Splicing factor SRSF1 is indispensable for regulatory T cell homeostasis and function

Graphical abstract

Highlights

- Deficiency of SRSF1 in Treg cells causes early lethal systemic autoimmune disease
- Treg cells from Srsf1-KO mice exhibit aberrant homeostasis and impaired function
- Srsf1-KO Treg cells display plasticity and produce aberrantly inflammatory cytokines
- Srsf1-KO Treg cells exhibit elevated glycolytic metabolism and mTOR activity

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In brief
Katsuyama and Moulton show that splicing factor SRSF1 is indispensable for the homeostasis and function of Treg cells. Its conditional absence in Treg cells causes profound autoimmunity and organ inflammation by elevating glycolytic metabolism and production of proinflammatory cytokines. The study offers insights into the pathogenesis of autoimmune disease.

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Splicing factor SRSF1 is indispensable for regulatory T cell homeostasis and function

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SUMMARY

The ability of regulatory T (Treg) cells to control the immune response and limit the development of autoimmune diseases is determined by distinct molecular processes, which are not fully understood. We show here that serine/arginine-rich splicing factor 1 (SRSF1), which is decreased in T cells from patients with systemic lupus erythematosus, is necessary for the homeostasis and proper function of Treg cells, because its conditional absence in these cells leads to profound autoimmunity and organ inflammation by elevating the glycolytic metabolism and mTORC1 activity and the production of proinflammatory cytokines. Our data reveal a molecular mechanism that controls Treg cell plasticity and offer insights into the pathogenesis of autoimmune disease.

INTRODUCTION

Regulatory T (Treg) cells are pivotal in maintaining self-tolerance to prevent fatal autoimmune disease (Pilts and Rudensky, 2016; Shevach, 2018; Wing et al., 2019). Defects in homeostasis, maintenance, and stability are key contributors to Treg cell dysfunction in mice and humans with autoimmune disease. Molecules and mechanisms that control Treg cell homeostasis and function are therefore critical determinants of Treg cell integrity and may serve as therapeutic targets for autoimmune disease (Kasper et al., 2016; Sharabi et al., 2018).

Instability and plasticity contribute to the defective function of CD4+CD25+Foxp3+ Treg cells and are known to play important roles in the pathogenesis of autoimmunity (Overacre and Vignali, 2016). Recent studies have shown that acquisition of effector-like properties and the production of proinflammatory cytokines from Treg cells is associated with not only their dysfunction but also pathogenic potential. It has been shown that Treg cells from patients with multiple sclerosis produce more interferon-γ (IFN-γ) compared to those from healthy controls (Sumida et al., 2018), and IFN-γ-producing Treg cells exhibit suppressive dysfunction (Domínguez-Villar et al., 2011). Also, interleukin-17 (IL-17)-producing cells originating from Foxp3+ T cells have a key role in the pathogenesis of autoimmune arthritis (Komatsu et al., 2014). Furthermore, IL-17-producing Treg cells were found to be increased in the peripheral blood of patients with systemic lupus erythematosus (SLE) and associated with lupus nephritis and neuropsychiatric lupus (Jiang et al., 2019). Metabolic programs have key roles in the function and stability of Treg cells. Treg cells rely on oxidative phosphorylation (OXPHOS) rather than glycolysis (Michalek et al., 2011; Newton et al., 2016). The mechanistic target of rapamycin (mTOR) pathway plays central roles in the glycolytic metabolism of Treg cells (Gerriets et al., 2016; Wei et al., 2016). Higher glycolytic metabolism induced by excessive mTOR complex 1 (C1) activity causes abnormal proinflammatory cytokine production from Treg cells and Treg dysfunction (Yu et al., 2018). However, the underlying mechanisms that control these pathways to regulate the stability and plasticity of Treg cells remain unclear.

Serine/arginine-rich splicing factor 1 (SRSF1) is the prototype member of the highly conserved serine/arginine (SR) family of RNA-binding proteins (Das and Krainer, 2014). SRSF1 controls post-transcriptional gene expression via pre-mRNA alternative splicing, mRNA stability, and translation (Howard and Sanford, 2015). While SRSF1 is a key controller of gene expression, very little is known of its role in the immune system and in immune-mediated disease. By discovery approaches, we identified SRSF1 to bind to the mRNA of the CD3 zeta (ζ) gene and promote its normal expression in human T cells (Moulton and Tsokos, 2010; Moulton et al., 2008). We demonstrated that SRSF1 is decreased in T cells from patients with SLE with severe disease (Kono et al., 2018; Moulton et al., 2013), which exhibit an overactive T cell phenotype (Katsuyama et al., 2018; Moulton and Tsokos, 2015; Tsokos, 2011). In agreement with these reports of its role in human autoimmune disease, we showed that selective deletion of SRSF1 in total T cells in mice leads to T cell hyperactivity and systemic autoimmune in vivo (Katsuyama et al., 2019). While SRSF1 is an emerging molecule in the control of gene regulation and immune-mediated disease, its role in regulatory T cells is unknown.

Here, we show that mice that lack Srsf1 in Treg cells develop early lethal systemic autoimmunity with peripheral organ inflammation. In the absence of SRSF1, Treg cells assume a...
proinflammatory phenotype and are unable to control lymphocyte activation. We find that Srsf1-deficient Treg cells display increased glycolytic metabolism and activity of the mTOR signaling pathway and produce proinflammatory cytokines. Our studies establish a crucial role of SRSF1 in the survival, integrity, and function of Treg cells and suggest that the deficiency of SRSF1 in Treg cells contributes to the pathogenesis of autoimmune disease. We propose that SRSF1 is requisite for the proper function of Treg cells and prevents their conversion to proinflammatory cells.

RESULTS

Srsf1 deficiency in Treg cells leads to spontaneous early-onset fatal systemic autoimmune disease

To evaluate the role of SRSF1 in Treg cells, we crossed Srsf1-flox mice with the Foxp3YFP-Cre mice to generate Treg conditional Foxp3YFP-CreSrsf1+/-- wild-type (WT), Foxp3YFP-CreSrsf1+/- heterozygous (HET), and Foxp3YFP-CreSrsf1-flox/flox homozygous knockout (KO) mice. Treg Srsf1-KO mice succumbed to an early-onset fatal systemic autoimmune disease at 3–4 weeks of age (Figure 1A). Mice showed severely stunted in growth and significantly reduced body weight (Figures 1B and 1C). Importantly Treg Srsf1-KO mice developed systemic autoimmunity. The titers of serum autoantibodies, including anti-double stranded DNA (anti-dsDNA) and anti-histone, were significantly higher in KO mice (Figure 1F). Notably, mice developed massive inflammatory cell infiltrations in the lungs with hemorrhages and destruction of alveolar structures (Figure 1G). Mice also exhibited inflammatory cell infiltration in the liver (Figure 1G). Flow cytometric analysis revealed significantly increased infiltrations of T cells and neutrophils in the lungs (Figure 1H). These results indicate that SRSF1 is essential for Treg cell physiology and that its loss leads to fatal systemic autoimmune inflammatory disease.

Deletion of Srsf1 in Treg cells leads to uncontrolled immune cell activation

Aberrant Treg homeostasis and/or function leads to unchecked immune cell activation (Georgiev et al., 2019).
Given that the Treg Srsf1-KO mice succumb to autoimmune disease, we examined their peripheral lymphoid tissues for immune cell phenotype and function and found significantly expanded T cell populations in the spleen (Figure 2A), increased frequencies of CD44lo/CD62Llo effector/effector memory (EM) CD4 and CD8 T cell populations, and decreased proportions of CD62Lhi/CD44lo naive CD4 and CD8 T cells in both spleen and peripheral lymph nodes (PLN) of Treg Srsf1-KO mice compared to WT mice (Figures 2B, 2C, S1A, and S1B). Deletion of Srsf1 in Treg cells also led to increased production of proinflammatory cytokines. When stimulated ex vivo, CD4 T cells produced significantly higher IFN-γ and IL-4 cytokines compared to WT mice (Figure 2D and E). Consistent with the observed systemic autoimmunity, FasGL7+ germinal center (GC) B cells were expanded in the spleens from KO mice (Figures 2F and 2G). To evaluate the effect of competitive WT Treg cells over the KO Treg cells, we performed mixed bone marrow chimera transfer experiments into RAG−/− mice. We observed that bone marrow chimera recipients exhibited milder phenotypic features with signs of peripheral T cell activation and autoimmunity without lethal systemic inflammation, suggesting that Treg cells from WT mice were able to rescue the severe phenotype observed in the Treg Srsf1-KO mice (Figures S2A and S2B). These data indicate that Treg-specific deletion of Srsf1 leads to an uncontrolled peripheral immune cell activation and systemic autoimmune disease in vivo.

**SRSF1 is essential for not only survival but also function of Treg cells**

SRSF1 is a pro-survival factor and controls genes involved in cell survival, and its deletion in cell lines in vitro leads to apoptosis (Bielli et al., 2014; Gautrey and Tyson-Capper, 2012; Li et al., 2005). We examined the Treg cell populations in Treg Srsf1-KO mice and found that the frequencies of Treg cells were dramatically reduced in the spleen, mesenteric lymph nodes (MLN), and peripheral lymph nodes (Figures 3A and 3B). In the thymus, the frequencies of CD25+Foxp3+ cells among single-positive CD4 T cells were comparable between WT and Treg Srsf1-KO mice (Figure S3A), although the thymus was significantly reduced in size.
and cellularity in the KO mice. In addition, we observed increased frequencies of early apoptotic cells during induced Treg (iTreg) differentiation from naive CD4 T cells from Treg Srsf1-KO mice compared to those from WT mice (Figures 3C and 3D), with no significant difference in Foxp3 expression (Figure S3B). We also found that natural Treg (nTreg) cells from Treg Srsf1-KO mice exhibit increased frequencies of dead cells compared to nTreg cells from WT mice (Figure S4A). To confirm that the survival defects of Treg cells in the Treg-Srsf1-KO mice, we utilized Srsf1-deficient Treg cells from T-cell-Srsf1-KO (dLckCre Srsf1flox/flox) mice to obtain sufficient numbers. Treg suppressive function is associated with a number of molecules, including cytotoxic T lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 protein (LAG3), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), inducible costimulator (ICOS), Helios, CD39, and CD103 (Lu et al., 2017; Suffia et al., 2005; Vocanson et al., 2010). Srsf1-deficient Treg cells displayed lower frequencies of CTLA4+ Treg cells and higher expression of PD-1 and CD39, whereas the expression levels of CD103, ICOS, GIfTR, Helios, and LAG3 were comparable to Treg cells from WT mice (Figures 4A and 4B). When we cultured fluorescence-activated cell sorting (FACS)-sorted nTreg cells with CD3 and CD28 antibodies for T cell receptor (TCR) engagement, Foxp3 expression was also comparable between WT and Srsf1-deleted Treg cells (Figures S5A and S5B).

To evaluate the role of SRSF1 in Treg function, we performed in vitro and in vivo Treg suppression experiments. To avoid the effects of systemic inflammation in the Foxp3YFP-Cre Srsf1flox/flox Treg-Srsf1-KO mice, we performed these experiments using Treg cells from the dLckCre Srsf1flox/flox T cell Srsf1-KO mice. We confirmed that the frequencies of CD4+CD25+ cells and CD25+Foxp3+ Treg cells in T cell Srsf1-KO mice were comparable to those in WT mice (Figures S5C–S5F). CD4+CD25+CD127lo nTreg cells from T cell Srsf1-KO mice were unable to suppress the proliferation of conventional CD4 T (Tconv) cells in 7-day

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Figure 3. SRSF1 is essential for survival of Treg cells

(A) Plots show gating strategies and CD4+CD25+Foxp3+ Treg cells in spleen, MLN, and PLN from WT and Treg Srsf1-KO mice.

(B) Graphs show frequencies of CD4+CD25+ Foxp3+ Treg cells (spleen: n = 5 each; MLN and PLN: n = 4 each).

(C) Naive CD4 T cells were isolated from pooled spleen and PLN cells from WT and Treg Srsf1-KO mice and cultured under Treg differentiation conditions. Cells were collected and analyzed by flow cytometry on days 1–3. Plots show 7AAD and Annexin V staining.

(D) Graph shows the percentage of early apoptotic (7AAD−Annexin V+) cells among CD4 T cells (n = 3–6).

*p < 0.05, **p < 0.005, and ***p < 0.0005, unpaired t test (B) or one-way ANOVA with Tukey’s multiple comparisons test (D); mean ± SEM.
in vitro co-culture assays (Figures 4C and 4D). To investigate the function of Srsf1-deficient Treg cells in vivo, we adoptively transferred sorted WT or T cell Srsf1-KO mice, and expression levels of Treg-associated molecules in CD4+CD25+Foxp3+ Treg cells were analyzed by flow cytometry. Dot plots (left) and histograms (right) show representative data of frequencies and mean fluorescence intensity (MFI) of markers in gated Treg cells.

(B) Graphs show the percentage of CTLA4+−, PD1+−, and CD39+− cells gated on Treg cells and quantification of MFI of CD103, ICOS, GITR, LAG3, and Helios (n = 4–10 each).

(C) Spleen cells from WT or T cell Srsf1-KO mice were isolated and conventional CD4 T (Tconv) cells and Treg cells (CD4+CD25+CD127lo) were sorted by flow cytometry. Tconv cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with Treg cells at increasing ratios for 7 days, and proliferation of Tconv cells was analyzed by flow cytometry. Representative plots are shown.

(D) Graph shows proliferation of Tconv cells (n = 8 WT, n = 10 KO).

(E) B6 mice were given 2.5% dextran sodium sulfate (DSS) in water to induce colitis for 8–10 days. One day before the initiation of DSS administration, PBS or flow-cytometry-sorted Treg cells (CD4+CD25+CD127lo) from WT or T cell Srsf1-KO mice were injected into B6 mice. Graph shows body weight of mice from five independent experiments (n = 6 each).

(F) Representative image of colon after DSS administration for 8–10 days. Graph shows colon length of WT Treg and KO Treg group (n = 6 WT, n = 5 KO).

(G) Naive CD4 T cells were adoptive transferred into Rag1−−−− mice to induce colitis together with PBS or sorted Treg cells (CD4+CD25+CD127lo) from WT or T cell Srsf1-KO mice. Graph shows time course of body weight of Rag1−−−− recipient mice (n = 4–5 each).

*p < 0.05; **p < 0.005; and n.s., no significant difference, two-way ANOVA test (D, E, and G) and unpaired t test (B and F); mean ± SEM.

Figure 4. Srsf1-deficient Treg cells exhibit impaired phenotype and suppressive function

(A) Spleen cells were isolated from WT and T cell Srsf1-KO mice, and expression levels of Treg-associated molecules in CD4+CD25+Foxp3+ Treg cells were analyzed by flow cytometry. Dot plots (left) and histograms (right) show representative data of frequencies and mean fluorescence intensity (MFI) of markers in gated Treg cells.

To validate that SRSF1 plays an important role in Treg function and integrity.

Srsf1 deficiency leads to an aberrant transcriptomics profile in Treg cells

To better understand the molecular landscape controlled by SRSF1, we performed RNA sequencing of ex-vivo-stimulated
nTreg cells from WT and T cell Srsf1-KO mice followed by transcriptomics data analysis. We found that deletion of Srsf1 led to significant transcriptional alterations in the Srsf1-deficient Treg cells. A total of 218 genes were differentially expressed (DE) in KO compared to WT Treg cells, with 65 genes upregulated and 153 genes downregulated at the 2-fold cutoff and 23 genes up and 8 genes down at the 4-fold cutoff (p < 0.05) (Figures 6A and 6B). Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed pathways involved in leukocyte differentiation, T cell activation, regulation of cell motility, and positive regulation of cytokine production (Figures 6C and 6D). Among these, the expression levels of multiple cytokines and chemokines, including IL-17A, IL-17F, CCL22, CCL20, CXCR3, and CCL17, were found to be elevated, and other genes, including SATB1, SCD1, and DAPL1, were decreased (Figure 6E). There was no significant difference in the expression levels of IL-10, IL-35, or transforming growth factor β (TGF-β). These data suggest that SRSF1 controls genes involved in activation, differentiation, migration, and cytokine production in Treg cells.

**Srsf1 deficiency leads to Treg cell plasticity with a hyper-glycolytic metabolism and acquisition of an aberrant proinflammatory phenotype rescued by rapamycin**

Recent studies have shown that the activated phenotype and production of proinflammatory cytokines from Treg cells is associated with their dysfunction in autoimmune diseases (Pandiyan and Zhu, 2015). Because the transcriptomics profiling indicated that SRSF1 controls genes involved in activation and cytokine production in Treg cells, we investigated cytokine production from the Srsf1-deficient Treg cells. We found that Srsf1-deficient Treg cells produced higher levels of proinflammatory cytokines, including IFN-γ, IL-17A, and IL-4, compared to WT Treg cells (Figures 7A and 7B). It is known that metabolic reprogramming influences the activation and function of Treg cells. Glycolytic metabolism promotes T helper cell type 1 (Th1) and Th17 differentiation (Peng et al., 2016; Shi et al., 2011). Moreover, higher glycolysis leads to abnormal proinflammatory cytokine production from Treg cells and Treg dysfunction (Sun et al., 2017). Therefore, we assessed the metabolic profiles of WT and Srsf1-deficient Treg cells. By glycolysis stress test assays, we found that Srsf1-deficient Treg cells had a significantly higher maximum glycolytic rate than WT Treg cells (Figures 7C and 7D). Activation of the mTORC1 pathway is known to induce higher glycolytic metabolism and abnormal proinflammatory cytokine production from Treg cells and Treg dysfunction (Sun et al., 2017). Therefore, we evaluated the activity of the mTORC1 pathway. We found increased expression of phosphorylated S6 (pS6) protein levels, indicating an increased activity of the mTORC1 pathway (Figure 7E). Importantly, treatment with the mTOR inhibitor rapamycin reverted abnormal production of IFN-γ from Srsf1-deficient Treg cells (Figures 7F and 7G). There was no difference in IL-17A.
Figure 6. Srsf1-deficient Treg cells display an aberrant transcriptomics profile

Total T cells were isolated from spleens of 8- to 10-week-old WT and T cell Srsf1-KO mice (n = 3 each). Natural Treg (nTreg) cells (CD4+CD25+CD127lo) were sorted by flow cytometry. nTreg cells were stimulated with anti-CD3, anti-CD28, and recombinant (r)IL-2 for 72 h.

(A) RNA-sequencing data analysis shows differentially expressed (DE) genes with log2 fold change (FC) differences at p < 0.05.

(B) Volcano plot showing upregulated and downregulated genes in KO nTreg cells.

(C) Table showing the description, count, and adjusted p-value of differentially expressed genes.

(D) Diagram showing the network of T cell differentiation, immune response, T cell activation, and cytokine production.

(E) Heatmap showing the expression levels of genes in WT and KO Treg cells.

Note: (legend continued on next page)
production or Foxp3 expression after treatment with rapamycin in Srsf1-deleted iTreg cells (Figures S7A and S7B). Furthermore, we found that Srsf1 mRNA expression levels as analyzed by RNA-sequencing data were comparable among naïve CD4 T cells, effector CD4 T cells, and nTreg cells from WT mice (Figure S7C). These data suggest that SRSF1 regulates T cell plasticity, and in its absence, Treg cells convert to effector-like proinflammatory cells.

**DISCUSSION**

In this study, we demonstrate that selective deletion of the essential RNA-binding protein SRSF1 in Treg cells leads to lethal systemic inflammation and autoimmunity. SRSF1 is not only necessary for Treg cell survival but also essential for a number of gene programs that are important in Treg function. Loss of SRSF1 in Treg cells results in impaired suppressive function and conversion to a proinflammatory phenotype. Glycolytic metabolism and mTOR pathway activity are increased in Srsf1-deficient Treg cells. Importantly, rapamycin treatment reverts the abnormal proinflammatory cytokine production. Thus, we show roles for SRSF1 in the control of Treg cell physiology and its potential role in the pathogenesis of autoimmune disease.

Recent advances in our understanding of molecules that regulate Treg survival and function have yielded insights into potential new therapeutic strategies for autoimmune diseases, including SLE (Mizui and Tsokos, 2018). There are conflicting reports on the numbers and function of Treg cells in autoimmune diseases and SLE, with some studies reporting defects in numbers and/or function and others reporting no differences (La Cava, 2018). Heterogeneity in patient cohorts, inclusion criteria, thresholds for disease activity, and differences in gating strategies for Treg cells account for these ambiguous results. A recent meta-analysis of a large number of studies, however, does point to reduced numbers of Treg cells in active patients with SLE (Li et al., 2019). Furthermore, recent clinical trials with low-dose IL-2 therapy boosted Treg numbers and improved disease activity in patients with SLE, indicating that targeting Treg cells is a valuable strategy to treat systemic autoimmune disease (He et al., 2016; von Spee-Mayer et al., 2016). Therefore, identifying molecules and mechanisms that promote Treg homeostasis and function is important. Our current study with Treg-specific conditional Srsf1-KO mice reveals that SRSF1 is crucial for not only Treg survival but also their integrity and function.

Recent studies have shown that increased activity of the mTOR pathway negatively affects Treg function (Chapman and Chi, 2015). Hyperactivation of the mTOR pathway induces higher glycolytic metabolic characteristics in Treg cells, which also leads to Treg dysfunction and production of proinflammatory cytokines (Apostolidis et al., 2016; Liu et al., 2010). Consistent with these reports, mTOR activation is implicated in rheumatic diseases (Perl, 2016, 2017), and rapamycin, an inhibitor of the mTOR pathway, promotes the expansion of functional Treg cells (Battaglia et al., 2006). Importantly, a recent clinical trial of rapamycin in patients with SLE has shown promising results (Lai et al., 2018). In this study, we demonstrate that the deletion of SRSF1 alters metabolic characteristics and leads to the activated phenotype of Treg cells via activation of the mTOR pathway. Therefore, while we have previously shown that decreased levels of SRSF1 are associated with worse disease activity of patients with SLE (Moulton et al., 2013), here, we have uncovered a link between SRSF1 and Treg function.

Here, we found that Srsf1-deficient Treg cells exhibit significantly decreased expression of CTLA-4, which is crucial for the suppressive function of Treg cells (Wing et al., 2008). Because a constitutively active allele of the kinase Akt in Treg cells leads to an overall dampening of the Treg cell gene signature, including reduced expression of CTLA-4 (Haxhinasto et al., 2008), mTOR pathway activation might contribute to reduced expression levels of CTLA-4 in Srsf1-deficient Treg cells. However, PTEN-deficient Treg cells, which exhibited an activated mTOR pathway, showed higher expression levels of ICOS, PD-1, and GITR, whereas the expression of CTLA-4 was normal (Shrestha et al., 2015), suggesting the existence of other mechanisms by which SRSF1 controls CTLA-4 expression and reduces CTLA-4 expression may be another mechanism of impaired function of Srsf1-deficient Treg cells independent of the mTOR pathway. There might be other molecules controlled by SRSF1 that directly regulate the suppressive function of Treg cells independently of the phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR pathway. Furthermore, how altered expression levels of immunosuppressive molecules including low CTLA-4 and high PD1 levels are associated with the impaired suppressive activity of Srsf1-deficient Treg cells in the context of inflammation needs further investigation.

Given the impaired homeostasis and survival of SRSF1-deficient Treg cells and ensuing autoimmunity, it would be important to carefully assess the survival/proliferation of these Treg cells in *in vitro* and *in vivo* functional studies and, furthermore, assess how these Treg cells respond to growth factor signals, including IL-2. Furthermore, it is important to identify the mechanisms underlying the impaired homeostasis and function to understand the role of SRSF1 in autoimmunity. Accordingly, we have recently shown that SRSF1 controls the expression of the anti-apoptotic gene Bcl-xL and that Bcl-xL levels are decreased in Srsf1-deficient T cells (Katsuyama et al., 2020). Furthermore, Bcl-xL is known to be important for development of functional regulatory CD4 T cells (Sharabi et al., 2010). We are currently pursuing mechanistic studies to evaluate the role of SRSF1/Bcl-xL-related pathways in Treg homeostasis and function. While the deficiency of SRSF1 leads to increased apoptosis, and this is certainly one aspect of its role in Treg homeostasis, it likely controls Treg function in addition through the control of genes involved in signaling and cytokine production, as we have recently shown (Katsuyama et al., 2019).
Also, further studies are required to assess the impact of SRSF1 on the thymic development of Treg cells. This will be better delineated using mice that lack SRSF1 in Treg cells but do not display severe inflammation or in mice with inducible deletion of SRSF1 so as to induce SRSF1 deletion in non-infant mice. Compared to homozygous Treg Srsf1-KO mice, Srsf1-HET mice display a significantly mild phenotype and normal frequencies of peripheral Treg cells, suggesting that thymic development in these mice may be normal. Yet, these Srsf1-HET mice exhibit signs of peripheral immune activation (Figures 5C–5F), suggesting that Treg cells are functionally defective. These data also suggest that there may be a gene-dose effect of the heterozygous versus homozygous deficiency of SRSF1 on Treg cell development and phenotype. This is pertinent from a translational standpoint, because while T cells from patients with SLE exhibit reduced levels of SRSF1, they do not completely lack the gene. Therefore, Srsf1-HET mice may be valuable tools to study Treg defects and translate findings to humans.

An interesting aspect of the systemic inflammation in the Treg Srsf1-KO mice is tissue involvement, with the lungs being a major site of severe inflammation. Given the differential expression of chemokine/receptor genes, it is likely that SRSF1 controls migratory capacity and tissue tropism of Treg cells, which determine tissue-specific inflammation. It would be important to evaluate the functional dynamics of Srsf1-deleted Treg cells to assess whether these Treg cells are dividing and producing inflammatory cytokines in situ. Since the metabolic cues are likely different across these different sites, they may induce different Treg cell functional outcomes. Also, while we observe a skewed proinflammatory cytokine phenotype of the Treg cells, we did not observe differences in the Treg signature cytokines, including IL-10 and TGF-β. While low CTLA4 expression may play a role in the Srsf1-mediated impairment of Treg function, the differential expression of chemokine/receptor genes suggests aberrant homing capacity and tissue localization of these Treg cells as potential mechanisms of defect. Additionally, from our results so far, it appears that SRSF1 does not control FoxP3 expression, because we do not see significant effect on FoxP3 levels after TCR engagement in nTreg cells or iTreg cells. Rather, SRSF1,
through its control of apoptosis-related genes such as Bcl-xL, CTLA4, mTOR signaling/inflammatory cytokine pathways, chemokine/receptor genes, and metabolism, likely controls Treg homeostasis, tissue-tropism, and suppressive function.

While we have evaluated cytokines in the experiments with rapamycin treatment, in further studies, we plan to assess other cytokines, including, among others, IL-4. We also plan to evaluate the mRNA levels of proinflammatory cytokines DE in the transcriptomics analyses. In the experiments to assess Treg function, we sorted cells based on the surface markers CD4+CD25+CD127- cells as nTreg cells. However, due to the severe systemic inflammation in the KO mice, these populations may contain activated T cells. Future evaluations using fluorescent-reporter-based sorting of cells, especially those from Srsf1-HET mice, would be valuable to overcome these issues.

Given the severe autoimmune phenotype in mice with Treg-specific deficiency of SRSF1, it is crucial to examine the expression levels of SRSF1 between Treg cells and conventional/effector T cells and the signals/factors that control its expression at the molecular level. We found that Srsf1 mRNA levels are comparable between naive CD4, effector CD4, and nTreg cells (Figure S7C). The differences in phenotype we observed in our total T cell (Lck-Cre) Srsf1-KO (Katsuyama et al., 2019) versus Treg (FoxP3 Cre) Srsf1-KO mice is likely because of the timing of deletion. Under distal Lck-Cre promoter control, the Srsf1 gene is deleted after thymic development, whereas in Treg-specific mice, it is deleted during early thymic development. Furthermore, while we have previously found its decreased levels in total T cells from patients with SLE (Katsuyama et al., 2019), studies are needed to evaluate SRSF1 levels in Treg cells from patients with SLE. While genome-wide association studies (GWASs) in SLE have not specifically reported any SNPs in the Srsf1 locus, we have considered environmental triggers, inflammatory milieu (cytokines), and hormones as potential factors in its regulation.

We have initiated investigation on the underlying factors that control SRSF1 expression. We have shown that ubiquitin-induced proteasomal degradation contributes to the downregulation of SRSF1 in T cells from normal individuals and patients with SLE (Moulton et al., 2014). We have ongoing studies investigating the role of estrogen in the regulation of SRSF1 at the transcriptional, post-transcriptional, and post-translational levels. In addition, post-transcriptional elements, including microRNAs, have been reported to modulate SRSF1 expression (Ramanujan et al., 2021; Sokol et al., 2018). In addition to its expression levels, the activity of SRSF1 and other SR proteins is controlled by phosphorylation, and these proteins are dephosphorylated by ceramide-induced protein phosphatase 1 (PP1). Ceramides are lipid metabolites generated by sphingomyelin hydrolysis, induced by various environmental triggers such as UV radiation, inflammatory cytokines (including tumor necrosis factor [TNF]), and cytotoxic drugs (Chalfant et al., 2002; Pettus et al., 2002). Thus, further studies are needed to delineate the precise role of these factors in regulating SRSF1 expression specifically in distinct T cell subsets.

In conclusion, we have uncovered a crucial role of SRSF1 in Treg homeostasis and function. Loss of SRSF1 leads to activation of the mTOR pathway, hyper-glycolytic metabolic phenotype, and proinflammatory cytokine production in Treg cells. Given the aberrant expression and function of SRSF1 in human systemic autoimmune disease, our findings suggest that low SRSF1 levels in Treg cells may play an important role in the pathogenesis of autoimmunity and implicate a therapeutic target for autoimmune diseases.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109339.

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**AUTHOR CONTRIBUTIONS**

T.K. and V.R.M. conceptualized the work, designed experiments, performed experiments, analyzed and interpreted data, wrote the manuscript, and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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# KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-mouse CD8a     | Biolegend | Cat #100705 |
|                     |         | RRID: AB_312744 |
| Anti-mouse CD25     | Biolegend | Cat #102012 |
|                     |         | RRID: AB_312861 |
| Anti-mouse CD44     | Biolegend | Cat #103020 |
|                     |         | RRID: AB_493683 |
| Anti-mouse CD62L    | Biolegend | Cat #104412 |
|                     |         | RRID: AB_313099 |
| Anti-mouse CD90.2   | Biolegend | Cat #140310 |
|                     |         | RRID: AB_10643586 |
| Anti-mouse TCR-β    | Biolegend | Cat #109205 |
|                     |         | RRID: AB_313428 |
| Anti-mouse IL-4     | Biolegend | Cat #504109 |
|                     |         | RRID: AB_493320 |
| Anti-mouse IL-17A   | Biolegend | Cat #504109 |
|                     |         | RRID: AB_493320 |
| Anti-mouse IFN-γ    | Biolegend | Cat #505818 |
|                     |         | RRID: AB_893526 |
| Biotin anti-mouse CXCR5 | Biolegend | Cat #145510 |
|                     |         | RRID: AB_2562126 |
| Anti-mouse CD39     | Biolegend | Cat #143803 |
|                     |         | RRID: AB_11219591 |
| Anti-mouse CD103    | Biolegend | Cat #121413 |
|                     |         | RRID: AB_1227503 |
| Anti-mouse CD127    | Biolegend | Cat #135031 |
|                     |         | RRID: AB_2564216 |
| Anti-mouse CTLA4    | Biolegend | Cat #106305 |
|                     |         | RRID: AB_313254 |
| Anti-mouse GITR     | Biolegend | Cat #120208 |
|                     |         | RRID: AB_439726 |
| Anti-mouse ICOS     | Biolegend | Cat #107705 |
|                     |         | RRID: AB_313334 |
| Anti-mouse PD1      | Biolegend | Cat #135213 |
|                     |         | RRID: AB_10689633 |
| Anti-mouse LAG3     | Biolegend | Cat #125207 |
|                     |         | RRID: AB_2133344 |
| Anti-mouse Ly6G     | Biolegend | Cat #127605 |
|                     |         | RRID: AB_1236488 |
| Anti-mouse CD11b    | Biolegend | Cat #101205 |
|                     |         | RRID: AB_312788 |
| Anti-mouse GL7      | Biolegend | Cat #144614 |
|                     |         | RRID: AB_2563292 |
| Annexin V           | Biolegend | Cat #640941 |

(Continued on next page)
| REAGENT or RESOURCE                      | SOURCE              | IDENTIFIER               |
|-----------------------------------------|---------------------|--------------------------|
| 7AAD                                    | Biolegend           | Cat #420404              |
| Anti-mouse CD4                          | eBioscience         | Cat #46-0041-82          | RRID: AB_11150050 |
| Anti-mouse Foxp3                        | eBioscience         | Cat #12-5773-82          | RRID: AB_465936 |
| Anti-mouse CD95                         | BD Bioscience       | Cat #12-5773-82          | RRID: AB_465936 |
| Purified anti-mouse CD3ε                | Biolegend           | Cat #100302              | RRID: AB_312667 |
| Purified anti-mouse CD28                | Biolegend           | Cat #102112              | RRID: AB_312877 |
| Purified anti-mouse IFN-γ               | Biolegend           | Cat #505706              | RRID: AB_315394 |
| Purified anti-mouse IL-4                | Biolegend           | Cat #504108              | RRID: AB_315322 |
| CD16/32                                 | Biolegend           | Cat #101302              | RRID: AB_312801 |
| Anti-S6 ribosomal protein phospho-Ser 235 and Ser 236 | Cell Signaling Technology | Cat #2211S |
| Anti-S6 ribosomal protein               | Cell Signaling Technology | Cat #2217S |
| Goat anti-rabbit IgG horseradish peroxidase (HRP) | Cell Signaling Technology | Cat #7074 |
| Goat anti-mouse IgG horseradish peroxidase (HRP) | Abcam               | Cat #97023               |
| β-actin                                 | Sigma-Aldrich       | Cat #A5316               |

### Chemicals, peptides, and recombinant proteins

| REAGENT or RESOURCE                      | SOURCE              | IDENTIFIER               |
|-----------------------------------------|---------------------|--------------------------|
| Rapamycin                               | Millipore           | Cat #553210-100ug        |
| Phorbol myristic acid (PMA)             | Sigma-Aldrich       | Cat #P1585               |
| Ionomycin                               | Sigma-Aldrich       | Cat #I9657               |
| Mitomycin C                             | Sigma-Aldrich       | Cat #M4287               |
| Dextran Sodium sulfate                  | MP Biomedicals      | Cat #MP216011025         |
| Recombinant TGF-β                       | R&D systems         | Cat #240-B-010           |
| Recombinant IL-2                        | R&D systems         | Cat #402-ML-100          |

### Critical commercial assays

| REAGENT or RESOURCE                      | SOURCE              | IDENTIFIER               |
|-----------------------------------------|---------------------|--------------------------|
| Annexin binding buffer                   | Biolegend           | Cat #422201              |
| Zombie Aqua Fixable Viability kit        | Biolegend           | Cat #423102              |
| Carboxyfluorescein succinimidyl ester Cell division Tracker Kit | Biolegend | Cat #423801 |
| Mouse regulatory T cell staining kit     | eBioscience         | Cat #88-8118-40          |
| Fixation/Permeabilization solution kit   | BD Bioscience       | Cat #554714              |
| Golgi Stop protein transport inhibitor   | BD Bioscience       | Cat #554724              |
| ACK lysing buffer                        | Fisher Scientific   | Cat #1049201             |
| Mouse CD4+CD62L+ T cell isolation kit    | Miltenyi Biotec     | Cat #130-106-643         |
| Mouse Pan T cell isolation kit II        | Miltenyi Biotec     | Cat #130-095-130         |
| ECL detection reagent                    | GE Healthcare        | Cat #RPN2209             |
| ECL prime detection reagent              | GE Healthcare        | Cat #RPN2236             |

### Deposited data

| REAGENT or RESOURCE                      | SOURCE              | IDENTIFIER               |
|-----------------------------------------|---------------------|--------------------------|
| Raw and analyzed RNA-seq data           | This paper          | GEO: GSE173268           |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents and resources should be directed to and will be fulfilled by the lead contact, Dr. Vaishali Moulton (vmoulton@bidmc.harvard.edu).

Materials availability
B6.Srsf1^fl/fl (B6-Srsf1-flox) strain generated in this study will be deposited to Jackson Laboratory. B6.Srsf1^fl/fl.Lckcre and B6.Srsf1^fl/fl.Foxp3YFP-cre mice generated in this study will be made available on request with a complete Materials Transfer Agreement and we may require a payment if there is potential for commercial application.

Data and code availability
The RNA-sequencing data from this study have been deposited at the NCBI Gene Expression Omnibus (GEO) database under accession number GSE173268.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6J (stock 000664), B6.129S4-Srsf1-flox (stock 018020), B6.d.Lck.Cre (stock 012837), B6.Foxp3YFP-cre (stock 016959) and RAG1^-/- (stock 002216) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). B6.Srsf1^fl/fl (B6-Srsf1-flox) mice were generated by backcrossing the B6.129S4-Srsf1-flox mice with C57BL/6J mice for twelve generations. They were then bred with B6.d.Lck.Cre or B6.Foxp3YFP-cre mice to generate B6.Srsf1^fl/fl d.LckCre (T cell Srsf1-KO) mice or B6.Srsf1^fl/fl Foxp3YFP-cre (Treg Srsf1-KO) mice, respectively. Treg Srsf1-KO mice were used at 3-4 weeks old. In most studies with Treg Srsf1-KO mice, sex-matched male and female WT littermates were used as controls. All mice were maintained in the specific pathogen free animal facility at Beth Israel Deaconess Medical Center (BIDMC). All studies were approved by the Institutional Animal Care and Use Committee at BIDMC.

METHOD DETAILS

Tissue processing and cell isolation
Spleens and lymph nodes were homogenized using a syringe plunger and mesh cell strainer. RBC lysis was performed with ACK lysing buffer. All cell cultures were in RPMI complete medium (RPMI plus 10% FBS plus penicillin and streptomycin antibiotics). Blood samples from mice were collected by tail vein incision and capillary tube collection, and serum separated by centrifugation. For histopathology, tissues were immediately fixed in 10% formalin overnight, processed in an automated tissue processor, embedded into paraffin blocks and sent to the BIDMC Histopathology core for sectioning and slide staining with hematoxylin and eosin (H and E).
Induced (i)Treg differentiation assays
Naive CD4 T cells were isolated from spleens using the CD4+CD62L+ T cell isolation kit (Miltenyi Biotec) and cultured with anti-mouse CD3 (0.5 μg/ml) and anti-mouse CD28 (1 μg/ml) in the presence of recombinant (r)TGF-β (1ng/ml) and rIL-2 (20ng/ml) for 72 hours. For western blotting to detect phosphorylated (p)-S6, additional stimulation with anti-mouse CD3 (5 μg/ml) and anti-mouse CD28 (5 μg/ml) for 5min was performed.

Flow cytometry
Cells were washed with phosphate buffered saline (PBS) and stained with Zombie aqua viability dye. Surface staining was performed in FACS staining buffer (PBS plus 2% fetal bovine serum (FBS)) with Fc block on ice for 20 min. For intracellular cytokine staining, cells were stimulated for 4h in culture medium with PMA (100ng/ml), Ionomycin (1 μM) in presence of Monensin (1 μM). Cells were surface stained followed by fixation and permeabilization. Appropriate antibodies were used for intracellular staining for cytokines or transcription factors. Flow cytometry data were acquired on CytoFLEX LX and analyzed with FlowJo software. All procedures were performed according to the manufacturer’s instructions.

Autoantibody detection
For anti-double stranded (ds)DNA and anti-Histone ELISA, Immulon II plates (Dynatech) precoated with BSA were coated individually with 50 μg/ml calf thymus DNA (Sigma-Aldrich) or 50μg/ml calf thymus histone (Sigma-Aldrich) respectively. Serum was diluted and assayed for autoantigen reactivity against the plates described above by incubation overnight at 4°C. Bound IgG was detected with a goat polyclonal HRP-anti-mouse IgG detection antibody (SouthernBiotech) and visualized at 450nm using a tetramethylbenzidine (TMB) substrate (Sigma-Aldrich).

Mixed bone marrow chimeras
Rag1−/− mice, 12-14 weeks of age were irradiated once at a total dose of 300rad for 4.25 s. Bone marrow cells from donor CD45.2 WT or Treg Srsf1−KO mice were mixed in a 1:1 ratio with BM cells from WT CD45.1 B6 mice and intravenously injected into the tail vein of irradiated Rag1−/− recipient mice post irradiation (a total of 8 × 10^6 cells/mouse). Recipient mice were euthanized 7-8 months after transfer and spleen cells analyzed by flow cytometry.

RNA sequencing
Total T cells were isolated from spleens of 8-10-week-old WT and T cell Srsf1−KO mice (n = 3 each). CD4+CD25+CD127lo natural (n) Treg cells were sorted by flow cytometry. nTregs were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) antibodies and rIL-2 (10 ng/ml) for 72h. Cells were collected, and total RNA was extracted using the RNeasy mini kit (QIAGEN) and submitted for RNA sequencing to the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute (DFCI). Libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kits according to the manufacturer’s protocols. Samples were sequenced on an Illumina NextSeq500 run with single-end 75-bp reads. Data analyses were performed using the VIPER pipeline by the DFCI Core.

Western blot
Total protein was extracted from cells after lysis with radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproducts), electrophoresed on NuPAGE 4%–12% Bis-Tris gels (Life Technologies) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% (wt/vol) nonfat milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) or Hikari solution A (Nacalai Tesque) at 4°C overnight or at room temperature for 2h for β-actin antibody. Membranes were washed three times with TBS-T, incubated with HRP-conjugated secondary antibody (1:2000 for ECL detection reagents or 1:4000 for ECL prime detection reagents; GE Healthcare) for 1h, washed three times with TBS-T, developed with ECL reagents, and visualized by a Fujifilm LAS-4000 imager or a Bio-Rad ChemiDoc XRS imager. Densitometry was performed using Quantity One software (Bio-Rad).

In vitro suppression assays
Total T cells were isolated from spleens by magnetic assisted cell sorting (MACS), using the Pan T cell isolation kit (Miltenyi Biotech). Cells were stained with fluorescent antibodies and CD4+CD25+CD127hi conventional CD4 T cells (Tconv) and CD4+CD25+CD127lo Tregs were sorted by flow cytometry (BD FACSria II). Tconv cells (6 × 10^4 cells/well) were labeled with CFSE and co-cultured with Treg cells at increasing ratios, in presence of Mitomycin C treated splenocytes (12 × 10^4 cells/well) with anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) and crosslinker (goat anti hamster IgG, 10 μg/ml, MP Biomedicals) for seven days. Proliferation of Tconv cells was analyzed by flow cytometry.

In vivo Treg suppression assays
Tconv cells and Tregs were sorted by flow cytometry as described above. For the dextran sulfate sodium (DSS) colitis induction, B6 mice at 14-18 weeks of age were administered 2.5% DSS (molecular weight 36–50 kDa; MP Biomedicals) in water for 7-10 days to induce colitis, and PBS or 7 × 10^5 Tregs from WT or T cell Srsf1−KO mice were injected intraperitoneally one day before the starting DSS. Body weight was measured every day for 8 days. Colon length was measured on the day of euthanasia. For the CD4 T cell...
transfer-induced colitis model, Rag1<sup>−/−</sup> mice were injected intravenously with 4 × 10⁵ naive CD4 T cells along with either PBS or 2 × 10⁶ Tregs from WT or T cell Srsf1-KO mice. Mice were weighed and examined once a week for 14 weeks.

**Metabolism assays**
Cell-Tak Cell and Tissue Adhesive (Fisher Scientific) was used for coating plates and 0.15 × 10⁶ iTregs were seeded in each well. Extracellular acidification rate (ECAR) was measured using an 8-well XFp extracellular Flux Analyzer (Seahorse) following the Glycolytic stress test protocol.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
GraphPad Prism (version 6) was used for statistical analysis. Student’s two-tailed t test, Mann-Whitney U test, and one-way analysis of variance (ANOVA) were used to calculate statistical significance among groups. All statistical details of experiments can be found in the figure legends. A p value of less than 0.05 was considered significant. Individual p values can be found in the figures.
Supplemental information

Splicing factor SRSF1 is indispensable for regulatory T cell homeostasis and function

Takayuki Katsuyama and Vaishali R. Moulton
Figure S1. Activated effector/effecter memory phenotype of CD8 T cells from Treg Srsf1-KO mice. Related to Figure 2. Spleen cells were isolated from 3-4 week-old WT and Treg Srsf1-KO mice and analyzed by flow cytometry. (A) Plots show CD62L and CD44 staining gated on live CD8 T cells in spleen and peripheral lymph nodes (PLNs) from WT and KO mice. (B) Graphs show percent naïve (CD62Lhi CD44lo) and effector memory (CD62Llo CD44hi) cells among CD8 T cells in spleen and PLNs of WT and Treg Srsf1-KO (n=5 each) (B: unpaired t-test, *p<0.05, **p<0.005, ***p<0.0005 mean± SEM)
Figure S2. Activated cellular phenotype and defects of Tregs in RAG⁻/⁻ bone marrow chimera recipients. Related to Figure 2. Bone marrow cells from CD45.2 WT or Treg Srsf1-KO mice were mixed in a 1:1 ratio with BM cells from WT CD45.1 B6 mice and adoptively transferred into irradiated Rag1⁻/⁻ recipient mice. Spleen cells were isolated from recipients and analyzed by flow cytometry. (A) Plots show CD69 staining gated on live CD4 T cells. Graph shows percent CD69⁺CD4 T cells (n=WT:6, KO:7). (B) Plots show PD1 and CXCR5 staining gated on live CD4 T cells. Graph shows percent CXCR5⁺PD1⁺ Tfh cells (n=WT:6, KO:7). (A and B: unpaired t-test, *p<0.05, mean± SEM).
**Figure S3.**

**A.**

![Flow cytometry plot](image)

FoxP3 expression is comparable in iTregs derived from WT and Treg Srsf1-KO mice. Related to Figure 3. A. Cells were isolated from thymus of WT and Treg Srsf1-KO mice. Plots and the graph show CD25^+Foxp3^+ Tregs gated on single positive CD4 T cells (n=WT:5, KO:6).

**B.**

![Graph](image)

Naïve CD4 T cells were sorted from pooled spleen and PLN cells from WT and Treg Srsf1-KO mice, and cultured under Treg differentiation conditions. Cells were collected and analyzed by flow cytometry on day1, day2 and day3. Graphs show FoxP3 expression mean fluorescence intensity of CD25^+Foxp3^+ Treg cells among CD4 T cells (n=3-12) (unpaired t-test, mean± SEM, n.s. not significant).
Figure S4. Survival of Srsf1-deleted Tregs. Related to Figure 3. (A) Spleen cells were isolated from WT and Treg Srsf1-KO mice. Flow plots show Zombie Aqua (ZAqua+) positive dead cells gated on CD4+CD25+Foxp3+ Tregs (n=WT:5, KO:4). (B) Bone marrow cells from CD45.2 WT or Treg Srsf1-KO mice were mixed in a 1:1 ratio with bone marrow cells from WT CD45.1 B6 mice and adoptively transferred into irradiated RAG1−/− recipient mice. Spleen cells were isolated from recipients and analyzed by flow cytometry. Plots show YFP+ CD45.2 cells gated on live CD4 T cells. Graph shows percent CD4+YFP+ Tregs (n=WT:6, KO:7). (A and B: unpaired t-test, *p<0.05, ***p<0.0005, mean±SEM).
Figure S5.

(A) Spleen cells were isolated from WT and T cell Srsf1-KO mice. Tregs (CD4+CD25+CD127-) were sorted by flow cytometry and cultured with CD3 and CD28 antibodies and recombinant IL-2 for 72 hours. Foxp3 MFI in CD4+CD25+Foxp3+ Tregs were analyzed by flow cytometry. Graph shows Foxp3 MFI gated on Tregs (n=3 each).

(B) Graph shows Foxp3 expression post-TCR engagement and the frequencies of CD25+CD4 and CD25+Foxp3+CD4 T cells are comparable between WT and T cell-Srsf1-KO mice. Related to Figure 4. (A and B) Spleen cells were isolated from WT and T cell Srsf1-KO mice. Tregs (CD4+CD25+CD127-) were sorted by flow cytometry and cultured with CD3 and CD28 antibodies and recombinant IL-2 for 72 hours. Foxp3 MFI in CD4+CD25+Foxp3+ Tregs were analyzed by flow cytometry. Graph shows Foxp3 MFI gated on Tregs (n=3 each).

(C-F) Spleen cells were isolated from WT and T cell-Srsf1-KO mice. Cells were surface stained for CD4 and CD25, and intracellular stained for Foxp3 and analyzed by flow cytometry. Graph shows frequencies of CD25+CD4 T cells (n=5 each). Plots and graph show frequencies of CD25+Foxp3+ cells gated on CD4+ T cells (E&F) (n=5 each) (B, D&F: unpaired t-test, n.s.: no significant difference, mean± SEM).
Figure S6. Decreased CTLA4+ Tregs from Treg Srsf1-HET mice. Related to Figure 5. (A) Spleen cells were isolated from WT and Treg Srsf1-HET mice, and expression levels of CTLA4 in CD4+CD25+Foxp3+ Tregs were analyzed by flow cytometry. Plots show representative data of frequencies of CTLA4 in gated Tregs. (B) Graph shows the percentage of CTLA4+ cells gated on Tregs (n=WT:4, HET:5) (B: unpaired t-test, *p<0.05, mean± SEM).
Figure S7. IL-17A production and Foxp3 expression after Rapamycin treatment in iTregs. *Srsf1* mRNA expression in distinct T cell subsets. Related to Figure 7. Naïve CD4 T cells were isolated from spleens of mice, and cultured under Treg differentiation conditions for 72h to generate induced Treg cells (iTregs). (A) After 72h, iTregs were cultured for additional 4h with PMA plus Ionomycin with or without Rapamycin (10nM). Cells were collected, surface stained, fixed and permeabilized for intracellular cytokine staining. Plots show IL-17A gated on live Tregs, and Foxp3 gated on live CD4 T cells. (B) Graphs show average data from n=4 mice. (C) Gene expression of *Srsf1* mRNA as measured by transcripts per kilobase million (TPM) in RNA-sequencing data from naïve CD4 T cells, effector CD4 T cells and Treg cells from WT mice. (B: One-way ANOVA with Tukey’s correction, n.s.: not significant, mean± SEM).