

Mammalian target of rapamycin inhibitors permit regulatory T cell reconstitution and inhibit experimental chronic graft-versus-host disease

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Abstract

Chronic graft-versus-host disease (GVHD) remains a major late complication of allogeneic bone marrow transplantation (BMT). In a previous study, impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD using MHC class II-deficient (H2-Ab1 KO) B6 into C3H model and CD4⁺ T cells isolated from chronic GVHD mice caused chronic GVHD when administered into the secondary recipients. In this study, we evaluated the kinetics of regulatory T cell (Treg) reconstitution in wild type B6 into C3H model. After myeloablative conditioning, host Tregs disappeared rapidly, followed by expansion of Tregs derived from the donor splenic T cell inoculum. However, the donor splenic T cell-derived Treg pool contracted gradually and was almost completely replaced by newly generated donor bone marrow (BM)-derived Tregs in the late post-transplant period. Next, we compared the effects of cyclosporine (CSA) and mammalian target of rapamycin (mTOR) inhibitors on Treg reconstitution. Administration of CSA significantly impaired Treg reconstitution in the spleen and thymus. In contrast, BM-derived Treg reconstitution was not impaired in mTOR inhibitor-treated mice. Histopathological examination indicated that mice treated with CSA, but not mTOR inhibitors, showed pathogenic features of chronic GVHD on day 120. Mice treated with CSA, but not mTOR inhibitors until on day 60 developed severe chronic GVHD followed by adoptive transfer of the pathogenic CD4⁺ T cells isolated from H2-Ab1 KO into C3H model. These findings indicated that long-term use of CSA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD. The choice of immunosuppression, such as calcineurin inhibitor-free GVHD prophylaxis with mTOR inhibitor, may have important implications for the control of chronic GVHD after BMT.

Introduction

Chronic GVHD is the most serious late complication after allogeneic HSCT, but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined (1-3). GVHD prophylaxis using calcineurin inhibitors, such as cyclosporine (CSA) and tacrolimus, reduces the expansion of effector T cells by blocking interleukin (IL)-2 and prevents acute GVHD, but fails to reduce chronic GVHD (4, 5). Administration of CSA for up to 24 months, longer than the standard 6 months of CSA, also did not decrease the risk of chronic GVHD (6). Several studies have indicated that the efficacy and safety of mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAPA), in refractory chronic GVHD patients (7-10). However, a recent randomized trial showed that the combination of RAPA and tacrolimus as GVHD prophylaxis failed to reduce chronic GVHD compared with tacrolimus and methotrexate (11).

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) have been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. A series of animal studies indicated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells (12-14). Based on the role of Tregs in the prevention of GVHD and on their dependence on IL-2, there is considerable concern regarding the impact of blocking IL-2 signaling or IL-2 production by the immunosuppressive agents used for prophylaxis of GVHD. Zeiser *et al.* reported that Tregs showed relative resistance to RAPA as a result of reduced usage of the mTOR pathway and functional PTEN, a negative regulator of the phosphatidyl-inositol 3-kinase (PI3K)/Akt/mTOR pathway in Tregs compared with conventional T cells (15). In contrast to CSA, RAPA allowed expansion of adoptively transferred Treg cells and led to reduction of

alloreactive T cell expansion when animals received Treg treatment in combination with RAPA. They also showed that a combination of RAPA plus IL-2 increased both expansion of donor natural Tregs and conversion of induced Tregs from donor conventional T cells, and suppressed acute GVHD (16). These animal data suggest that RAPA and CSA have differential impacts on peripheral Tregs following BMT.

IL-2 signaling is pivotal for Treg homeostasis in the periphery and is also essential for naturally occurring Treg development in the thymus (17-19). T cell repopulation following BMT is composed of two subsets: T cells derived from the donor splenic T cell inoculum and newly arising T cells from bone marrow (BM) inoculum. It has been shown that Tregs from the former pathway play an important role in acute GVHD, whereas, no previous study evaluated whether use of CSA for an extended period affects donor BM-derived Treg generation. We hypothesized that BM-derived Tregs comprise the long-term peripheral Treg pool and that CSA, but not mTOR inhibitors, causes impaired BM-derived Treg reconstitution, which has a negative impact on chronic GVHD. In the present study, we therefore evaluated effects of different immunosuppressants on two distinct Treg expansion reconstitution pathways and on the development of chronic GVHD.

Materials and methods

Mice

Female C57BL/6 (B6: H-2^b, CD45.2⁺) and C3H/HeN (C3H: H-2^k) mice were purchased from Charles River Japan (Yokohama, Japan) or from the Okayama University mouse colony (Okayama, Japan). B6-Ly5a (H-2^b, CD45.1⁺) and C3.SW (H-2^b, CD45.2⁺) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient H2-Ab1^{-/-} mice (B6.129-H2-Ab1^{tm1Gru} N12) were from Taconic Farms (Germantown, NY) (20). Mice between 8 and 18 weeks of age were maintained under specific pathogen-free conditions and received normal chow and hyperchlorinated drinking water after transplantation. All experiments involving animals were approved by the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

Bone marrow transplantation (BMT)

Mice received transplants according to the standard protocol described previously (21, 22). Briefly, recipient mice received two split doses of either 500 cGy (allogeneic C3H and C3.SW recipients) or 650 cGy (syngeneic B6 recipients) total-body irradiation (TBI) 3 – 4 h apart. Recipients were injected with 10×10⁶ T cell-depleted bone marrow (TCD-BM) cells plus 1 or 2×10⁶ whole spleen cells from B6 donors. [H2-Ab1^{-/-} → C3H] chimeras were produced by reconstituting lethally irradiated C3H mice with 5×10⁶ TCD-BM cells from H2-Ab1^{-/-} mice, as described previously (23). T cell depletion was performed using anti-CD90–microbeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients

on day 0.

Immunosuppressive treatment

Rapamycin (RAPA) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Everolimus (RAD) and CSA were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Everolimus emulsion was dissolved in distilled water at a concentration of 625 µg/ml and administered to recipients by oral gavage at a dose of 5 mg/kg. RAPA and CSA were given as suspensions in carboxymethylcellulose sodium salt: CMC (C5013; Sigma-Aldrich, St. Louis, MO) at a final concentration of 0.2% CMC. RAPA and CSA were administered to recipients by peritoneal injection at doses of 0.5 and 20 mg/kg, respectively(15, 24). Immunosuppressive treatments were performed once daily, starting on day 0 and continuing until death or end of the observation period (day 110 – 125).

Adoptive transfer

Splenocytes were isolated from [*H2-Ab1*^{-/-} → C3H] chimeras 6–11 weeks after TCD-BMT. CD4⁺ T cells were negatively selected from splenocytes by depletion of CD8⁺, DX5⁺, CD11b⁺, Ter-119⁺, and B220⁺ cells using the AutoMACS system, as described previously (23). A total of 2×10⁷ CD4⁺ T cells per mouse were injected intravenously into recipients following immunosuppressive therapy for 70 days after BMT.

Assessment of GVHD

Following BMT, survival was monitored daily, and weight changes were assessed twice per week. The degree of clinically acute GVHD was assessed twice per week using a scoring

system that sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described previously (22). Shaved skin from the interscapular region (approximately 2 cm²), liver, and salivary gland specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. Skin slides were scored on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop-out (0 – 2 for each category; the maximum score was 10) (21). Liver slides were scored based on bile duct injury and inflammation (0 – 4 for each category), and the maximum score was 8 (25). Salivary gland slides were scored based on atrophy and inflammation (0 – 3 for each category), and the maximum score was 6. All slides were scored by pathologists (T.K. and T.T.) blind to experimental group.

Immunohistochemistry

Immunohistochemical staining for Foxp3 and CD3 was performed using the high polymer (HISTOFINE simple stain, NICHIREI, Tokyo, Japan) method. Anti-Foxp3 (eBioscience) and anti-CD3 (Abcam, Cambridge, MA) were used to identify Tregs and effector T cells, respectively.

Flow cytometry

The mAbs used were unconjugated anti-CD16/32(2.4G2); FITC-, PE-, PerCP-, or APC-conjugated anti-mouse CD4, CD25, CD45.1, CD45.2, H-2^b, H-2^d (BD Pharmingen, San Diego, CA); and Foxp3 (eBioscience, San Diego, CA), as described previously (26). A Foxp3 staining kit™ (eBioscience) was used for intracellular staining. Cells were analyzed on a

FACSAria flow cytometer with FACSDiva software (BD Immunocytometry Systems, San Diego, CA).

Mixed leukocyte reaction

CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ T cells, and CD11c⁺ DC were magnetically separated by AutoMACS using microbeads from a CD4⁺CD25⁺ regulatory T cell isolation kit and CD11c microbeads. CD4⁺CD25⁻ T cells (5×10^4 per well) together with various numbers of CD25⁺CD4⁺ T cells ($0 - 5 \times 10^4$ per well) were cultured with irradiated (30 Gy) CD11c⁺ DC as stimulators for 72 h in 96-well round-bottomed plates. Cells were pulsed with ³H-thymidine (1 μCi [0.037 MBq] per well) for a further 16 h (27). Proliferation was determined using Topcount NXT (Packard Instruments, Meriden, CT).

Statistics

Data are given as means ± SEM. The survival curves were plotted using Kaplan–Meier estimates. Group comparisons of pathology scores were performed using the Mann–Whitney *U* test. Comparative analysis of cell ratios was performed by the unpaired two-tailed Student's *t*-test or Welch's *t*-test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Kinetics of Treg reconstitution after allogeneic bone marrow transplantation

We first examined whether Tregs intermixed in the graft persist in the host for long periods post-BMT using the major histocompatibility complex (MHC)-mismatched model of BMT. Lethally irradiated C3H (H-2^k) recipient mice received 10×10^6 TCD-BM cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without $1 - 2 \times 10^6$ spleen cells from B6 (H-2^b,CD45.2) mice. All of the recipients of allogeneic C3H TCD-BM cells from B6 mice and syngeneic mice survived and were resistant to induction of GVHD. Although 100% of the animals that received allogeneic BM plus 2×10^6 spleen cells died by day 35 with clinical and histopathological signs of severe GVHD, the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed mild clinical signs of GVHD and 60% survived by day 120 (Fig. 1A); the following experiment was performed in this setting. Flow cytometric analysis of donor cell chimerism in the spleen 3 weeks after allogeneic BMT showed that $98.8\% \pm 0.7\%$ of spleen cells were derived from the donor in mice, thus confirming complete donor cell engraftment. Host Tregs, as determined by CD4⁺Foxp3⁺H-2^{k+}, were not detected in the spleen on day 21 post-transplantation (data not shown). On day 21 post-transplantation, the majority of CD4⁺Foxp3⁺ Tregs were derived from CD45.2⁺ splenic T cells ($83.4\% \pm 2.2\%$), suggesting that splenic T cell-derived Tregs underwent homeostatic and/or alloantigen-driven expansion (Fig. 1B) and the absolute number of Tregs in the spleens of the recipients of BM plus Sp cells was significantly higher than in TCD-BM recipients. From day 21 onward, due to GVHD-induced lymphopenia, the absolute number of Tregs in the spleens of recipients of BM plus Sp cells was lower than in TCD-BM recipients (Fig. 1C). The rate of CD45.2⁺ splenic T cell-derived Tregs in CD4⁺Foxp3⁺ Treg decreased gradually and most

CD4⁺Foxp3⁺ Treg were CD45.1⁺ BM-derived (93.2%) on day 125 post-transplantation (Fig. 2A). The rate of CD45.1⁺ BM-derived Tregs in the mesenteric lymph nodes (MLN) was also increased and became dominant in the late post-transplant period (Fig. 2B). To exclude strain-dependent artifacts, we next evaluated the kinetics of Treg reconstitution in the B6 (H-2^b) into C3.SW (H-2^b) MHC-compatible, multiple minor histocompatibility antigen (miHA)-incompatible model of SCT. The kinetics of Treg reconstitution in the spleen was similar and most CD4⁺Foxp3⁺ Tregs were derived from CD45.1⁺ BM (97%) on day 90 post-transplantation (Fig. 2C). These findings indicated that the peripheral Treg pool was restored first by expanded splenic T cell-derived mature Treg and then by new Tregs generated from donor BM-derived progenitors. Next, to examine the function of newly arising Tregs, purified CD4⁺CD25⁺ T cells on day 120 post-transplantation were assessed for their ability to inhibit proliferation by responding syngeneic CD4⁺CD25⁻ B6 T cells. Their suppressive activity was virtually indistinguishable from that of Tregs obtained from normal B6 mice (Fig. 2D). Taken together, Treg_s generated from donor BM-derived progenitors comprise the long-term peripheral Treg pool and exhibit immunosuppressive activity.

CSA, but not mTORinhibitors, hampered reconstitution of BM-derived Treg

Coenen *et al.* reported that 28 days of CSA administration hampered Treg homeostasis in normal mice (28). We examined whether use of CSA for an extended period affected the long-term peripheral Treg pool after BMT. C3H recipient mice were transplanted from B6 donor mice (as shown in Fig. 1) and received i.p. injection of CSA, mTOR inhibitor (rapamycin; RAPA), or vehicle control daily from day 0. We analyzed the effects of CSA and RAPA on the Treg compartment at 21, 35, 56, and 110 days post-HCT. Mice treated with

CSA or RAPA showed the same Treg reconstitution pattern as those treated with vehicle solution. On day 21 post-transplantation, the majority of CD4⁺Foxp3⁺ Tregs in the spleen were CD45.2⁺ splenic T cell-derived cells but the Treg compartments were dominated by BM-derived cells on days 56 and 110 post-transplantation in all three groups (Fig. 3A). In the MLN, these three groups also showed similar Treg reconstitution kinetics (Fig. 3B). There were no differences in the absolute numbers of Treg among the three groups on day 21. From day 21 onward, however, the absolute numbers of Tregs in the CSA-treated mice were lower than those in control mice both in the spleen (day 56: 1.3 ± 0.4 vs. $4.6 \pm 0.8 \times 10^5$, $P < 0.01$, day 110: 10.4 ± 1.4 vs. $16.7 \pm 2.4 \times 10^5$, $P < 0.05$, Fig. 4A) and in the MLN (day 56: 1.3 ± 0.5 vs. $7.4 \pm 1.6 \times 10^4$, $P < 0.03$, day 110: 2.9 ± 1.0 vs. $4.9 \pm 1.9 \times 10^5$, $P = 0.46$, Fig. 4B). Especially in the thymus, mice treated with CSA showed a marked reduction in the absolute numbers of Tregs compared to those treated with vehicle control (day 110: 4.6 ± 1.8 vs. $25.7 \pm 5.0 \times 10^4$, $P < 0.01$, Fig. 4C). In contrast to CSA, mice treated with RAPA showed no reduction in the absolute numbers of Tregs and no differences compared to control mice in the spleen or MLN at any time point post-transplantation (Fig. 4A, B). The absolute numbers of newly arising Tregs in the thymus were also not reduced in mice treated with RAPA (Fig. 4C). We next examined the effects of another mTOR inhibitor, everolimus (RAD), which exhibits greater polarity than RAPA and has been approved in Europe for use as an immunosuppressant for prevention of cardiac and renal allograft rejection. Reconstitution of newly arising Tregs in the thymus was not impaired in mice treated with RAD, and there were no differences in the absolute numbers of spleen Tregs compared to control mice on day 110 (spleen: 15.4 ± 2.5 vs. $16.6 \pm 2.4 \times 10^5$, $P = 0.73$, Supplemental data Fig. 1A, thymus: 17.4 ± 3.2 vs. $25.7 \pm 5.0 \times 10^4$, $P = 0.26$, Supplemental data Fig. 1B). These findings

suggested that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Treg_s.

CSA, but not mTOR inhibitors, increased liability to chronic GVHD

Recent studies revealed the association of reduced Treg frequency in patients with chronic GVHD. In the present study, we examined histopathological change in CSA-treated mice where reconstitution of BM-derived Tregs was impaired. The skin of CSA-treated mice showed pathogenic features of chronic GVHD (Fig. 5A), and pathogenic scores revealed significantly exacerbated chronic GVHD pathology compared to those treated with vehicle control (Fig. 5B; 5.5 ± 0.8 vs. 1.6 ± 0.3 , $P < 0.01$). A dry mouth is one of the distinctive features of chronic GVHD. Lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of CSA-treated mice (Fig. 5A) and pathological scores were significantly higher in CSA-treated mice than in the controls (Fig. 5C; 4.0 ± 0.5 vs. 1.8 ± 0.1 , $P < 0.01$). CSA-treated mice showed bile duct injury and fibrosis in the portal area and peripheral mononuclear cell infiltration in the liver and pathological scores of the liver also tended to be worse in CSA-treated mice, as compared to those treated with vehicle control, although it was not statistically significant (Fig 5D). In contrast to CSA, mice treated with RAPA showed no pathogenic features of chronic GVHD and there were no differences in pathogenic skin and salivary gland scores, as compared to control mice (Fig. 5B skin 0.75 ± 0.4 vs. 1.6 ± 0.3 , $P = 0.18$, Fig. 5C salivary gland ; 1.25 ± 0.2 vs. 1.78 ± 0.1 , $P = 0.08$). Immunohistochemical staining for Foxp3 and CD3 revealed that CD3⁺ T cells infiltrated in the skin tissue of all three groups, and RAD-treated mice showed abundant infiltration by CD3⁺ T cells and Foxp3⁺ cells (Fig. 6A). In contrast to RAD, Foxp3⁺ cells were scarcely

found in skin tissue of CSA-treated mice. The ratio of Foxp3 Tregs per 100 CD3⁺ lymphocytes in the skin tissue of CSA-treated mice was significantly lower than those in RAD-treated mice (3.23 ± 0.4 vs. 19.5 ± 4.4 , $P < 0.05$). CSA-treated mice tended to show poorer survival, as compared to those treated with mTOR inhibitors or vehicle control (CSA 27.6% vs. control 54.2%, RAD 57.1%, RAPA 61.5%, $P = 0.28$, Supplemental data Fig. 2). These findings suggested that CSA, but not mTOR inhibitors, hampered the reconstitution of BM-derived Treg and increased liability to chronic GVHD.

We next tested liability to chronic GVHD in CSA-treated mice using adoptive transfer experiments. Previously, Sakoda *et al.* demonstrated that impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD (Fig. 7A) (23). Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient (H2-Ab1^{-/-}) B6 mice ([H2-Ab1^{-/-} → C3H]). These mice developed disease conditions that showed all of the clinical and histopathological features of human chronic GVHD. CD4⁺ T cells isolated from chronic GVHD mice ([H2-Ab1^{-/-} → C3H] 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) (23). In the current study, C3H mice were transplanted from B6 donors as shown in Fig. 1 and were orally administered CSA, RAPA, or vehicle solution until 60 days post-BMT, when none of the recipients showed significant signs of chronic GVHD. To test liability to chronic GVHD, these C3H recipient mice with B6-derived antigen presenting cells received adoptive transfer of [H2-Ab1^{-/-} → C3H] CD4⁺ T cells (Fig. 7B). As shown in Fig. 7C and 7D, adoptive transfer of pathogenic CD4⁺ T cells caused severe weight loss (CSA $81.1 \pm 4.1\%$ vs. control $94.5 \pm 2.1\%$, $P < 0.05$, CSA $81.1 \pm 4.1\%$ vs. RAPA $98.9 \pm 1.5\%$, $P < 0.01$) and chronic

GVHD in CSA-treated mice, with a mortality rate of 83%. RAPA-treated mice and controls showed resistance to induction of chronic GVHD by transfer of pathogenic CD4⁺ T cells; the 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-transplant 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 two subsets, T cells derived from peripheral-expanded mature T cells and newly arising T cells from bone marrow stem cells, using two 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-transplant 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 three 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 to those receiving mTOR inhibitors or PBS on day 110. CSA blocks nuclear factor of activated T cells (NF-AT) 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 NF-AT-dependent Foxp3 promoter activity (36). In contrast, rapamycin-sensitive downstream targets of PI3K 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, while 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, while 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 ATG (46) may have important implications for the control of chronic GVHD after BMT.

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Figure legends

Figure 1. Regulatory T cell reconstitution after allogeneic BMT.

Lethally irradiated C3H(H-2^k) recipient mice received 10×10^6 T cell-depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without $1 - 2 \times 10^6$ spleen cells from B6 (H-2^b,CD45.2) mice. The syngeneic group received transplantation from C3H mice. (A) Survival: the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed a survival rate of 60% by day 120. Open circle, syngeneic; closed circle, TCD-BM cells only; triangle, -with 1×10^6 spleen cells; square, -with 2×10^6 spleen cells. (B) Origin of CD4⁺Foxp3⁺ Treg in the spleen on day 21 post-transplantation: CD45.2⁺ splenic T cell-derived (white bars) and CD45.2⁻ BM-derived (black bars) are shown. (C) The absolute numbers of Treg in the recipients of BM plus Sp cells (triangles) and TCD-BM (closed circles) are shown. Each group consisted of 7 – 25 mice. The means (\pm SE) of each group are shown. Data are from a representative of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 2. Donor BM-derived progenitors comprise the long-term peripheral Treg pool.

Lethally irradiated C3H recipients were transplanted as in Fig. 1: (B6 \rightarrow C3H). The rates of CD45.2⁺ spleen cell-derived (broken lines) and CD45.2⁻ BM-derived (solid lines) Treg in CD4⁺Foxp3⁺ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 \rightarrow C3H) mice at various time points after BMT and cells were analyzed by FACS. (C) Lethally irradiated C3.SW (H-2^b) recipients were transplanted from B6 (H-2^b) donors. The rates of CD45.2⁺ splenic T cell-derived (broken lines) and CD45.2⁻ BM-derived

(solid lines) Treg in CD4⁺Foxp3⁺ Treg in the spleen are shown. Each group consisted of 20 – 23 mice. The means (\pm SE) of each group are shown. Data are from a representative of at least two independent experiments. (D) CD25⁺CD4⁺ Treg were purified from the spleens of (B6 \rightarrow C3H) mice (on day 120) or naïve B6 (WT). B6 CD4⁺CD25⁻ T cells (Tcon) together with various numbers of Treg were cultured with irradiated C3H CD11c⁺ DC as stimulators for 72 h. Proliferative activities were determined by monitoring ³H-thymidine uptake.

Figure 3. Effects of CSA and mTOR inhibitors on the Treg compartment.

Lethally irradiated C3H recipients were transplanted from B6 donor mice as shown in Fig. 1 and received i.p. injections of CSA (closed squares), mTOR inhibitor (rapamycin, RAPA; open triangles), or vehicle control (open squares) daily from day 0 to 110. The rates of CD45.2⁺ splenic T cell-derived (broken lines) and CD45.2⁻ BM derived (solid lines) Treg in CD4⁺Foxp3⁺ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 \rightarrow C3H) mice at various time points after BMT and cells were analyzed by FACS. Each group consisted of 16 – 23 mice. The means (\pm SE) of each group are shown. Data are from a representative of at least two independent experiments.

Figure 4. CSA, but not mTOR, inhibitors hampered reconstitution of BM-derived Treg.

(B6 \rightarrow C3H) mice received i.p. injections of CSA (black bars), mTOR inhibitor (rapamycin, RAPA; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A), MLN (B), and thymus (C) are shown. Each group consisted of 19–26 mice. The means (\pm SE) of each group are shown. Data are from a representative of at least two independent experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 5. CSA, but not mTOR, inhibitors increased the likelihood of chronic GVHD.

(A) Histological findings of the skin (a – d), liver (e – h), and salivary glands (i – l) (on day 120) from (B6 → C3H) mice given CSA, mTOR inhibitor (RAPA, RAD), or vehicle control. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (b); fibrosis in the portal area and peripheral mononuclear cells infiltrates in the liver (f); and fibrosis and atrophy of acinar tissue in the salivary glands (j) were observed (original magnification: $\times 100$) Pathological scores of skin (B), salivary gland (C) and liver (D) . The data are expressed as means \pm SE. Data are from a representative of at least two independent experiments. $*P < 0.05$; $**P < 0.01$.

Figure 6. CSA, but not mTOR, reduces Treg infiltration in skin tissue.

(A) Lethally irradiated C3H recipients were transplanted from B6 donor mice as shown in Fig. 1 and received vehicle control (a, d), CSA (b, e), or mTOR inhibitor (RAD; c, f), daily from day 0 to 120. Immunohistochemical staining was performed using anti-Foxp3 (a-c) and anti-CD3 (d-f) antibodies on day 120. Arrows indicate Foxp3 positive cells. (B) The ratio of Foxp3 Tregs per 100 CD3⁺ lymphocytes. The number of CD3 and Foxp3 cells was counted in all the high-power fields. Results are expressed as mean \pm SD. Pictures and data are from a representative of two independent experiments. ($n = 3-4$ per group). $*P < 0.05$.

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 were transplanted from B6 donors as shown in Fig. 1 and were 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 (C) and overall survival (D). Data from three similar experiments are combined ($n = 8 - 12$ per group). The data are expressed as means \pm SE. * $P < 0.05$; ** $P < 0.01$.

Supplemental data Figure 1. Another mTOR inhibitor, everolimus (RAD), allowed reconstitution of BM-derived Treg.

(B6 → C3H) mice received CSA (black bars), the mTOR inhibitor everolimus (RAD; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A) and thymus (B) are shown. Each group consisted of 4 – 10 mice. The means (\pm SE) of each group are shown. Data are from a representative of at least two independent experiments. * $P < 0.05$; ** $P < 0.01$.

Supplemental data Figure 2. Kaplan-Meier curves showing the survival of recipient mice administered CSA, RAPA, RAD or vehicle solution.

Lethally irradiated C3H recipients were transplanted from B6 donor mice as shown in Fig. 1 and received CSA, RAPA, RAD, or vehicle control, daily from day 0 to 120. Data from two

similar experiments are combined. ●, control, n=45; ■, CSA, n=44; ○, RAPA, n=34; ▲, RAD, n=21. There was no significant difference among the four groups.

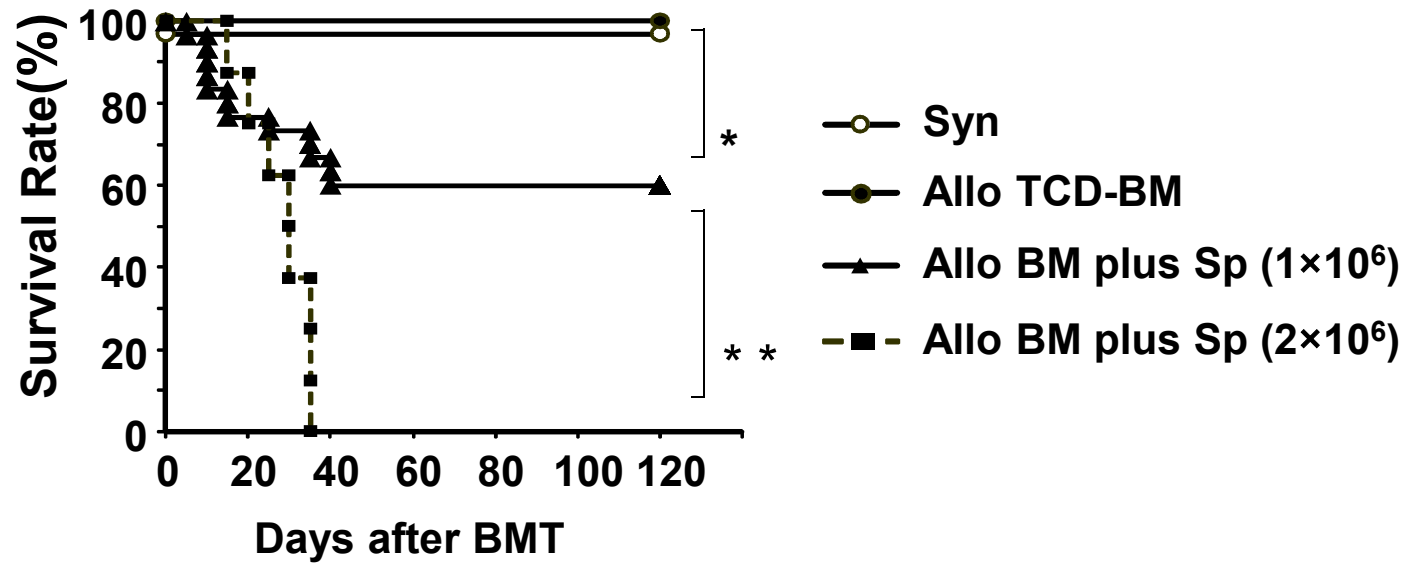
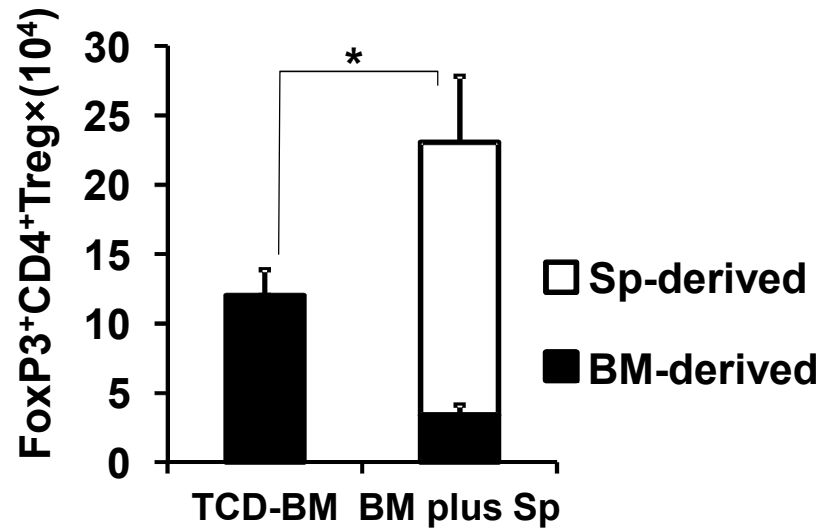
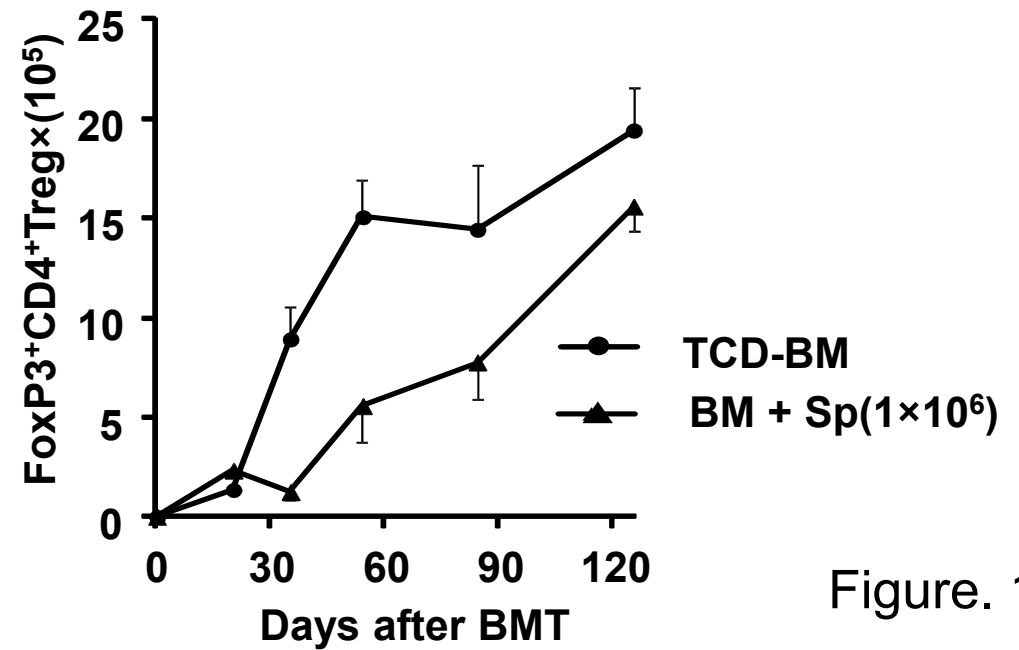
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Figure. 1

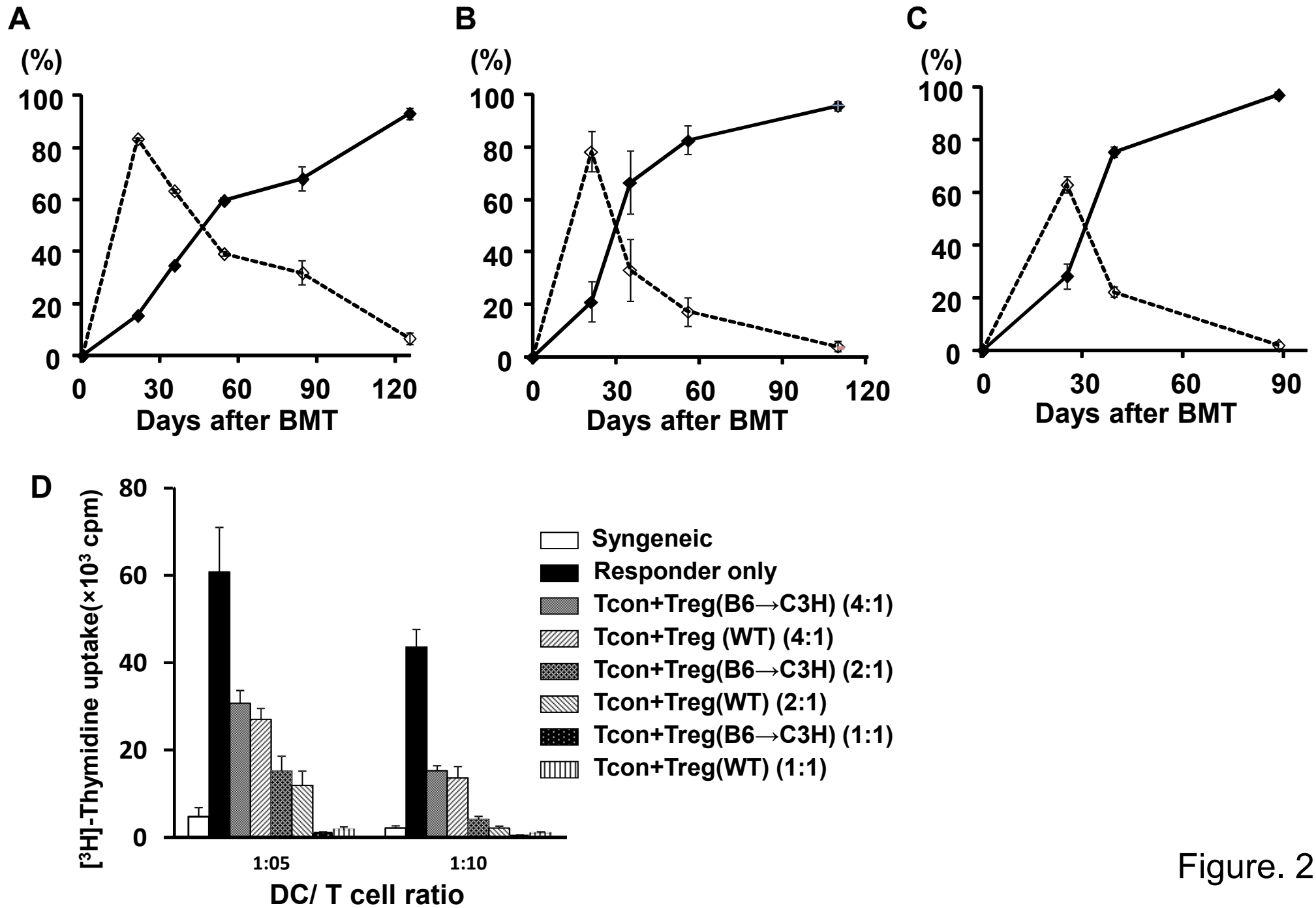


Figure. 2

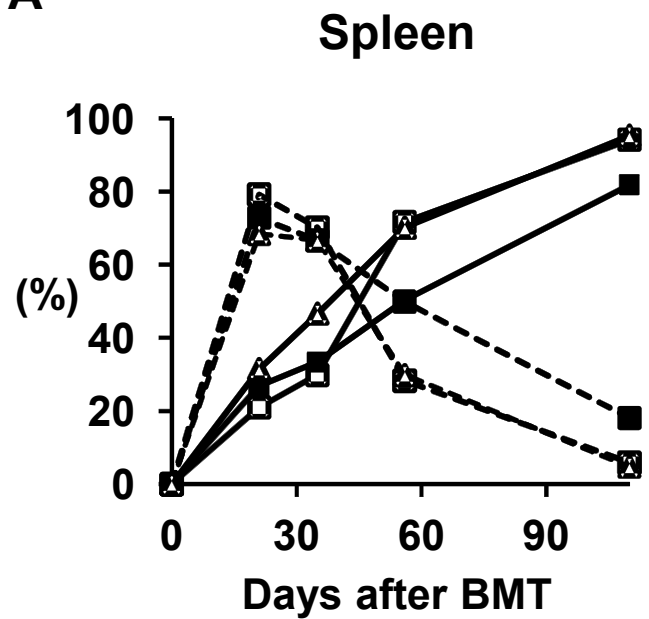
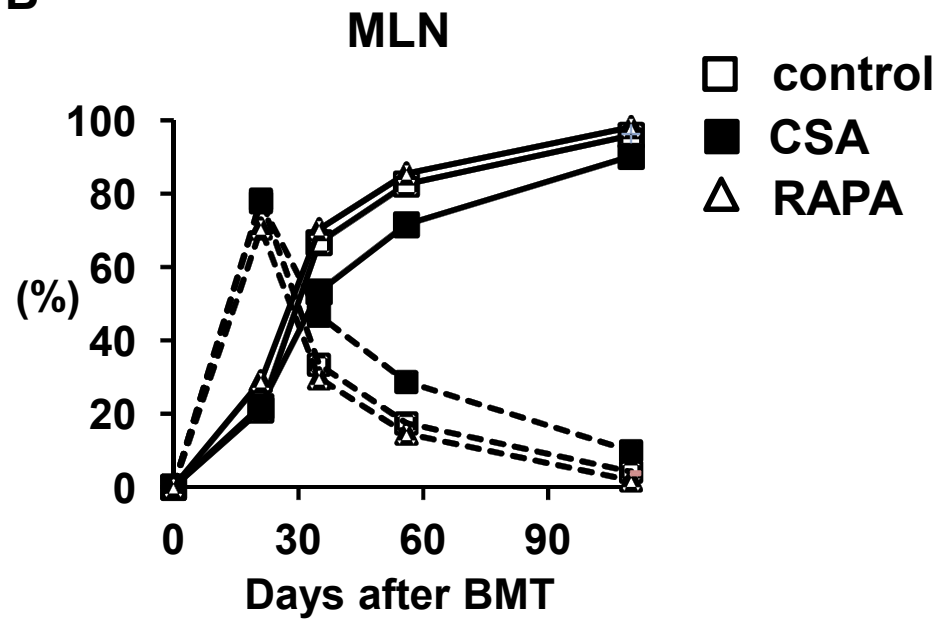
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Figure. 3

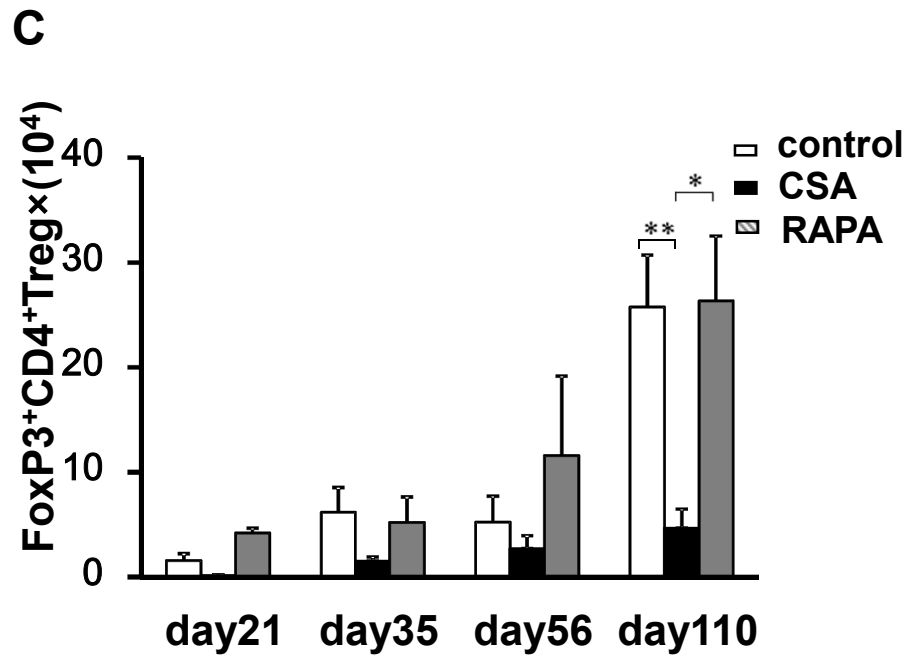
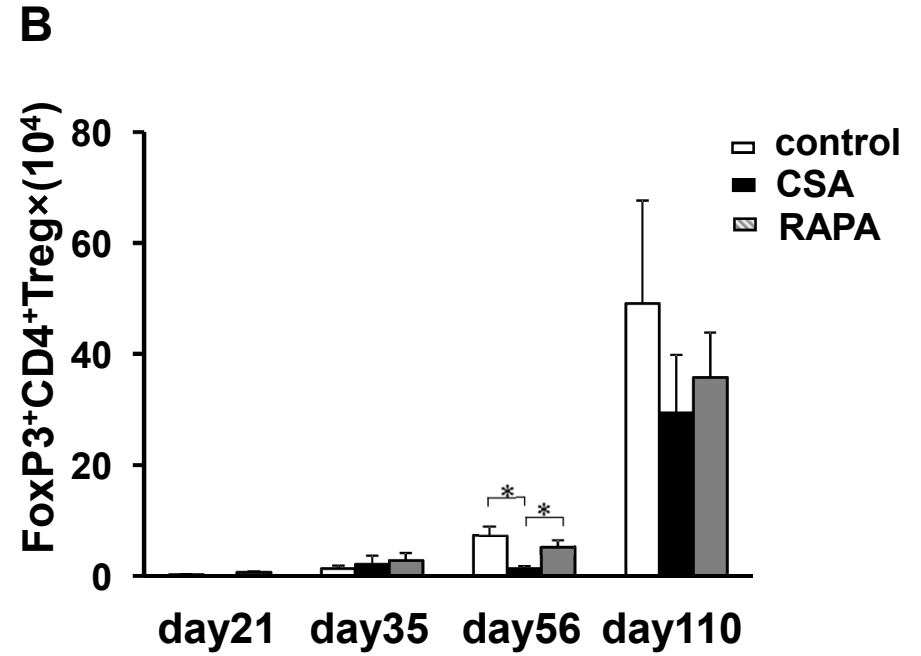
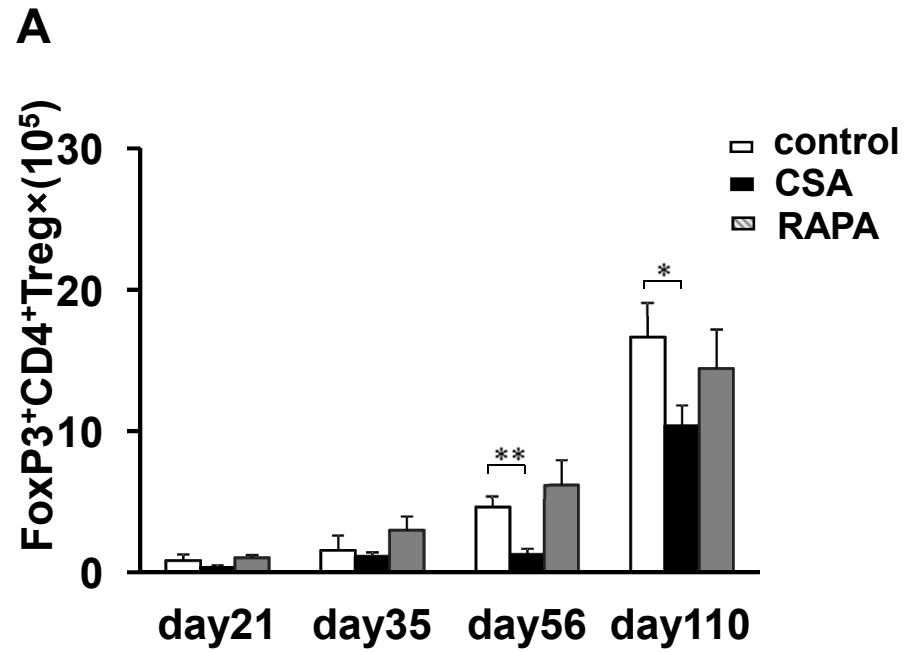


Figure. 4

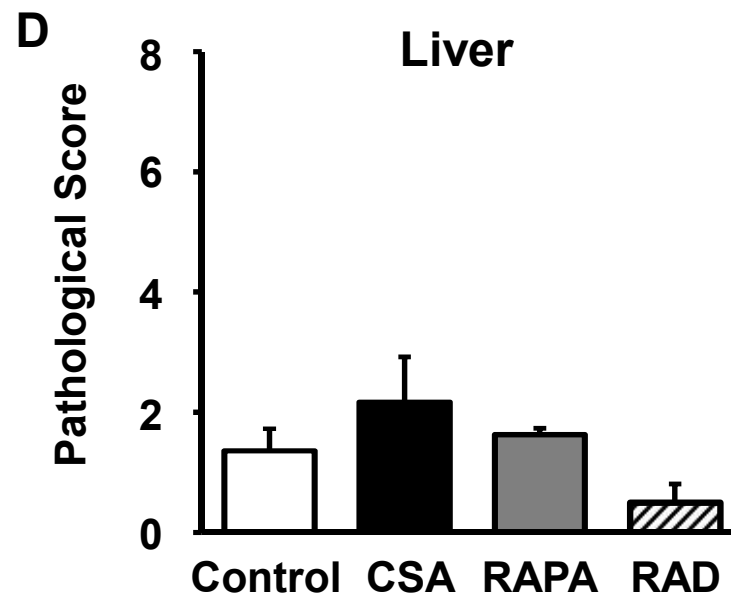
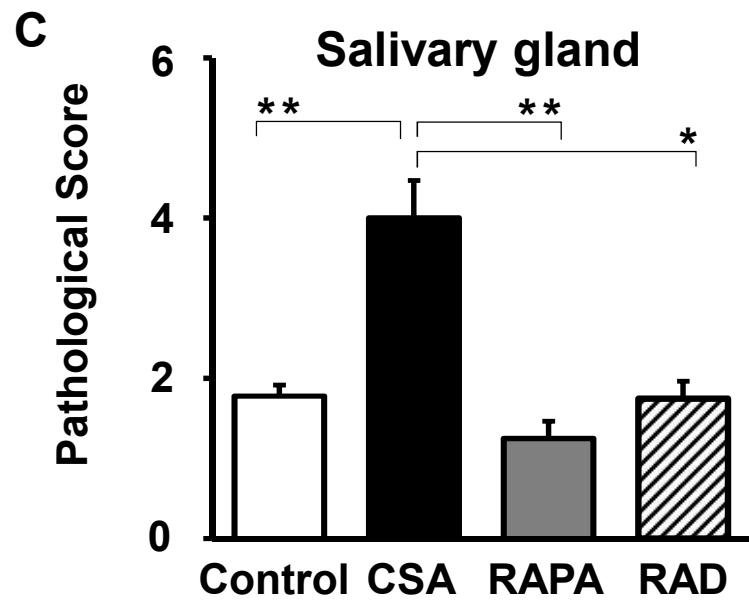
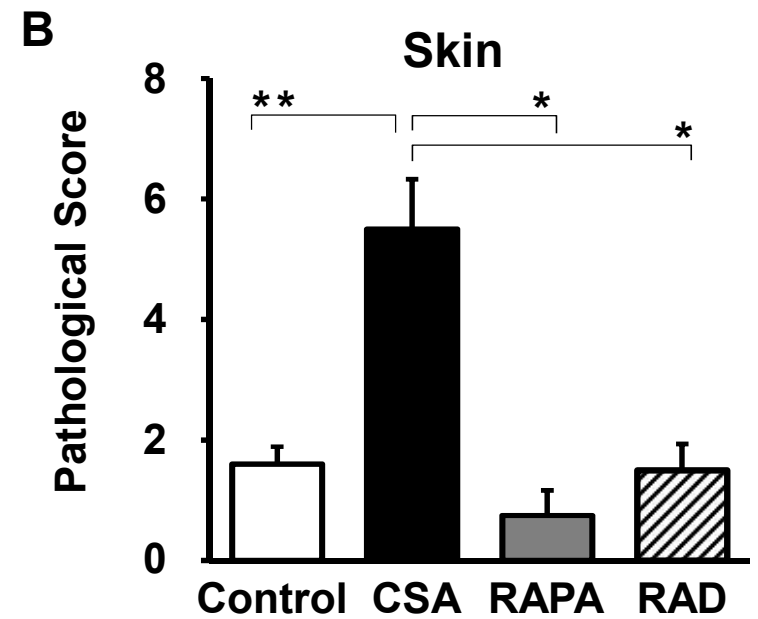
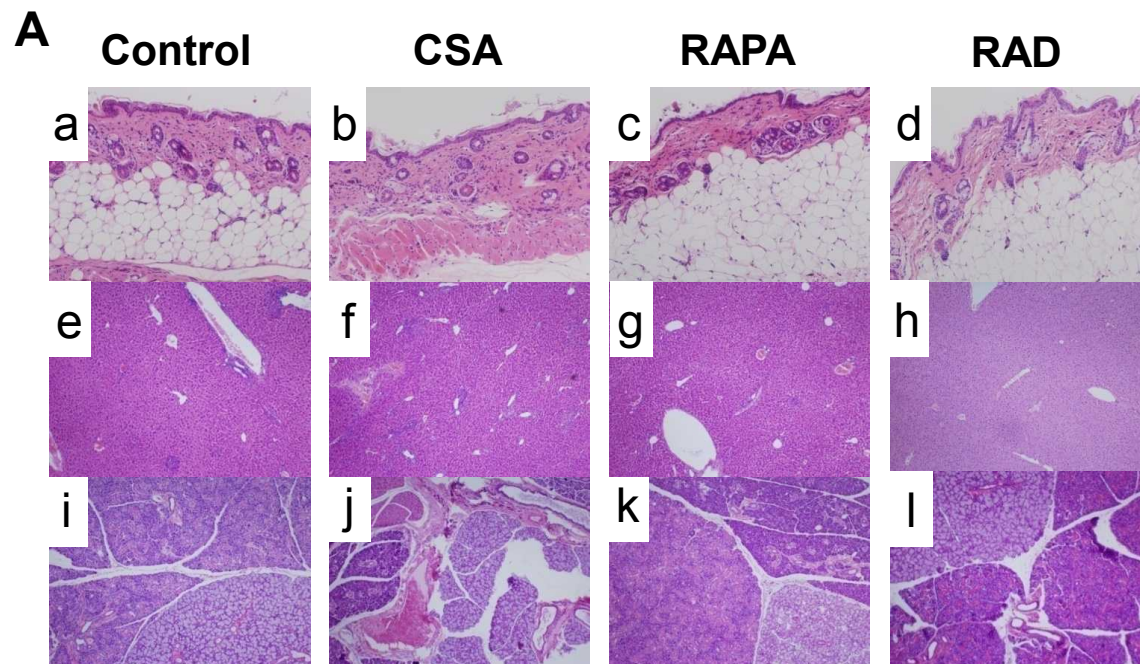


Figure. 5

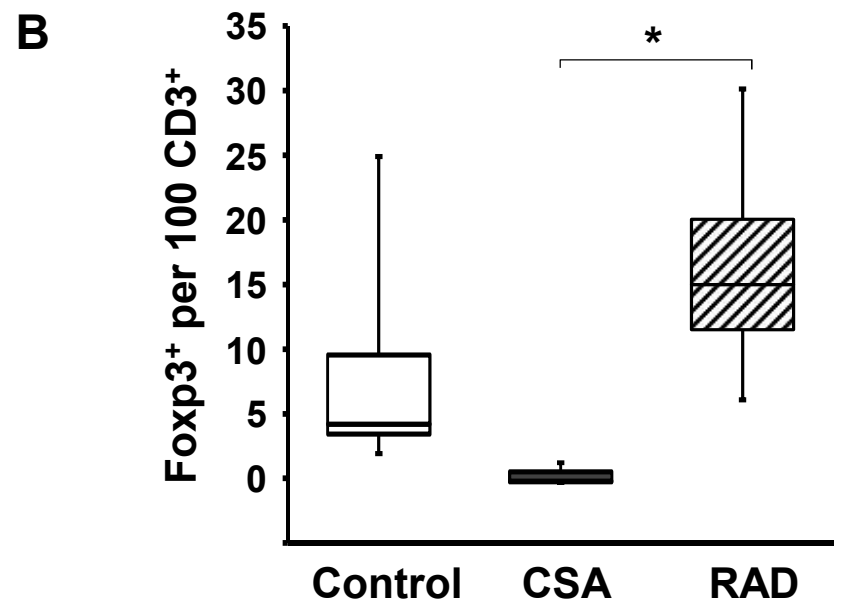
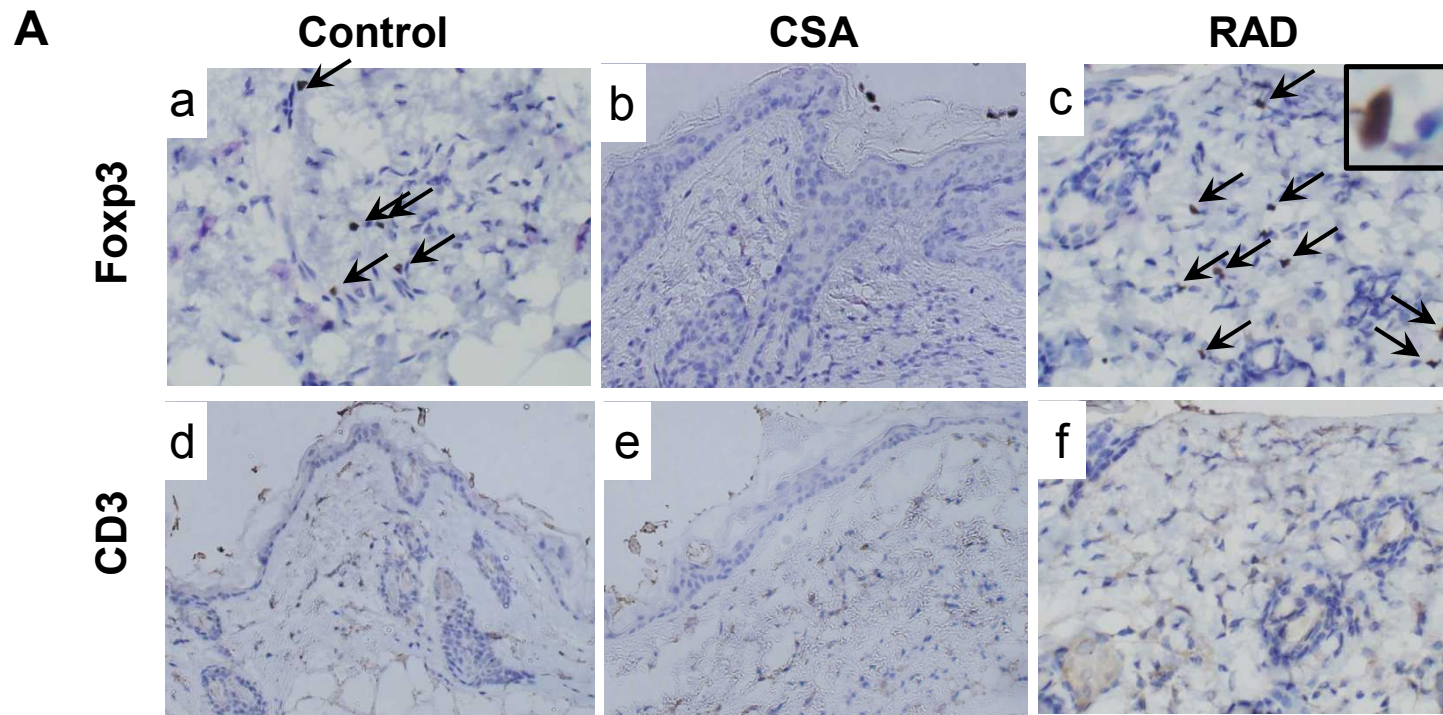
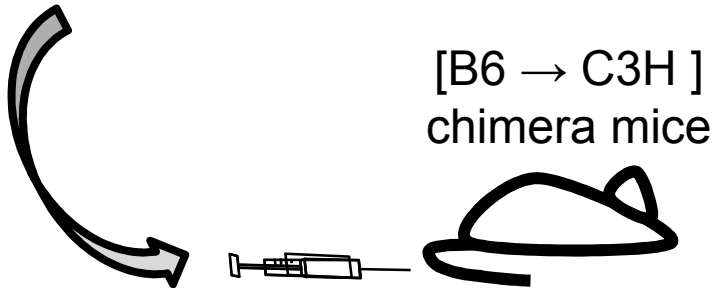
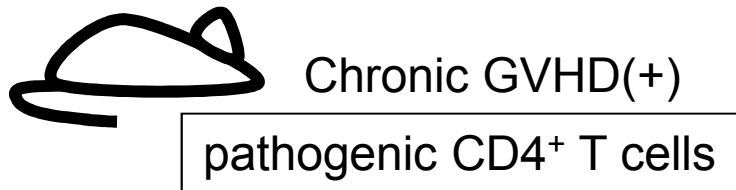


Figure. 6

A

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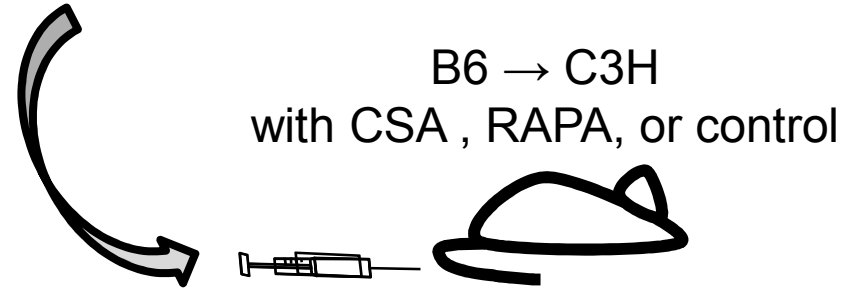
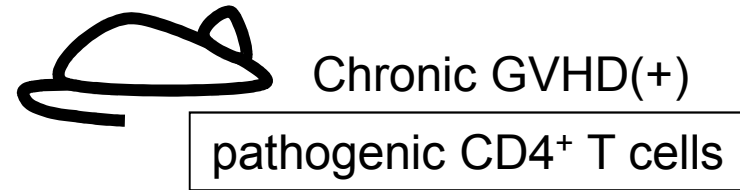


Adoptive transfer

Chronic GVHD(+)

B

B6 classII^{-/-} → C3H



Adoptive transfer

Chronic GVHD(+)?

Figure. 7

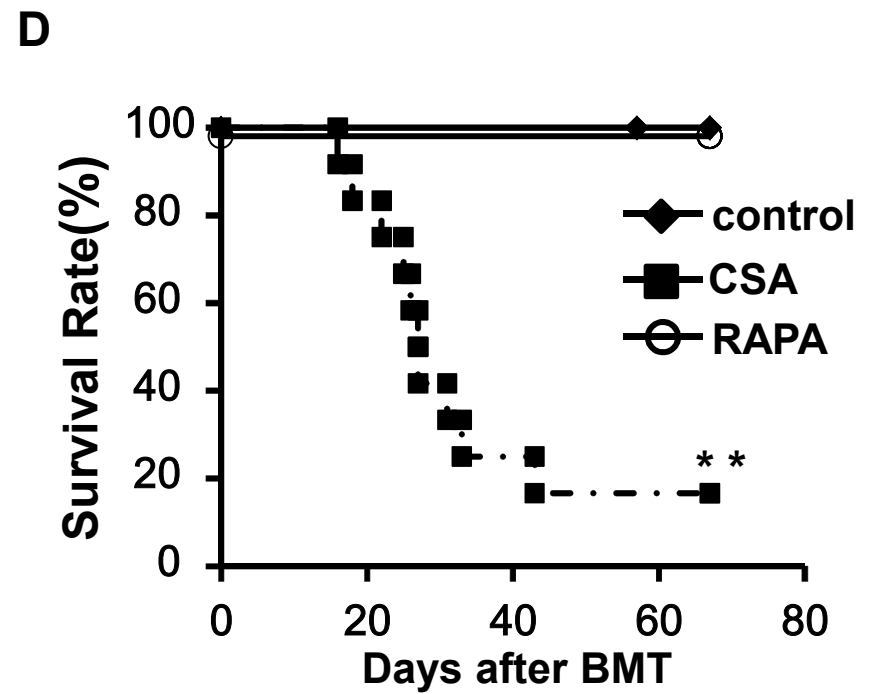
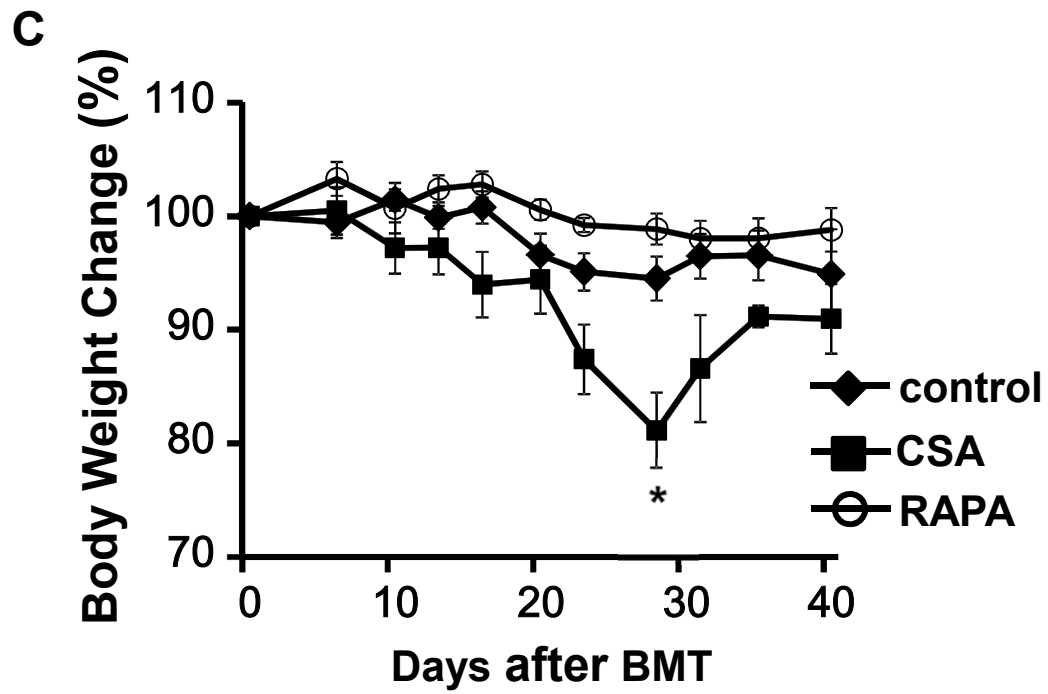
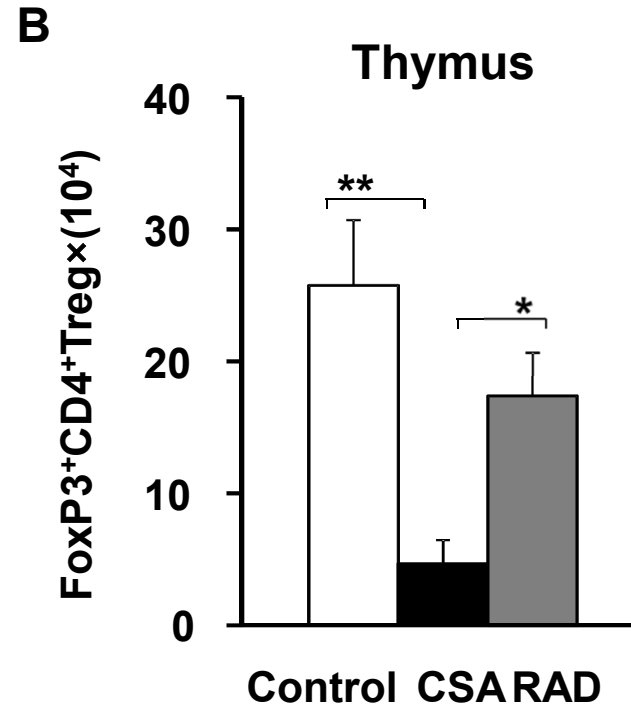
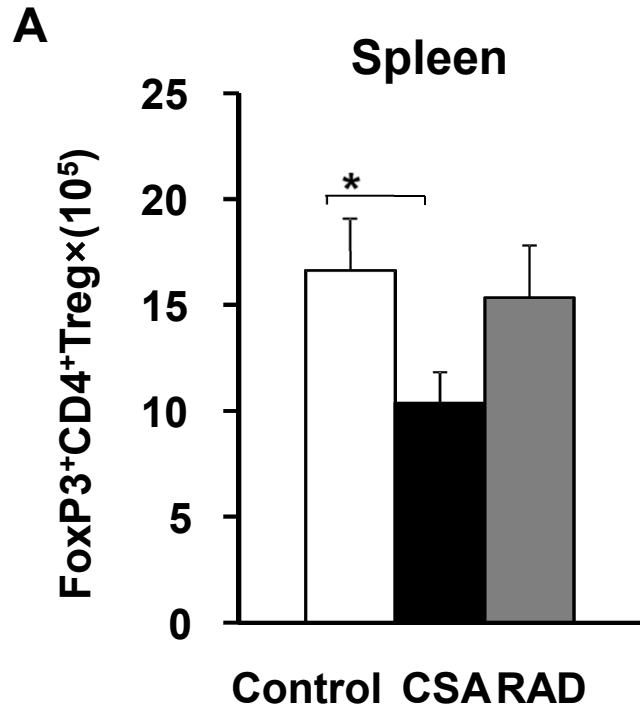
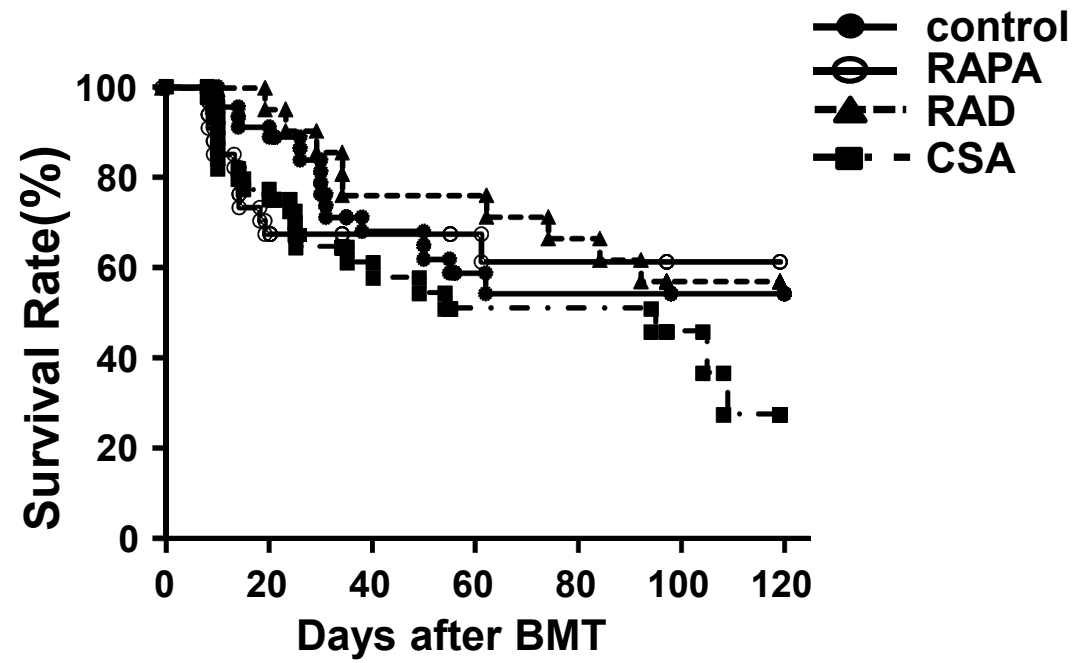


Figure. 7



Supplemental data Figure. 1



Supplemental data Figure. 2