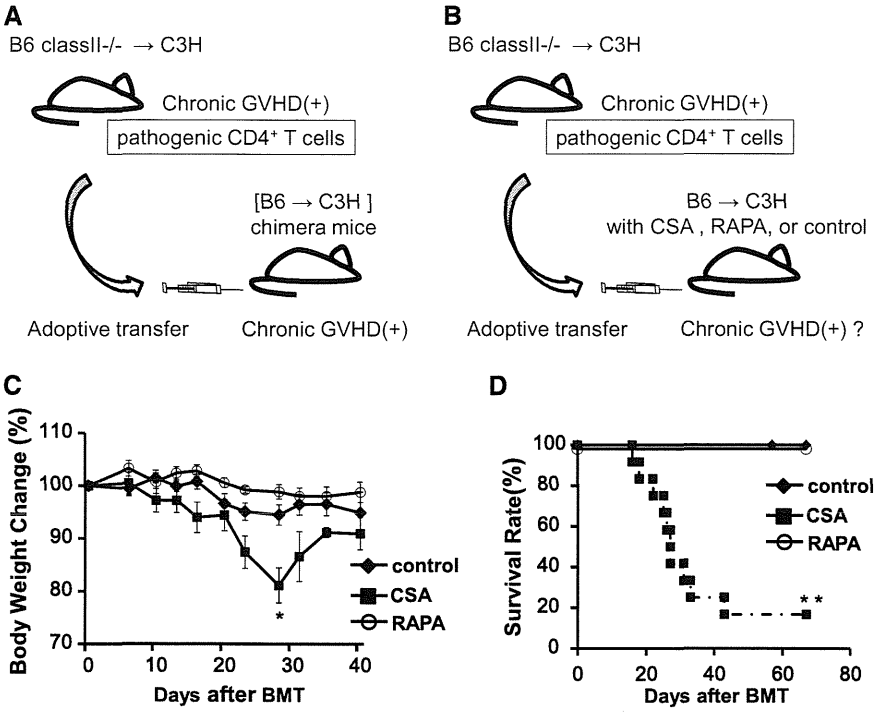

Figure 7. Adoptive transfer of pathogenic CD4+ T cells caused severe chronic GVHD. (A) Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient (H2-Ab1$^{-/-}$) B6 mice. These mice developed chronic GVHD and CD4+ T cells isolated from chronic GVHD mice ([H2-Ab1$^{-/-}$→C3H] CD4+ T cells) were primarily donor reactive. These pathogenic CD4+ T cells cause chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium ([B6→C3H] chimeras). (B) C3H recipient mice underwent transplantation from B6 donors as shown in Figure 1 and received CSA, RAPA, or vehicle solution until 60 days post BMT. These C3H recipient mice received adoptive transfer of [H2-Ab1$^{-/-}$→C3H] CD4+ T cells. Body weight change is shown in (C) and overall survival is shown in (D). Data from 3 similar experiments are combined (n = 8 to 12 per group). The data are expressed as means ± SE. *$P < .05$; **$P < .01$.

survival rate on day 62 after adoptive transfer was 100%. Taken together, these data demonstrated that CSA, but not mTOR inhibitors, increased liability to chronic GVHD.

DISCUSSION

Patients with chronic GVHD have a lower frequency of Tregs when compared with patients without chronic GVHD [29-32]. Experimental BMT demonstrated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]; however, it is not known whether Tregs in the grafts persist into the late post-transplantation period and play a role in preventing chronic GVHD. Mastuoka et al. prospectively monitored CD4+ T cell subsets and showed that thymic generation of naïve Treg was markedly impaired and Treg levels subsequently declined in patients with prolonged CD4+ lymphopenia [32]. This resulted in a relative Treg deficiency, which was associated with a high incidence of extensive chronic GVHD. In the present study, we monitored Treg reconstitution kinetics in the spleen, MLN, and thymus according to 2 subsets, T cells derived from peripheral-expanded mature T cells and newly arising T cells from bone marrow stem cells, using 2 mouse BMT models because this is difficult to examine in a human setting. The results indicated that host Tregs disappeared rapidly in mice receiving allogeneic T cells early in the early post-transplantation period, consistent with a previous report [33]. In addition, this study showed that splenic T cell–derived Treg initially occupy a niche in lymphopenic transplantation recipients, suggesting that mature Treg underwent homeostatic and/or alloantigen-driven expansion. However, the donor splenic T cell–derived Treg pool contracted gradually and Tregs generated from donor BM-derived progenitors comprised the long-term peripheral Treg pool. The BM-derived Treg compartment was functionally competent, as determined by in vitro lymphoid suppression, indicating that these cells play a role in post-BMT immune tolerance.

Coenen et al. reported that 28 days of treatment with CSA resulted in a reduction in thymic generation of CD4+Foxp3+ T cells and peripheral CD25+Foxp3+ T cells in normal mice [28]. We assessed whether CSA affects the peripheral Treg pool after allogeneic BMT; on day 21, there were no differences in the absolute numbers of Tregs among 3 groups, and CSA had no impact on early Treg reconstitution. Consistent with our observations, Setoguchi et al. reported that in contrast to the requirement of IL-2 for physiological expansion of CD4+CD25+ Treg cells in normal nonlymphopenic mice, homeostatic proliferation in a lymphopenic environment appears to be IL-2-independent [19]. Zeiser et al. also reported that CSA administration has only a minor impact on the expansion of adoptively transferred CD4+CD25+ T cells on day 7 post transplantation [34]. However, whether prolonged use of CSA affects the long-term peripheral Treg pool has not been reported. Our data showed that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Tregs. The numbers of Tregs in the spleen, thymus and tissue were significantly reduced in mice receiving CSA in comparison with those receiving mTOR inhibitors or PBS on day 110. CSA blocks nuclear factor of activated T cells translocation into the nucleus by inhibiting calcineurin phosphatase activity [35]. CSA inhibits the thymic generation of Tregs by impairment of TCR signaling and by reducing nuclear factor of activated T cells–dependent Foxp3 promoter activity [36]. In contrast, rapamycin-sensitive downstream targets of phosphatidyl-inositol 3-kinase are IL-2-independent, and

rapamycin affects neither the initial signal transduction upon TCR triggering nor the thymic generation of Treg [37]. Immunosuppressive drugs have different mechanisms of promoting immune suppression and our data revealed different effects on the long-term peripheral Treg pool after allogeneic BMT.

Although mouse models of chronic GVHD have provided important insights into pathophysiology of this disease, one factor that confounds the translation of findings in mouse models to the human disease is that time course of development of chronic GVHD is more rapid in most mouse models than in human. Another factor is that most patients are given immunosuppressive therapy to prevent acute GVHD [38], and these medications might influence the development of chronic GVHD. In this study, histopathological examination revealed that CSA-treated mice showed pathogenic features of chronic GVHD, whereas those treated with mTOR inhibitors showed no significant differences compared with control mice. This is the first report that long-term use of CSA induces chronic GVHD in transplant-recipient mice. This may have been due to induction of autoreactive T cells by CSA [39,40]. Wu et al. reported that CSA contributes to chronic GVHD in experimental models, which was ascribed to the disruption of clonal-deletion mechanisms in the thymus, resulting in the export of autoreactive T cells [41]. The present study demonstrated another mechanism by which CSA impaired Treg reconstitution. Adoptive transfer of the pathogenic $CD4^+$ T cells caused severe chronic GVHD in CSA-treated mice, whereas mTOR inhibitor-treated and control mice showed resistance to induction of chronic GVHD. These findings suggest that the increased liability to chronic GVHD in CSA-treated mice might be due to limited reconstitution of BM-derived Treg cells; further mechanistic studies will be required to determine if this is truly causative rather than merely an association.

Here, we assessed the differential effects of CSA and mTOR inhibitors on the long-term peripheral Treg pool after allogeneic BMT. Our findings indicated that, in contrast to mTOR inhibitors, CSA compromises homeostasis in peripheral immune compartments and thymic generation of $CD4^+CD25^+Foxp3^+$ T cells. GVHD prophylaxis with mTOR inhibitor and calcineurin inhibitor failed to reduce chronic GVHD [11,42-45]. The choice of calcineurin inhibitor–free GVHD prophylaxis with mTOR inhibitors, such as mTOR inhibitors plus IL-2 [16] or mTOR inhibitors plus antithymocyte globulin [46] may have important implications for the control of chronic GVHD after BMT.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.11.018.

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