receive sustained allostimulation. Consistent with this hypothesis, a greater number of activated $CD4^+$ T cells and $CD8^+$ T cells ($CD62L^{low}$) were contained in the spleen of BDF_1→DBA/2 chimeras and positive controls (BDF_1→BDF_1 mice), compared with BDF_1→B6 mice (Fig. 4). Therefore, our result

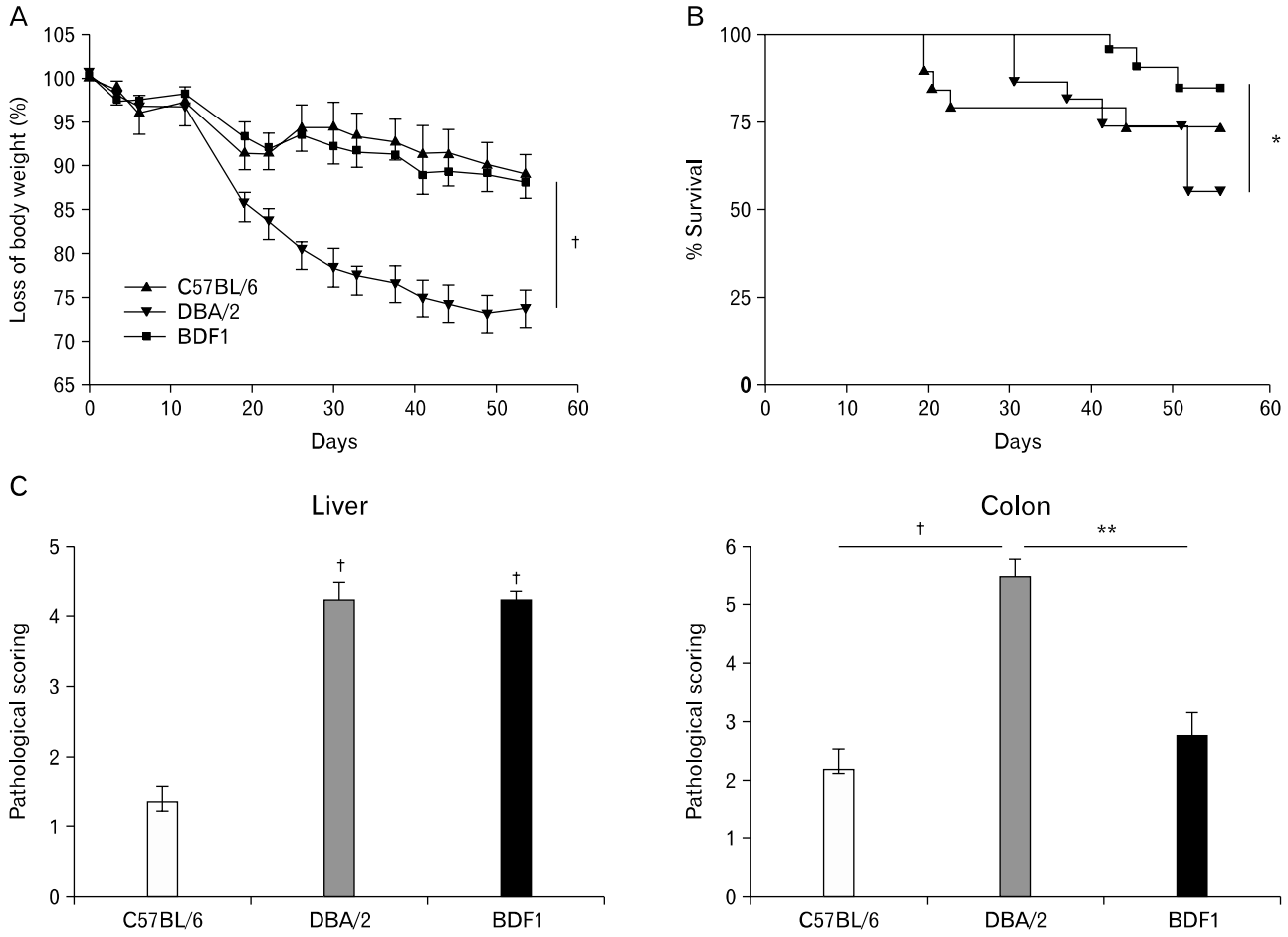

Figure 2. MHC disparity on nonhematopoietic cells exacerbates aGVHD. Eighty-four days after BM reconstitution, aGVHD was induced by transferring 8×10^7 B6 spleen/lymph node cells into three sets of mice (n=20 per group). (A) Loss of body weight. (B) Percent of survival. (C) Pathological scores for livers and colons. Organs were harvested at day 54 after disease induction (n=5~8 per group). $^{\dagger}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$ between the indicated groups.

suggests that alloreactive T cells in $BDF_1\rightarrow B6$ chimeras may remain quiescent after allogenic host hemtopoietic cells are removed.

The $DBA/2\rightarrow BDF_1$ cGVHD model has been extensively utilized to study how alloimmunity breaks B-cell tolerance to self molecules (21). Using this model, we wanted to examine the effect of MHC disparity on nonhematopoietic cells on autoimmunity and donor cell engraftment. cGVHD was induced by transferring DBA/2 spleen/lymph node cells into $BDF_1\rightarrow B6$, $BDF_1\rightarrow DBA/2$, and $BDF_1\rightarrow BDF_1$ chimeras. As shown in Fig. 5A, DBA/2 donor T cells were equally potent in inducing production of IgG1 anti-DNA autoAb in all of the three sets of chimeras, suggesting that MHC disparity on hematopoietic cells are sufficient for but MHC disparity on nonhematopoietic cells are not involved in the development of cGVHD. Furthermore, it seems that mortality due to cGVHD was not influenced by MHC disparity on nonhematoietic cells: there was no difference in the rate of mortality between $BDF_1\rightarrow DBA/2$ and $BDF_1\rightarrow B6$ chimeras (Fig. 5B). Histopathological analysis showed that the kidney of either $BDF_1\rightarrow DBA/2$ or $BDF_1\rightarrow B6$ chimeras had severe glomerulonephritis (Fig. 5C). Nontherless, engraftment of donor lymphocytes, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, was significantly higher in $BDF_1\rightarrow DBA/2$ than in $BDF_1\rightarrow B6$ chimeras, although the two groups had no difference in the percent of total donor cell engraftment (Fig. 6). Levels of donor lymphocyte engraftment were positively correlated with the extent of donor $CD8^+$ T cell activation (Fig. 7). These results sug-

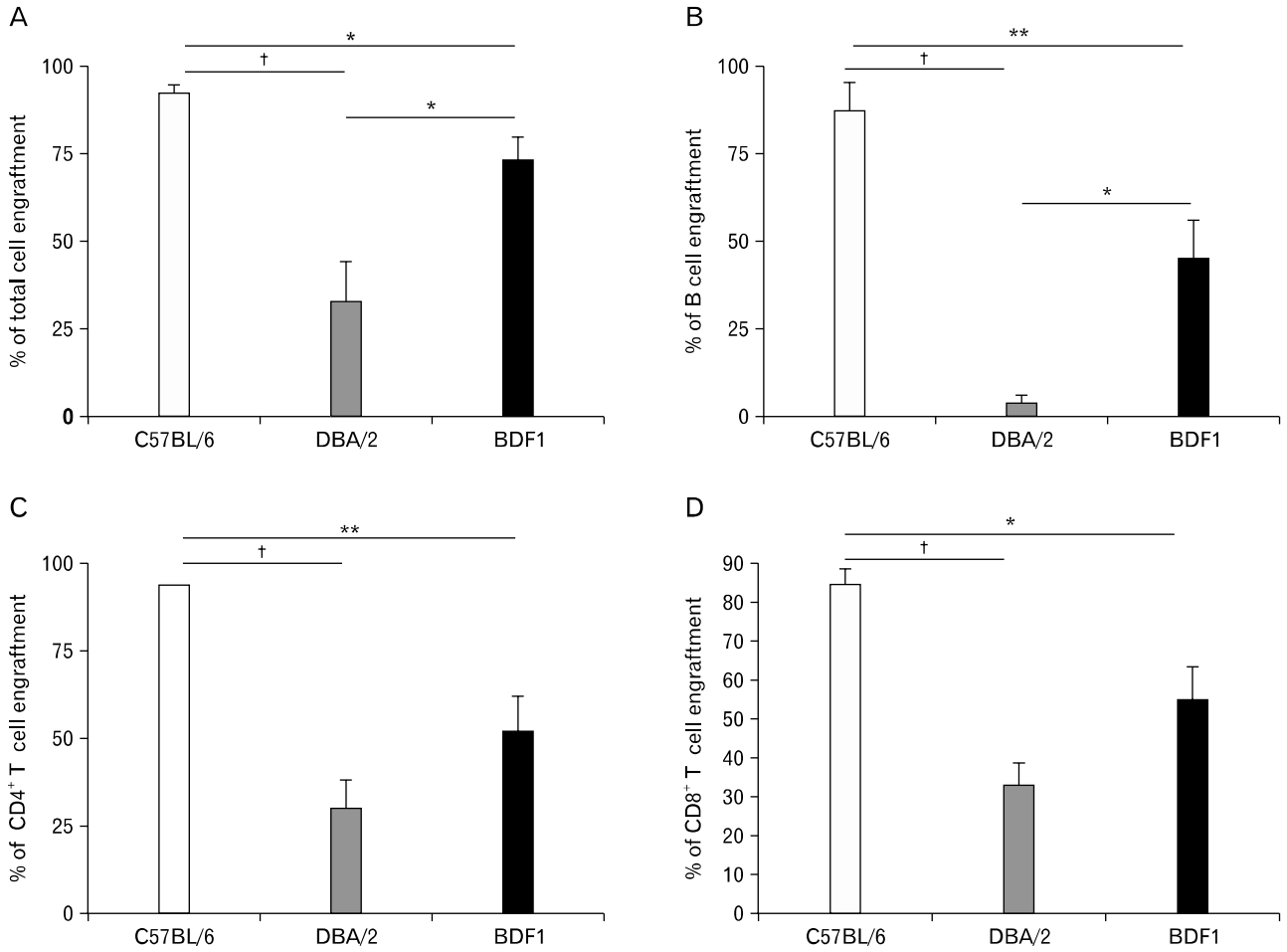

Figure 3. MHC match on nonhematopoietic cells increases donor cell engraftment in aGVHD. Eighty-four days after BM reconstitution, aGVHD was induced by transferring 8×10^7 B6 spleen/lymph node cells into three sets of mice (n=20 per group). Splenocytes were harvested at day 54 after disease induction and stained with anti-H-2K^d plus anti-CD4, anti-CD8 or B220. (A) Percent of total donor cells. (B) Percent of donor B cells. (C) Percent of donor CD4$^+$ T cells. (D) Percent of donor CD8$^+$ T cells (n=5~8 per group). $^\dagger p<0.001$, $^{**}p<0.01$ and $^*p<0.05$ between the indicated groups.

gest that MHC match on nonhematopoietic cells is critical in donor cell engraftment, as shown in the B6→BDF$_1$ aGVHD model.

DISCUSSION

In the B6→BDF$_1$ aGVHD model, theoretically, MHC-haplomismatched host APCs can not only stimulate donor T cells through interactions between allo-MHC and TCRs but also present processed alloAgs or self-Ags to donor T cells in the context of self-MHC. Our results suggest that, even though GVHD is initiated by donor T cells after receiving allostimulation by host APCs, through the direct alloreactive pathway (22), presentation of nonhematopoietically derived alloAgs by donor APCs is a prerequisite for the propagation of the disease. It seems that (BDF$_1$→B6) chimeras experience transient cachexia (which sometimes results in mortality) with reduced target-tissue injury over time, reflecting an early, limited graft-versus-host response. This interpretation is consistent with results obtained from various MHC-matched or-mismatched aGVHD models (23-27).

It is still controversial whether the expression of nonhematopoietically derived alloAgs is required for CD4-mediated aGVHD. Two sharp contrast results have been reported (13,20). Teshima *et al.* (13) demonstrated in the MHC-disparate bm12 →[B6→B6.MHC II$^{-/-}$] BM chimera model that alloAg ex-

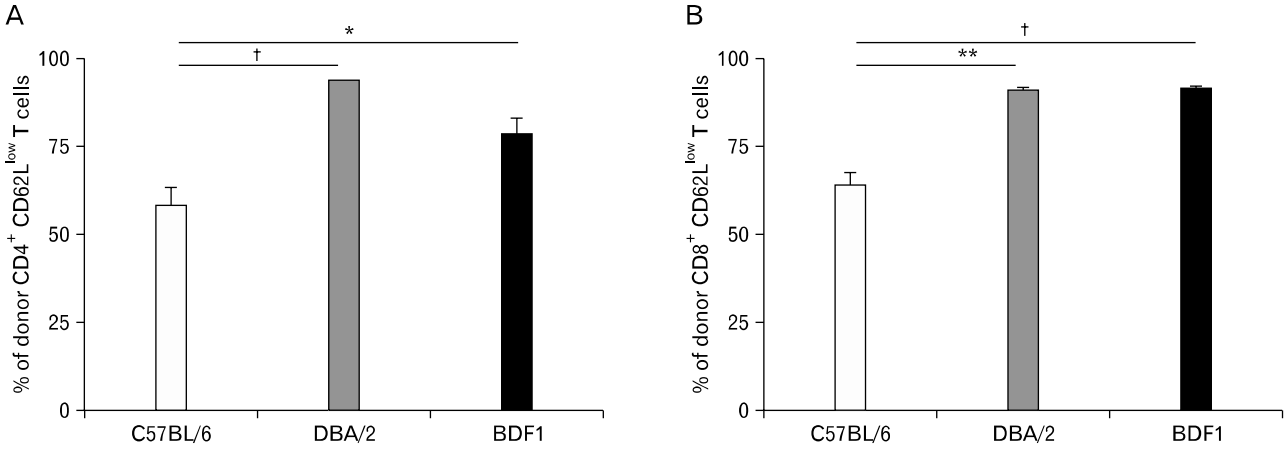

Figure 4. MHC match on nonhematopoietic cells decreases activation of donor T cells in aGVHD. Eighty-four days after BM reconstitution, aGVHD was induced by transferring 8×10^7 B6 spleen/lymph node cells into three sets of mice (n=20 per group). Splenocytes were harvested at day 54 after disease induction and stained with anti-H-2K^d plus anti-CD62L and anti-CD4 or anti-CD8. (A) Percent of donor CD4$^+$CD62L^{low} T cells. (B) Percent of donor CD8$^+$CD62L^{low} T cells (n=8 per group). $^\dagger p<0.001$, $^{**}p<0.01$ and $^*p<0.05$ between the indication groups.

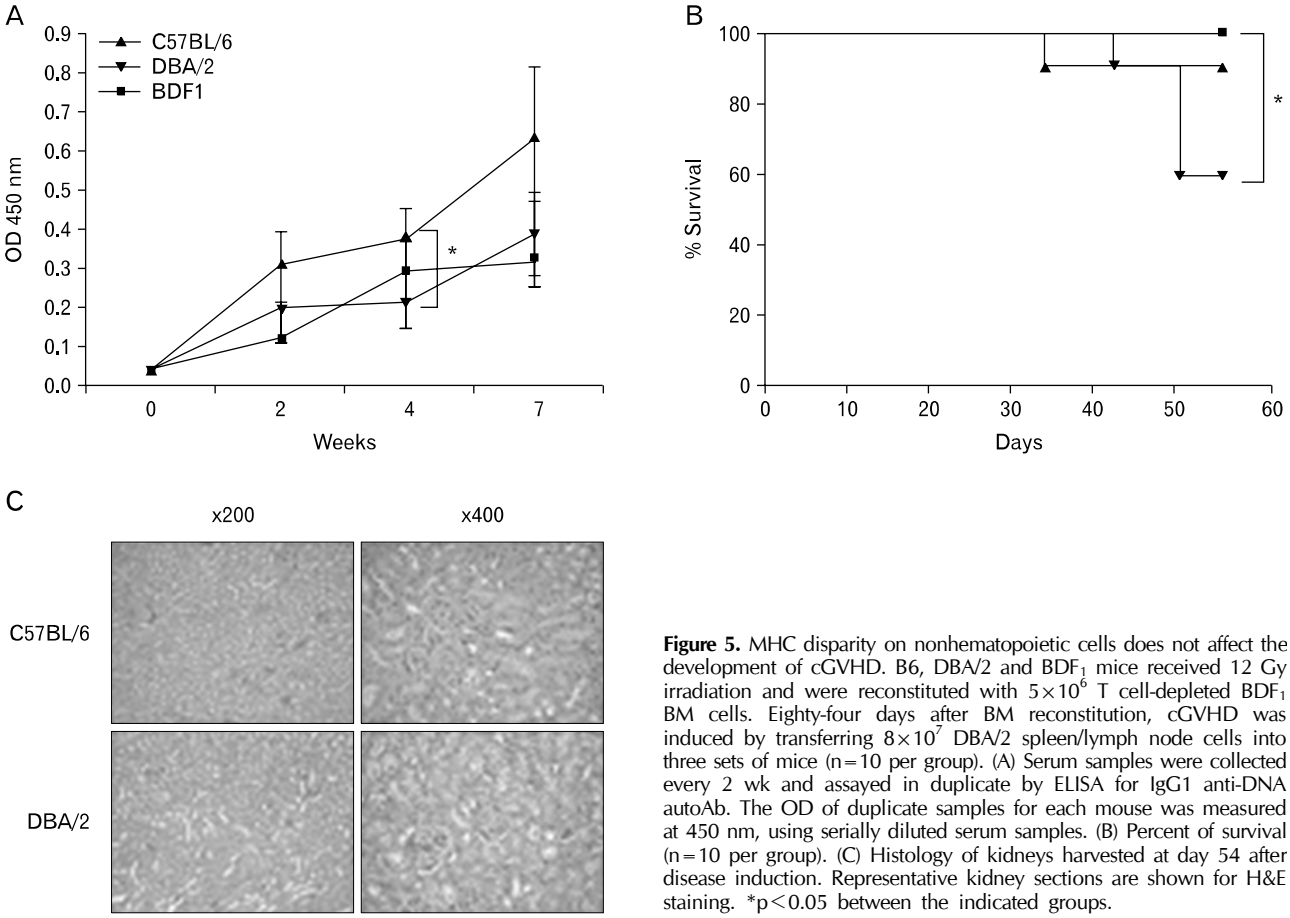

Figure 5. MHC disparity on nonhematopoietic cells does not affect the development of cGVHD. B6, DBA/2 and BDF$_1$ mice received 12 Gy irradiation and were reconstituted with 5×10^6 T cell-depleted BDF$_1$ BM cells. Eighty-four days after BM reconstitution, cGVHD was induced by transferring 8×10^7 DBA/2 spleen/lymph node cells into three sets of mice (n=10 per group). (A) Serum samples were collected every 2 wk and assayed in duplicate by ELISA for IgG1 anti-DNA autoAb. The OD of duplicate samples for each mouse was measured at 450 nm, using serially diluted serum samples. (B) Percent of survival (n=10 per group). (C) Histology of kidneys harvested at day 54 after disease induction. Representative kidney sections are shown for H&E staining. $^*p<0.05$ between the indicated groups.

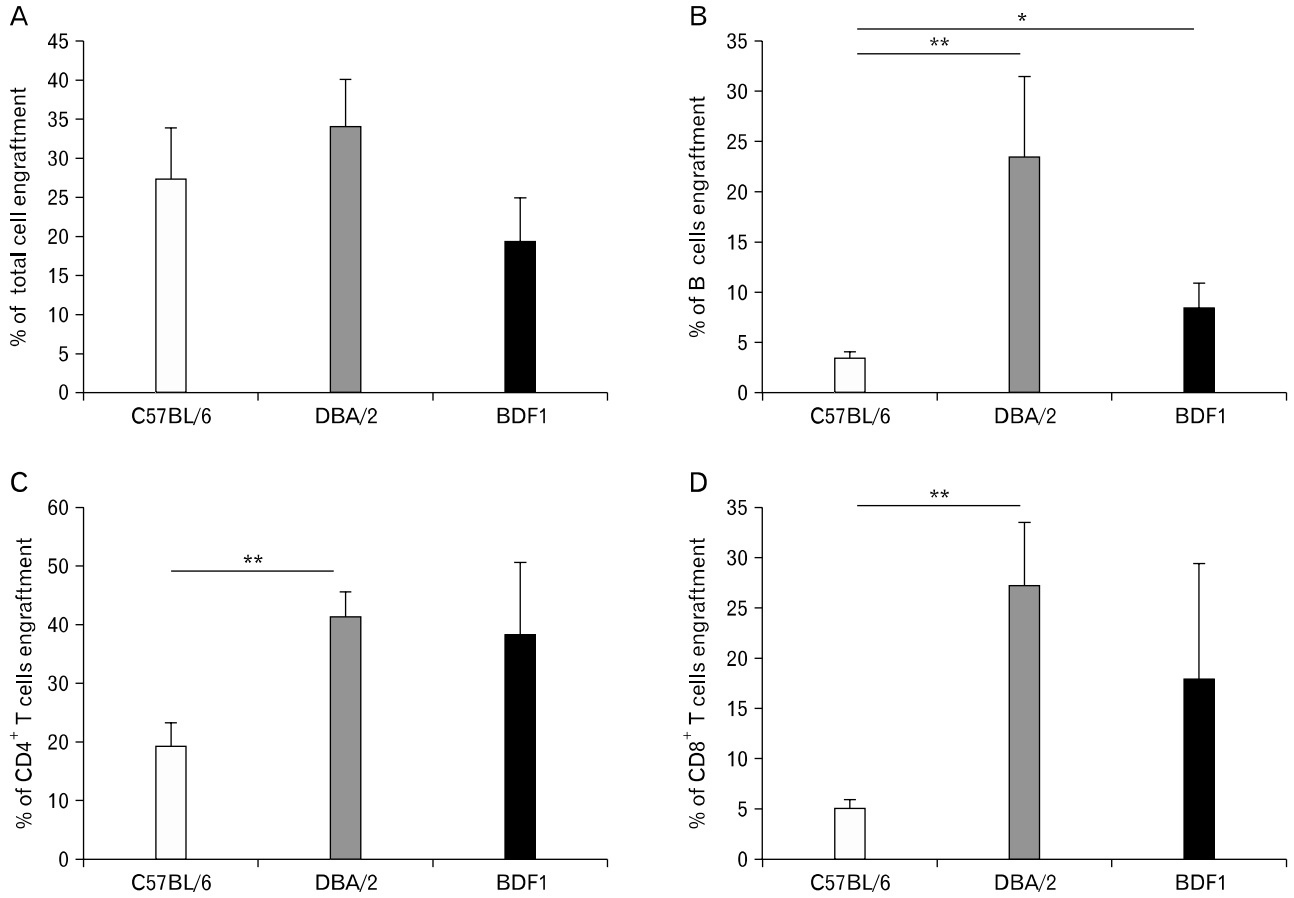

Figure 6. MHC match on nonhematopoietic cells increases donor lymphocyte engraftment in cGVHD. Eighty-four days after BM reconstitution, cGVHD was induced by transferring 5×10^6 DBA/2 spleen/lymph node cells into three sets of mice (n=20 per group). Splenocytes were harvested at day 54 after disease induction and stained with anti-H-$2K^b$ plus anti-CD4, anti-CD8 or B220. (A) Percent of total donor cells. (B) Percent of donor B cells. (C) Percent of donor $CD4^+$ T cells. (D) Percent of donor $CD8^+$ T cells (n=5~8 per group). $^{**}p<0.01$ and $^{*}p<0.05$ between the indicated groups.

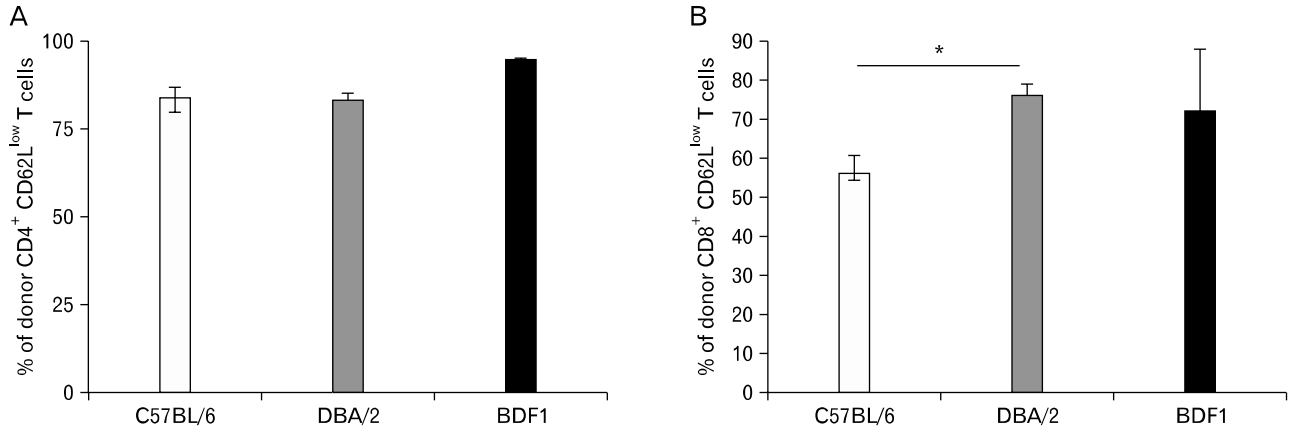

Figure 7. Donor lymphocyte engraftment correlates with donor $CD8^+$ T cell activation in cGVHD. Eighty-four days after BM reconstitution, aGVHD was induced by transferring 5×10^6 DBA/2 spleen/lymph node cells into three sets of mice (n=10 per group). Splenocytes were harvested at day 54 after disease induction and stained with anti-H-$2K^b$ plus anti-CD62L and anti-CD4 or anti-CD8. (A) Percent of donor $CD4^+$ $CD62L^{low}$ T cells. (B) Percent of donor $CD8^+CD62L^{low}$ T cells (n=8 per group). $^{*}p<0.05$ between the indicated groups.

pression by the hematopoietic compartment alone was sufficient to obtain lethal GVHD, whereas Jones *et al.* (20) demonstrated significantly diminished B6 $CD4^+$ T cell-mediated GVHD development when recipient BALB.B mHAs were exclusively derived from cells of the hematopoietic compartment. This discrepancy may be due to differences in the allogeneic T cell response directed across MHC versus mHA barriers, most notably the involvement of a much higher alloreactive $CD4^+$ T cell precursor frequency in the former situation (28). In bm12→B6 (20) and similar models (18,29-32), such a vigorous T cell response can rapidly generate high levels of proinflammatory cytokines responsible for initiating acute tissue damage in the early post-hematopoietic stem cell transplantation period. However, our results suggest that alloreactive T cells generated by the exposure to allogeneic host APCs are insufficient to drive fully developed GVHD in the absence of preconditioning. Therefore, proinflammatory cytokines produced as a result of preconditioning such as total body irradiation play a critical role in the induction of GVHD, presumably in synergy with a donor T cell response or/and by helping the broadening of the pool of alloreactive donor T cells (33-35). In either case, the data suggest that alloreactive donor T cells generated from either the direct pathway (by allogeneic host APCs) or the indirect pathway (nonhematopoietically derived alloAgs are presented by host or donor APCs) mediate GVHD with great help of proinflammatory cytokines triggered by preconditioning.

It is well known that GVHD, in particular, cGVHD, has clinical manifestations of autoimmune disorders (36-38). Our observations showing that BDF_1→B6 chimeras did not display aGVHD after transfer of B6 T cells suggest that allogeneic host APCs is insufficient to break self-tolerance. Since GVHD-induced autoreactivity is donor APC-dependent in irradiated recipient mice (39), and BDF_1 APCs can present self-Ags to donor B6 T cells in BDF_1→B6 chimeras, proinflammatory cytokines produced as a result of preconditioning may facilitate autoimmunity by donor T cells. In such irradiated models, autoimmunity is evolved as aGVHD is progressing (24). In our cGVHD model, alloreactive donor T cells ($CD4^+$ T cells) rapidly broke B cell tolerance regardless of MHC match on nonhematopoietic cells. Therefore, a certain genetic combination of MHC disparity between donors and recipients may be a risk factor for SLE-like cGVHD, especially in patients with transient or chronic states of mixed chimerism. However, clinical correlates of these observations should be established.

In the B6→BDF_1 aGVHD model, alloreactive donor $CD8^+$ T cells kill host hematopoietic cells for donor cell engraftment. Surprisingly, donor cell engraftment is not positively correlated with the severity of GVHD. This observation suggests that donor-recipient genetic combinations may exist where donor T cells do not induce GVHD with intact donor cell engraftment and thus intact graft-versus-leukemia effects. It will be important to search for a genetic factor that governs donor cell engraftment.

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CONFLICTS OF INTEREST

The author have no financial conflict of interest.

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