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Mst1/Mst2 Regulate Development and Function of Regulatory T Cells through Modulation of Foxo1/Foxo3 Stability in Autoimmune Disease

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Foxp3 expression and regulatory T cell (Treg) development are critical for maintaining dominant tolerance and preventing autoimmune diseases. Human MST1 deficiency causes a novel primary immunodeficiency syndrome accompanied by autoimmune manifestations. However, the mechanism by which Mst1 controls immune regulation is unknown. In this article, we report that Mst1 regulates Foxp3 expression and Treg development/function and inhibits autoimmunity through modulating Foxo1 and Foxo3 (Foxo1/3) stability. We have found that Mst1 deficiency impairs Foxp3 expression and Treg development and function in mice. Mechanistic studies reveal that Mst1 enhances Foxo1/3 stability directly by phosphorylating Foxo1/3 and indirectly by attenuating TCR-induced Akt activation in peripheral T cells. Our studies have also shown that Mst1 deficiency does not affect Foxo1/3 cellular localization in CD4 T cells. In addition, we show that Mst1+/− mice are prone to autoimmune disease, and mutant phenotypes, such as overactivation of naive T cells, splenomegaly, and autoimmune pathological changes, are suppressed in Mst1−/− bone marrow chimera by cotransplanted wt Tregs. Finally, we demonstrate that Mst1 and Mst2 play a partially redundant role in Treg development and autoimmunity. Our findings not only identify Mst kinases as the long-searched-for factors that simultaneously activate Foxo1/3 and inhibit TCR-stimulated Akt downstream of TCR signaling to promote Foxp3 expression and Treg development, but also shed new light on understanding and designing better therapeutic strategies for MST1 deficiency-mediated human immunodeficiency syndrome.

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Regulatory T cells (Tregs) play crucial roles in preserving immunological self-tolerance and immune homeostasis by suppressing aberrant or excessive immune responses, such as autoimmune diseases and allergies. Tregs express the signature forkhead family transcription factor Foxp3. Mutations in Foxp3 and FoxP3 lead to autoimmune disorders in Scurfy mice and in humans, respectively (1). Overexpression of Foxp3 in peripheral CD4+ CD25+ T cells enhances the suppressive function and conversion of naive CD4+ T cells to Tregs (2, 3). However, the molecular mechanisms underlying the regulation of Foxp3 expression are not fully understood.

Foxo1 and Foxo3 (Foxo1/3) transcription factors are required for Foxp3 expression and Treg development (4, 5). Foxo1−/− Foxo3−/− mice have impaired Treg development and function and display severe autoimmune disorders (5, 6). Foxo1/3 activity is negatively regulated by Akt, which can phosphorylate Foxo1/3 and promote their nuclear export and degradation (7–9). Sustained Akt activity inhibits de novo Foxp3 expression and Treg development (10, 11), probably through inactivation of Foxo1/3. PI3K–Akt signaling can be stimulated by TCR and is required for the survival and homeostasis of T cells (12). However, Foxp3 expression and Treg development also require TCR/CD28 stimulation. Therefore, in developing T cells, Akt activity has to be maintained at a proper level that is low enough to guarantee Foxo1/3 activity to promote Foxp3 expression and Treg differentiation, but high enough to ensure T cell survival. Such a factor or factors acting downstream of TCR signaling to specifically attenuate Akt activity have not yet been identified.

Mst1 and Mst2 (Mst1/2) are two mammalian homologs of Drosophila hpo encoding a Ser/Thr kinase (13). Mst1+/−/Mst2−/− mice develop spontaneous tumors in liver, colon, and ethyl-N-nitrosourea–induced lymphomas/leukemias (14–18). Mst1 induces apoptosis through caspase-mediated proteolytic activation and histone H2B phosphorylation (19), or by enhancing Foxo1/3 nuclear entry through phosphorylation of Foxo1 and Foxo3 at S212 and S207, respectively, in fibroblasts and granule neurons (20, 21). Mst1/2 proteins may also promote apoptosis by interacting and suppressing Akt activation in...
cancer cells (22). However, T cells from Mst1-deficient mice and human patients exhibit enhanced apoptosis (23–25) in addition to the mutant phenotypes of impaired adhesion and migration in mice (25–29), suggesting that Mst1 may exert different cellular functions in tissue/cell type–specific manners (28). Both patients and mice with homozygous Mst1-null mutations display autoimmune manifestations (23, 24, 29), indicating critical roles of Mst1 in autoimmune diseases. However, the mechanistic contribution of Mst1/2 to disease remains elusive.

In this study we have identified Mst1 as an important factor that promotes Foxp3 expression and Treg development/function and simultaneously attenuates TCIR-stimulated Akt activation. Mst1 deficiency impairs Foxp3 expression and Treg development/function owing to excessive degradation of Foxo1/3. Mechanistic study reveals that Mst1 enhances Foxo1/3 stability directly by phosphorylating Foxo1/3 and indirectly by antagonizing TCR-induced Akt activation; however, it does not seem to regulate Foxo1/3 cellular localization in T cells. Furthermore, we show that mutant phenotypes, such as overactivation of naïve T cells, splenomegaly, and autoimmune pathological changes, in Mst1−/− mice are suppressed in the Mst1−/− bone marrow chimera by cotransplanted wt Tregs. Finally, we demonstrate that Mst1/2 play a partially redundant role in Treg development. Therefore, our findings indicate that Mst1/2 are important for controlling Treg development and preventing autoimmune disease in mice, but also shed new light on our understanding of Mst1 deficiency–mediated human immunodeficiency syndrome.

Materials and Methods

Mice

Mst1−/− mice and Mst1+/−Lck-cre were described previously (26). The generation of Mst2−/− mice is described in Supplemental Fig. 1. All mice except for B6.SJL and Rag1−/− from the Jackson Laboratory were maintained on a 129/Sv genetic background and raised in a specific pathogen–free facility. Experiments were conducted with consent from the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University, Shanghai, China.

Plasmid and Abs

The Foxo1, Foxo3, and Foxo3S207A expression vectors and Mst1 RNA interference (shMst1) plasmid were described previously (20, 21). The Foxo1S212A was generated by site-directed mutagenesis and verified by sequencing. The MIGR1-Flag-Foxo3 retrovirus vector was generated by cloning Flag-tagged-Foxo3 cDNA into MIGR1. The Foxo3 luciferase reporter plasmid was a gift from Yun-Cai Liu (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Abs against the following proteins were used for our studies: CD4, CD8, CD44, CD62L, Foxp3, IL-2, IL-4, IFN-γ, and Actin (Santa Cruz); GAPDH (Beyotime).

Histology

Tissue processing and frozen section and immunofluorescent microscopic analysis were performed as described elsewhere (30). The analysis for infiltration foci was described previously (31).

Autoantibody detection and immunofluorescent microscopy

For the anti-nuclear Ab (ANA) test, Hep-2 cell and monkey liver cells (EUROIMMUN) were immunofluorescently stained with wt and Mst1−/− sera. For the extractable nuclear Ab test, an ENA kit (EUROIMMUN) was used according to the manufacturer’s instructions. To analyze Foxo1/3 localization, fresh isolated peripheral lymphocytes for (Foxo1) or MIGR1-Flag-Foxo3–infected CD4 T cells (for Foxo3) were immunofluorescently stained with the appropriate Abs.

Cell purification and flow cytometry analysis

CD4+ T cells and CD4+CD25+ Tregs were purified by using the Mouse CD4 kit or CD4+CD25+ Treg cells kit (11461D, 11463D; Invitrogen). CD4+CD25+ cells were purified by depleting CD25+ cells with an anti-CD25/Anti-Rat IgG kit (11035; Invitrogen), followed by further purification using a Mouse CD4 kit. Intracellular FACS for Foxp3/cytokines was conducted using a kit (00-5523; E Bioscience) or performed as described elsewhere (32). 7-Aminoactinomycin D (Sigma-Aldrich) was used to label dead cells. All samples were analyzed with a Calibur or a Cyan FACS machine and FlowJo software (Treestar).

Retroviral transduction

293T cells were cotransfected with MIGR1-Flag-Foxo3 (or MIGR1) and pCL packaging plasmid using Lipofectamine 2000, and recombinant viruses were harvested 48 h later. Purified peripheral CD4+ T cells were first stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 48 h, then infected with retrovirus in the presence of 8 μg/ml polybrene by centrifuging cells at 1000 × g for 60 min at room temperature. At 48 h post infection, cells were collected for immunofluorescent staining.

![FIGURE 1. T cell activation and differentiation in Mst1−/− mice. (A) Flow cytometry analysis of the expression of CD44 and CD62L in CD4 T cells from spleen and brachial lymph node (bLN) of 1-y-old wt and Mst1−/− mice. Numbers in quadrants indicate percent of cells in each. (B) Representative gross anatomy of spleen (upper panel) and submandibular gland lymph node (lower panel) from wt and Mst1−/− mice at 1.5 y of age. (C) Quantification of BrdU incorporation in CD4 and CD8 T cells from spleen (left two panels) and bLN (right two panels) in wt and Mst1−/− mice. (D) Intracellular FACS analysis of the expression of IL-2, IL-4, IFN-γ, and IL-17 in splenic CD4 T cells from 8-wk-old wt and Mst1−/− mice after stimulation for 6 h with PMA and ionomycin. Data in (A), (B), and (D) are representative of at least three independent experiments. Values in (C) represent the means ± SEM of three separate experiments. *p < 0.05, **p < 0.01.]
Quantitative PCR

Total RNA was extracted from purified peripheral CD4+ T cells with TRIzol (Invitrogen). RT-PCR was carried out with TaKaRa RNA PCR Kit (AMV) according to the manufacturer’s instructions. Quantitative PCR reactions were performed on Mx3000P (Stratagene) with the Fast SYBR Green QPCR Master Mix (Stratagene), and data were analyzed with MxPro software. Expression of Gapdh was used as internal control for real-time PCR. The primer sequences are as follows: Foxo1-F, 5'-AACAGCCTCCTTCATTC-3' ; Foxo1-R, 5'-TCTTGCCCTTCACTTTGTCCCAG-3'; Foxo3-F, 5'-AACAGCCTCCTTCATTC-3'; Foxo3-R, 5'-TCTTGCCCTTCACTTTGTCCCAG-3'; Gapdh-L1, 5'-TGGGTCTTACCCCCAATGTGTC-3'; Gapdh-R1, 5'-GGAGTTGCTGTTGAAGTCGCAG-3'; N4r2-F, 5'-ATCATCAGAGGGTGGGCAGAGAAG-3'; N4r2-R, 5'-TGGGTCTTCACCCCCAATGTGTC-3'; N4r3-F, 5'-AAATGTAGGCTGAAAGTGTCG-3'; N4r3-R, 5'-TGGGTCTTACCCCCAATGTGTC-3'; N4r4-F, 5'-TTGGTTGCGTTCAGCAGAAG-3'; N4r4-R, 5'-TGGGTCTTACCCCCAATGTGTC-3'.

Treg suppression assay

A total of 6 × 10^6 splenic CD4+CD25− cells labeled with CFSE (4) were cultured with mitomycin C–treated T cell–depleted splenocytes (4 × 10^4) and Con A (2 μg/ml) in the presence of 3 × 10^5 or 1.5 × 10^6 Tregs (spleen CD4+CD25−) for 72 h and then analyzed by FACS. The suppressive capacity of Tregs was calculated using the following formula: [100 × (1 - %CFSElow CD4+CD25− T cells in coculture/%CFSElow CD4+CD25− T cells alone)], as described previously (33). For the bone marrow chimera experiment, Mst1+/− mice were backcrossed to C57BL/6 for seven generations. Bone marrow cells (1 × 10^5) isolated from the femurs of wt or Mst1+/− mice were injected separately or coinjected (ratio wt:mutant = 1:4) into the tail vein of irradiated Rag1−/− (7 Gy) recipients. The congenic markers CD45.1 and CD45.2 were used to distinguish cells from different donors in the chimeras. Data were collected 6–7 wk later.

Adaptive transfer of Tregs

Spleen CD4+CD25− Tregs were purified from the congenic CD45.1+ C57BL/6 wt mice, and 3 × 10^5 CD45.1+ wt Tregs were cotransplanted with the Mst1−/− bone marrow into the irradiated Rag1−/− (7 Gy) recipients. Then, 2 wk later, 3 × 10^5 wt Tregs were transferred into the same recipients through i.p. injection. The mice were sacrificed and analyzed 6–7 wk after bone marrow transplantation.

**FIGURE 2.** Impaired Foxp3 expression, development, and function of Mst1−/− Tregs. (A–D) Flow cytometry plots of Foxp3+ CD4 SP cells in thymus [(A) and (B), left panels] and Foxp3+ CD4 T cells in spleen [(C) and (D), left panels] from 1-wk-old [(A) and (C)] or 6- to 8-wk-old [(B) and (D)] wt and Mst1−/− mice, and frequency (middle panel) and number (right panel) of Tregs (at least four mice per group). (E) Flow cytometry plots of Foxp3+CD4 SP cells in thymus of 6- to 8-wk-old Mst1+/− and Mst1−/−Lck-cre mice, and frequency and number of Tregs (n = 3 mice per group). (F) Flow cytometry analysis of CFSE dilution in wt CD4+CD25− T cells cultured alone or cocultured with wt or Mst1−/− CD4+CD25− Tregs at a ratio of 1:1 (left panels) and suppression of wt CD4+CD25− T cells by wt and Mst1−/− Tregs at a ratio of 2:1 and 4:1 (right panels). Numbers in left panels indicate the percentage of divided wt CD4+CD25− T cells. (G) Quantification of Foxp3 expression in wt and Mst1−/− thymic Tregs, presented as mean fluorescence intensity (MFI) of wt and Mst1−/− Foxp3+ CD4 SP thymocytes. (H) FACS analysis (left panel) and quantification (right panel) of Foxp3 expression in splenic CD4+CD25− T cells stimulated with anti-CD3/anti-CD28 in the presence of 1 ng/ml of TGF-β for 3 d. (I) Foxp3+ cells converted from wt or Mst1−/− splenic CD4+CD25− T cells after stimulation with anti-CD3/anti-CD28 in the presence of indicated concentrations of TGF-β for 3 d. Numbers above outlined areas in left panels indicate percent of Foxp3+ T cells in CD4+ T cells. Data are from one representative of three independent experiments. Values in (A)–(F) (right two panels) and (G)–(I) represent the means ± SEM of three separate experiments. *p < 0.05, **p < 0.01.
**FIGURE 3.** Mst1 regulates Foxp3 expression by modulating Foxo1/3 stability rather than affecting their subcellular location in CD4 T cells. (A) Quantification of luciferase activity in 293T cells transfected with a luciferase reporter plasmid containing the Foxp3 promoter together with an empty, Mst1, Foxo3, Foxo3S207, or both Mst1 and Foxo3 or Foxo3S207 expression vectors. (B) Quantification of luciferase activity in Jurkat T cells electroporated with a luciferase reporter plasmid containing the Foxp3 promoter together with Foxo3 expression and/or shMst1 knockdown plasmids. (C and D) Representative immunofluorescence images of wt and Mst1−/− lymph node CD4 T cells (C) or Tregs (D) with nuclear (upper panels) or cytoplasmic Foxo1 (lower panels) after staining with the Abs indicated. (E) Representative immunofluorescence images of peripheral wt and Mst1−/− CD4 T cells expressing exogenous Flag-tagged Foxo3 stained with anti-Flag. (F) Western blot analysis for the distribution of endogenous Foxo1 and Foxo3 proteins after subcellular fractionation of peripheral CD4 T cells from wt and Mst1−/− mice. (G) Western blot analysis of Foxo1/3 and p-Foxo1/3 protein levels and densitometry quantification of p-Foxo1 and Foxo1 in splenic CD4 T cells freshly isolated from 6-wk-old wt and Mst1−/− mice. (H) Western blot analysis of Foxo1 in CD4 SP thymocytes (upper panels) and Tregs (lower panels) from wt and Mst1−/− mice. (I) Western blot analysis of Foxo1/3 of the CD4 T cells in (G) treated with 25 mM MG132 for 2 h in vitro after isolation. (J) Western blot analysis of exogenous Foxo1 and Foxo1S212A in 293T cells transfected with Myc-Foxo1 or Myc-Foxo1S212A expression plasmids together with GFP alone or with Mst1-HA or Mst1K59R-HA expression plasmids. (K) Experiments similar to those represented in (J) were performed for analysis of exogenous Foxo3 and Foxo3S207A. (L) The 293T cells were transfected with plasmids expressing Myc-Foxo1 or Myc-Foxo1S212A (left) or Flag-Foxo3 or Flag-Foxo3S207A (right) together with GFP and Mst1-HA expression plasmids. At 24 h (Foxo3/Foxo3S207A) or 48 h (Foxo1/Foxo1S212A) after transfection, the cells were treated with 50 μg/ml CHX for different periods, as indicated, followed by Western blot analysis with Abs as indicated. GFP was used for transfection efficiency control. (M) Quantitative analysis of Foxo1, Foxo1S212A, Foxo3, and Foxo3S207A proteins in (L). The density of each band was plotted against incubation time with the
Luciferase-based reporter assay

The 293T cells were transfected with Foxp3 promoter luciferase reporter plasmid together with Foxo3, Mst1, and β-gal expression vectors with Lipofectamine 2000. Jurkat T cells were transfected with empty, shMst1, or Foxo3 expression plasmids separately, or shMst1 and Foxo3 expression plasmids together by electroporation, using a GENE PULSER II (Biorad). Cells were collected 40 h after electroporation and analyzed as described previously (34).

Subcellular fractionation and immunoblotting

Cellular fractionation was performed according to the Nuclear Extraction Protocol (Invitrogen). Immunoblotting was performed as described previously (26), and images were collected with Tanon-5200 or by x-ray film exposure. For quantification of protein levels, appropriate film exposures were scanned and the density of bands was determined with Image J and normalized to band intensity for GAPDH or GFP.

Statistical analysis

Statistical analysis was conducted using an unpaired *t* test by GraphPad Prism 4. A *p* value < 0.05 was considered significant.

Results

Excessive activation and differentiation of T cells in Mst1−/− mice

Mst1 deficiency resulted in a slight but significant increase in the proportion of CD62L+/CD44hi effector T cells in secondary lymphoid organs of young (6–9 wk) Mst1−/− mice (25, 26). This phenotype was further enhanced (Fig. 1A), and splenomegaly and lymphadenopathy (Fig. 1B) were observed in aged Mst1−/− mice. BrdU pulse labeling experiments showed that the proliferation of Mst1−/− peripheral T cells was significantly enhanced (Fig. 1C). To determine whether Mst1 deficiency affects T cell function, we evaluated the cytokine expression profile of Mst1−/− peripheral T cells. In comparison with their wt counterpart, a higher fraction of Mst1−/− splenic CD4 T cells produced effector cytokines such as IL-2, IL-4, IL-17, and IFN-γ after stimulation by PMA/ionomycin (Fig. 1D). These results suggest that Mst1−/− peripheral T cells are overactivated.

Mst1 deficiency impairs the development and functions of Tregs

Tregs are a subset of CD4 T cells that have an important responsibility in preventing the overactivation of other T cells and are essential in maintaining self-tolerance (35). To understand the role of Mst1 in the regulation of Tregs, we investigated whether Mst1 regulates Treg development and function of Mst1−/− Treg mice in vivo. Our study showed that both the proportion and the number of Tregs in Mst1−/− mice decreased significantly (Fig. 2D) compared with those in wild-type (wt) Treg mice. These results demonstrate that Mst1 is required for thymic Treg development.

Consistent with impaired thymic Treg development, the percentage and number of peripheral Foxp3+ Tregs in 1-wk-old Mst1−/− mice decreased significantly (Fig. 2C). However, the proportion of peripheral Foxp3+ Tregs in adult Mst1−/− mice was restored to a level similar to or even higher than that in the controls (Fig. 2D). Further study showed that a higher fraction of peripheral Tregs in adult Mst1−/− mice were labeled by BrdU (Supplemental Fig. 2A). Therefore, this enhanced proliferation, which could be a result of T cell homeostasis in adult Mst1−/− mice, may lead to an increase in the proportion of peripheral Tregs. Nonetheless, the total number of peripheral Tregs was still significantly lower than that in the controls (Fig. 2D), possibly owing to impaired T cell egress from the thymus (26) and/or impaired conversion of induced Tregs from peripheral T cells (Fig. 2D).

To further investigate whether Mst1 regulates Treg development in a cell-intrinsic manner, we examined Treg development in the thymus of Mst1fl/fl-Lck-cre mice. Our results showed that both the proportion and the number of Tregs in Mst1fl/fl-Lck-cre mice were also significantly decreased (Fig. 2E).

Finally, we analyzed the function of Mst1−/− Tregs by performing an in vitro suppression assay. Results showed that Mst1−/− Tregs were less potent in suppressing the proliferation of naïve T cells than were wt Tregs (Fig. 2F). Furthermore, we showed that Mst1-deficient bone marrow–mediated autoimmune pathological changes were rescued by wt Tregs, suggesting impaired suppression function of Mst1−/− Tregs in vivo. Taken together, our findings demonstrate that Mst1 is required for the development and suppressive functions of Tregs.

Mst1 deficiency impairs Foxp3 expression and Treg conversion from naive T cells induced by TGF-β

To understand the mechanisms underlying the impaired development and function of Mst1−/− Tregs, we evaluated Foxp3 expression, a hallmark of Tregs, by intracellular FACS analysis. The result revealed that Foxp3 expression was reduced in Mst1−/− Tregs (Fig. 2G), suggesting that Mst1 regulates Treg development by modulating Foxp3 expression.

TGF-β is a prominent regulator for inducing Treg conversion from naive T cells via upregulating Foxp3 expression (36). To further confirm Mst1-mediated regulation of Foxp3 expression, we examined Foxp3 induction and conversion in wt naïve T cells with TGF-β/TCR costimulation. The results revealed that the mean fluorescence intensity of Mst1−/− Tregs stained with anti-Foxp3 was weaker than that of wt counterparts (Fig. 2H). Our analysis also showed that compared with wt counterparts, almost 2-fold fewer Mst1−/− CD4+CD25+ T cells were converted into Foxp3+ Tregs when induced with TGF-β (Fig. 2I). These results indicate that Mst1 is required for optimal Foxp3 expression and Treg conversion from naive T cells.

Ablation of Mst1 does not affect subcellular localization of Foxo1/3 in T cells

Foxp3 transcription is coordinately regulated by multiple transcription factors, such as NFAT, CREB, Ets-1, Smad, and Nrf2, and Foxo 1/3 (37). However, our studies showed that Mst1 deficiency had no effect on the phosphorylation of CREB, Ets-1, and Smads; nuclear entry of NFAT; and Nrf2 mRNA level in peripheral T cells (Supplemental Fig. 2E–G). These results rule out the possibility that Mst1 modulates Foxp3 expression by affecting the transcriptional activities of these transcription factors.

Two transcription factors, Foxo1/3, are required for Foxp3 expression and Treg development (4, 5). Mst1 phosphorylates Foxo1/3 and promotes their nuclear entry in fibroblasts and granule neurons (20, 21). Therefore, we assumed that Mst1 regulates Foxp3 expression and Treg development by modulating subcellular localization of Foxo1/3. To verify our assumption, we first examined whether Mst1 could enhance Foxo3-mediated Foxp3 expression. Our study showed that cotransfection with Foxo3 and Mst1 led to a significantly higher luciferase expression compared with the transfection with Foxo3 only in 293T cells. We found that a S207A mutation in Foxo3 dramatically reduced its transcriptional activity.
and cotransfection of Mst1 with Foxo3S207A had no effect on transcriptional activities of Foxo3S207A (Fig. 3A). We also found that reducing the level of endogenous Mst1 protein by short hairpin RNA in Jurkat T cells resulted in a significant decrease of luciferase activity driven by Foxp3 promoter (Fig. 3B, Supplemental Fig. 2B), further confirming the effect of Mst1 on Foxo3-mediated Foxp3 expression. These results demonstrate that Mst1 modulates Foxo3-mediated Foxp3 expression.

To further understand the molecular mechanisms by which Mst1 regulates Foxp3 expression, we evaluated the subcellular localization of Foxo1 proteins in peripheral Mst1−/− T cells and Tregs, respectively, by immunofluorescent staining. Surprisingly, we found that the percentages of Mst1−/− and wt peripheral CD4 T cells containing cytoplasmic Foxo1 were very similar (68.4% ± 3.4 and 68.8% ± 4, respectively) (Fig. 3C, Supplemental Fig. 2C). The percentage of Mst1−/− Tregs having cytoplasmic Foxo1 was also not significantly different from that of control Tregs, although many fewer Tregs contain cytoplasmic Foxo1 proteins (Fig. 3D, Supplemental Fig. 2C). Owing to unavailability of Foxo3 Abs for immunofluorescent staining, the subcellular localization of Foxo3 was examined in peripheral CD4 T cells transduced with Foxo3-expressing retrovirus, and 100% mutant and control peripheral T cells (n = 30, respectively) examined had cytoplasmic Foxo3 (Fig. 3E). To further confirm the above result, subcellular fractionation/Western blot analysis was performed. The study showed that the majority of Foxo1 and Foxo3 proteins were located in the cytoplasm in both Mst1−/− and wt peripheral CD4 T cells (Fig. 3F). These results demonstrate that Mst1 deficiency does not affect the cellular distribution of Foxo1/3 proteins in peripheral CD4 T cells and Tregs.

Mst1 regulates Foxo1/3 protein stability

Our subcellular fractionation/Western blot analysis also revealed that protein levels of Foxo1/3 were dramatically reduced in Mst1−/− peripheral T cells, suggesting that Mst1 deficiency may affect Foxo1/3 protein expression. Further Western blot analysis with whole-cell lysate also showed that both total protein level and the phosphorylation of Foxo1/3 in Mst1−/− peripheral CD4 T cells were dramatically reduced compared with these measures in controls (Fig. 3G). The reduction of Foxo1 protein level was also observed in Mst1−/− Tregs and thymic CD4 SP cells (Fig. 3H). These results confirm that Mst1 is required for regulating Foxo1/3 protein expression.

To investigate the mechanisms underlying the regulation of Foxo1/3 expression, we quantified Foxo1/3 mRNAs in Mst1−/− and wt T cells. The Foxo1/3 mRNA levels in Mst1−/− T cell were found to be comparable to those in the wt controls (Supplemental Fig. 2D). Then, we asked if Mst1 deficiency affects Foxo1/3 expression at the translational and/or posttranslational level. Results showed that Foxo1/3 protein levels in the Mst1−/− and wt control T cells were similar when the cells were treated with a proteasome inhibitor, MG132, to inhibit proteasome-dependent protein degradation (Fig. 3I). Furthermore, we found that Foxo1/3 protein levels were higher in 293T cells cotransfected with hemagglutinin (HA)–tagged Mst1 (Fig. 3J, 3K, left). These experiments demonstrate that Mst1 stabilizes Foxo1/3 proteins rather than regulates Foxo1/3 expression at the transcriptional level in mouse T cells.

Mst1 stabilizes Foxo1 and Foxo3 proteins by phosphorylating S212 and S207, respectively

Foxo1/3 are degraded by proteasomes after Akt-mediated phosphorylation (8, 9). Because Mst1 can phosphorylate Foxo1 and Foxo3 at S212 and S207, respectively (20, 21), and the S207A mutation in Foxo3 abolished its transcripational activity toward the Foxp3 gene (Fig. 3A), we investigated whether phosphorylation by Mst1 is required for maintaining protein stability. The results showed that cotransfection of Mst1 resulted in higher protein levels of Foxo1 and Foxo3, but had no effect on those of non–phosphorylation-mimic mutants, Foxo1S212A and Foxo3S207A (Fig. 3J, 3K, middle). Furthermore, cotransfection of Mst1K59R, a kinase-inactive form, did not enhance but instead reduced the expression of Foxo1/3 (Fig. 3J, 3K, right). These results demonstrate that Mst1 kinase activity is required to maintain the stability of Foxo1/3.

Finally, we confirmed the differential stability of the exogenous Foxo1 and Foxo1S212A in 293T cells treated with cycloheximide (CHX), which specifically inhibits protein synthesis. Foxo1S212A levels decreased dramatically after 3 h of CHX treatment, whereas Foxo1 remained unchanged at the same time point. (Fig. 3L, 3M, left). Similar results were also obtained for Foxo3 and Foxo3S207A (Fig. 3L, 3M, right). Taken together, these results strongly suggest that phosphorylation of Foxo1/3 by Mst1 is necessary for maintaining Foxo1/3 stability. Foxo1/3 degradation is one of the factors leading to impaired Foxp3 expression in Mst1−/− Treg development.

Mst1 stabilizes Foxo1 and Foxo3 protein by inhibiting Akt activation

Foxp3 expression and Treg development require signaling from TCR. Both Mst1 and Akt can be activated by TCR stimulation (38, 39). However, constitutively activated Akt inhibits Foxp3 expression and Treg development (10). Because Mst1 and Akt

![FIGURE 4](http://www.jimmunol.org) Overactivation of Akt in Mst1−/− peripheral CD4 T cells. (A and B) Western blot analyses and densitometry quantification of the phosphorylation of Akt (S473) (A) and phosphorylation of Foxo1(T24) (B) in wt and Mst1−/− peripheral CD4 T cells stimulated with TCR (anti-CD3/anti-CD28) for the indicated times. For ease of comparing Foxo1 phosphorylation, a similar level of Foxo1 protein was loaded for the left panel. (C) Western blot (left panel) and quantitative (right panel) analysis of Foxo1 protein level in wt and Mst1−/− peripheral CD4 T cells stimulated with anti-CD3/anti-CD28 for 2 h in the presence of Ly294002 (15 μM) or DMSO. Blots shown are representative of three experiments with similar results. Values in (A)–(C) represent the means ± SEM of three separate experiments. *p < 0.05, **p < 0.01.
FIGURE 5. Mst1 deficiency–mediated autoimmunity/inflammation in mice. (A) The wt and Mst1−/− mice at 6 mo of age. (B) H&E staining of sections of lacrimal (2 mo) and submandibular (6 mo) glands of wt and Mst1−/− mice. (C) Quantification of infiltration in lacrimal glands from mice of indicated genotypes and ages. (D) Immunofluorescence staining of frozen sections of lacrimal glands from 6-mo-old mice of indicated genotypes with anti-CD4, -CD8, and -B220, respectively. (E) Immunofluorescence photomicrograph of commercial slides stained with sera from wt and Mst1−/− mice for the ANA test. (F) The Mst1fl/fl and Mst1fl/fl-Lck-cre mice at 6 mo of age. (G) Photomicrograph of H&E-stained section of lacrimal glands from the mice in (F). (H–J) Development of immune pathological changes in the Mst1−/− or wt or wt/Mst1−/− mixed chimeras 6–7 wk after transplantation. Representative photograph of the chimeric mice (H), spleens and colons (I), and H&E-stained colon sections (J) from the mice in (H). (K) Flow cytometry of effector (CD44hiCD62Llo) and naive (CD44loCD62Lhi) CD4 T cells in the brachial lymph node from sublethally irradiated Rag1−/− mice 6–7 wk after transplantation with (a) wt (CD45.1), (b) Mst1−/− (CD45.2), or (c) wt/Mst1−/− mixed (1:4) bone marrows. (L) Flow cytometry of Tregs (Foxp3+) in
proteins interact and inhibit reciprocally in cancer cells (22) and activated Akt also destabilizes Foxo1/3 (8), we hypothesized that Mst1 may also maintain Foxo1/3 stability through inhibiting TCR-induced Akt activation. To test this possibility, we examined the activation status of Akt in Mst1−/− peripheral CD4 T cells after TCR stimulation. Our results showed that Akt was more activated in Mst1−/−-expressing cells than in controls upon TCR stimulation (Fig. 4A). To further confirm the effect of Mst1 deficiency-mediated overactivation of Akt on Foxo1/3 proteins, we evaluated the Akt-specific phosphorylation of Foxo1. Our study showed that phosphorylation of Foxo1 at T24 was significantly increased in Mst1−/− CD4 T cells after TCR stimulation (Fig. 4B). Finally, we demonstrated that inhibiting Akt activity could partially rescue Mst1 deficiency-mediated degradation of the Foxo1 protein (Fig. 4C). All these results suggest that Mst1 may promote Foxo3 expression and Treg development by inhibiting Akt activation in mice.

Mst1-deficient mice are prone to autoimmune disease

Genetic abnormalities that affect Tregs cause or predispose to autoimmunity (40). Consistent with the mutant phenotypes of impaired development/function of Mst1−/− Tregs and overactivation of peripheral Mst1−/− T cells, Mst1-deficient mice were found to be prone to autoimmune disease. Mst1-deficient mice tended to develop skin lesions around the eyes as early as 2 mo of age. These abnormalities became much more severe as the mice aged (Fig. 5A, Supplemental Fig. 3A, Table I). The infiltrations of mononuclear cells were detected in the lacrimal and submandibular glands of Mst1−/− mice at the age of 2 and 6 mo, respectively (Fig. 5B, 5C), and in other tissues such as lung and liver at the age of 1 y (Supplemental Fig. 3B). The number of infiltration foci increased as the mice aged (Fig. 5C, Supplemental Fig. 3C), and both CD4/CD8 T cells and B cells were easily detected in the infiltrated areas (Fig. 5D). We also found that the circulating ANA, a critical marker for autoimmunity, was present in 78% (7/9) of sera of half-year-old Mst1−/− mice, but were absent in the control group (Fig. 5E). In addition, an ENA test demonstrated that anti-nucleosome and anti-dsDNA Abs were present in 100% and 43%, respectively, of ANA-positive Mst1−/− sera (Table II). Collectively, our data demonstrate that Mst1−/− mice are prone to developing autoimmune disease.

Next we further investigated whether Mst1-deficient T cells were sufficient to cause autoimmune diseases in mice. Our study showed that Mst1−/−/Lck−cre mice (26), in which Mst1 is specifically deleted in T cells, developed skin lesions around the eyes and infiltration of mononuclear cells in the lacrimal gland at a lower frequency and later ages compared with Mst1−/− mice (Fig. 5E, 5F, 5G, Supplemental Fig. 3D). These results demonstrate a T cell–intrinsic role of Mst1 in protecting mice from autoimmune disease.

Mst1-deficient cell-mediated autoimmunity/inflammation in Rag1−/− mice can be suppressed by wt Tregs

To further confirm the role of Mst1-deficient cells in autoimmunity, we evaluated the ability of Mst1-deficient cells to induce autoimmunity or inflammation by generating Mst1−/−/CD45.2, wt (CD45.1), or Mst1−/−/wt mixed bone marrow chimera mice, respectively. Our studies showed that skin lesions around eyes and splenomegaly observed in Mst1−/− mice were observed only in Mst1−/− chimera, but not in mixed or wt chimera (Fig. 5H, 5I). Furthermore, we found that Mst1−/− chimera exhibited severe colitis, which was observed neither in mixed chimera nor in unmanipulated 1-y-old Mst1-deficient mice (Fig. 5I, 5J). Next, we determined whether wt cells from bone marrow would suppress the activation of Mst1−/− naive T cells. The results revealed that the majority of peripheral T cells in the Mst1−/− chimera were activated (CD44hiCD62Llo) effector T cells (Fig. 5K, Supplemental Fig. 3E). In contrast, the proportion of activated peripheral Mst1−/− T cells in the recipients of Mst1−/−/wt mixed chimera was similar (in brachial lymph nodes) to that of wt counterparts (Fig. 5Kc, Supplemental Fig. 3E) or dramatically reduced (in spleen, Supplemental Fig. 3G, 3H). These results strongly suggest that autoimmunity or inflammation induced by Mst1-deficient cells and activation of peripheral Mst1−/− T is not due to an intrinsic defect or defects of effector T cells but can be suppressed by the presence of wt cells from the bone marrow cotransplanted.

We also analyze the development of Mst1−/− Tregs in mixed chimera. FACS analysis showed that the proportion of Mst1−/− thymic Tregs (FoxP3+CD45.1−) was ~7.5-fold lower than that of the wt counterpart (FoxP3+CD45.1+) in mixed chimera, although the majority of thymic CD4+ SP cells (FoxP3+CD45.1+) were from Mst1−/− bone marrow (Fig. 5L, Supplemental Fig. 3F). Consistent with the impaired thymic Mst1−/− Treg development, the proportion of Mst1−/− peripheral Tregs was also ~2- to 3.5-fold lower than that of the wt control in peripheral lymphoid organs of the mixed chimera (Fig. 5L, Supplemental Fig. 3F). These results demonstrate that Mst1−/− bone marrow contributes much less than the coinfected wt counterpart to thymic Tregs in mixed chimera and further confirm the intrinsic functions of Mst1 in Treg development.

Tregs suppress excessive immune responses and are critical for maintaining dominant tolerance. Because the development of Mst1−/− Tregs was impaired in both Mst1-deficient mice (Fig. 2A–D) and Mst1−/−/wt mixed chimera (Fig. 5L) and because wt cells differentiating from wt bone marrow suppress autoimmunity/inflammation in the chimera (Fig. 5H–J), we hypothesized that the autoimmune disease in Mst1−/− chimera was due to the abnormality of Mst1−/− Tregs. To test this hypothesis, wt Tregs were transferred into Mst1−/− bone marrow–reconstituted Rag1−/− mice, and inflammation and peripheral T cell activation status were analyzed 6–7 wk later. Our results showed that transplantation of wt Tregs rescued autoimmune pathological changes, including skin lesions around the eyes, colitis, and body weight loss, and suppressed the activation of peripheral T cells and thymus, spleen, and brachial lymphoid of the wt/Mst1−/− mixed chimera in (Kc). (M–P) Development of immune pathological changes in the sublethally irradiated Rag1−/− mice 6–7 wk after transplantation with Mst1−/− bone marrow in the presence or absence of adoptive transferred wt Tregs. Representative photograph of the mice (M), spleens and colons (N), and H&E-stained colon sections (O) from the mice in (M). (P) Statistical analysis of body weight of the chimeric mice in (M). The body weight of each mouse at 28 d after bone marrow transplantation was defined as 100%. n = 3 for each group. Images and flow cytometry plots shown are representative of at least three experiments with similar results. White and black arrows in (A), (B), (F), (G), (H), and (M) indicate skin lesions around the eyes and mononuclear cell infiltration, respectively. Each dot in (C) represents an individual mouse. Values in (G) and (P) represent the means ± SEM. *p < 0.05, **p < 0.01.
spleenomegal of the Mst1−/− chimera (Fig. 5M–P, Supplemental Fig. 3I, 3J).

Altogether, the results above strongly suggest that abnormality of Mst1−/− Tregs is the main cause of autoimmunity in Mst1-deficient mice.

### Table II. Spectrum of anti-nuclear Abs

<table>
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<tr>
<th>Anti-nuclear Abs</th>
<th>Genotype wt</th>
<th>Mst1−/−</th>
<th>Genotype wt</th>
<th>Mst1−/−, %</th>
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<tbody>
<tr>
<td>nRNP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Smn</td>
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<tr>
<td>SSA</td>
<td>—</td>
<td>—</td>
<td>dsDNA</td>
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<td>—</td>
<td>—</td>
<td>Nucleosome</td>
<td>100 (7/7)</td>
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<td>Ro-52</td>
<td>—</td>
<td>—</td>
<td>Histone</td>
<td>—</td>
</tr>
<tr>
<td>Scl-70</td>
<td>—</td>
<td>—</td>
<td>Rib-P</td>
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—, Not detected in 7 wt or Mst1−/− mice.

Redundant functions of Mst1 and Mst2 in Treg development

Mouse Mst1/2 proteins share 76% identical amino acids and are functionally redundant for embryonic development and tumorigenesis (15, 16, 41). Mst1−/− mice displayed compromised development and functions of Tregs and inflammatory autoimmunity disease (Figs. 2, 5), but Mst2−/− mice did not (Supplemental Fig. 1E and data not shown). These results suggest that Mst1 plays more important roles in regulating Treg development and autoimmune pathological changes than does Mst2, but it does not rule out the possibility that Mst1/2 function redundantly in these processes. To test this possibility, we generated Mst1fl/flMst2−/−Lck-cre mice in which both Mst1/2 were deleted in T cells (Fig. 6A). Further analysis revealed that Mst2 did have redundant roles in the control of Treg development and immunopathological changes. We found that deleting one allele each in Mst1 and Mst2 genes had no effect on the proportion of thymic Tregs, but deleting one allele of Mst1 and two alleles of Mst2 resulted in a substantial reduction in the proportion of thymic Tregs (Fig. 6B). This effect is more prominent when the proportions of thymic Tregs in Mst1fl/+Mst2−/−Lck-cre and Mst1fl/flMst2−/−Lck-cre mice were compared (Fig. 6B). Our findings demonstrate a redundant function for Mst1/2 in the control of thymic Treg development.

Histological analyses revealed more severe lymphocyte infiltration in lacrimal glands in Mst1fl/flMst2−/−Lck-cre mice than in Mst1fl/+Mst2−/−Lck-cre mice, in addition to skin lesions around the eyes in Mst1fl/flMst2−/−Lck-cre and Mst1fl/flMst2−/−Lck-cre mice (Fig. 6C, 6D). FACS analysis showed that the percentages of activated peripheral T cells (CD44hiCD62Llo) from Mst1fl/flMst2−/−Lck-cre and Mst1fl/flMst2−/−Lck-cre mice were very similar (Fig. 6E), suggesting that Mst1 plays a major role in controlling the activation of peripheral T cells. However, 47.52% ± 0.75% of peripheral CD4 T cells became activated T cells in Mst1fl/flMst2−/−Lck-cre mice, whereas only 28.13% ± 0.40% were activated T cells in Mst1fl/flMst2−/−Lck-cre mice (Fig. 6E), strongly suggesting that Mst1/2 also plays a redundant role in the control of activation of peripheral T cells.

![FIGURE 6. Redundancy of Mst1 and Mst2 in Treg development.](http://www.jimmunol.org/)

Western blot analysis of protein expression of Mst1 and Mst2 in Mst1fl/fl Mst2−/−Lck-cre and Mst1fl/flMst2−/−Lck-cre mice. Blots shown are representative of three experiments with similar results. (B) Scatter plot showing the mean frequency of thymic Tregs (Foxp3+ CD4 SP cells) in the thymus of 6- to 8-wk-old mice of the indicated genotypes. (C and D) Representative microscopic images of H&E staining of lacrimal gland sections (C) and scatter plot showing the mean number of infiltration foci per gland (D) from 6-mo-old mice of the indicated genotypes. (E) Scatter plot showing the mean frequency of effector (CD44hiCD62Llo) T cells in the spleen from 6- to 8-wk-old mice of the indicated genotypes. Each dot in the scatter plots represents an individual mouse. n = 4 mice for each genotype. Data are representative of three independent experiments. 1+/−2+/−, 1+/−2−/−, 1−/−2+/−, and 1−/−2−/− represent Mst1fl/+Mst2−/−Lck-cre, Mst1fl/flMst2−/−Lck-cre, Mst1fl/flMst2−/−Lck-cre, and Mst1fl/flMst2−/−Lck-cre mice, respectively. **p < 0.01.

![FIGURE 7. Proposed model by which Mst1 regulates Foxp3 expression and Treg development through maintaining Foxo activity in direct and indirect ways.](http://www.jimmunol.org/)
In summary, our findings demonstrate that Mst1/2 play partially redundant roles in the control of thymic Treg development and immunopathological changes, and also suggest that the role of Mst1 is more dominant than that of Mst2. Given the function redundancy of Mst1 and Mst2 mentioned earlier, it is conceivable to postulate that Mst1 and Mst2 may share a similar mechanism in regulation of Treg development and immunopathology (Fig. 7).

Discussion

In this report, we demonstrate that Mst1 regulates Foxp3 expression and Treg development/function by enhancing the stability of Foxo1/3 proteins through phosphorylating Foxo1/3 and attenuating Akt activation. Previous studies reported that Mst1 regulated Foxo activity by phosphorylating Foxo proteins and promoting their nuclear accumulation in nonhematopoietic cells (20, 21). However, we found that Mst1 deficiency did not significantly change the localization of Foxo proteins, but rather led to their excessive degradation in thymic CD4 SP cells, peripheral T cells, and Tregs. Lower levels of Foxo proteins were also observed in Mst1-deficient human PBMCs and peripheral T cells of mice from one laboratory (23, 24, 42), but the underlying mechanisms are unknown. In this article, we demonstrate that Mst1 regulates Foxo1/3 stability through phosphorylation in the lymphoid T cells and that excessive degradation of Foxo proteins impairs Foxp3 induction and Treg development in Mst1−/− mice. Our findings not only support the idea that Mst1/2 exerts its various cellular functions by regulating different downstream targets in tissue- and/or cell type–specific manners (28), but also uncovers a new mechanism by which Mst1 regulates Foxo signaling.

TCR/CD28 stimulation is required for Foxp3 expression and Treg development, but also activates the PI3K–Akt pathway, which inhibits de novo Foxp3 expression and Treg development. To explain this paradoxical phenomenon, it has been proposed that undefined factors specifically attenuate TCR/CD28-induced Akt activation and therefore maintain Foxo proteins in activating state to induce Foxp3 expression (37). In this article, we have, to our knowledge, for the first time identified Mst1 as one of such search-for factors. Mst1 can be activated by TCR/CD28 stimulation in T cells (25, 38). We have found that Mst1 deficiency not only results in destabilization of Foxo1/3 proteins but also increases Akt activation in peripheral T cells. On the basis of these results, we propose that upon TCR/CD28 stimulation, activated Mst1 maintains sufficient Foxo activity required for Foxp3 expression and Treg development by directly or indirectly stabilizing Foxo proteins through phosphorylation and inhibition of TCR/CD28-induced Akt activation (Fig. 7). Because Mst1 and Akt are reciprocally inhibitory (22, 43), it is conceivable that they may repress each other to orchestrate the fine balance of TCR signals needed to regulate Foxp3 expression and Treg development.

Foxo1 has been demonstrated to be a pivotal regulator of Treg function (44). In this article, we have demonstrated that Mst1 deficiency dramatically reduces the protein level of Foxo1 (Fig. 3H) in Mst1−/− Tregs. Thus, it seems likely that Mst1 deficiency–mediated reduction of Foxo1 proteins could be one explanation for impaired suppressive function of Mst1−/− Tregs. Mst1 regulates LFA-1 activation (38). Because LFA-1−/− Tregs also show suppressive defects in vivo and in vitro, it is possible that reduction of the suppressive function of Mst1−/− Tregs is in part due to Mst1 deficiency–mediated inability to activate LFA-1 integrin. During the revision of this manuscript, Tomiyama et al. (45) have reported that Mst1−/− Tregs display defective suppressor function and are impaired in their interactions with Ag-presenting dendritic cells owing to inefficient formation of an immunological synapse via LFA-1/ICAM-1 (45). However, the effect of Mst1 deficiency on Treg development seems unlikely to be a secondary effect of impaired LFA-1 integrin activation because the mutant Treg phenotype of Mst1-deficient mice is different from that of LFA-1 deficiency. LFA-1−/− mice have a reduced frequency of Tregs in peripheral lymphoid organs but an increased frequency of Tregs in the thymus. In contrast, Mst1−/− mice exhibit a reduced number of Tregs in both the thymus and the periphery. The reduction of Foxo1/3 proteins in Mst1−/− thymic CD4 SP cells (Fig. 3H) may account for the impaired Treg development in the thymus.

Human Mst1 deficiency leads to a novel primary immunodeficiency syndrome including autoimmune manifestations (23, 24). Ueda et al. (29) also reported autoimmune disease in aged (1-yr-old) Mst1-deficient mice. In this study, we found that some novel and severe autoimmune phenotypes, such as skin lesions around the eyes, could be detected in our Mst1−/− mice as early as 2 mo of age, in addition to the lymphocyte infiltration in the multiple organs of aged mutant mice. This discrepancy could have resulted because different strains of mice were used for the studies. It has been implied that impaired thymocyte selection was responsible for autoimmune disease in Mst1−/− mice (29). However, we showed in this article that excessive T cell activation, splenomegaly, and immune disease manifestations, including skin lesions around the eyes and severe colitis, were observed only in Mst1−/−/wt chimera, but not in the Mst1−/−/wt mixed chimera. Our study demonstrates that Mst1 deficiency–mediated autoimmunity or inflammation can be suppressed by the presence of wt cells derived from cotransplanted bone marrow, therefore suggesting that autoimmunity in Mst1−/− mice may not be intrinsic to Mst1−/− effector T cells. Indeed, all autoimmune pathology phenotypes, activation of peripheral T cells, and splenomegaly of the Mst1−/−/chimera were rescued by cotransplanted wt Tregs (Fig. 5M-P, Supplemental Fig. 3I, 3J). This result strongly suggests that loss of dominant tolerance mediated by Tregs in Mst1−/− mice is the main reason leading to T cell activation and probably autoimmunity phenotypes. Our findings that all Mst1 deficiency–mediated autoimmunity or inflammation can be suppressed by the presence of wt cells from mixed bone marrow or cotransplanted wt Tregs may provide help in designing a strategy to cure immunodeficiency syndrome in Mst1-deficient patients.

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Disclosures

The authors have no financial conflicts of interest.

References