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Maintenance of $CD8^+$ T-cell anergy by $CD4^+CD25^+$ regulatory T cells in chronic graft-versus-host disease

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Abbreviations: aGVHD, acute graft-versus-host disease; BDF1, $(CD57BL/6 \times DBA/2)F_1$; cGVHD, chronic GVHD; T_{reg} cell, regulatory T cell

Abstract

In a murine model of systemic lupus erythematosus (SLE)-like chronic graft-versus-host disease (cGVHD), donor $CD8^+$ T cells rapidly fall into anergy to host cells, while donor $CD4^+$ T cells hyperactivate B cells and break B-cell tolerance to self-Ags in the recipient mouse. The functional recovery of donor $CD8^+$ T cells can result in the conversion of cGVHD to acute GVHD (aGVHD), indicating that donor $CD8^+$ T-cell anergy is a restriction factor in the development of cGVHD. In this report, we present evidence that donor $CD4^+CD25^+$ regulatory T cells (T_{reg} cells) are critical in maintaining the donor $CD8^+$ T-cell anergy and thus suppressing the development of aGVHD in mice that are naturally prone to cGVHD. Our results provide a novel insight into the role of T_{reg} cells in determining cGVHD versus aGVHD.

Keywords: apoptosis; B-lymphocytes; clonal anergy; graft vs host disease; T-lymphocytes, cytotoxic; T-lymphocytes, regulatory

Introduction

One model that has been used to examine graft-versus-host disease (GVHD) involves transfer of parental T cells into unconditioned F_1 hybrid mice. In this combination of mouse strains, the recipient develops GVHD because the host T cell can't actively resist the donor T cell. For example, $(C57BL/6 \times DBA/2)F_1$ (BDF_1) mice develop acute GVHD (aGVHD) or chronic GVHD (cGVHD) by transfer of T cells from C57BL/6 mice or the other parental strain, DBA/2, respectively (Murphy, 2000). aGVHD is due to the attack of host cells by cytotoxic donor $CD8^+$ T cells and is characterized by the elimination of host lymphocytes and a profound immunodeficiency (Pals *et al.,* 1984; Via *et al.,* 1987; Rus *et al.,* 1995). In contrast to aGVHD, cGVHD is caused by alloreactive donor $CD4^+$ T cells that hyperactivate host B cells, resulting in breaking self-tolerance (Morris *et al.,* 1990; Rus *et al.,* 1995). Donor $CD8^+$ T cells of DBA/2 origin fall into anergy in BDF_1 recipients (Kim *et al.,* 2006a). Interestingly, breaking of donor $CD8^+$ T cell-anergy results in the conversion of a chronic form of GVHD to an acute form, indicating that donor $CD8^+$ T cell-anergy is a restriction factor for the development of cGVHD (Pals *et al.,* 1984; Via *et al.,* 1987; Kim *et al.,* 2006a). At present, it is not known what controls $CD8^+$ T-cell anergy in cGVHD.

$CD8^+$ T-cell anergy is induced by exposure to high doses of chronic Ags (Redmond and Sherman, 2005; Redmond *et al.,* 2005), whereas clonal deletion, another mechanism of peripheral tolerance, is promoted by continuous exposure to low doses of Ags (Redmond *et al.,* 2005). It is interesting in this context that transgenic donor $CD8^+$ T cells with allospecificity against a host MHC alloantigen become anergic shortly after massive deletion (Zhang *et al.,* 1996; Bimalangshu *et al.,* 1999). In cGVHD, donor $CD8^+$ T cells follow a similar fate- most donor $CD8^+$ T cells are deleted and the residual apoptosis-resistant cells become anergic (Kim *et al.,* 2006a). Even though anergic $CD8^+$ T cells are different from regulatory T cells (T_{reg} cells) in that proliferation of anergic $CD8^+$ T cells are impaired after exposure to Ags, anergic $CD8^+$ T cells share many features of T_{reg} cells such as secretion of IL-10 and inhibition of other Ag-specific T cells (Zhang *et al.,* 1996; Bimalangshu *et al.,* 1999). Recent studies have shown that, by enforcing costimulation through 4-1BB or

GITR, the induction of anergy in CD8$^+$ T cells can be prevented in transplant models (Wilcox *et al.*, 2004; Kim *et al.,* 2006a).

In this study, we investigated the role of donor CD4$^+$CD25$^+$ T_{reg} cells in maintaining CD8$^+$ T-cell anergy in cGVHD. Our data demonstrate that donor T_{reg} cells can block activation of donor CD8$^+$ T cells and subsequently prevent this T-cell subset from causing aGVHD. These findings reveal a new mechanism for the pathogenesis of cGVHD versus aGVHD.

Materials and Methods

Mice

Female DBA/2 (H-2^d) and BDF_1 (H-2$^{b/d}$) mice, 6-8 wk of age, were purchased from Orient (Seoul, Korea). All mice were maintained in pathogen-free conditions. These studies were approved by the Institutional Animal Care Committee.

Antibodies and reagents

The following FITC-, phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, or biotin-conjugated Abs to mouse cell surface molecules were purchased from BD Biosciences Pharmingen (San Diego, CA): CD4, CD8, CD62L, and H-2K^b. Streptavidin-Cy (cychrome) was also purchased from BD Biosciences Pharmingen. Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG_1 was purchased from Southern Biotechnology (Birmingham, AL).

Induction of cGVHD

Single-cell suspensions in PBS were prepared from the spleens and lymph nodes of normal DBA/2 parental donors, filtered through a sterile mesh (BD Falcon, San Diego, CA) and washed. After the erythrocytes were lysed in hemolysis buffer (144 mM NH_4Cl and 17 mM Tris-HCl, pH 7.2), the remaining cells were resuspended at 8×10^7 cells/0.2 ml in PBS. cGVHD was induced by transfer of 8×10^7 of DBA/2 parental cells into the tail vein of normal, unirradiated BDF_1 mice. To induce cGVHD with parental cells depleted of T_{reg} cells, T_{reg} cells were removed by anti-CD25-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA) from DBA/2 spleen/lymph node cells. The remaining cells (8×10^7) were transferred into BDF_1 mice to induce cGVHD.

Flow cytometry

The spleens of cGVHD mice were harvested 3 wk after parental cell transfer. After lysis of the erythrocytes, the splenocytes were preincubated in a blocking buffer (PBS containing 2.4G2 antibody/0.2% BSA/0.1% sodium azide), and then incubated with the relevant antibodies for 30 min at 4^{o}C. Finally, they were washed twice with staining buffer (PBS containing 0.2% BSA/0.1% sodium azide) and analyzed by FACS (BD Biosciences Pharmingen, Mountain View, CA).

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^{o}C with 100 μl of salmon sperm DNA (Sigma Aldrich, St. Louis, MO) at a concentration of 10 μg/ml. After blocking with 2% BSA, the plates were incubated with 100 μl of 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 were 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 of the 3,5,3', 5'-tetramethylbenzidine (TMB) substrate for 15 min (Pierce, Rockford, IL), 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 (EG & G Wallac, Turku, Finland).

Per-cell-based CTL assay

CTL assays were modified as described previously (Wilcox *et al.,* 2004). Splenoytes from cGVHD mice were stained with anti-H-2K^b and anti-CD8 to count donor CD8$^+$ T cells. Splenocytes containing equal numbers of donor CD8$^+$ T cells were used as effector cells to compare the cytotoxicities of single cells between experimental groups. EL-4 cells (1×10^5) were used as a target cell.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between experimental groups.

Results

The activity of alloantigen-specific CTLs is associated with the development of cGVHD versus aGVHD in the parent-into-F_1 model of GVHD (Pals *et al.,* 1984; Via *et al.,* 1987). Whereas increasing the CTL activity of donor CD8$^+$ T cells, e.g., treatment of IL-12 (Via *et al.,* 1994) and *in vivo* ligation of GITR (Kim *et al.,* 2006), changes the type of GVHD from a

chronic form to an acute form, its downregulation induces cGVHD instead of aGVHD (Shustov *et al.,* 2000; Via *et al.,* 2001). However, it is not known whether T_{reg} cells can control the development of GVHD by regulating the activity of donor $CD8^+$ T cells. We induced cGVHD by transferring total or T_{reg} cell-depleted DBA/2 spleen/lymph node cells into unirradiated BDF_1 mice. Three weeks after disease induction, we measured levels of anti-DNA IgG_1 autoantibody, a predominant immunoglobulin isotype in cGVHD (Kim *et al.,* 2005; 2006b). Introduction of T_{reg} cell-depleted parental cells induced significantly lower levels of anti-DNA IgG_1 in the recipient than did introduction of total parental cells (Figure 1). This result was surprising, because alloantigen-specific T_{reg} cells have been shown to be able to inhibit cGVHD (Zheng *et al.,* 2004; Kim *et al.,* 2005). In these cases, it is thought that a large number of T_{reg} cells can inhibit the activity of alloantigen-specific $CD4^+$ T cells that are prerequisite for breaking self-tolerance of B cells. Based on this and another fact that donor $CD8^+$ T cells, if activated instead of being anergic, can inhibit the development of cGVHD by attacking host B cells (Kim *et al.,* 2005; 2006a), we reasoned that T_{reg} cells existing in the donor cell population might suppress the CTL activity of alloantigen-specific donor $CD8^+$ T cells, resulting in the inhibition of cGVHD. Indeed, we observed a massive deletion of B cells in the absence of donor T_{reg} cells (Figure 2). Therefore, deletion of B cells provides an explanation for the basal levels of anti-DNA IgG_1 produced in BDF_1 mice that received T_{reg} cell-depleted parental cells.

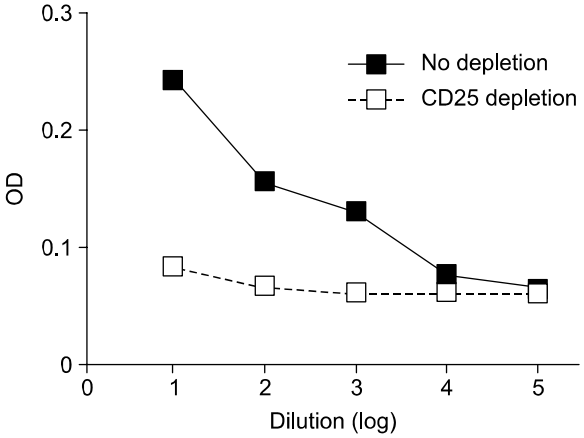

Figure 1. Inhibition of autoantibody production by donor T_{reg} cells in cGVHD. cGVHD was induced by transferring 8×10^7 of total or T_{reg} cell-depleted DBA/2 spleen/lymph node cells into BDF_1 mice. Three weeks after transfer of parental cells, serum samples were collected, and assayed in duplicate by ELISA for IgG_1 anti-DNA autoantibody. The optical OD of duplicate samples for each mouse was measured at 450 nm, using serially-diluted serum samples. OD values are means $\pm$ SE of $n = 5$ per group and are representative of two independent experiments.

We next examined whether T_{reg} cells can regulate the activity of alloantigen-specific donor $CD8^+$ T cells. First, we looked at the expression of CD62L, which is downregulated on T cells after activation. Three weeks after disease induction, a majority of donor $CD8^+$ T cells consisted of activated cells expressing low levels of CD62L in the spleen of BDF_1 recipients that received T_{reg} cell-depleted parental cells (Figure 3A and B). Consistent with our previous data (Kim *et al.,* 2006a), at this time, most donor $CD8^+$ T cells had a naive phenotype (expressing high levels of CD62L) in BDF_1 recipients that received total parental cells. This result indicates that the absence of donor T_{reg} cells induces a sustained activation of donor $CD8^+$ T cells in the

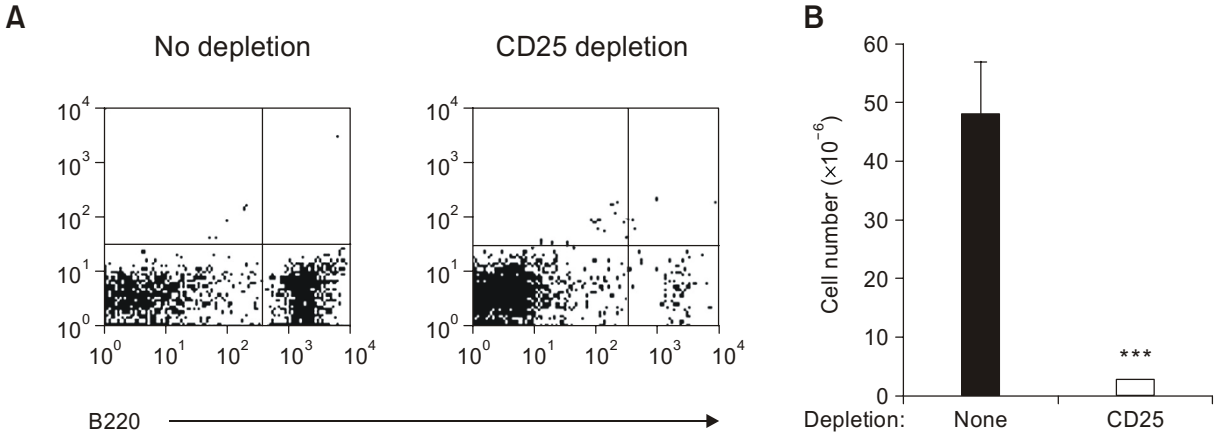

Figure 2. Deletion of B cells in the absence of donor T_{reg} cells. Three weeks after disease induction, splenocytes were prepared and counted by staining with anti-B220. (A) Representative FACS plots. (B) Counts of B cells ($n = 5$ per group). ***$P < 0.001$, between the two groups. Experiments were performed two times and similar results were obtained.

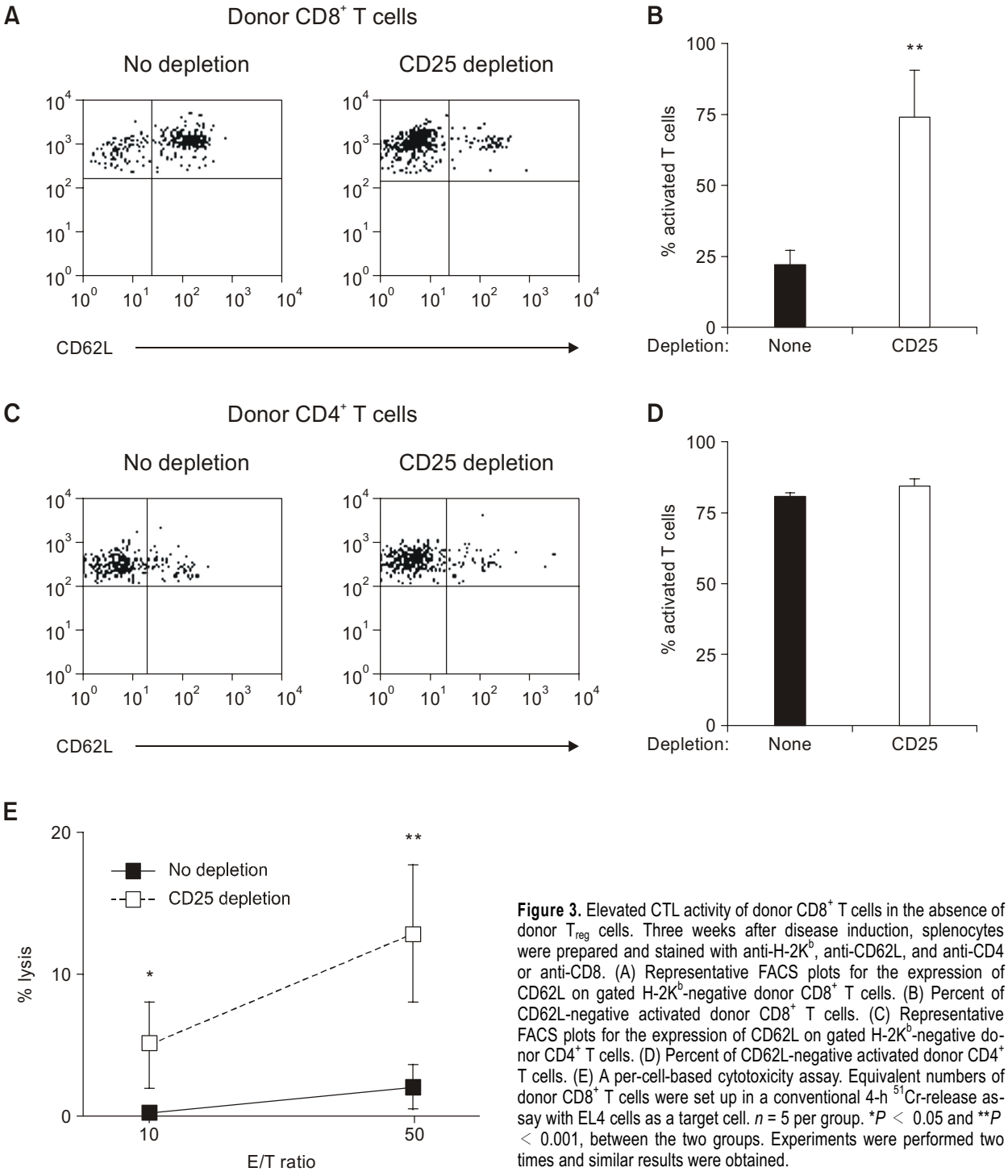

Figure 3. Elevated CTL activity of donor $CD8^+$ T cells in the absence of donor T_{reg} cells. Three weeks after disease induction, splenocytes were prepared and stained with anti-H-$2K^b$, anti-CD62L, and anti-CD4 or anti-CD8. (A) Representative FACS plots for the expression of CD62L on gated H-$2K^b$-negative donor $CD8^+$ T cells. (B) Percent of CD62L-negative activated donor $CD8^+$ T cells. (C) Representative FACS plots for the expression of CD62L on gated H-$2K^b$-negative donor $CD4^+$ T cells. (D) Percent of CD62L-negative activated donor $CD4^+$ T cells. (E) A per-cell-based cytotoxicity assay. Equivalent numbers of donor $CD8^+$ T cells were set up in a conventional 4-h ^{51}Cr-release assay with EL4 cells as a target cell. n = 5 per group. *$P < 0.05$ and **$P < 0.001$, between the two groups. Experiments were performed two times and similar results were obtained.

recipient. The activation status of donor $CD4^+$ T cells were similar between the two experimental groups (Figure 3C and D). Consistently, a per-cell-based cytotoxicity assay demonstrated that there were increased levels of cytotoxicity against alloantigens in donor $CD8^+$ T cells from BDF_1 mice that received T_{reg} cell-depleted parental cells (Figure 3E). In agreement of others (Pals *et al.,* 1984; Via *et al.,* 1987; Rus *et al.,* 1995) and our previous data (Kim *et al.,* 2006a), a minimal level of cytotoxicity was observed in BDF_1 mice that received total parental cells. Taken together, our results suggest that T_{reg}

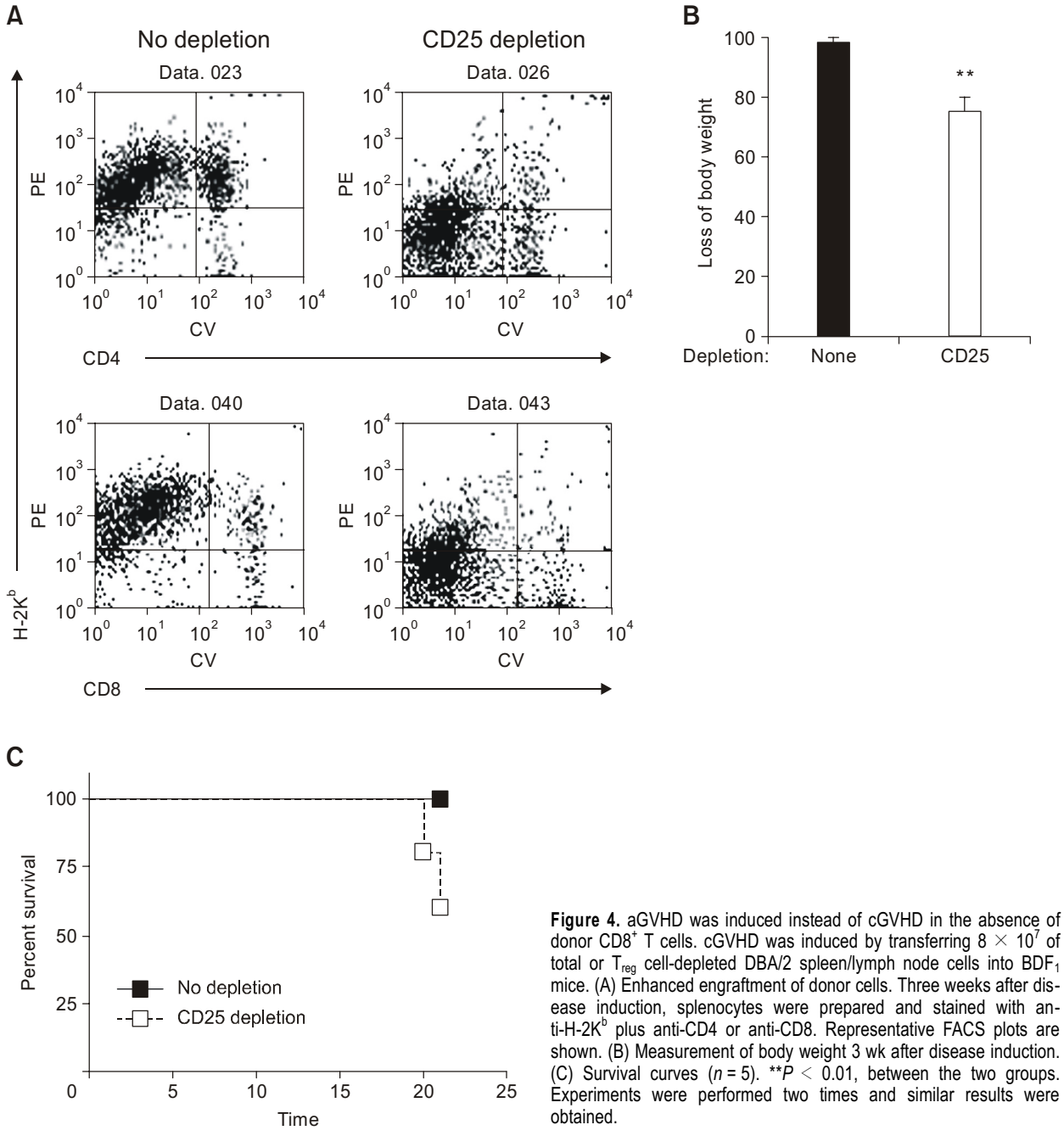

Figure 4. aGVHD was induced instead of cGVHD in the absence of donor $CD8^+$ T cells. cGVHD was induced by transferring 8×10^7 of total or T_{reg} cell-depleted DBA/2 spleen/lymph node cells into BDF_1 mice. (A) Enhanced engraftment of donor cells. Three weeks after disease induction, splenocytes were prepared and stained with anti-H-$2K^b$ plus anti-CD4 or anti-CD8. Representative FACS plots are shown. (B) Measurement of body weight 3 wk after disease induction. (C) Survival curves ($n = 5$). $^{**}P < 0.01$, between the two groups. Experiments were performed two times and similar results were obtained.

cells inhibit the activity of donor $CD8^+$ T cells rather than of donor $CD4^+$ T cells in cGVHD induced by T_{reg} cell-containing parental cells.

Finally, we investigated whether increase in the CTL activity of donor $CD8^+$ T cells could result in the development of aGVHD in BDF_1 mice that received T_{reg} cell-depleted parental cells. Indeed, we observed clinical signs of aGVHD, as manifested by profuse diarrhea and hunched posture in BDF_1 mice that received T_{reg} cell-depleted parental cells. Consistently, we found other signs of aGVHD, such as the engraftment of donor cells and a severe loss of body weight in BDF_1 mice that received T_{reg} cell-depleted parental cells (Figure 4A and B). In one experiment, 60% BDF_1 mice that received T_{reg} cell-depleted parental cells were dead by week 3 after disease induction (3 out 5 mice), while control mice that received total parental cells were healthy (Figure 4C). Overall, the data presented here suggest that donor T_{reg} cells play a critical role in

suppressing the CTL activity of donor CD8$^+$ T cells against recipient alloantigens, which may be a key step in the pathogenesis of cGVHD.

Discussion

T_{reg} cells play a key role in maintaining immunological tolerance to self-Ags, tumor Ags, and alloantigens (Sakaguchi, 2005). Even though it is well known that T_{reg} cells can prevent both aGVHD (Hoffman *et al.,* 2002; Taylor *et al.,* 2002; Edinger *et al.,* 2003) and cGVHD (Zheng *et al.,* 2004; Kim *et al.,* 2005), little is known regarding their involvement in the pathogenesis of cGVHD versus aGVHD. In human cGVHD, there is evidence showing that T_{reg} cells are positively associated with cGVHD (Clark *et al.,* 2004), although contradictory data have been reported more recently (Miura *et al.,* 2004). There is also circumstantial evidence that T_{reg} cells are involved in the conversion of aGVHD to cGVHD; aGVHD has been shown to progress into cGVHD by rapamycin (Blazar *et al.,* 1999) which is highly effective in inducing the proliferation of T_{reg} cells *in vitro* (Battaglia *et al.,* 2005). To our knowledge, however, our data are the first demonstration that T_{reg} cells can inhibit the development of aGVHD in mice that are naturally prone to cGVHD by suppressing the activity of donor CD8$^+$ T cells.

The induction of CD8$^+$ T-cell anergy by T_{reg} cells is involved in immune evasion of tumors (Ercolini *et al.,* 2005; Yu *et al.,* 2005) and in chronic viral infection (Boettler *et al.,* 2005). It is an interesting question how T_{reg} cells can preferentially regulate CD8$^+$ T cells rather than CD4$^+$ T cells in our cGVHD model. It seems that the size of T_{reg} cells is important. Because infusion of a large number of T_{reg} cells cultured *in vitro* can inhibit the production of autoantibody in cGVHD that can't occur without the help of CD4$^+$ T cells (Zheng *et al.,* 2004; Kim *et al.,* 2005), it is possible that the infused T_{reg} cells can inhibit the activity of CD4$^+$ T cells as well as CD8$^+$ T cells. From these observations, it may be inferred that there is a hierarchy of T-cell subsets in the susceptibility to the suppressive activity of T_{reg} cells. Then, how do donor CD8$^+$ T cells have a higher susceptibility than donor CD4$^+$ T cells to the suppressive activity of donor T_{reg} cells in cGVHD? One possibility is that in the DBA/2 $\rightarrow$ F_1 cGVHD model, donor CD8$^+$ T cells have a higher responsiveness to allostimulation than donor CD4$^+$ T cells, being more susceptible to the regulation of T_{reg} cells. A second possibility is that the responsiveness to cytokines determines the susceptibility to the suppressive activity of T_{reg} cells (Yu *et al.,* 2005). For example, CD4$^+$ T cell-subsets are different in the susceptibility to the suppressive activity of T_{reg} cells (Cosmi *et al.,* 2004). Since Th2 cells are preferentially activated in cGVHD (De Wit *et al.,* 1993), it is possible that under the environments enriched with Th2 cytokines, Th2 cells are more resistant to the suppressive activity of T_{reg} cells than are Th1 and CD8$^+$ T cells (Cosmi *et al.,* 2004). A third possibility is that T_{reg} cells maintain the anergy of donor CD8$^+$ T cells escaping activation-induced cell death (Kim *et al.,* 2006a). This hypothesis is supported by the observation that a majority of residual donor CD8$^+$ T cells exhibit an activation phenotype in cGVHD mice that received T_{reg} cell-depleted parental cells (Figure 3). It seems that there is no difference in the degree of activation-induced cell death of donor CD8$^+$ T cells in mice that received either total or T_{reg} cell-depleted parental cells (our unpublished data). Therefore, a small number of residual CD8$^+$ T cells may be the main factor that drives cGVHD to aGVHD in the absence of T_{reg} cells in mice that are naturally prone to cGVHD. The hypotheseses, however, are not mutually exclusive.

Even though the model used for this study has been questioned regarding its relevance to human cGVHD (Lee *et al.,* 2005), our result may have an implication in treatment of aGVHD. Treatment of aGVHD by blocking the activity of donor CD8$^+$ T cells (e.g., infusion of T_{reg} cells and administration of rapamycin) may increase the chance for aGVHD to develop into cGVHD (Blazar *et al.,* 1999; Shustov *et al.,* 2000). Therefore, we need to find methods to inhibit the activity of both donor CD4$^+$ T cells and CD8$^+$ T cells to effectively prevent or treat aGVHD.

In summary, this study demonstrated that T_{reg} cells maintain donor CD8$^+$ T-cell anergy in cGVHD. cGVHD occurs as a default consequence of the suppression of aGVHD by T_{reg} cells. Our result also warrants further studies to reveal mechanisms of how T_{reg} cells differentially regulate T-cell subsets, which will ultimately find its value in treatment of GVHD.

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