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Interleukin-21–Producing c-Maf–Expressing CD4+ T Cells Induce Effector CD8+ T Cells and Enhance Autoimmune Inflammation in Scurfy Mice

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Objective. **FoxP3 induces Treg cells and prevents autoimmune diseases. However, the precise mechanisms of FoxP3 in the prevention of autoimmune diseases remain unknown. We undertook this study to determine the regulatory roles of FoxP3 in autoimmune inflammation by using FoxP3-mutant sf mice.**

Methods. **We characterized interleukin-21 (IL-21)–producing cells in sf mice. We examined the underlying mechanisms of enhanced IL-21 production in sf mouse CD4+ T cells. We examined the roles of IL-21 and CD8+ T cells in autoimmune inflammation in sf mice using IL-21 receptor (IL-21R)–deficient sf mice.**

Results. **IL-21–producing c-Maf+CD4+ T cells, which were distinct from Th17 cells, were increased in sf mice. Increased c-Maf expression was involved in enhanced IL-21 production in sf mouse CD4+ T cells. Experiments using bone marrow chimeric mice showed that lack of cell-extrinsic suppression by FoxP3+ Treg cells, but not cell-intrinsic defects in FoxP3 in sf mouse CD4+ T cells, was mainly involved in the development of IL-21–producing c-Maf+CD4+ T cells in sf mice. IL-21R deficiency prolonged survival and reduced multiorgan autoimmune inflammation in sf mice. Moreover, IL-21R deficiency decreased short-lived effector CD8+ T cells in the lung in sf mice. Furthermore, depletion of CD8+ T cells inhibited lung inflammation in sf mice, suggesting that CD8+ T cells are critical for inducing lung inflammation in sf mice.**

Conclusion. **Unique IL-21–producing c-Maf+ CD4+ T cells develop in the absence of FoxP3+ Treg cells, induce short-lived effector CD8+ T cells, and enhance multiorgan autoimmune inflammation in sf mice.**

FoxP3 is a master regulator of Treg cells, and FoxP3+ Treg cells are considered to play crucial roles in the prevention of autoimmune diseases (1–3). Sf mice have a frameshift mutation in the FoxP3 gene, totally lack Treg cells, and have autoimmune diseases leading to death from multiorgan inflammation (skin, lungs, and liver) by age 4 weeks (1,4,5). In humans, patients with mutations in the FoxP3 gene develop a rare, recessive disorder called IPEX syndrome (immune dysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome) (6). It has been shown that CD4+ T cells play a critical role in the pathogenesis of autoimmune diseases in sf mice (7) and that T cell receptor–mediated signaling and costimulatory signaling are required for the development of the sf phenotype (8,9). However, the mechanisms by which FoxP3-mutant sf mice develop autoimmune inflammation in various organs are still largely unknown.

Interleukin-21 (IL-21) exhibits pleiotropic effects on tumor immunity, antiviral immunity, and autoimmunity (10–13). It has been shown that IL-21 is involved in the development of a number of autoimmune disease models, including type 1 diabetes mellitus in NOD mice (14) and lupus-like pathology in sanroque mice (15) and BXSB.Yaa mice (16). In addition, a blockade of IL-21 signaling has been demonstrated to ameliorate murine

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models of rheumatoid arthritis and lupus (17,18). However, although a variety of cytokine genes including IL-21 have been shown to be up-regulated in sf mice (19,20), the roles of IL-21 in the development of autoimmune inflammation in sf mice have not been investigated.

IL-21 is produced by many cell types including Th17 cells (21–24), follicular helper T (Tfh) cells (25), Th2 cells (26), IL-6– or IL-21–stimulated CD4+ T cells (24), and natural killer T cells (27). Regarding the transcriptional control of IL-21 expression, it has been shown that the genetic loss of c-Maf, which is highly expressed in Th17 cells, results in reduced IL-21 production in Th17 cells (28). Consistent with findings in c-Maf–deficient mice, we have previously shown that c-Maf directly binds the promoter and conserved noncoding sequence 2 (CNS-2) enhancer of the IL-21 gene and induces IL-21 production in CD4+ T cells (29). Moreover, we have shown that FoxP3 binds to the IL-21 promoter and the CNS-2 enhancer and suppresses c-Maf–induced expression of IL-21 (29). However, the in vivo roles of FoxP3 in the regulation of IL-21 production in CD4+ T cells still remain unclear.

Therefore, in the present study, we examined the regulatory roles of FoxP3 in the development of IL-21–producing CD4+ T cells and autoimmune inflammation by using sf mice. Our results indicate that unique IL-21–producing c-Maf+CD4+ T cells develop in the absence of FoxP3+ Treg cells, induce short-lived effector CD8+ T cells, and enhance autoimmune inflammation in the lung, skin, and liver in sf mice.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River Japan. IL-21 receptor (IL-21R)–deficient mice (30) were backcrossed over 8 generations onto C57BL/6 mice. CD45.1+ background scurfy mice were kind gifts from Dr. S. Hori (RIKEN). All mice were housed in microisolator cages under specific pathogen–free conditions. The Chiba University Animal Care and Use Committee approved animal procedures used in this study.

Reagents. Antibodies to CD3 (145-2C11), CD28 (37.51), CD44 (IM7), CD62L (MEL-14), B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CXCR5 (2G8), Fas (Jo2), IL-4 (11B11), interferon-γ (IFNγ) (XMG1.2), and IL-17A (TC11-18H10) were purchased from BD Biosciences. Antibodies to CD45.1 (A20), CD45.2 (104), killer cell lectin-like receptor subfamily G member 1 (KLR-G1) (2F1), and IL-7Rα (SB/199) were purchased from BioLegend. Antibodies to GL-7 (GL-7), programmed death 1 (RMP1-30), FoxP3 (FJK-16s), c-Maf (sym0F1), T-bet (eBio4B10), GATA-3 (TWAJ), and retinoic acid receptor–related orphan nuclear receptor γt (RORγt) (AFKJS-9) were purchased from eBioscience. Murine IL-6 was purchased from PeproTech. Human transforming growth factor β (TGFβ) and mouse IL-21R/Fc chimera were purchased from R&D Systems.

Cell isolation. CD4+ T cells and naive CD4+ T cells were isolated using a CD4+ T cell isolation kit II and a CD4+ CD62L+ T cell isolation kit II (Miltenyi Biotec), respectively.

Cell culture. CD4+ T cells were stimulated with plate-bound anti-CD3ε monoclonal antibody (mAb) (1 μg/ml) in the presence of anti-CD28 mAb (1 μg/ml) (anti-CD3/CD28 mAb). Where indicated, CD4+ T cells were stimulated with soluble anti-CD3ε mAb (5 μg/ml) in the presence of irradiated CD3-depleted splenocytes as antigen-presenting cells (APCs). Cells were cultured under neutral conditions (10 μg/ml anti–IL-4 mAb and 10 μg/ml anti-IFNγ mAb), Th1 cell–polarizing conditions (10 ng/ml IL-2, 1 ng/ml IL-12, and 10 μg/ml anti–IL-4 mAb), Th2 cell–polarizing conditions (10 ng/ml IL-2, 10 ng/ml IL-4, and 10 μg/ml anti-IFNγ mAb), Th17 cell–polarizing conditions (0.5 ng/ml IL-6, 1 ng/ml TGFβ, 10 μg/ml anti–IL-4 mAb, and 10 μg/ml anti-IFNγ mAb), or Tfh-like cell–polarizing conditions (100 ng/ml IL-6, 10 μg/ml anti–IL-4 mAb, and 10 μg/ml anti-IFNγ mAb).

Retrovirus vectors and retrovirus-mediated gene expression. Retrovirus vectors MSCV-IRES-GFP (MIG), MIG-FoxP3, MSCV-IRES-Thy1.1 (MIT), MIG-c-Maf, and MIT-c-Maf have been described previously (29). MIG and MIT are bicistronic retrovirus vectors that express green fluorescent protein (GFP) and Thy1.1, respectively. Given that L-Maf mutant lacking a transcription activation domain (L-b-Zip) acts as a dominant-negative mutant of L-Maf (31–33), we made c-Maf mutant lacking a transcription activation domain (Maf dTA; a fragment encoding amino acids 125–370 of c-Maf) as a dominant-negative form of c-Maf. We then introduced Maf dTA into MIT vector (MIT-Maf-dTA). The retrovirus-mediated transduction to CD4+ T cells was performed as described previously (29).

Intracellular cytokine and transcription factor staining. Intracellular cytokine staining of IL-21 was performed as described previously (34). For transcription factor staining, cells were stained with fixable viability dye (Invitrogen) and then fixed and permeabilized with a FoxP3 staining buffer set (eBioscience) according to the manufacturer's instructions. Fluorescence-activated cell sorting profiles were analyzed using FlowJo software (Tree Star).

Real-time polymerase chain reaction (PCR) analysis. Quantitative PCR was performed with a StepOnePlus real-time PCR system (Applied Biosystems) as described previously (29).

Mixed bone marrow chimeras. Bone marrow cells obtained from scurfy mice (CD45.1+ background mice) and C57BL/6 wild-type (WT) mice (CD45.2+ background mice) (2×10^6 cells each) were mixed and injected intravenously into C57BL/6 WT mice (CD45.2+ background mice) 1 hour after total body irradiation (9.5 Gy). Naive CD4+ T cells were purified from chimeric mice 10–12 weeks after bone marrow reconstitution by using an EasySep mouse naive CD4+ T cell isolation kit (StemCell Technologies). CD45.1+ sf mouse naive CD4+ T cells were separated from CD45.2+ WT mouse naive CD4+ T cells by using fluorescein isothiocyanate (FITC)–conjugated anti-CD45.1 and anti-FITC MACS beads (Miltenyi Biotec).

Computed tomography (CT) scanning. The lungs of the mice were scanned by using a Latheta (LCT-200) experi-

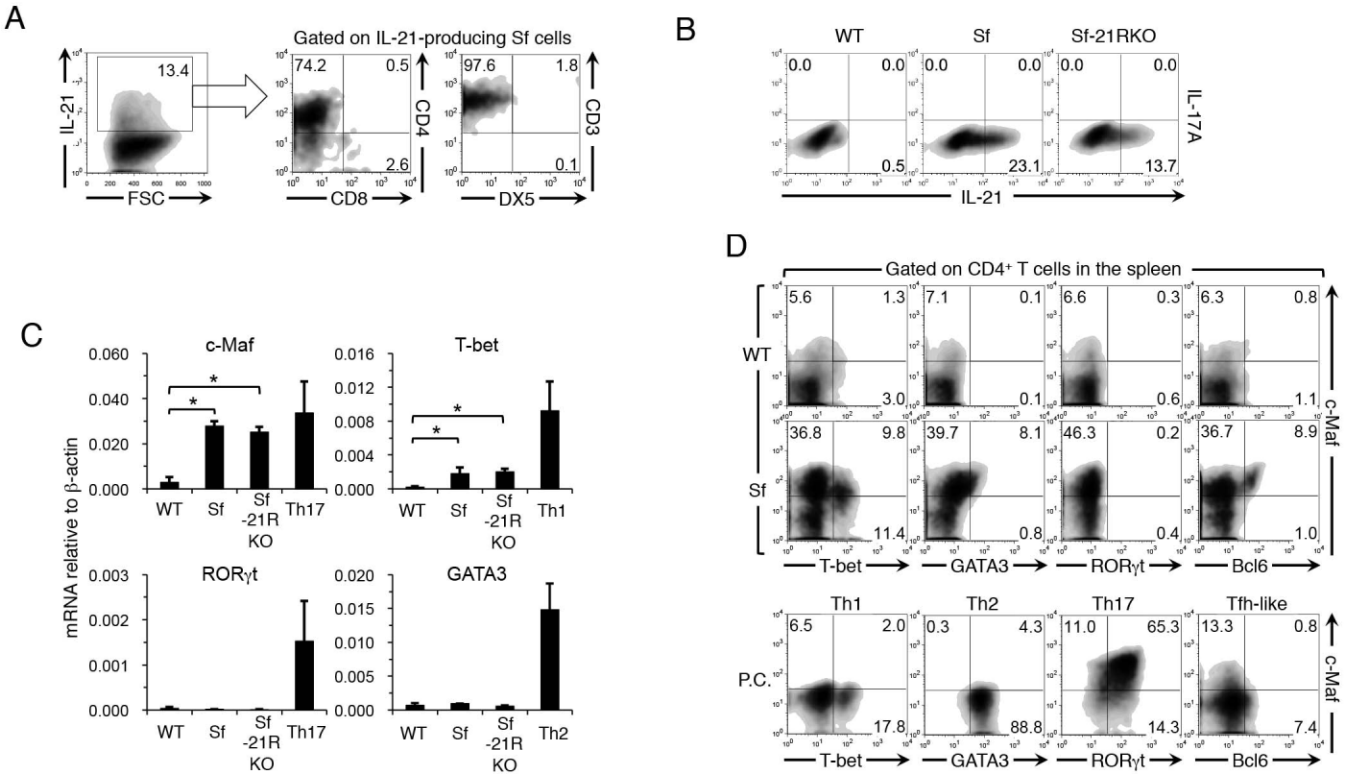

Figure 1. CD4+ T cells in sf mice express c-Maf and produce interleukin-21 (IL-21). **A,** Lymph node cells of sf mice were stimulated with anti-CD3/CD28 monoclonal antibody (mAb) for 7 days, and the expression of CD4 versus CD8 and of CD3 versus DX5 gating on IL-21–producing cells was evaluated by fluorescence-activated cell sorting. **B,** CD4+ T cells from lymph nodes of sf mice, IL-21 receptor–knockout (IL-21R–KO) sf mice (Sf-21RKO mice), and wild-type (WT) mice were stimulated with anti-CD3/CD28 mAb under neutral conditions, and the expression of IL-21 and IL-17A was assessed. Results shown are representative of 4 independent experiments. **C,** Expression of c-Maf, retinoic acid receptor–related orphan nuclear receptor γt (RORγt), T-bet, and GATA-3 mRNA in CD4+ T cells from sf mice, IL-21R–KO sf mice, and WT mice was assessed by quantitative polymerase chain reaction. Th1 cells (for T-bet), Th2 cells (for GATA-3), and Th17 cells (for c-Maf and RORγt) generated from WT mouse CD4+ T cells were used as positive controls (P.C.). Values are the mean ± SD (n = 5 mice for each genotype). $* = P < 0.05$. **D,** Expression levels of c-Maf, T-bet, GATA-3, RORγt, and Bcl-6 in splenic CD4+ T cells from sf mice and WT mice were analyzed. Th1 cells, Th2 cells, Th17 cells, and follicular helper T (Tfh)–like cells were used as positive controls. Results shown are representative of 4 independent experiments.

mental animal CT system (Aloka). The CT scanner was calibrated according to the manufacturer's protocols. The CT scan was done at 120-μm intervals. The lung parenchyma was segmented from each of the CT image volumes, and a histogram was generated from the lung CT values in Hounsfield Units.

Histologic analysis of the lung. Lung inflammation was quantified in a blinded manner by using a scoring system based on a previous report (35), including alveolar collapse, thickness of alveolar septa, alveolar fibrin deposition, and neutrophil infiltration graded on a scale of 0 to 4. In each tissue sample, 5 random areas were scored and the mean value was calculated. The histologic score is the sum of all 4 parameters.

Depletion of CD8+ T cells in vivo. Pregnant IL-21RHet$^{sf/x}$ female mice were given a single 500 μg dose of anti-CD8 mAb on day 15 postfertilization. Seven days after parturition, newborn male mice from these carriers were injected intraperitoneally with 250 μg of anti-CD8 mAb twice a week until analysis.

Statistical analysis. Data are summarized as the mean ± SD. The statistical analysis of the results was performed using an unpaired *t*-test. *P* values less than 0.05 were considered significant. Differences in survival were analyzed with Kaplan-Meier estimates, and groups were compared using the log rank test.

RESULTS

Development of IL-21–producing c-Maf–expressing CD4+ T cells in sf mice. T cells in FoxP3-mutant sf mice have been shown to express messenger RNAs (mRNAs) for many cytokines including IL-21 (19). However, the mechanisms underlying enhanced

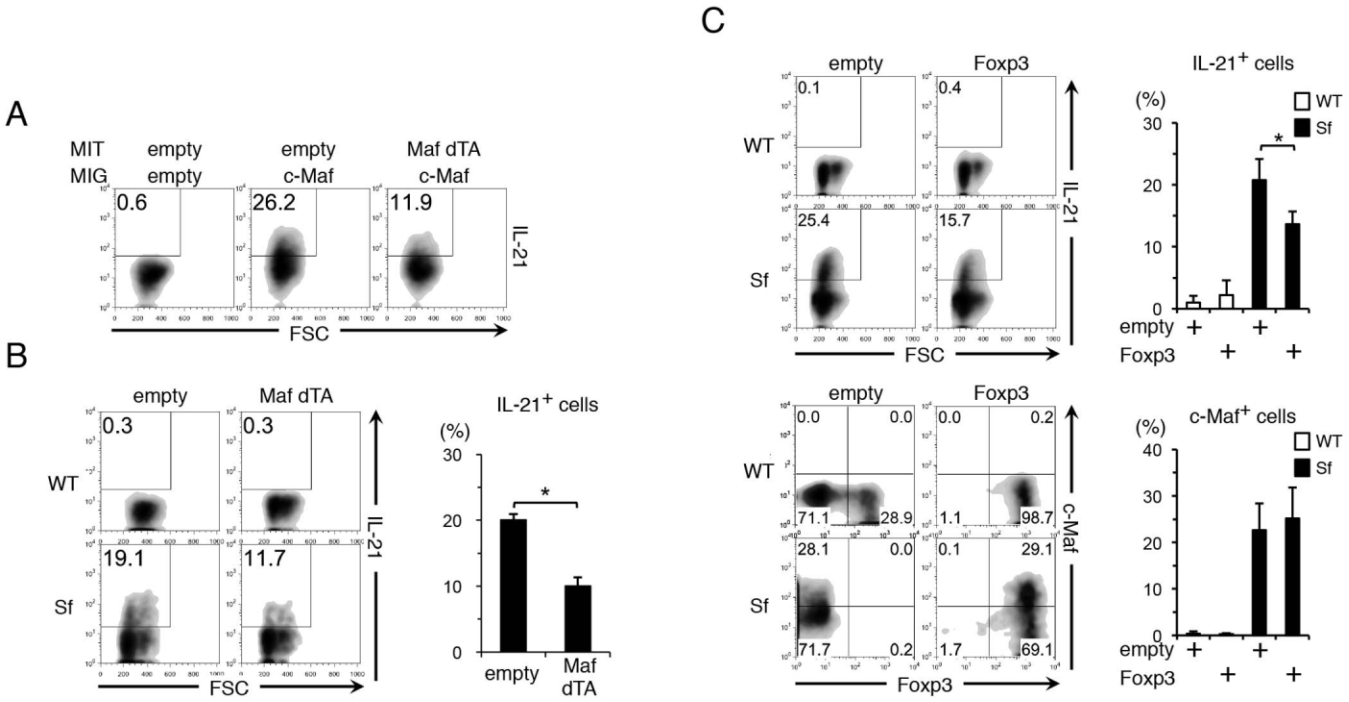

Figure 2. The transcription factor c-Maf induces enhanced IL-21 production in sf mouse CD4+ T cells. **A,** Naive CD4+ T cells from WT mice were stimulated with anti-CD3/CD28 mAb and coinfected with retroviruses of either MSCV-IRES-GFP (MIG) (empty vector) or MIG-c-Maf (c-Maf) and either MSCV-IRES-Thy1.1 (MIT) (empty vector) or MIT-Maf dTA (Maf dTA). Cells were cultured under neutral conditions for an additional 4 days, and the expression of IL-21 in green fluorescent protein–positive (GFP+) Thy1.1+ cells was evaluated. Results shown are representative of 5 independent experiments. **B,** CD4+ T cells from WT and sf mice were stimulated with anti-CD3/CD28 mAb and infected with retroviruses of MIT or MIT-Maf dTA. Cells were cultured under neutral conditions for an additional 4 days, and frequencies of IL-21–producing cells in Thy1.1+ cells were evaluated. Bars show the mean ± SD (n = 3 experiments). ∗ = $P < 0.05$. **C,** CD4+ T cells from WT and sf mice were stimulated with anti-CD3/CD28 mAb and infected with retroviruses of either MIG or MIG-FoxP3 for 24 hours. Cells were cultured under neutral conditions for an additional 4 days, and the expression of IL-21 versus FSC and of c-Maf versus FoxP3 gating on GFP+ cells was evaluated. Cell sorting results are representative of 3 independent experiments. Bars show the mean ± SD (n = 3 experiments). ∗ = $P < 0.05$. See Figure 1 for other definitions.

IL-21 expression and its role in the development of autoimmune inflammation in sf mice remain unknown. We first used intracellular cytokine staining to investigate the cell types that produce IL-21 in sf mice. We found that CD4+ T cells predominantly produced IL-21 upon stimulation with anti-CD3/CD28 mAb (Figure 1A). Sf mouse CD4+ T cells produced significantly greater amounts of IL-21 than did WT mouse CD4+ T cells, but did not produce IL-17A (Figure 1B). These IL-21–producing CD4+ T cells were negative for CXCR5 (data not shown), suggesting that these cells are different from Tfh cells. Because IL-21 itself enhances the production of IL-21 in CD4+ T cells (24), we also examined IL-21 production in IL-21R–knockout (KO) sf mice. IL-21 production was modestly decreased in IL-21R–KO sf mouse CD4+ T cells as compared to that in sf mouse CD4+ T cells (Figure 1B), suggesting that IL-21–producing CD4+ T cells develop in sf mice independently of the autocrine loop of IL-21 production.

We also examined the expression of transcription factors, which affect the differentiation of CD4+ T cells, in sf, IL-21R–KO sf, and WT mouse CD4+ T cells. Notably, the expression levels of c-Maf mRNA in sf mouse CD4+ T cells and IL-21R–KO sf mouse CD4+ T cells were significantly higher than those in WT mouse CD4+ T cells (both $P < 0.05$) (n = 5 mice in each group) and were comparable to those in Th17 cells (Figure 1C). Messenger RNA expression for T-bet, a master regulator of Th1 cells, was also significantly up-regulated in sf mouse CD4+ T cells and IL-21R–KO sf mouse CD4+ T cells (both $P < 0.05$) (Figure 1C). On the other hand, mRNA expression for RORγt, a lineage-specifying transcription factor of Th17 cells, was not significantly up-regulated in sf mouse CD4+ T cells and IL-21R–KO sf mouse CD4+ T cells compared to WT mouse CD4+ T cells (Figure 1C). We confirmed the enhanced expression of c-Maf proteins in sf mouse CD4+ T cells at single-cell levels by intracellular stain-

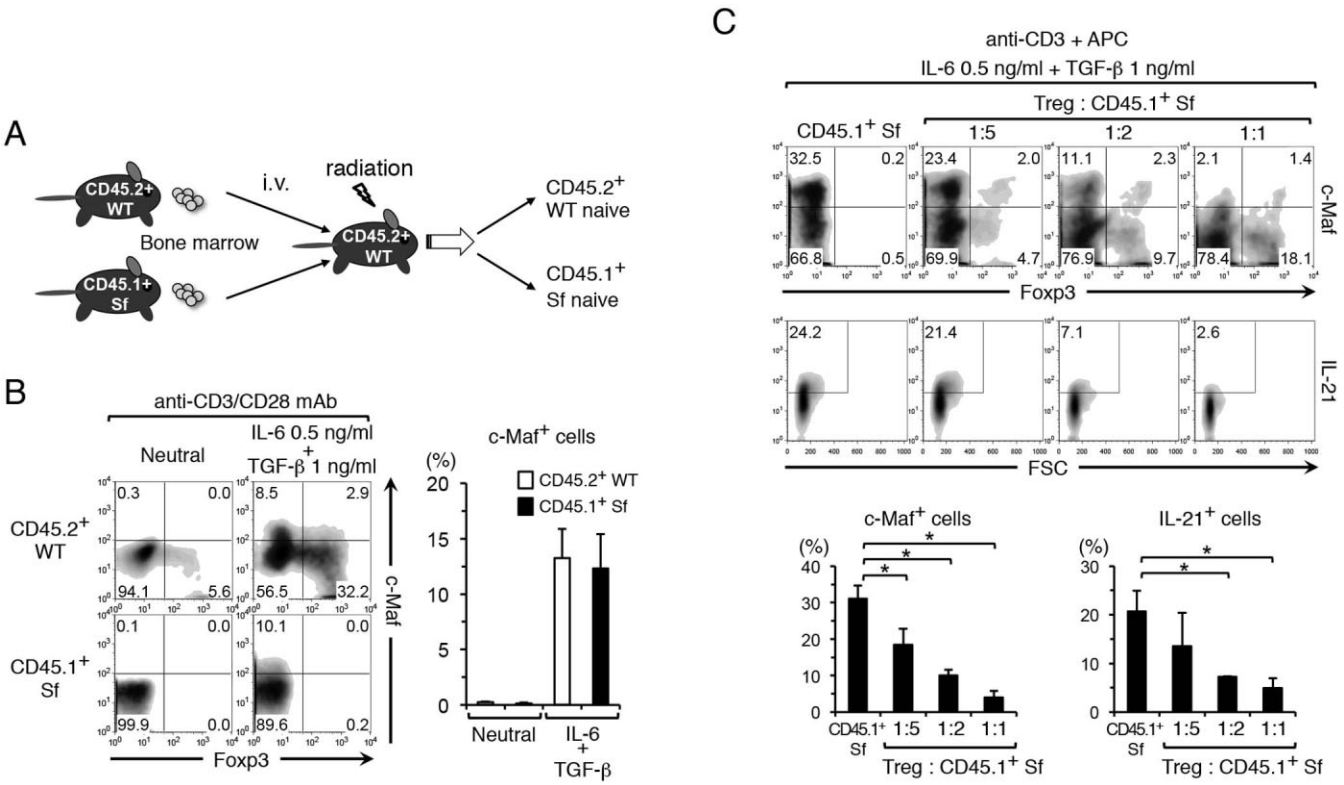

Figure 3. FoxP3+ Treg cells suppress the development of IL-21–producing c-Maf+CD4+ T cells by a cell-extrinsic mechanism. **A,** Bone marrow cells obtained from sf mice (CD45.1+) and WT mice (CD45.2+) were mixed and injected intravenously (IV) into irradiated WT mice (CD45.2+). Ten to 12 weeks after bone marrow reconstitution, CD45.1+ sf mouse naive CD4+ T cells and CD45.2+ WT mouse naive CD4+ T cells were purified from the chimeric mice. **B,** CD45.1+ sf mouse naive CD4+ T cells and CD45.2+ WT mouse naive CD4+ T cells were stimulated with anti-CD3/CD28 mAb in the presence or absence of IL-6 and transforming growth factor β (TGFβ) for 3 days, and the expression of c-Maf versus FoxP3 was evaluated by flow cytometry. Bars show the mean ± SD (n = 3 experiments). **C,** CD45.1+ sf mouse naive CD4+ T cells were stimulated with soluble anti-CD3 mAb plus irradiated antigen-presenting cells (APCs) in the presence of IL-6 and TGFβ for 3 days. CD4+CD25+ Treg cells isolated from WT mice were added to the culture at indicated ratios. The expression of c-Maf versus FoxP3 and of IL-21 versus FSC was evaluated. Bars show the mean ± SD (n = 3 experiments). $* = P < 0.05$. See Figure 1 for other definitions.

ing (Figure 1D). We found that part of c-Maf+CD4+ T cells in sf mice expressed T-bet, GATA-3, or Bcl-6, but not RORγt (Figure 1D). In contrast, conventional Th1 cells and Th2 cells did not express c-Maf, but Th17 cells expressed high levels of c-Maf and RORγt (Figure 1D). We also found that >95% of c-Maf+CD4+ T cells in sf mice exhibited a $CD44^{high}CD62L^{low}$ memory phenotype (data not shown). Taken together, these results indicate that sf mouse CD4+ T cells express high levels of c-Maf and produce large amounts of IL-21 and that IL-21–producing c-Maf+CD4+ T cells in sf mice are different from conventional Th17 cells.

Induction of IL-21 production in sf mouse CD4+ T cells by c-Maf. Previous studies have shown that c-Maf induces IL-21 production in CD4+ T cells (28,29). We therefore examined whether c-Maf expression is involved in IL-21 production in sf mouse CD4+ T cells by using a dominant-negative mutant of c-Maf lacking a transcription activation domain (Maf dTA). As expected, retrovirus-mediated induction of Maf dTA inhibited c-Maf–mediated IL-21 production in WT mouse CD4+ T cells (Figure 2A). As shown in Figure 2B, retrovirus-mediated induction of Maf dTA significantly decreased IL-21 production in sf mouse CD4+ T cells ($P < 0.05$) (n = 3 experiments). These results suggest that increased c-Maf expression is involved in enhanced IL-21 production in sf mouse CD4+ T cells.

FoxP3 intrinsically inhibits IL-21 production in sf mouse CD4+ T cells. We have previously shown that FoxP3 inhibits IL-21 production by binding to the regulatory elements of the IL-21 gene in CD4+ T cells (29). To determine whether FoxP3 regulates IL-21 produc-

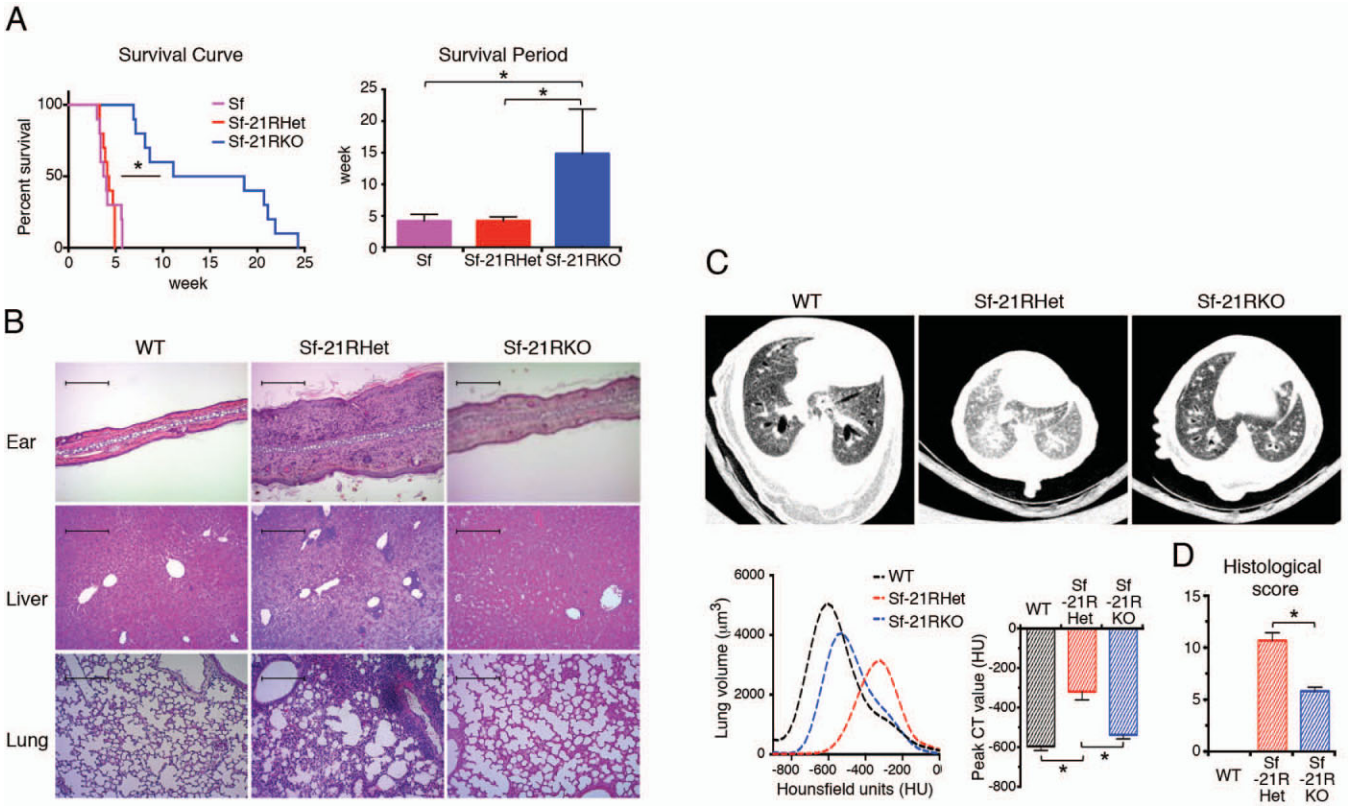

Figure 4. IL-21R–KO sf mice exhibit prolonged survival and reduced inflammatory cell infiltration in the skin, lung, and liver. **A,** Survival rates (left) and survival period (right) of sf mice, IL-21R$^{+/-}$ sf mice (Sf-21RHet mice), and IL-21R–KO sf mice. Left, $* = P < 0.01$ for IL-21R–KO sf mice versus both IL-21R$^{+/-}$ sf mice and sf mice, by log rank test (n = 10 mice per group). Right, Values are the mean ± SD (n = 10 mice per group). $* = P < 0.01$ by analysis of variance and unpaired *t*-test. **B,** Representative photomicrographs of the ear, liver, and lung of 3-week-old WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice. Hematoxylin and eosin stained; bars = 200 μm. **C,** Top, Representative computed tomography (CT) images of the lung. Bottom, Distribution of representative CT values in the pulmonary parenchyma (left), and mean ± SD peak CT values in 3-week-old WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice (n = 3 mice per group) ($* = P < 0.05$) (right). **D,** Lung histologic scores of 3-week-old WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice, quantified as described in Materials and Methods. Values are the mean ± SD (n = 3 mice per group). $* = P < 0.05$. See Figure 1 for other definitions.

tion and c-Maf expression in sf mouse CD4+ T cells, we examined the effect of the enforced expression of FoxP3 on IL-21 production in sf mouse CD4+ T cells. Consistent with our previous study (29), retrovirus-mediated FoxP3 induction in sf mouse CD4+ T cells significantly inhibited IL-21 production ($P < 0.05$) (n = 3 experiments) (Figure 2C). In contrast, retrovirus-mediated FoxP3 induction in sf mouse CD4+ T cells did not inhibit the expression of c-Maf (Figure 2C). These results suggest that the lack of FoxP3 may intrinsically increase IL-21 production in sf mouse CD4+ T cells without affecting c-Maf expression.

Development of IL-21–producing c-Maf+CD4+ T cells is suppressed by FoxP3+ Treg cells via a cell-extrinsic mechanism. To further determine the regulatory role of FoxP3 in the development of IL-21–producing c-Maf+CD4+ T cells in sf mice, we generated mixed bone marrow chimeric mice (CD45.2+ WT mice) having CD45.1+ sf mouse bone marrow cells and CD45.2+ WT mouse bone marrow cells (Figure 3A). Inflammatory lesions did not develop, and IL-21–producing CD4+ T cells did not increase in the chimeric mice (data not shown). When CD45.2+ WT mouse naive CD4+ T cells and CD45.1+ sf mouse naive CD4+ T cells were isolated from the chimeric mice and stimulated with anti-CD3/CD28 mAb under neutral conditions, c-Maf was not induced even in sf mouse CD4+ T cells (Figure 3B). In addition, c-Maf was similarly induced by sf mouse CD4+ T cells and WT mouse CD4+ T cells in the presence of IL-6 and TGFβ (Figure 3B). As expected, FoxP3+CD4+ T cells developed in CD45.2+ WT mouse CD4+ T cells but not in CD45.1+

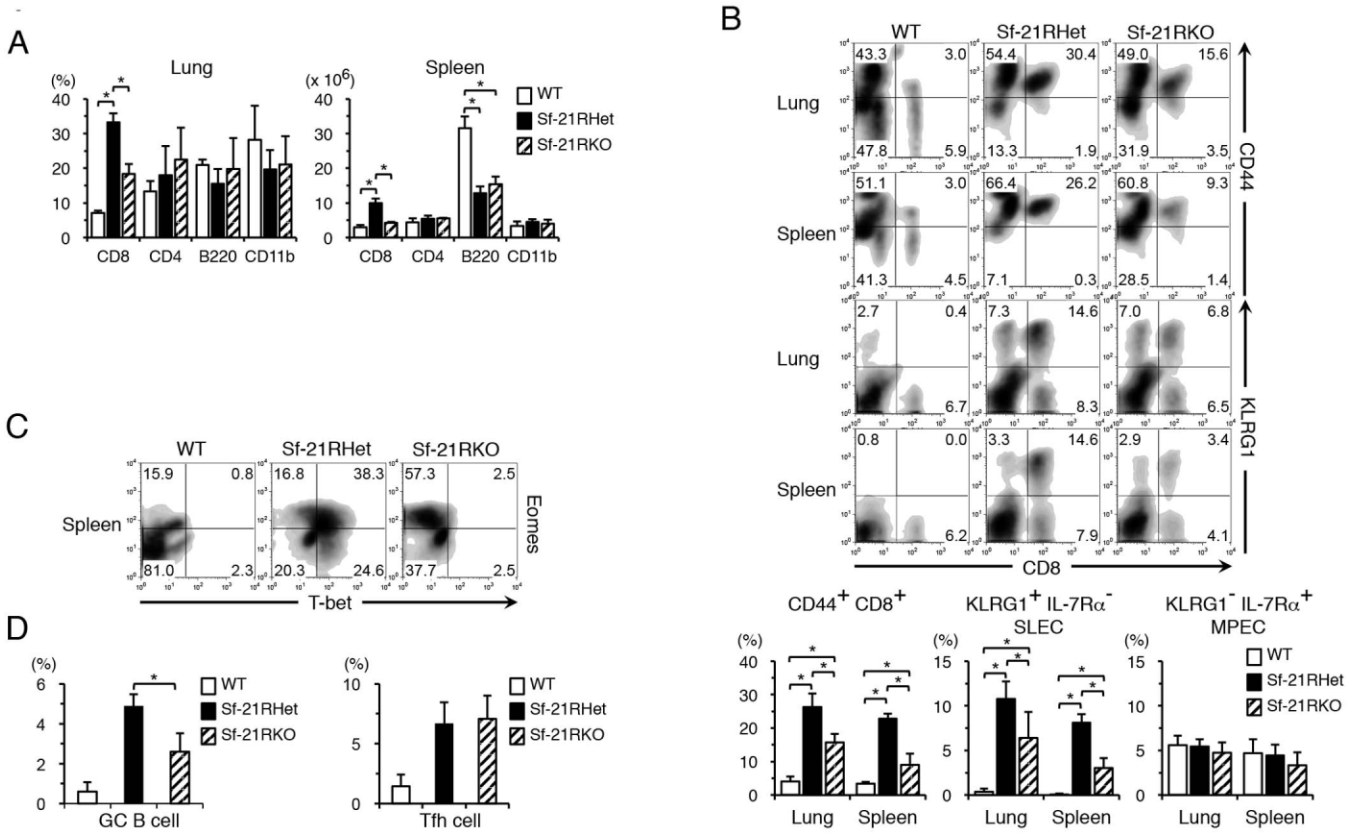

Figure 5. IL-21R signaling increases short-lived effector CD8+ T cells (SLECs) in the lungs of sf mice. **A,** Percentages of CD8+, CD4+, B220+, and CD11b+ (Mac-1+) cells in the lungs and spleens of WT mice, IL-21R$^{+/-}$ sf mice (Sf-21RHet mice), and IL-21R–KO sf mice, assessed by fluorescence-activated cell sorting (FACS). **B,** Representative FACS profiles of CD8 versus either CD44 or killer cell lectin-like receptor subfamily G member 1 (KLR-G1) (top) and frequencies of CD44+CD8+ T cells, KLR-G1+IL-7Rα− short-lived effector CD8+ T cells, and KLR-G1−IL-7Rα+ memory precursor effector CD8+ T cells (MPECs) in the lungs and spleens of WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice (bottom). **C,** Representative FACS profiles of T-bet versus Eomesodermin (Eomes) in splenic CD8+ T cells from WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice. **D,** Frequencies of GL-7+Fas+ germinal center (GC) B cells in splenic B220+ cells and of CXCR5+ programmed death 1–positive Tfh cells in splenic CD4+ T cells. Bars show the mean ± SD (n = 4 mice per group). ∗ = $P < 0.05$. See Figure 1 for other definitions.

sf mouse CD4+ T cells (Figure 3B). These results suggest that cell-intrinsic defects in FoxP3 in sf mouse CD4+ T cells may not be significantly involved in the development of c-Maf+CD4+ T cells in sf mice.

On the other hand, the addition of CD25+ Treg cells from WT mice dose-dependently inhibited the expression of c-Maf and the production of IL-21 in CD45.1+ sf mouse CD4+ T cells that were stimulated with soluble anti-CD3 mAb plus irradiated APCs in the presence of IL-6 and TGFβ (Figure 3C). Taken together, these results suggest that the lack of cell-extrinsic suppression by FoxP3+ Treg cells results in the development of IL-21–producing c-Maf+CD4+ T cells in sf mice.

Prolonged survival and reduced inflammatory cell infiltration in the skin, lung, and liver of IL-21R–KO sf mice. To examine the role of the enhanced production of IL-21 in the pathophysiology of sf mice, we next analyzed the survival of sf mice, IL-21R–KO sf mice, and IL-21R$^{+/-}$ sf mice. As shown in Figure 4A, the survival of IL-21R–KO sf mice was significantly prolonged compared with that of IL-21R$^{+/-}$ sf mice or sf mice ($P < 0.01$) (n = 10 mice per group). The mean survival period of IL-21R–KO sf mice was 3.5-fold longer than that of IL-21R$^{+/-}$ sf mice (mean ± SD 14.8 ± 7.0 weeks versus 4.2 ± 0.6 weeks; $P < 0.01$). Consistently, inflammatory infiltrates in the ear, lung, and liver at age 3 weeks were less pronounced in

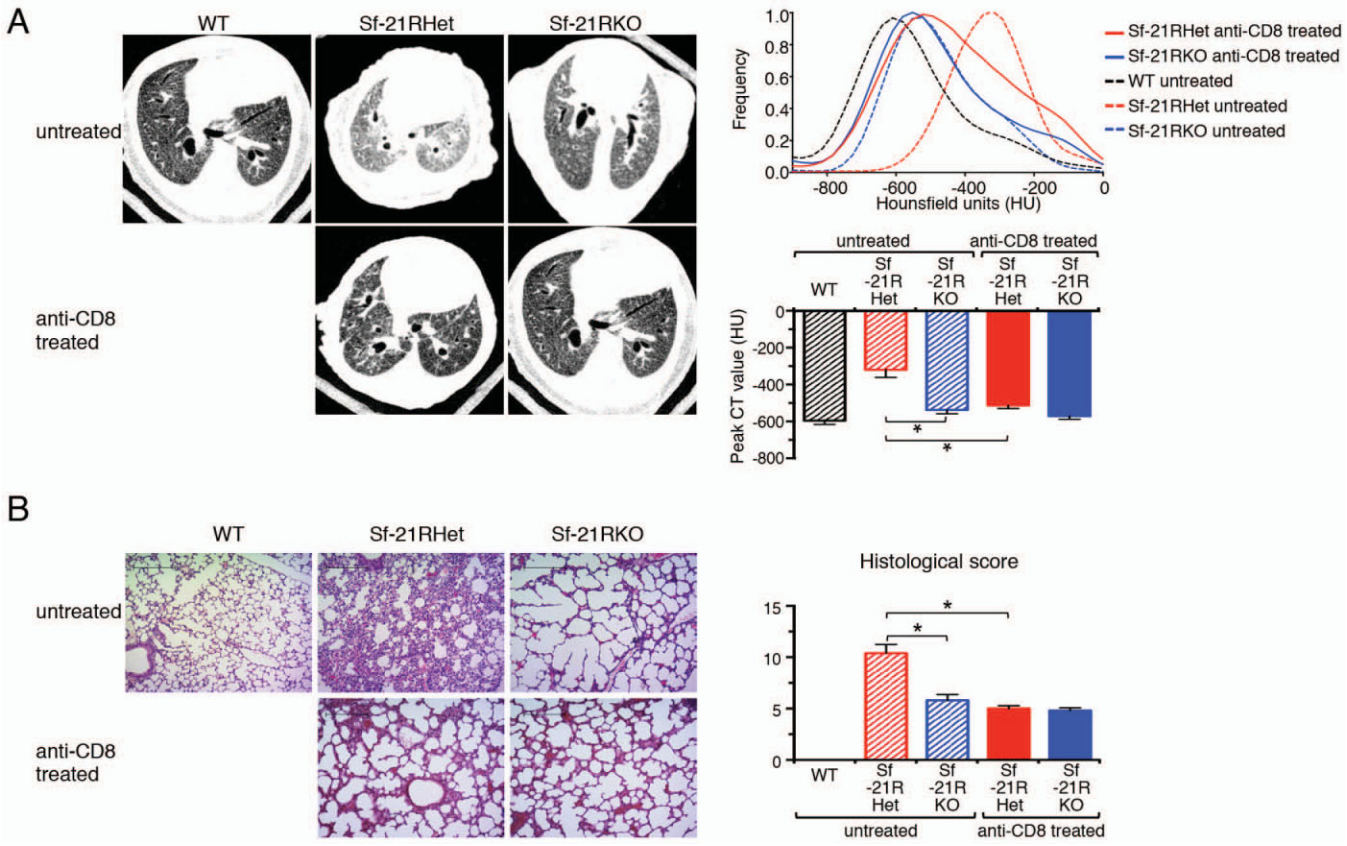

Figure 6. Depletion of CD8+ T cells ameliorates lung inflammation in sf mice. **A,** Left, Representative computed tomography (CT) images of the lung. Right, Distribution of representative CT values in the pulmonary parenchyma (top), and mean ± SD peak CT values in anti-CD8 mAb–treated or untreated IL-21R$^{+/-}$ sf mice (Sf-21RHet mice) and IL-21R–KO sf mice (n = 3 mice per group) (∗ = $P < 0.05$) (bottom). Age-matched WT mice were used as a control. **B,** Left, Representative photomicrographs of the lung of anti-CD8 mAb–treated or untreated IL-21R$^{+/-}$ sf mice and IL-21R–KO sf mice. Hematoxylin and eosin stained; bars = 200 μm. Right, Lung histologic scores of anti-CD8 mAb–treated or untreated IL-21R$^{+/-}$ sf mice and IL-21R–KO sf mice. Age-matched WT mice were used as a control. Values are the mean ± SD (n = 3 mice per group). ∗ = $P < 0.05$. See Figure 1 for other definitions.

IL-21R–KO sf mice than in littermate IL-21R$^{+/-}$ sf mice (Figure 4B). CT scan of the lung revealed that ground-glass opacities were reduced in IL-21R–KO sf mice compared with IL-21R$^{+/-}$ sf mice (Figure 4C). Quantitative analysis of the lung intensity by CT scan showed that the lung parenchyma in IL-21R$^{+/-}$ sf mice exhibited significantly increased intensities compared to those in IL-21R–KO sf mice and WT mice ($P < 0.05$) (n = 3 mice per group) (Figure 4C). In addition, quantification of lung histology showed that lung inflammation was significantly attenuated in IL-21R–KO sf mice compared to IL-21R$^{+/-}$ sf mice ($P < 0.05$) (Figure 4D). These results indicate that IL-21R signaling is significantly involved in the induction of autoimmune inflammation in multiple organs (especially in the lung) and in the shortened survival in sf mice.

Short-lived effector CD8+ T cells in the lungs of sf mice are increased by IL-21R signaling. To determine the effector cell populations that are involved in lung inflammation in sf mice, we next examined cell populations in the lung and spleen of WT mice, IL-21R$^{+/-}$ sf mice, and IL-21R–KO sf mice. As shown in Figure 5A, the frequency of CD8+ T cells in the lung was significantly increased in IL-21R$^{+/-}$ sf mice compared with that in WT mice ($P < 0.05$) (n = 4 mice per group). Importantly, the frequency of CD8+ T cells in the lung was significantly decreased in IL-21R–KO sf mice compared with that in IL-21R$^{+/-}$ sf mice ($P < 0.05$) (Figure 5A). Among CD8+ T cells, CD44+ cells were significantly decreased in the lung in IL-21R–KO sf mice compared with IL-21R$^{+/-}$ sf mice (Figure 5B), consistent with a previous finding that CD44+CD8+ T cells

are increased in IL-21–transgenic mice (36). In addition, KLR-G1+IL-7Rα− short-lived effector CD8+ T cells (37,38) were significantly decreased in IL-21R–KO sf mice compared to IL-21R$^{+/-}$ sf mice (Figure 5B).

In contrast, numbers of KLR-G1−IL-7Rα+ memory precursor effector CD8+ T cells were not significantly different among IL-21R$^{+/-}$ sf mice, IL-21R–KO sf mice, and WT mice (Figure 5B). Furthermore, T-bet was significantly down-regulated in IL-21R–KO sf mouse CD8+ T cells compared to IL-21R$^{+/-}$ sf mouse CD8+ T cells (Figure 5C), consistent with previous findings that IL-21 up-regulates T-bet expression in CD8+ T cells (39) and that high T-bet expression induces short-lived effector CD8+ T cells (36). Taken together, these results suggest that IL-21R signaling enhances the expansion of short-lived effector CD8+ T cells, possibly via the induction of T-bet in sf mice.

Because IL-21 is produced by Tfh cells and promotes the differentiation of germinal center (GC) B cells (40), we next examined the frequency of Tfh cells and GC B cells in IL-21R$^{+/-}$ sf mice, IL-21R–KO sf mice, and WT mice. As shown in Figure 5D, GC B cells were significantly reduced in IL-21R–KO sf mice compared to IL-21R$^{+/-}$ sf mice. On the other hand, the numbers of Tfh cells did not differ significantly between IL-21R$^{+/-}$ sf mice and IL-21R–KO sf mice.

Amelioration of lung inflammation in sf mice by depletion of CD8+ T cells. To determine the role of CD8+ T cells in lung inflammation in sf mice, we examined the effect of in vivo depletion of CD8+ T cells on lung inflammation in sf mice. IL-21R–KO sf mice, IL-21R$^{+/-}$ sf mice, and WT mice were treated with anti-CD8 mAb from day 15 postfertilization, and CT scan and histologic analysis of the lung were performed at age 3 weeks. In this experimental protocol, the number of CD8+ T cells in the spleen was reduced by ~90%, whereas the number of CD4+ T cells was not significantly reduced. The quantitative analysis of lung intensity by CT scan showed that lung inflammation was significantly decreased by treatment with anti-CD8 mAb in IL-21R$^{+/-}$ sf mice ($P < 0.05$) (n = 3 mice per group) (Figure 6A). In addition, lung histology showed that lung inflammation was significantly decreased by treatment with anti-CD8 mAb in IL-21R$^{+/-}$ sf mice ($P < 0.05$) (Figure 6B). Furthermore, histologic scores did not differ between anti-CD8 mAb–treated IL-21R$^{+/-}$ sf mice and IL-21R–KO sf mice. These results suggest that CD8+ T cells induced by IL-21 are critical for inducing lung inflammation in sf mice.

DISCUSSION

In this study, we show that unique IL-21–producing c-Maf+CD4+ T cells develop in FoxP3-mutant sf mice, and that IL-21 produced by c-Maf+CD4+ T cells induces short-lived effector CD8+ T cells and accelerates autoimmune inflammation in the lung, skin, and liver in sf mice. We found that sf mouse CD4+ T cells express high levels of c-Maf and produce large amounts of IL-21, and that these cells are different from conventional Th17 cells in that sf mouse CD4+ T cells do not express RORγt or produce IL-17A (Figure 1). Our findings also suggest that increased c-Maf expression induces enhanced IL-21 production in sf mouse CD4+ T cells (Figure 2). Furthermore, we found that IL-21R deficiency results in prolonged survival and reduced multiorgan inflammation (Figure 4) as well as in decreased numbers of short-lived effector CD8+ T cells in the lung (Figure 5) in sf mice. Finally, we found that administration of anti-CD8 mAb reduced lung inflammation in sf mice (Figure 6). These results indicate that IL-21–producing c-Maf+CD4+ T cells are importantly involved in inducing autoimmune inflammation in sf mice, presumably through induction of short-lived effector CD8+ T cells.

We show that the lack of cell-extrinsic suppression by FoxP3+ Treg cells is mainly involved in the development of IL-21–producing c-Maf+CD4+ T cells in sf mice. We found that the enforced expression of FoxP3 modestly decreased IL-21 production in sf mouse CD4+ T cells, but it did not inhibit c-Maf expression in sf mouse CD4+ T cells (Figure 2). Importantly, in the experiments using mixed bone marrow chimeric mice, c-Maf expression was not significantly enhanced in sf mouse CD4+ T cells compared to WT mouse CD4+ T cells when these naive CD4+ T cells were stimulated with anti-CD3/CD28 mAb in the presence of IL-6 and TGFβ (Figure 3). On the other hand, CD25+ Treg cells from WT mice dose-dependently inhibited the development of c-Maf+CD4+ T cells from naive sf mouse CD4+ T cells when these cells were stimulated with anti-CD3 mAb plus irradiated APCs in the presence of IL-6 and TGFβ (Figure 3). Taken together, these results suggest that FoxP3 does not intrinsically suppress c-Maf induction in CD4+ T cells, but that FoxP3+ Treg cells suppress the development of IL-21–producing c-Maf+CD4+ T cells by a cell-extrinsic mechanism. Among many mechanisms suggested for Treg cell–mediated suppression (41), we speculate that Treg cells possibly suppress the development of IL-21–producing c-Maf+CD4+ T cells by in-

hibiting APC function through down-regulation of CD80 and CD86 on APCs via CTLA-4.

Our results suggest that c-Maf is involved in enhanced IL-21 production in sf mouse CD4+ T cells. We found that enforced expression of a dominant-negative c-Maf significantly inhibited IL-21 production in sf mouse CD4+ T cells (Figure 2B). We have previously shown that enforced expression of c-Maf induces the expression of IL-21 in CD4+ T cells by binding to its promoter and enhancer (29). Our finding is also consistent with a previous finding that IL-21 production is impaired in c-Maf–deficient CD4+ T cells (28). Further studies are needed to identify other factors involved in IL-21 production and to elucidate the relative contribution of these factors to enhanced IL-21 production in sf mouse CD4+ T cells.

We show that IL-21R signaling induces autoimmune inflammation in multiple organs in sf mice through the induction of short-lived effector CD8+ T cells. We found that lack of IL-21R signaling prolonged survival and reduced multiorgan inflammation, including that in the lung, in sf mice (Figure 4). Moreover, we found that CD8+ T cells were increased in the lung in sf mice and that lack of IL-21R signaling decreased CD8+ T cells, especially short-lived effector CD8+ T cells, in the lung in sf mice (Figure 5). Furthermore, depletion of CD8+ T cells significantly inhibited lung inflammation in sf mice (Figure 6). Importantly, the severity of lung inflammation in IL-21R$^{+/-}$ sf mice was decreased by the depletion of CD8+ T cells to a degree similar to that in IL-21R–KO sf mice (Figure 6). These results suggest that IL-21R signaling induces the proliferation and/or survival of short-lived effector CD8+ T cells in autoimmune inflammation in sf mice.

Regarding the relationship between IL-21 and CD8+ T cell function, it has been reported that CD44+ memory CD8+ T cells are increased in IL-21–transgenic mice (36). In addition, it has been shown that a lack of IL-21 signaling in CD8+ T cells results in the reduction of pathogen-specific CD8+ T cells and the failure to control viral infection (37,42–45). Moreover, it has been demonstrated that the enforced expression of IL-21 in pancreatic beta cells leads to cytotoxic CD8+ T cell differentiation via the induction of T-bet and results in type 1 diabetes mellitus (39). In contrast, it has been reported that IL-21 supports the expansion of CD8+ T cells with suppressive function and broadly suppresses the activation of CD4+ T cells in lupus-like diseases in BXSB.Yaa mice (46). These findings suggest that IL-21 regulates the function of effector CD8+ T cells as well as that of suppressor CD8+ T cells, and that the roles of IL-21 in autoimmunity may depend on the relative contribution of these subsets to each autoimmune disease model.

Our findings in the experiments of CD8+ T cell depletion (Figure 6) suggest that CD8+ T cells are critical for inducing lung inflammation in sf mice. In contrast, Blair et al have reported that the depletion of CD8+ T cells in scurfy sparse fur double-mutant mice (which have 2 point mutations of FoxP3 and the ornithine transcarbamoylase [*otc*] gene) on a 129/Rl background did not significantly alter the extent or course of sf mouse phenotypes (7). The discrepancy between the findings of Blair et al and our findings may be caused by the difference in the genetic background of mice or the additional mutation in the *otc* gene.

We also found that GC B cells are significantly decreased in IL-21R–KO sf mice compared to IL-21R$^{+/-}$ sf mice (Figure 5D). This is consistent with a finding that IL-21 plays an important role in optimal proliferation of GC B cells (40). Because it has been reported that hypergammaglobulinemia is a clinical symptom in sf mice (4) and that the lack of B cells improves sf mouse phenotypes (47), the decrease in GC B cells may be involved in the prolonged survival of IL-21R–KO sf mice.

Sf mice are natural mutant mice with disease resembling the human IPEX syndrome. Although the main features of IPEX syndrome are early-onset type 1 diabetes mellitus, severe enteropathy, eczema, and hypothyroidism, pulmonary involvement is sometimes observed and related to cause of death (6). Consistent with the sf mouse phenotype, it has been shown that frequencies of memory/effector CD4+ T cells and CD8+ T cells are increased in patients with IPEX syndrome (48), suggesting that IL-21 may be involved in the increase in memory/effector CD8+ T cells in these patients. Although further studies are needed, our results suggest that IL-21/IL-21R signaling could be a therapeutic target for patients with IPEX syndrome.

In conclusion, we have shown that IL-21–producing c-Maf+CD4+ T cells develop in the absence of FoxP3+ Treg cells, and that IL-21 produced by c-Maf+CD4+ T cells enhances proliferation of effector CD8+ T cells, leading to exacerbation of interstitial lung inflammation in sf mice. These findings should provide a new insight into the mechanism of autoimmune inflammation caused by the lack or dysfunction of Treg cells.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Suto and Nakajima had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. T. Iwamoto, Suto, Takatori, Suzuki, Nakajima.

Acquisition of data. T. Iwamoto, Suto, Tanaka.

Analysis and interpretation of data. T. Iwamoto, Suto, Takatori, Suzuki, I. Iwamoto.

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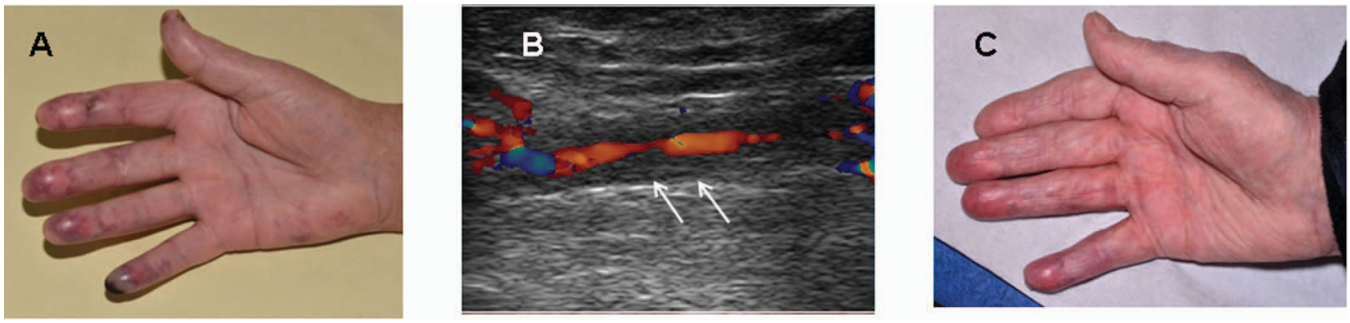
Clinical Images: Digital necrosis revealing giant cell arteritis



The patient, a 78-year-old woman, presented with ischemia of the right hand, necrosis of the proximal phalanx of the fifth finger, and necrosis and subungual necrosis of the second, third, and fourth fingers (**A**). She had experienced a 15-kg weight loss over the prior 6 months. Her medical history was unremarkable. The patient did not have a cardiac murmur, but her temporal pulses were diminished. Laboratory test results included a C-reactive protein level of 68 mg/liter. Test results for antineutrophil cytoplasmic antibodies, cryoglobulins, and antiphospholipid antibodies were normal, and findings on serologic tests for viral infection (human immunodeficiency virus 1, hepatitis B virus, hepatitis C virus, and cytomegalovirus) were negative. Blood cultures were negative. Doppler ultrasonography showed bilateral stenosis of the subclavian and axillary arteries, with features of inflammation (**arrows** in **B**). This stenosis was confirmed by computed tomographic angiography. Giant cell arteritis was suspected, and the results of a temporal artery biopsy confirmed the diagnosis. Treatment with low molecular weight heparin, aspirin, glucocorticoids, and iloprost was initiated. The inflammatory parameters rapidly improved, but the necrosis worsened. Repeat Doppler ultrasonography revealed an occlusion of the right subclavian and ulnar arteries, which was confirmed by angiography. Subclavian angioplasty was performed, with subsequent gradual improvement and complete healing by 6 months after the procedure (**C**). Digital ischemia is a rare initial manifestation of giant cell arteritis. In this case, the hand ischemia was related to reduced arterial blood flow secondary to proximal axillary artery stenosis. Medical and surgical treatment led to healing.

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