

# Engagement of SLAMF3 enhances CD4<sup>+</sup> T-cell sensitivity to IL-2 and favors regulatory T-cell polarization in systemic lupus erythematosus

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Edited by Dennis A. Carson, University of California, San Diego, La Jolla, CA, and approved June 29, 2016 (received for review April 5, 2016)

**Signaling lymphocytic activation molecule family 3 (SLAMF3/Ly9) is a coregulatory molecule implicated in T-cell activation and differentiation. Systemic lupus erythematosus (SLE) is characterized by aberrant T-cell activation and compromised IL-2 production, leading to abnormal regulatory T-cell (Treg) development/function. Here we show that SLAMF3 functions as a costimulator on CD4<sup>+</sup> T cells and influences IL-2 response and T helper cell differentiation. SLAMF3 ligation promotes T-cell responses to IL-2 via up-regulation of CD25 in a small mothers against decapentaplegic homolog 3 (Smad3)-dependent manner. This augments the activation of the IL-2/IL-2R/STAT5 pathway and enhances cell proliferation in response to exogenous IL-2. SLAMF3 costimulation promotes Treg differentiation from naïve CD4<sup>+</sup> T cells. Ligation of SLAMF3 receptors on SLE CD4<sup>+</sup> T cells restores IL-2 responses to levels comparable to those seen in healthy controls and promotes functional Treg generation. Taken together, our results suggest that SLAMF3 acts as potential therapeutic target in SLE patients by augmenting sensitivity to IL-2.**

SLE | autoimmunity | IL-2 | Treg | SLAMF3

**S**ignaling lymphocytic activation molecule family members (SLAMF1-9) are type I transmembrane glycoprotein cell surface receptors that deliver downstream signals on their engagement (1). SLAMF3 (CD229/Ly9) is expressed on T cells, B cells, NK cells, macrophages, and dendritic cells (2). SLAMF3 acts as a self-ligand through the binding of the N-terminal Ig domain of SLAMF3 (3).

SLAMF3 has been proposed to be involved in the immunopathogenesis of systemic lupus erythematosus (SLE), a multisystem autoimmune disease characterized by a loss of tolerance to endogenous antigen, leading to autoantibody production and to a wide range of clinical manifestations (4). The SLAMF3 encoding gene is located on chromosome 1 within 1q23, a region known to be associated with increased susceptibility for SLE development (5, 6), and polymorphisms of SLAMF3 have been described in SLE families (7, 8). SLAMF3-deficient mice spontaneously develop SLE-associated autoantibodies (9) and display impaired T-cell proliferation and compromised antigen-driven IL-2 production (10).

T cells are key players in the pathogenesis of SLE. Impaired IL-2 production by activated T cells is a hallmark of both murine and human SLE (4, 11–13). Previous studies have shown that CD4<sup>+</sup> T cells from SLE patients display lower levels of CD25, the  $\alpha$  chain of the IL-2 receptor (IL-2R), compared with healthy subjects (14–16). IL-2 helps maintain peripheral immune self-tolerance and plays a critical role in the differentiation, survival, and function of regulatory T cells (Tregs) (4, 12). Tregs express the nuclear transcription factor forkhead box protein 3 (FoxP3) and the IL-2R $\alpha$  chain CD25. IL-2-deficient mice develop a severe lupus-like phenotype, characterized by diminished number of Tregs (17). Most reports on human SLE have shown abnormal numbers/function of Tregs in the periphery (18–22). Replenishment of IL-2 in lupus-prone mice and in human SLE patients ameliorates disease manifestations through the expansion of Tregs (16, 23–27).

The beneficial effect of low-dose IL-2 treatment has been characterized in graft-vs.-host disease (GVHD) and hepatitis C-associated vasculitis, where it augments the numbers of functional Tregs in the periphery after treatment (28–30).

Here we demonstrate that SLAMF3 costimulation enhances CD4<sup>+</sup> T-cell sensitivity to IL-2 by up-regulating the IL-2R $\alpha$  chain in a small mothers against decapentaplegic homolog 3 (Smad3)-dependent manner, resulting in the generation of functional Tregs. In CD4<sup>+</sup> T cells isolated from SLE patients, engagement of SLAMF3 restores the sensitivity of IL-2R to IL-2 and the generation of competent Tregs.

## Results

**Anti-SLAMF3 Acts as a Coactivator of CD4<sup>+</sup> T Cells.** We examined the effect of SLAMF3 ligation on the IL-2/IL-2R/STAT5 pathway in naïve CD4<sup>+</sup> T cells. IL-2 signals through the IL-2R, which is composed of three chains: CD25 (IL-2R $\alpha$ ), CD122 (IL-2R $\beta$ ), and CD132 (IL-2R common  $\gamma$ -chain). Binding of IL-2 to its receptor leads to phosphorylation of the STAT5A/B transcription factors (31). Compared with CD28 costimulation or to CD3/isotype control, SLAMF3 costimulation increased the expression of CD25 on the cell surface after 18 h of stimulation (Fig. 1A and Fig. S1).

SLAMF3-induced expression of CD25 is regulated at the transcriptional level, as shown by greater levels of CD25 mRNA after anti-CD3/anti-SLAMF3 stimulation compared with

## Significance

**Systemic lupus erythematosus (SLE) is characterized by compromised IL-2 production and regulatory T-cell function. Studies in human SLE and in murine lupus models report that IL-2 replenishment ameliorates clinical lupus manifestations. Here we show that engagement of signaling lymphocytic activation molecule family 3 (SLAMF3), a coregulatory receptor of T cells, restores the sensitivity of SLE CD4<sup>+</sup> T cells to IL-2, increasing their response to exogenous IL-2 via up-regulation of the IL-2R $\alpha$  subunit. Moreover, activation of naïve CD4<sup>+</sup> T cells with a monoclonal antibody directed against SLAMF3 promotes T helper cell differentiation toward a suppressive phenotype. These data suggest that the SLAMF3 receptor may be a promising therapeutic target in SLE.**

Author contributions: D.C., M.P.K., K.K.-T., and G.C.T. designed research; D.C., M.P.K., K.K.-T., N.Y., S.J.B., M.M., M.K., and J.R.S. performed research; D.C., M.P.K., and K.K.-T. contributed new reagents/analytic tools; D.C., M.P.K., K.K.-T., N.Y., S.J.B., M.M., V.C.K., and G.C.T. analyzed data; and D.C., M.P.K., and G.C.T. wrote the paper.

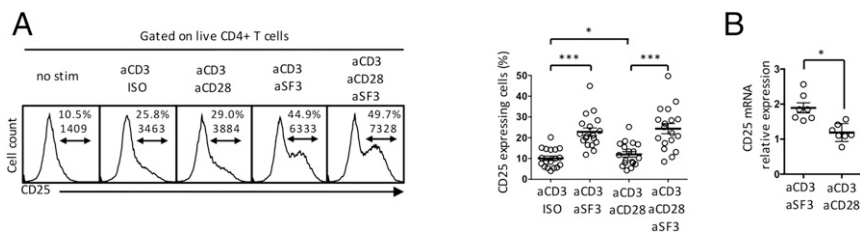
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605081113/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605081113/-DCSupplemental).



**Fig. 1.** SLAMF3 costimulation promotes the expression of the IL-2 receptor CD25 on human naïve CD4<sup>+</sup> T cells. (A) Naïve CD4<sup>+</sup> T cells were stimulated for 18 h, after which the expression of CD25 was assessed by flow cytometry. Shown are representative flow panels [top number, frequency (%); bottom number, mean fluorescence intensity (MFI)] and cumulative data (mean  $\pm$  SEM);  $n = 18$ . (B) Naïve CD4<sup>+</sup> T cells were stimulated for 6 h. CD25 mRNA was assessed by real-time quantitative RT-PCR (qRT-PCR) and is expressed as CD3/SLAMF3 or CD3/CD28 normalized to CD3/IgG isotype control. Data are mean  $\pm$  SEM;  $n = 7$ .

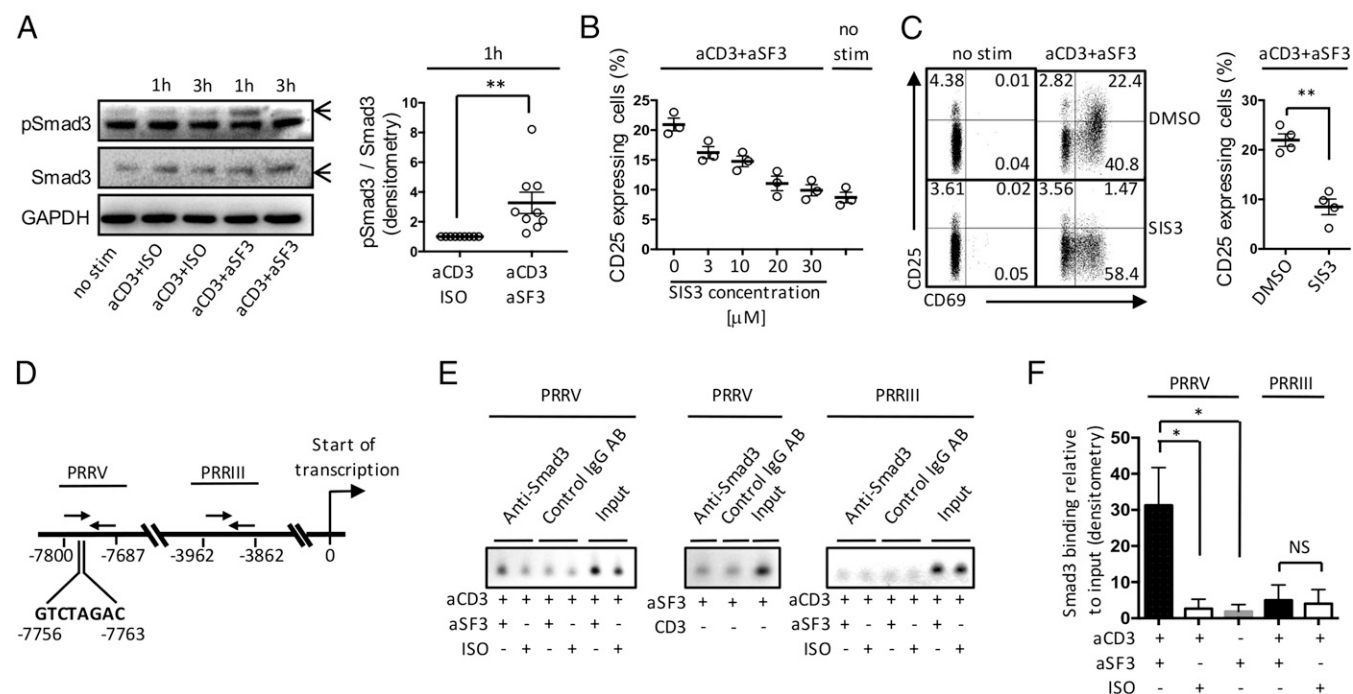
anti-CD3/anti-CD28 treatment (Fig. 1B). Examination of the promoter region of *CD25* for transcription factor binding sites identified Smad3 as a candidate capable of controlling *CD25* transcription (31).

We examined phosphorylation of Smad3 after stimulation of naïve CD4<sup>+</sup> T cells with anti-CD3 and anti-SLAMF3 monoclonal antibodies (mAbs) and found increased pSmad3 after 1 h (Fig. 2A), with no differences in the amount of total Smad3 protein. A specific inhibitor of Smad3 (SIS3) phosphorylation and Smad3-mediated cellular signaling, with no effect on Smad2, p38 MAPK, ERK, or PI-3K signaling (32), reduced SLAMF3-driven CD25 expression on naïve CD4<sup>+</sup> T cells in a dose-dependent manner (Fig. 2B). This occurred without abolishing cell activation, with expression of CD69 barely affected by SIS3 (Fig. 2C).

To confirm these findings, we used two small interfering RNAs (siRNAs) to knock down Smad3 in human CD4<sup>+</sup> T cells, which resulted in an 80–90% reduction in Smad3 expression (Fig. S2A). Compared with cells transfected with control siRNA,

Smad3 siRNA-transduced cells displayed a significant decrease in CD25 expression after 18 h of stimulation with anti-SLAMF3 mAb (Fig. S2B), suggesting that CD25 expression in response to SLAMF3 activation depends on Smad3. In accordance with the previous experiment, Smad3 siRNA CD4<sup>+</sup> T-cell activation was not completely abrogated, with CD69 expression still increased after stimulation (Fig. S2B).

To confirm that SLAMF3 favors *CD25* gene expression in an Smad3-dependent manner, we performed chromatin immunoprecipitation (ChIP) assays in Jurkat cells to examine the binding of Smad3 to *CD25* gene regulatory regions. Previous studies described six upstream positive regulatory regions (PRRs) that control the transcription of *CD25* gene (31, 33). Among these six PRRs, Smad3 is known to be able to bind to PRRV in response to TGF $\beta$ 1 and TCR engagement (34). Accordingly, we examined the binding of Smad3 to the regulatory PRRV region of *CD25* gene in response to SLAMF3 costimulation. PRRIII was used as



**Fig. 2.** CD25 up-regulation following SLAMF3 coengagement is Smad3-dependent. Expression of pSmad3, total Smad3, and GAPDH by stimulated naïve CD4<sup>+</sup> T cells was assessed by Western blot analysis. (A) Representative blot showing the expression of pSmad3 and total Smad3 at 1 h and 3 h of stimulation, and cumulative data showing the densitometry ratio of pSmad3 over total Smad3 after 1 h of stimulation. Data are mean  $\pm$  SEM;  $n = 9$ . (B) Naïve CD4<sup>+</sup> T cells were stimulated for 18 h in the presence of increasing concentrations (3–30  $\mu$ M) of Smad3 inhibitor SIS3, after which CD25 expression was assessed by flow cytometry. Data are mean  $\pm$  SEM;  $n = 3$ . (C) CD25 and CD69 expression was assessed after 18 h of stimulation in the presence of SIS3 (30  $\mu$ M). Data are mean  $\pm$  SEM;  $n = 4$ . (D) Schematic representation of the PRRIII and PRRV of the *CD25* gene. Numbers indicate forward (right arrows) and reverse (left arrows) primer positions relative to the start of *CD25* gene transcription. The Smad3 putative binding site (GTCTAGAC) position is depicted as well. (E) ChIP data showing the binding of Smad3 to PRRV (Left and Middle) and PRRIII (Right) regions of *CD25* in response to indicated stimulations. (F) Cumulative data of Smad3 binding relative to input (mean  $\pm$  SEM);  $n = 3$ –4.

a negative control, because this region displays no Smad3-binding element (Fig. 2D). Compared with CD3/isotype control stimulation, SLAMF3 coengagement increased the binding of Smad3 to PRRV without binding to PRRIII (Fig. 2E and F). Moreover, stimulation of cells with anti-SLAMF3 mAb alone (i.e., without CD3 engagement) did not increase the binding of Smad3 to PRRV (Fig. 2E and F). Taken together, these data demonstrate that SLAMF3 coengagement promotes the phosphorylation of Smad3, which can then bind to the regulatory region of *CD25* gene and promote its transcription.

After IL-2 binds to its receptor, the IL-2/IL-2R/STAT5 pathway is activated (31). We measured the level of phosphorylated STAT5 (pSTAT5) to assess the intrinsic capacity of cells to produce IL-2 and activate the IL-2R/STAT5 signaling pathway in an autocrine fashion after TCR activation. We observed that SLAMF3 costimulation promoted the phosphorylation of STAT5 to a greater extent than CD28 (Fig. 3A and B). Activation of the pathway was not a direct effect of SLAMF3 cross-linking, as demonstrated by the fact that phosphorylation of STAT5 was almost entirely abrogated in the presence of an IL-2-neutralizing antibody (Fig. 3C).

These results also may be explained by endogenous IL-2 production. To address this possibility, we assessed the frequency of IL-2-producing cells by intracellular cytokine staining after 18 h of stimulation of naïve CD4<sup>+</sup> T cells. Contrary to CD28 coengagement, SLAMF3 did not increase IL-2 production significantly (Fig. 3D). Measurement of IL-2 in the supernatant of sorted naïve CD4<sup>+</sup> T cells stimulated for 18 h with anti-CD3 and costimulatory molecules by ELISA showed that CD28 was more potent than SLAMF3 in inducing IL-2 production (Fig. S3).

Compared with CD28 costimulation, SLAMF3-costimulated CD4<sup>+</sup> T cells displayed significantly increased proliferation in response to exogenous IL-2 (Fig. S4A and B). Proliferation of naïve CD4<sup>+</sup> T cells was limited in the absence of exogenous IL-2.

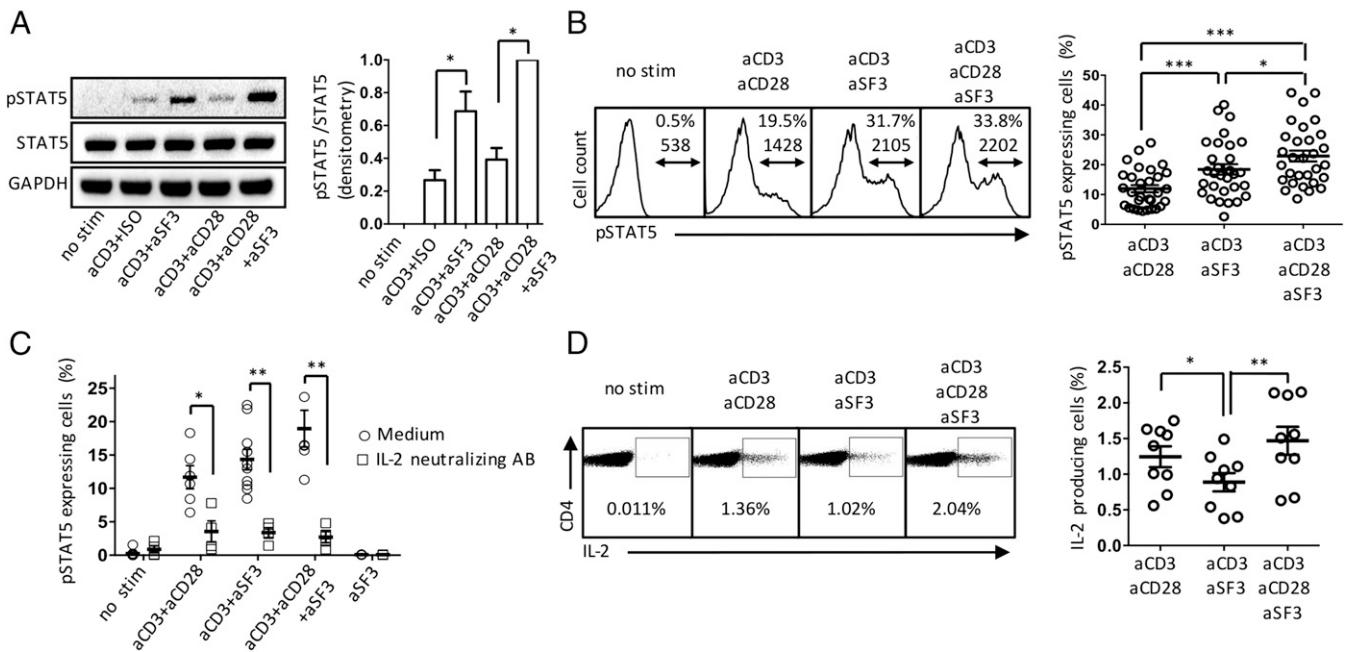
Of note, proliferation of naïve CD4<sup>+</sup> T cells in response to both SLAMF3 and CD28 revealed a synergistic effect even in the absence of IL-2 (Fig. S4A and B).

To ascertain whether anti-SLAMF3 antibody binds through specific interaction with SLAMF3 molecules on the cell surface, we silenced SLAMF3 in Jurkat cells using CRISPR/Cas9 (Fig. S5A) (35, 36). SLAMF3 knockout cells showed no difference in the expression of CD25 after 18 h of stimulation with anti-CD3 alone or with anti-CD3/anti-SLAMF3 (Fig. S5B), proving the specificity of the monoclonal anti-SLAMF3 antibody.

In summary, SLAMF3 costimulation promotes cell proliferation by increasing the response of naïve CD4<sup>+</sup> T cells to IL-2, not by increasing IL-2 cytokine production itself, but rather by up-regulating IL-2R and increasing activation of the IL-2/IL-2R/STAT5 pathway.

**Costimulation by Anti-SLAMF3 Favors Treg Differentiation of Naïve CD4<sup>+</sup> T Cells.** Because IL-2 is a key cytokine in T-cell homeostasis and activation, we examined the effect of SLAMF3 costimulation on CD4<sup>+</sup> T-cell differentiation. When naïve CD4<sup>+</sup> T cells were differentiated in Th1, Th2, and Th17 polarizing conditions, we observed decreased frequencies of IFN $\gamma$ -, IL-4-, and IL-17A-producing cells, respectively, in the presence of SLAMF3 ligation compared with CD28 ligation (Fig. 4A and B). In contrast, when naïve CD4<sup>+</sup> T cells were cultured under Treg polarizing conditions, SLAMF3 costimulation increased the frequency of CD25 and FoxP3 double-positive cells (Fig. 4A and B).

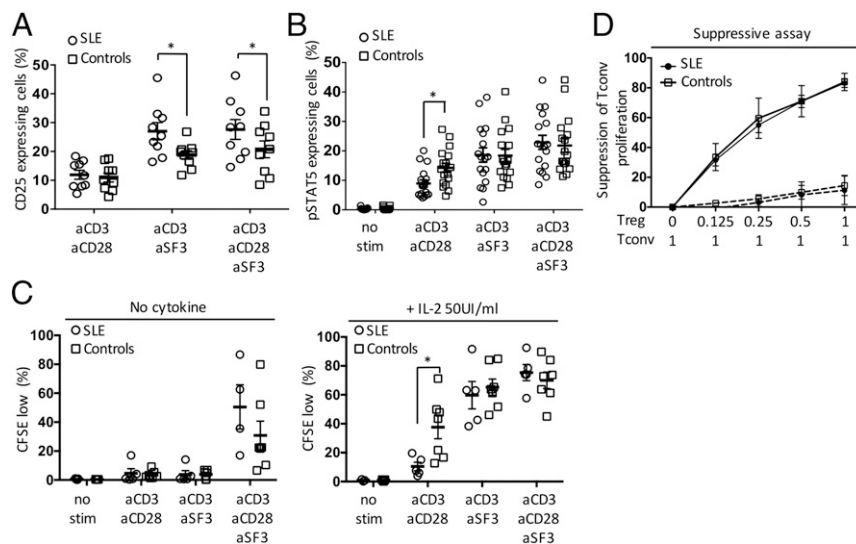
CD25 and FoxP3 expression, as well as STAT5 activation, are not unique to functional Tregs, and are also expressed by activated human effector T cells (37, 38). To clarify their function, we assessed the suppressive capacity of Tregs induced in the presence of SLAMF3 costimulation. Tregs induced in the presence of SLAMF3 ligation displayed a potent suppressive effect



**Fig. 3.** SLAMF3 coengagement promotes the activation of the IL-2/IL-2R/pSTAT5 pathway. Human naïve CD4<sup>+</sup> T cells were stimulated for 18 h, after which expression of pSTAT5 was assessed by Western blot analysis and flow cytometry. (A) Representative blot and cumulative densitometry ratio of pSTAT5 over total STAT5. Data are mean  $\pm$  SEM;  $n = 4$ . (B) Representative flow panels of pSTAT5 [top number, frequency (%); bottom number, MFI] and cumulative data (mean  $\pm$  SEM);  $n = 17$ . (C) Naïve CD4<sup>+</sup> T cells were stimulated for 18 h in the presence of an anti-IL-2 neutralizing antibody (2  $\mu$ g/mL). Data are representative of 6–10 subjects per group (mean  $\pm$  SEM). (D) Intracellular cytokine staining showing IL-2 production by naïve CD4<sup>+</sup> T cells after 18 h of stimulation. Shown are representative flow panels and cumulative data (mean  $\pm$  SEM);  $n = 9$ .







**Fig. 5.** SLAMF3 costimulation restores the IL-2 response in human naïve CD4<sup>+</sup> T cells and promotes Treg differentiation. (A and B) Naïve CD4<sup>+</sup> T cells from SLE and healthy controls were stimulated for 18 h, after which expression of CD25 (SLE,  $n = 9$ ; controls,  $n = 9$ ) (A) and pSTAT5 (SLE,  $n = 17$ ; controls,  $n = 17$ ) (B) was evaluated by flow cytometry. (C) Naïve CD4<sup>+</sup> T-cell proliferation was expressed as the percentage of CFSE-low cells after 6 d of stimulation (SLE,  $n = 4$ –5; controls,  $n = 5$ –7) without exogenous cytokine (Left) or in the presence of recombinant IL-2 (50 IU/mL) (Right). (D) Naïve CD4<sup>+</sup> T cells from SLE and controls were cocultured with anti-SLAMF3 mAb (solid line) or an isotype control (dashed line) and polarized under Treg conditions for 6 d. Their suppressive capacity was evaluated by measuring inhibition of Tconv proliferation (SLE,  $n = 3$ ; controls,  $n = 3$ ). Data are mean  $\pm$  SEM.

IL-2R-mediated phosphorylation of STAT5 (38, 39). Our experiments emphasize that SLAMF3 augments pSTAT5 expression, while promoting the differentiation of peripheral naïve CD4<sup>+</sup> T cells into polarized Tregs and inhibiting cytokine production by other differentiated T helper subsets. In a previous study, SLAMF3 costimulation was suggested to increase IL-17 production by CD4<sup>+</sup> T cells (40); however, in that study the anti-SLAMF3 antibody was used at a dose of 0.5  $\mu$ g/mL, which was not sufficient to increase cell proliferation (Fig. S8 A and B).

Our results suggest that SLAMF3 is involved in immune tolerance. A recent study has shown that *Ly9* (*SLAMF3*)-deficient mice develop spontaneous autoimmunity, and that in vitro anti-CD3- and anti-CD28-stimulated CD4<sup>+</sup> T cells from *Ly9*-deficient mice exhibit greater IFN $\gamma$  production compared with their wild type counterparts, suggesting SLAMF3 involvement in protection against autoimmunity (9).

Studies in SLE suggest that compromised IL-2 production is an important feature of the immune dysregulation involved in the pathogenesis of the disease. An impaired IL-2 level is associated with a reduced suppressive function of Tregs (4, 12, 16, 25). Moreover, IL-2 plays a role in mounting adequate cytotoxic responses. This process is compromised in SLE patients, contributing to the increased rate of infection, one of the leading causes of morbidity and mortality in SLE (41, 42). IL-2 also helps maintain peripheral immune self-tolerance by promoting activation-induced cell death (43), an important apoptotic process that contributes to the elimination of potentially autoreactive T cells. Here we show that SLAMF3 costimulation of SLE naïve CD4<sup>+</sup> T cells restores the response to IL-2 to the level observed in healthy controls. This is illustrated by (i) the normal frequency of pSTAT5-expressing cells after 18 h of stimulation, (ii) the normal proliferation of SLE naïve CD4<sup>+</sup> T cells in response to recombinant IL-2, and (iii) the generation of functional suppressive Tregs in the presence of SLAMF3 coengagement.

Recent case reports have emphasized that low-dose IL-2 administration could be beneficial in SLE (16, 27). Low-dose IL-2 treatment has been reported to have clinical value in patients with GVHD and hepatitis C-associated vasculitis by augmenting the numbers of functional Tregs in the periphery following treatment

(28–30). In this context, our findings suggest that treatment-targeting SLAMF3 may represent a promising therapeutic option in SLE as well as in other conditions in which IL-2 availability/response is compromised, such as GVHD. SLAMF3 stimulation could be beneficial in patients who exhibit resistance to IL-2 therapy or who cannot tolerate recombinant IL-2 treatment. Similarly, engagement of SLAMF3 could offer significant adjuvant therapeutic value to clinical trials in which in vitro expanded Tregs are transfused to patients with SLE, diabetes, and GVHD (44).

Some emerging questions merit further investigation. Foremost, our results suggest that anti-SLAMF3 antibody will favor the generation of Tregs from CD4<sup>+</sup> T cells, while inhibiting the development of proinflammatory CD4<sup>+</sup> T helper subsets. However, our study focused on CD4<sup>+</sup> T cells. SLAMF3 is also expressed on other hematopoietic cells (e.g., B cells and CD8<sup>+</sup> T cells) and plays an important role in cell–cell interaction; thus, examining the effect and the safety of the administration of an mAb directed against SLAMF3 in in vivo settings is warranted.

## Materials and Methods

More detailed information is provided in *SI Materials and Methods*.

**SLE Patients and Controls.** SLE patients ( $n = 42$ ) were diagnosed according to the American College of Rheumatology's classification criteria (45) and recruited from the Division of Rheumatology, Beth Israel Deaconess Medical Center. Age-, sex-, and ethnicity-matched healthy individuals served as controls. Disease activity was measured using the SLE Disease Activity Index scoring system (Table S1). This study was approved by the medical center's Institutional Review Board. Informed consent was obtained from each subject after the nature and possible consequences of the studies were explained.

**Cell Isolation.** Peripheral blood mononuclear cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium; Corning Life Sciences). T cells were isolated by negative selection (RosetteSep; Stem Cell Technologies). Naïve CD4<sup>+</sup> T cells were isolated using the Cell Isolation Kit II (Miltenyi Biotec) or by FACS sorting (CD4<sup>+</sup>CD8<sup>-</sup>CD45RO<sup>-</sup>CD25<sup>-</sup>; >97% purity) using a FACSAria II (BD Bioscience).

**Flow Cytometry.** Cells were stained for dead cells (Zombie Aqua/UV Fixable Viability Kit; Biolegend), and then labeled for surface antibodies (Table S2). Cells were permeabilized (Cytofix/Cytoperm; BD Biosciences) and stained for

cytokines. For phosphoproteins, cells were permeabilized using the PerFix EXPOSE Kit (Beckman Coulter). For FoxP3, cells were permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Data were acquired on an LSR II SORP digital cell analyzer (BD Biosciences) and analyzed using FlowJo version 10.0.8.

**T-Cell Stimulation.** Total or naïve CD4<sup>+</sup> T cells were stimulated in complete RPMI (supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin) with precoated antibodies (anti-CD3, 0.5 µg/mL; anti-CD28 and anti-SLAMF3, 5 µg/mL) for 18 h in the presence of Brefeldin A (GolgiPlug 1 µL/mL; BD Biosciences). Alternatively, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (0.5 µg/mL) for 6 h in the presence of Brefeldin A. In IL-2 neutralizing experiments, anti-IL-2 neutralizing antibody (R&D Systems) was added to the culture at a concentration of 2 µg/mL. Smad3 inhibitor SIS3 (EMD Millipore) was added to the cell culture at the indicated concentrations.

**Suppressive Assay.** Sorted naïve CD4<sup>+</sup> T cells were costimulated with anti-CD3 and anti-SLAMF3 in Treg polarizing conditions (i.e., in the presence of IL-2 and TGFβ) for 6 d. Differentiated cells were then cocultured with autologous carboxyfluorescein succinimidyl ester (CFSE)-labeled conventional T cells and

stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2. Autologous T-cell proliferation was assessed on day 5 by flow cytometry.

**Th1, Th2, Th17, and Treg Differentiation.** Freshly isolated naïve CD4<sup>+</sup> T cells were stimulated in complete RPMI with precoated (anti-CD3, 0.5 µg/mL; anti-CD28 and SLAMF3, 5 µg/mL) antibodies. Th1, Th2, Th17, and Treg polarizing conditions are described in Table S3. Cytokines were replenished after 72 h. On day 6, cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for the last 6 h of culture. Cytokines were purchased from Peprotech; neutralizing antibodies, from Biolegend.

**Statistics.** Statistical analyses were performed using the Mann–Whitney test (unpaired) or Wilcoxon matched-pair signed-rank test (paired). For multiple comparisons, analyses were performed using the Kruskal–Wallis test followed by Turkey's test (unpaired) or Friedman's test followed by Dunn's test (paired). All analyses were performed with GraphPad Prism version 6. Statistical significance was reported as follows: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**ACKNOWLEDGMENTS.** This work was supported by National Institutes of Health Grants P01 AI065687, R01 AI42269, and R37 AI49954, and by a SICPA Foundation grant (to D.C.).

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