Published in final edited form as:

Clin Immunol. 2016 December ; 173: 19–26. doi:10.1016/j.clim.2016.06.009.

Slamf6 negatively regulates autoimmunity

Ninghai Wang[#], **Marton Keszei**[#], **Peter Halibozek**[#], **Burcu Yigit**[#], **Pablo Engel**[‡], and **Cox Terhorst**[#]

[#]Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115

[‡]Department of Biomedical Sciences, Medical School, University of Barcelona, Barcelona, Spain

Abstract

The nine SLAM family (Slamf) receptors are positive or negative regulators of adaptive and innate immune responses, and of several autoimmune diseases. Here we report that the transfer of *Slamf6*$^{-/-}$ B6 CD4^{+} T cells into co-isogenic *bm12* mice causes SLE-like autoimmunity with elevated levels of autoantibodies. In addition, significantly higher percentages of Tfh cells and IFN-γ-producing CD4^{+} cells, as well as GC B cells were observed. Interestingly, the expression of the *Slamf6-H1* isoform in *Slamf6*$^{-/-}$ CD4^{+} T cells did not induce this lupus-like phenotype. By contrast, *Slamf1*$^{-/-}$ or *Slamf5*$^{-/-}$ CD4^{+} T cells caused the same pathology as WT CD4^{+} T cells. As the transfer of *Slamf [1+6]*$^{-/-}$ or *Slamf [1+5+6]*$^{-/-}$ CD4^{+} T cells induced WT levels of autoantibodies, the presence of Slamf1 was requisite for the induction of increased levels of autoantibodies by *Slamf6*$^{-/-}$ CD4^{+} T cells. We conclude that Slamf6 functions as an inhibitory receptor that controls autoimmune responses.

Keywords

Slamf6; GVH; Autoantibody; Tfh cells

1. Introduction

Systemic Lupus Erythematosus (SLE) is a chronic relapsing autoimmune disease, which is caused by interactions among genetic, hormonal and environmental factors and affects various end organs [1]. One main aspect of SLE is the breakdown in B and T cell tolerance leading to uncontrolled activation of effector T cells and self-reactive B cells which produce a large variety of autoantibodies. Genome-wide linkage scans have identified a large number of Lupus susceptibility loci in humans and mice [2], *e.g.* the mouse *Sle1b* locus on chromosome 1 [3, 4]. Approximately half of the *Sle1b* locus, a 0.9-Mb DNA segment, comprises the genes of the mouse Signaling Lymphocytic Activation Molecule Family of cell surface receptors (*Slamf* genes) [5]. Two haplotypes are found in inbred strains: *Slamf-*

Address correspondence and reprint requests to Drs. Ninghai Wang and Cox Terhorst, Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, 3 Blackfan Circle, Boston, MA, 02115, USA. nwang@bidmc.harvard.edu and cterhors@bidmc.harvard.edu.

Disclosures

The authors have no financial conflicts of interest.

haplotype 1 (*e.g.* in *B6*) and *Slamf-haplotype 2* (*e.g.* in *129, NZW, BALB/c*) [6]. Autoimmunity is thought to develop in *Sle1b* mice because of an epistatic interplay between one or more *Slamf-haplotype 2* genes with several *B6* genes [5, 7].

The nine receptors of the Slamf-family: CD150 (Slamf1), CD48 (Slamf2), CD229 (Slamf3). CD244 (Slamf4), CD84 (Slamf5), Ly108 (Slamf6), CD353 (Slamf8), and CD84-H1 (Slamf9) are differentially expressed on the surface of hematopoietic cells[8–10]. Binding of Slam family specific adaptors SAP and EAT-2 to the immunoreceptor tyrosine-based switch motifs (ITSMs) in the cytoplasmic tails of several Slamf receptors not only promotes immune responses, but also block the recruitment of the protein phosphatases SHP1/2 and lipid phosphatase SHIP1, which leads immune cell activation[8, 11–13]. On the other hand, SAP-deficient mice are unable to develop normal GC responses and generate humoral memory due to the profound defect in forming long-lasting T-B cell interaction in germinal center [14]. Slamf5 and Slamf6, which are highly expressed on T and B cells, are shown to partly contribute to SAP-mediated T-B cell adhesion [15]. Consistent with defect in humoral response, Lupus like-autoimmunity does not develop in Sle1b mice that lack SAP expression in T cells [16].

In this paper we examine the role of *Slamf6* gene in autoantibody production. Murine Slamf6 encodes three protein isoforms, i.e. Slamf6-1, Slamf6-2 and Slamf6-H1, which are generated by alternative exon usage [16, 17]. Introduction of one copy of a BAC-based transgene encoding Slamf6-H1 suppresses the spontaneous autoantibody production in *Sle1b* mice, which do not express this isoform [16, 17]. Furthermore, the transfer of *Sle1b* × *Slamf6-H1* B6 $CD4^+$ T cells into co-isogenic *bm12* recipients induces dramatically less autoantibodies than Sle1b $CD4^+$ T cells [17]. Unlike Slamf6-H1, Slamf6-1 and Slamf6-2 do not mitigate *Sle1b*-driven autoantibody formation [16, 17]. To examine here whether autoantibody production would be similarly regulated we adopted a well-established chronic graft versus host (GVH) model in which $CD4^+$ T cells from the same three B6 ($H\text{-}2^b$) Slamf-deficient mice are transferred into the co-isogenic *bm12* ($H\text{-}2^{bm12}$) mouse [18]. Because the $H\text{-}2^{bm12}$ mouse differs from C57BL/6 (H-2b) in three amino acids in its Class II MHC beta chain, donor $CD4^+$ T cells will be activated and differentiate into Tfh cells [16, 17, 19]. The later provide cognate help to host B cells, resulting in GC formation and autoantibody production with symptoms closely resembling SLE.

2. Materials and Methods

2.1. Mice

B6 WT and $B6.C\text{-}2^{bm12}$/KhEg (*bm12*) mice were obtained from the Jackson Laboratory. $Slamf5^{-/-}$, $Slamf6^{-/-}$, $Slamf[1+6]^{-/-}$ and $Slamf[1+5+6+]^{-/-}$ mice were generated from C57BL/6 ES cells [17, 20, 21]. $Slamf1^{-/-}[129xB6]$ was backcrossed ten times with B6 [22]. *BACSlamf6-H1* transgenic mice were generated as described previously [16]. $Slamf6^{-/-}$ × $BACSlamf6\text{-}H1^+$ mice were generated by crossing *BACSlamf6-H1* with $Slamf6^{-/-}$ mice. Age-matched female mice were used at 8–10 week. Animal protocols were approved by The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

2.2. Anti-Slamf receptor antibodies

Rat anti-mouse Slamf1 mAbb (9D1) is specific for the extracellular region of mouse Slamf1[23]. Mouse anti-mouse slamf6 mAbs (13G3) are specific for the extracellular region of mouse slamf6. Anti-Slamf1 and anti-Slamf6 used in our studies were purified by affinity chromatography (Harlan bioproducts for Science).

2.3. Murine transfer model of lupus

We adapted the *bm12* transfer model, as originally described by Morris *et al.* [17, 18]. To this end, 8–10 week-old naive female *bm12* mice were injected *i.p.* with 3×10^6 purified CD4+ T cells from WT and *Slamf5*$^{-/-}$, *Slamf6*$^{-/-}$, *Slamf[1+6]*$^{-/-}$ and *Slamf[1+5+6+]*$^{-/-}$ B6 mice.

For *in vivo* anti-Slamf6 treatment, recipients were injected *i.p.* with 80 μg of anti-Slamf6 antibody or 80 μg mouse Ig isotype control on day 14 after transfer of 3×10^6 WT CD4$^+$ T cells into female *bm12* mice.

2.4. Flow cytometry

Single-cell suspensions were prepared from spleens using standard procedures. After RBC lysis, cells were blocked with anti-CD16/32 Ab (2.4G2, Biolegend) and stained in FACS staining buffer (2.5% FBS, 0.05% sodium azide in PBS). The following antibodies were used: CD4 (L3T4), CD44 (IM7), CD62L (MEL-14, CD69 (H1.2F3), CD86 (GL-1), CD138 (281-1), B220 (RA3-6B2), FAS (Jo2), T-and B-cell activation antigen (GL-7), CXCR5 (2G8), and PD-1 (29F, 1A12) were purchased from eBioscience, BD, or BioLegend. Follicular T cells were stained as previously described [21] in a two-step process using Biotinylatd CXCR5 following by PE-labeled streptavidin in FACS staining buffer on ice. Dead cells were excluded upon DAPI uptake. Data were acquired with cytometer (LSRII: BD) and analyzed using FlowJo software (Tree Star).

Cytokine production was assessed with BD Cytofix/Cytoperm containing BD Golgi-Plug (BD Biosciences). Cells were restimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), Ionomycine (1μg/ml, sigma), and GolgiStop (1μl/ml, BD Biosciences) at 37°C in 5% CD2 for 4 hr. After surface staining, cells were fixed, permeabilized, and stained for IFN-γ (PE-anti-mouse IFN-γ, Biolegend), IL-4 (PE-anti-mouse IL-4, Biolegend) and IL-17 (PE-anti-mouse IL-17A, Biolegend). For intracellular staining IL-21, permeablized cells were incubated with IL-21R/Fc chimera (R&D systems) for 1 h at 4°C. Cells were then washed and stained with PE-conjugated affinity-purified $F(ab')_2$ fragment of goat anti-human Fc γ antibody (Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Viability was assessed using LIVE/DEAD Cell Viability Assays (Life Technologies).

2.5. ELISA

Titers of serum anti-nucleosome antibodies were determined by ELISA as described previously [16, 17]. In brief, met-BSA-precoated Immunolon plated were coated overnight with dsDAN and then with total histone solution. Samples were incubated on plates in various dilutions between 1:600 and 1:1,200, and then plates were washed, and autoantibodies were detected with anti-mouse IgG-HRPO (GE Healthcare).

Autoantibody titer was expressed as ELISA unit, comparing OD values of samples with a standard curve prepared with serial dilutions of ANA-positive NZM2410 serum pool. Antichromatin and anti-dsDNA titers were determined as for the antinucleosome levels. UV-irradiated Immunolon plates were incubated overnight with 3μg/ml chicken chromatin [24] or mung bean nuclease (New England Biolabs, Ins.)-treated dsDNA (Sigma-Aldrich. Anti-single-stranded DNA (ssDNA) was determined as describe previously [25]

2.6. Statistical analysis

Statistical significance was determined by unpaired *t*-test (two-tailed with equal SD) using Prism software (GraphPad, San Diego, CA, USA). The p value <0.05 was considered statistically significant.

3. Results

3.1. The transfer of Slamf6$^{-/-}$ CD4$^+$ T cells into co-isogenic bm12 mice induces a strong autoantibody response

To assess the overall role of the Slamf6 gene in autoantibody production we employed the chronic GVH model in which CD4$^+$ T cells isolated from female *Slamf6*$^{-/-}$ and B6 WT mice were transferred into co-isogenic female *bm12* mice, which differ in three amino acids in their MHC II. This transfer leads to activation of CD4$^+$ T cells and subsequent differentiation into Tfh cells [26]. These cells provide MHC class II-restricted cognate help to host B cells, resulting in GC formation and autoantibody production. Surprisingly, the *bm12* recipients of *Slamf6*$^{-/-}$ CD4$^+$ T cells had a markedly larger spleen and numbers of splenocytes [169 ± 10×10^6 vs. WT 74 ± 8 ×10^6] than recipients of WT CD4$^+$ T cells (Fig. 1A, 1B). Significantly higher levels of anti-dsDNA, anti-ssDNA and anti-chromatin antibodies were present in the serum of *bm12* mice after the transfer of *Slamf6*$^{-/-}$ CD4$^+$ T cells compared to recipients of WT cells (Fig. 1C–E). Consistent with the autoantibody titers, the frequency of CD4$^+$PD-1$^+$CXCR5$^+$ Tfh cells (Fig. 1F) and their absolute cell numbers [2.9 ± 0.6 ×10^6 vs. 0.3 ± 0.08×10^6] were increased in *bm12* recipients of *Slamf6*$^{-/-}$ CD4$^+$ T cells, as compared to WT CD4$^+$ T cell. As judged by expression of CD44, CD62 and CD69, the number of effector CD4$^+$ T cells was higher after the transfer of *Slamf6*$^{-/-}$ CD4$^+$ T cells compared to WT T cells. (Fig. 1G). Similarly, the frequency and absolute numbers of B220$^+$FAS$^+$GL-7$^+$ GC B cells in recipients of *Slamf6*$^{-/-}$ CD4$^+$ T cells were increased over WT (Fig. 1H and 3.85 ± 0.7 × 10^6 vs. 1.19 ± 0.1 ×10^6). More activated CD86$^+$ B cells were also found in recipients of *Slamf6*$^{-/-}$ CD4$^+$ T cells (Fig. 1I). We conclude that the lupus-related autoantibody production was at least as high as that obtained after transfer of CD4$^+$ T cells from the lupus prone *Sle1b* mouse into *bm12* mice [17].

3.2. Accumulation of IFN-γ-producing cells in recipients of Slamf6$^{-/-}$ CD4$^+$ T cells

As established in the literature, several cytokines produced by T cells have a profound impact on B cell development and influence the outcome of immune responses [27, 28]. We evaluated whether levels of different cytokine production is observed in donor CD4$^+$ T cells. The production of IFN-γ, IL-4, and IL-17 was examined by cytoplasmic staining of splenocytes that had been harvested four weeks after transfer of *Slamf6*$^{-/-}$ CD4$^+$ T cells into *bm12* recipients. Once again, the frequency of IFN-γ-producing CD4$^+$ T cells was

significantly higher after transfer of *Slamf6*$^{-/-}$ CD4$^+$ T cells than that of WT CD4$^+$ T cells (Fig. 1J, **Left panel**). In order to exclude the contribution of recipient IFN-γ producing CD4$^+$ T cells, IFN-γ producing CD4$^+$ T cells were further dissected into Slamf6 positive and Slamf6 negative IFN-γ producing CD4$^+$ T cells according to the expression of Slamf6. As expected, the majority of the IFN-γ-producing CD4$^+$ T cells were Slamf6 negative (Fig. 1J, **Right panel**). No difference of IL-4 and IL-17 production was observed (Fig. 1 K, 1L).

3.3. Introduction of one copy of the "suppressive isoform" Slamf6-H1 prevents autoantibody responses caused by Slamf6$^{-/-}$ CD4$^+$ T cells

The mouse Slamf6 gene was first reported to encode two distinct proteins: Slamf6-1 and Slamf6-2, which are generated by alternative exon usage [29]. We recently identified an additional protein isoform, Slamf6-H1[16, 17]. This isoform Slamf6-H1 is only expressed in B6 mice and plays a dominant role in suppressing the pathogenesis of SLE [17]. Therefore, we examined whether introduction of one copy of the isoform *Slamf6-H1* into the *Slamf6*$^{-/-}$ mice would suppress the T cell driven autoantibody production. (**See M&M**). Indeed, upon the transfer of *Slamf6*$^{-/-}$ × *tgBACSlamf6-H1* CD4$^+$ T cells, spleen weight (Fig. 2A) and the number of splenocytes [WT: 69 ± 6×10^6; *Slamf6*$^{-/-}$ 146 ± 11×10^6; *Slamf6*$^{-/-}$ × *BACSlamf6-H1* 71 ± 5 ×10^6], as well as the serum anti-ssDNA, anti-dsDNA and anti-chromatin autoantibodies were similar to those obtained after transfer of WT CD4$^+$ T cells (Fig. 2B–D). The transfer of *Slamf6*$^{-/-}$ × *tgBACSlamf6-H1* CD4$^+$ T cells reduced the frequency (Fig. 2E) and numbers [WT 0.43×10^6 ± 0.2×10^6; *Slamf6*$^{-/-}$ 2.6 ± 0.3 ×10^6; *Slamf6*$^{-/-}$ × *BACSlamf6-H1* 0.39 ± 0.2 ×10^6] of Tfh cells compared to *Slamf6*$^{-/-}$ CD4$^+$ T cells. The proportion of activated CD4$^+$ T cells, as judged by expression of CD62^{lo} and CD69^{hi}, was lower in the recipients of *Slamf6*$^{-/-}$ × *tgBACSlamf6-H1* CD4$^+$ T cells (Fig. 2F, 2G). Furthermore, lower numbers of GC B cells [WT 0.9 ± 0.05×10^6; *Slamf6*$^{-/-}$ 3.3 ± 0.07 ×10^6; *Slamf6*$^{-/-}$ × *BACSlamf6-H1* 0.8 ± 0.04 ×10^6], plasma cells and activated B cells (Fig. 2H–J) were found in the spleen of recipients of *Slamf6*$^{-/-}$ × *tgBAC Slamf6H-1* CD4$^+$ T cells. Taken together, the data indicate that the expression of Slamf6 H-1 counteracts the induction of autoimmunity in the absence of Slamf6.

3.4. CD4 T cell-intrinsic role of Slamf6 in autoimmune responses

Although the preceding observations strongly suggest that Slamf6-deficient CD4+ cells may be primarily responsible for the increased number of Tfh cells and GC B cells in recipients of *Slamf6*$^{-/-}$ CD4 T cells, it is plausible that other cell types may also be important for the altered response because besides T cells, B cells and other antigen presentation cells also express Slamf6 (**Immgen. org**). To determine whether the enhanced autoimmune responses was intrinsically caused by the absence of Slamf6 in CD4$^+$ T cells, we crossed *Slamf6*$^{-/-}$ mice with *bm12* mice to create *Slamf6*$^{-/-}$ × *bm12* recipients in which B cells and other APCs do not express Slamf6. Next, the reciprocal experiment was done in which B6 CD4$^+$ T cells were transferred into *Slamf6*$^{-/-}$ × *bm12* recipients. However, 28 days after the transfer the levels of serum autoantibodies (Fig. 3A–C) and the proportions of Tfh cells and GC B cells in the spleen were indistinguishable from the control group (Fig. 3E, 3G). Also, no discernible difference was observed in the expression of activation markers CD62, CD69 and CD86 on T cells, B cells and DCs between *Slamf6*$^{-/-}$ × *bm12* and *bm12* recipients (Fig. 1F, 1H **and data not shown**). Thus, these experiments suggested that stronger autoimmune

responses are driven by the absence of Slamf6 in $CD4^+$ T cells whilst the B cells, DCs and macrophages in the recipient *bm12* mice still express Slamf6.

3.5. Slamf1 is requisite for the autoantibody production induced by Slamf6$^{-/-}$ CD4$^+$ T cells

Like Slamf6, Slamf1 and Slamf5 are highly expressed on T and B cells [8–10], leading us to investigate potential effects of Slamf1 and Slamf5 on autoimmune response in chronic GVH model. Although the transfer of *Slamf1*$^{-/-}$ $CD4^+$ T cells caused a significant reduction in anti-ssDNA serum titers, the titers of anti-chromatin and anti-dsDNA IgG were only slightly reduced (Supplemental fig. 1B–D). In addition, no significant reduction of spleen size and frequencies of Tfh or GC B cells or activated T and B cells was detectable in recipients of *Slamf1*$^{-/-}$ $CD4^+$ T cells (Supplemental Fig. 1E–J). Similarly, the transfer of *Slamf5*$^{-/-}$ $CD4^+$ T cells has no influence on the development of Tfh cells and GC cells or autoantibody production (Supplemental Fig. 5). Clearly, neither Slamf1 nor Slamf5 alone can induce the high levels of autoantibodies in *bm12* recipient mice.

It has been shown that Slamf1 and Slamf6 synergistically regulate NK T cell development [20, 30] This prompted us to evaluate whether combined deletion of *Slamf[1+6]* or *Slamf[1+5+6]* in $CD4^+$ T cells can further influence GC formation and autoantibody production in *bm12* recipients. Surprisingly, transfer of *Slamf[1+6]*$^{-/-}$ or *Slamf[1+5+6]*$^{-/-}$ $CD4^+$ T cells did not increase numbers of splenocytes [WT: $80 \pm 6\times10^6$; *Slamf6*$^{-/-}$ $127 \pm 12\times10^6$; *Slamf[1+6]*$^{-/-}$ $88\pm 4\times10^6$; *Slamf[1+5+6]*$^{-/-}$ $58 \pm 5\times10^6$] in recipient *bm12* mice, and resulted in significantly lower serum levels of anti-dsDNA, anti-ssDNA and anti-chromatin antibodies compared to that after the transfer of *Slamf6*$^{-/-}$ $CD4^+$ T cells (Fig. 4A–C). After the transfer of *Slamf[1+6]*$^{-/-}$ or *Slamf[1+5+6]*$^{-/-}$ cells the proportion (Fig. 4D) and numbers of Tfh cells [WT: $0.47\pm 0.1\times10^6$; *Slamf6*$^{-/-}$ $3.7 \pm 0.5\times10^6$; *Slamf[1+6]*$^{-/-}$ $0.65 \pm 0.08\times10^6$; *Slamf[1+5+6]*$^{-/-}$ $0.44 \pm 0.06\times10^6$] were similar to those after transfer of WT cells. The proportion (Fig. 4E) and number of GC B cells were also similar to those in the WT control experiments [WT: $0.2 \pm 0.03\times10^6$; *Slamf6*$^{-/-}$ $1.3 \pm 0.2\times10^6$; *Slamf[1+6]*$^{-/-}$ $0.2 \pm 0.05\times10^6$; *Slamf[1+5+6]*$^{-/-}$ $0.1 \pm 0.02\times10^6$]. In addition, the frequencies of effector $CD4^+$ T cells and B cells in the recipients of *Slamf[1+6]*$^{-/-}$ and *Slamf[1+5+6]*$^{-/-}$ $CD4^+$ T cells were also similar to that in the transfer of WT $CD4^+$ T cells (Fig. 4F–H). Therefore, the absence of *Slamf[1+6]* and *Slamf[1+5+6]* in $CD4^+$ T cells appears to have similar effects.

We next wanted to determine the cytokine profile of $CD4^+$ T cells from the recipients of *Slamf1*$^{-/-}$, *Slamf6*$^{-/-}$, *Slamf[1+6]*$^{-/-}$ and WT $CD4^+$ T cells. CD4 cells were isolated at the peak of autoantibody production (day 28) in recipient mice and analyzed for IL-21, IL-17, IL-4 and IFN-γ production. While the frequency of IFN-γ^+ CD4 cells was increase in recipients of *Slamf6*$^{-/-}$ $CD4^+$ T cells (Fig. 1J and Fig. 5D), $CD4^+$ T cells lacking Slamf6 had the percentages of IL-21^+, IL-17^+ and IL-4^+ cells that were comparable with WT $CD4^+$ T cell transfer (Fig. 5A–C). Consistent with reduced autoantibody production, there was a dramatic reduction in IL-4^+, IL-17^+, and IL-21^+ $CD4^+$ T cells in the recipients of *Slamf[1+6]*$^{-/-}$ $CD4^+$ T cells (Fig. 5A–C). Contrary to cytokine profile in recipients of *Slamf[1+6]*$^{-/-}$ $CD4^+$ T cells, no significant differences were detected in the frequencies of IL-21, IL-17 and IFN-γ producing $CD4^+$ T cells except for lower IL-4^+ CD4 cells in the

recipients of *Slamf1*$^{-/-}$ CD4^{+} T cells (Fig. 5A–D). These findings showed that the expression of Slamf1 in Slamf6$^{-/-}$ CD4^{+} T cells is essential for the secretion of substantial amount of important cytokines including IL-21, which is required for enhanced autoimmune responses driven by *Slamf6*$^{-/-}$ CD4^{+} T cells.

3.6. Administration of anti-Slamf6 ameliorates autoantibody production

As the transfer of *Slamf6*$^{-/-}$ CD4^{+} T cells instigated autoimmunity, we reasoned that Slamf6-mediated inhibitory signaling *in vivo* might be initiated by treatment of mice with anti-Slamf6 antibody. Indeed, we observed that anti-Slamf6 antibody administered at day 14 after the transfer of WT CD4^{+} T cells had an significantly ameliorative effect on production of anti-chromatin, anti-ss-DNA, anti-dsDNA IgG (Fig. 6A–C). Consistent with reduction in autoantibodies, anti-Slamf6 treatment decreased the proportion of Tfh cells and GC B cells (Fig. 6D, 6E). Next we evaluated the effect of anti-Slamf1 antibody on autoimmune response in the same model. In contrast, we found a slight, but not significantly, increase in anti-chromatin and anti-ssDNA autoantibody production in anti-Slamf1-treated mice (Fig. 6F, 6G). In addition, increased Tfh cells and GC B cells were consistently detected, but the difference was not reach significance in anti-Slamf1-treated mice (Fig. 6I, 6J).

Taken together, anti-Slamf6 efficiently inhibits autoantibody production through a diminished differentiation of CD4 T cells and B cells into Tfh cells and GC B cells.

4. Discussion

The unexpected finding that the transfer of *Slamf6*$^{-/-}$ CD4^{+} T cells induces robust autoantibody responses with increasing frequencies of Tfh cells and GC B cells suggests that the interaction of Slamf6 between T cells and B cells is critical for the establishment of T-cell and B-cell tolerance. As SAP and SHP1/2 competitively bind to immunoreceptor tyrosine-based switch motifs [ITSM] on the cytoplasmic tail of Slamf6 [8, 9], the expression levels of SAP and SHP1/2 would determine the outcome of activated T cells during immune responses. We previously reported that SAP expression was down-regulated upon activation of T cells: 7 days post-stimulation, SAP protein was barely detected [31]. In contrast, the expression of SHP1 was markedly augmented in activated T cells (data now shown). We, therefore, hypothesize that the interaction of Slamf6 in the *bm12* model could recruit more SHP1 or SHP2 to immune synapses and suppress Tfh cell differentiation and GC formation because of relatively high expression of SHP1 and Slamf6 and low level of SAP expression during long-term activation of donor T cells and recipient B cells. This scenario mimics partial SAP deficiency in which Slamf6 is converted into a potential suppressive receptor in lupus development.

We have previously shown that anti-Slamf6-mediated engagement induced inhibitory effect on humoral immune responses [21]. To directly confirm that Slamf6 plays an inhibitory role in aberrant Tfh cell and GC B cell expansion we observed following transfer of Slamf6$^{-/-}$ CD4^{+} T cells, we employed the *bm12* transfer model with administration of anti-Slamf6 antibody. In marked contract to the enhanced Tfh and GC B cell differentiation and autoantibody production in the transfer of *Slamf6*$^{-/-}$ CD4^{+} T cells, anti-Slamf6 indeed inhibited autoimmune responses in the recipients of WT CD4^{+} T cell. Suppression

mechanism responsible for limiting Tfh cell and GC B cell differentiation in anti-Slamf6 treated mice is unclear. We hypothesize that crosslinking by anti-Slamf6 antibody may induce highly tyrosine-phosphorylation in ITSMs of Slamf6, which recruits inhibitory signaling molecules, such as SHP1, in T and B cells. Further experiments are necessary to investigate these possibilities.

As there is functional redundancy among Slam family member [20, 30], we next investigated whether combined deletion of *Slamf[1+6]* and *Slamf[1+5+6]* in $CD4^+$ T cells causes more severe diseases in the recipient mice. However, we were surprised to find that in contrast to the transfer of $Slamf6^{-/-}$ $CD4^+$ T cells, the absence of Slamf1 and Slamf6 in $CD4^+$ T cells greatly reduced autoimmune responses. It is not yet clear why the expression of Slamf1 is required for high autoantibody production in $Slamf6^{-/-}$ $CD4^+$ T cell-mediated autoimmunity. However, SAP and some Slamf receptors were demonstrated to be necessary for forming lasting mobile conjugate pairs of T and B cells in the germinal center [14, 15]. Therefore, we hypothesize that there are normal frequencies of antigen-specific T-B cell conjugation in the absence of *trans*-Slamf6 interactions, which allows T and B cell differentiation to Tfh cells and germinal center B cells. However, combined absence of Slamf[1+6] or Slamf[1+5+6] on T and B cells would compromise interaction of T cells and B cells. For example, published studies have described coordinated action of Slamf5 and Slamf6 in maintaining sustained T-B cell conjugate [15]. This is suggestive of the possibility that in this model $Slamf6^{-/-}$ $CD4^+$ T cell intrinsic role for highly autoimmune responses was dependent on the expression of Slamf1, which likely contribute to the sustaining T-B cell interaction.

In addition for sustaining T-B cell interaction, Slamf1 signaling is also required for cytokine production in CD4 cells [22, 32]. It is shown that Slamf1 is specifically required for IL-4 production by GC Tfh cells, which is necessary for optimal B cell help [33]. The data presented here is consistent with Slamf1 having a key role regulating IL-4 production. Furthermore, using this model, our studies unexpectedly revealed that although IL-21 and IL-17 production in the transfer of either $Slamf1^{-/-}$ or $Slamf6^{-/-}$ $CD4^+$ T cells was comparable with that of WT $CD4^+$ T cells, combined deletion of Slamf1 and Slamf6 in $CD4^+$ T cells had a profound negative effect on IL-21 and IL-17 secretion. The multitude of potential roles for IL-21 on T and B cell behavior has been documented [34, 35]. IL-21 has an important role in regulating T cell-dependent B cell responses, partly in cooperation with IL4 [36]. Both T and B cells express the receptor for IL-21, and the ligation of IL-21 receptors initiates signaling, which supports the differentiation and survival of Tfh cells and antibody-forming B cells. Additionally, IL-21 promotes the generation and population of Th17 cells [37]. Our results, described above, strongly suggest that Slamf1 and Slamf6 synergistically regulate IL-21, IL-17 and IL-4 production, which are critical for T-dependent humoral responses. It is therefore possible that the defect in the multiple key cytokine production also contributes to reduced autoimmune responses in the transfer of $Slamf[1+6]^{-/-}$ $CD4^+$ T cells into *bm12* recipient mice.

In conclusion, we have observed that the transfer of $Slamf6^{-/-}$ $CD4^+$ T cells into *bm12* mice induced robust autoantibody production and high Tfh cell and GC B cell development. Our results clearly show Slamf6 functions as an inhibitory receptor that controls autoimmune

responses. Moreover, the *Slamf6-H1* isoform is demonstrated to be a major player in suppressing development of lupus-like diseases. The outcomes of our studies also show that the adhesion molecule Slamf1 is requisite for the function of Slamf6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. George Tsokos and members of the Terhorst lab for helpful discussions and for a critical review of the manuscript.

The work was supported by National Institutes of Health Grants PO1 AI 065687 (to CT).

Abbreviations used

SLAMF	Signaling Lymphocyte Activation Molecule Family
SAP	SLAM-Associated Protein
SLE	systemic lupus erythematosus
Tfh cells	T follicular helper cells
GC B cells	Germinal Center B cells
GVH	Graft-versus-host

References

1. Tsokos GC. Systemic lupus erythematosus. The New England journal of medicine. 2011; 365(22): 2110–2121. [PubMed: 22129255]
2. Deng Y, Tsao BP. Advances in lupus genetics and epigenetics. Curr Opin Rheumatol. 2014; 26(5): 482–492. [PubMed: 25010439]
3. Morel L, Rudofsky UH, Longmate JA, Schiffenbauer J, Wakeland EK. Polygenic control of susceptibility to murine systemic lupus erythematosus. Immunity. 1994; 1(3):219–229. [PubMed: 7889410]
4. Mohan C, Alas E, Morel L, Yang P, Wakeland EK. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. The Journal of clinical investigation. 1998; 101(6):1362–1372.
5. Wandstrat AE, Nguyen C, Limaye N, Chan AY, Subramanian S, Tian XH, Yim YS, Pertsemlidis A, Garner HR Jr, Morel L, Wakeland EK. Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. Immunity. 2004; 21(6):769–780. [PubMed: 15589166]
6. Keszei M, Latchman YE, Vanguri VK, Brown DR, Detre C, Morra M, Arancibia-Carcamo CV, Paul E, Calpe S, Castro W, Wang N, Terhorst C, Sharpe AH. Auto-antibody production and glomerulonephritis in congenic Slamf1−/− and Slamf2−/− [B6.129] but not in Slamf1−/− and Slamf2−/− [BALB/c.129] mice. Int Immunol. 2011; 23(2):149–158. [PubMed: 21278219]
7. Carlucci F, Cortes-Hernandez J, Fossati-Jimack L, Bygrave AE, Walport MJ, Vyse TJ, Cook HT, Botto M. Genetic dissection of spontaneous autoimmunity driven by 129-derived chromosome 1 Loci when expressed on C57BL/6 mice. J Immunol. 2007; 178(4):2352–2360. [PubMed: 17277141]
8. Calpe S, Wang N, Romero X, Berger SB, Lanyi A, Engel P, Terhorst C. The SLAM and SAP gene families control innate and adaptive immune responses. Advances in immunology. 2008; 97:177–250. [PubMed: 18501771]

9. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. Annual review of immunology. 2011; 29:665–705.
10. Veillette A. Immune regulation by SLAM family receptors and SAP-related adaptors. Nature reviews Immunology. 2006; 6(1):56–66.
11. Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, van Schaik S, Notarangelo L, Geha R, Roncarolo MG, Oettgen H, De Vries JE, Aversa G, Terhorst C. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature. 1998; 395(6701):462–469. [PubMed: 9774102]
12. Dong Z, Veillette A. How do SAP family deficiencies compromise immunity? Trends in immunology. 2010; 31(8):295–302. [PubMed: 20650688]
13. Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, Schwartzberg PL, Crotty S. The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. Immunity. 2012; 36(6):986–1002. [PubMed: 22683125]
14. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. Nature. 2008; 455(7214):764–769. [PubMed: 18843362]
15. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, Wakeland EK, Germain RN, Schwartzberg PL. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity. 2010; 32(2):253–265. [PubMed: 20153220]
16. Keszei M, Detre C, Castro W, Magelky E, O'Keeffe M, Kis-Toth K, Tsokos GC, Wang N, Terhorst C. Expansion of an osteopontin-expressing T follicular helper cell subset correlates with autoimmunity in B6.Sle1b mice and is suppressed by the H1-isoform of the Slamf6 receptor. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2013; 27(8):3123–3131. [PubMed: 23629864]
17. Keszei M, Detre C, Rietdijk ST, Munoz P, Romero X, Berger SB, Calpe S, Liao G, Castro W, Julien A, Wu YY, Shin DM, Sancho J, Zubiaur M, Morse HC 3rd, Morel L, et al. A novel isoform of the Ly108 gene ameliorates murine lupus. The Journal of experimental medicine. 2011; 208(4): 811–822. [PubMed: 21422172]
18. Morris SC, Cohen PL, Eisenberg RA. Experimental induction of systemic lupus erythematosus by recognition of foreign Ia. Clin Immunol Immunopathol. 1990; 57(2):263–273. [PubMed: 2208807]
19. Shao WH, Eisenberg RA, Cohen PL. The Mer receptor tyrosine kinase is required for the loss of B cell tolerance in the chronic graft-versus-host disease model of systemic lupus erythematosus. J Immunol. 2008; 180(11):7728–7735. [PubMed: 18490777]
20. De Calisto J, Wang N, Wang G, Yigit B, Engel P, Terhorst C. SAP-Dependent and -Independent Regulation of Innate T Cell Development Involving SLAMF Receptors. Frontiers in immunology. 2014; 5:186. [PubMed: 24795728]
21. Wang N, Halibozek PJ, Yigit B, Zhao H, O'Keeffe MS, Sage P, Sharpe A, Terhorst C. Negative Regulation of Humoral Immunity Due to Interplay between the SLAMF1, SLAMF5, and SLAMF6 Receptors. Frontiers in immunology. 2015; 6:158. [PubMed: 25926831]
22. Wang N, Satoskar A, Faubion W, Howie D, Okamoto S, Feske S, Gullo C, Clarke K, Sosa MR, Sharpe AH, Terhorst C. The cell surface receptor SLAM controls T cell and macrophage functions. The Journal of experimental medicine. 2004; 199(9):1255–1264. [PubMed: 15123745]
23. Howie D, Okamoto S, Rietdijk S, Clarke K, Wang N, Gullo C, Bruggeman JP, Manning S, Coyle AJ, Greenfield E, Kuchroo V, Terhorst C. The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. Blood. 2002; 100(8):2899–2907. [PubMed: 12351401]
24. Cohen, PL.; Maldonado, MA. Animal models for SLE. In: Coligan, John E., et al., editors. Current protocols in immunology. 2003. Chapter 15:Unit 15 20
25. Walter JE, Rucci F, Patrizi L, Recher M, Regenass S, Paganini T, Keszei M, Pessach I, Lang PA, Poliani PL, Giliani S, Al-Herz W, Cowan MJ, Puck JM, Bleesing J, Niehues T, et al. Expansion of immunoglobulin-secreting cells and defects in B cell tolerance in Rag-dependent immunodeficiency. The Journal of experimental medicine. 2010; 207(7):1541–1554. [PubMed: 20547827]

26. Klarquist J, Janssen EM. The bm12 Inducible Model of Systemic Lupus Erythematosus (SLE) in C57BL/6 Mice. Journal of visualized experiments: JoVE. 2015; (105):e53319. [PubMed: 26554458]
27. Peng SL, Moslehi J, Craft J. Roles of interferon-gamma and interleukin-4 in murine lupus. The Journal of clinical investigation. 1997; 99(8):1936–1946. [PubMed: 9109438]
28. Kim K, Cho SK, Sestak A, Namjou B, Kang C, Bae SC. Interferon-gamma gene polymorphisms associated with susceptibility to systemic lupus erythematosus. Annals of the rheumatic diseases. 2010; 69(6):1247–1250. [PubMed: 19919944]
29. Peck SR, Ruley HE. Ly108: a new member of the mouse CD2 family of cell surface proteins. Immunogenetics. 2000; 52(1–2):63–72. [PubMed: 11132158]
30. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, Mamchak AA, Terhorst C, Bendelac A. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity. 2007; 27(5):751–762. [PubMed: 18031695]
31. Wu C, Sayos J, Wang N, Howie D, Coyle A, Terhorst C. Genomic organization and characterization of mouse SAP, the gene that is altered in X-linked lymphoproliferative disease. Immunogenetics. 2000; 51(10):805–815. [PubMed: 10970095]
32. Davidson D, Shi X, Zhang S, Wang H, Nemer M, Ono N, Ohno S, Yanagi Y, Veillette A. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. Immunity. 2004; 21(5):707–717. [PubMed: 15539156]
33. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, Barnett B, Crotty S. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). J Immunol. 2010; 185(1):190–202. [PubMed: 20525889]
34. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annual review of immunology. 2008; 26:57–79.
35. Ettinger R, Kuchen S, Lipsky PE. The role of IL-21 in regulating B-cell function in health and disease. Immunological reviews. 2008; 223:60–86. [PubMed: 18613830]
36. Al-Shami A, Spolski R, Kelly J, Fry T, Schwartzberg PL, Pandey A, Mackall CL, Leonard WJ. A role for thymic stromal lymphopoietin in CD4(+) T cell development. The Journal of experimental medicine. 2004; 200(2):159–168. [PubMed: 15263024]
37. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007; 448(7152):484–487. [PubMed: 17581588]

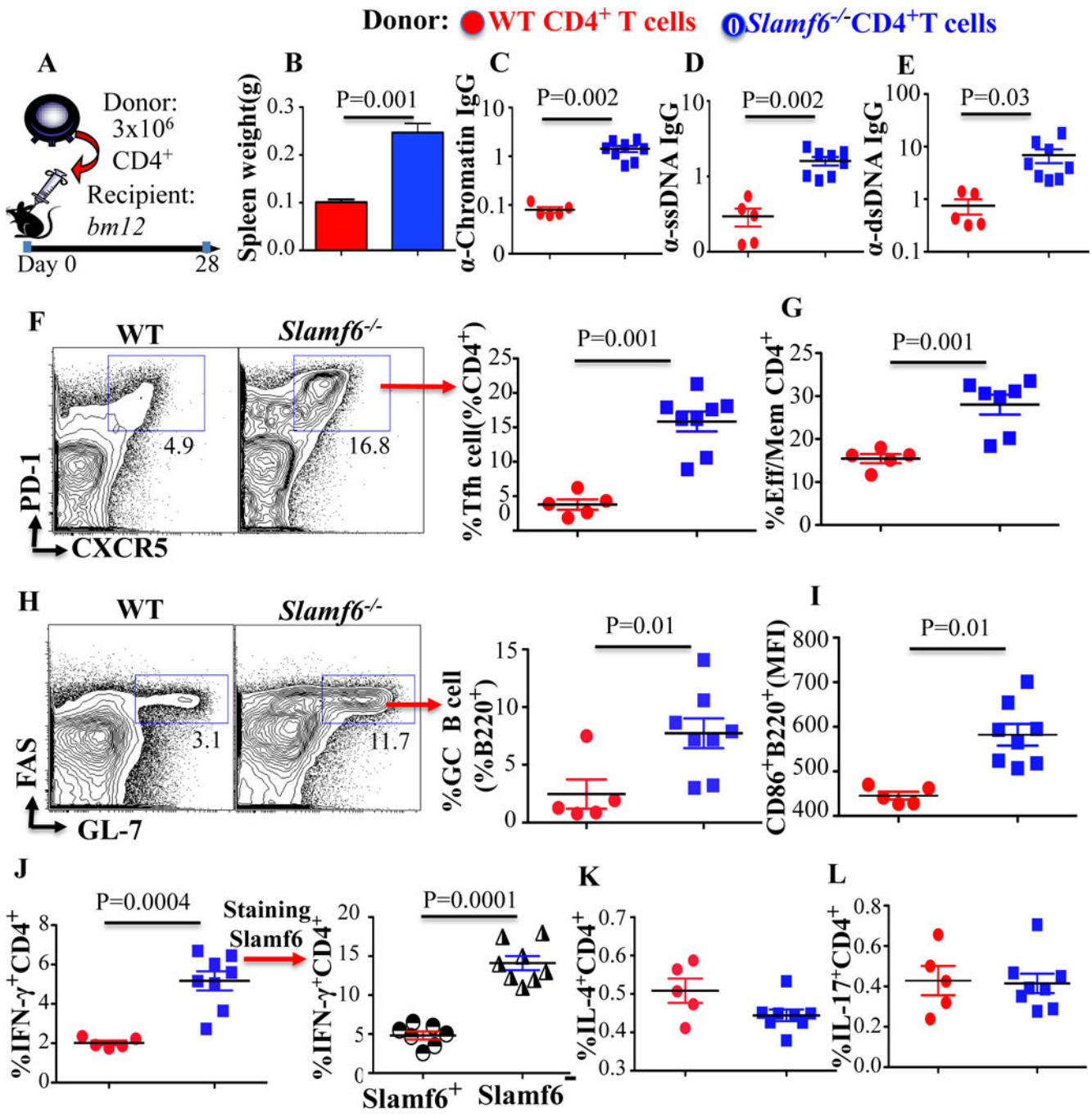

Figure 1. The transfer of *Slamf6*$^{-/-}$ CD4^{+} T cells into co-isogenic *bm12* female recipients induce Lupus-related autoantibody responses

A. 3×10^{6} CD4^{+} T cells isolated from *Slamf6*$^{-/-}$ and *WT* female mice were transferred into *bm12* female recipients by *i.p.* injection. After 4 weeks, the indicated recipient mice were scarified and their spleens and serum were analyzed.

B. Spleen weights.

C–E Anti-chromatin, Anti-ssDNA and Anti-dsDNA in the serum of recipient mice, as determined by ELISA and expressed as ELISA units (EU).

F. Representative Flow Cytometry plots (left Panel) and percentages (Right Panel) of $CD4^+PD\text{-}1^+CXCR5^+$Tfh cells.

G. Percentages of $CD4^+CD44^{hi}CD62^{lo}CD69^+$ effector/memory T cells.

H. Representative Flow Cytometry plots (left Panel) and percentages (Right Panel) of $B220^+GL\text{-}7^+FAS^+$ GC B cells.

I. Expression of CD86 (MFI) on $B220^+$ B cells.

J. Percentages of IFN-γ-producing $CD4^+$ T cells by intracellular staining (left) and Percentages of IFN-γ-producing $CD4^+$ T cells in $Slamf6^+CD4^+$ (whit and black circles) and $Slamf6^-CD4^+$cells (Triangle).

K. Percentages of IL-4-producing $CD4^+$ T cells.

L. Percentages of IL-17-producing $CD4^+$ T cells.

Data are representative of at lest three independent experiments.

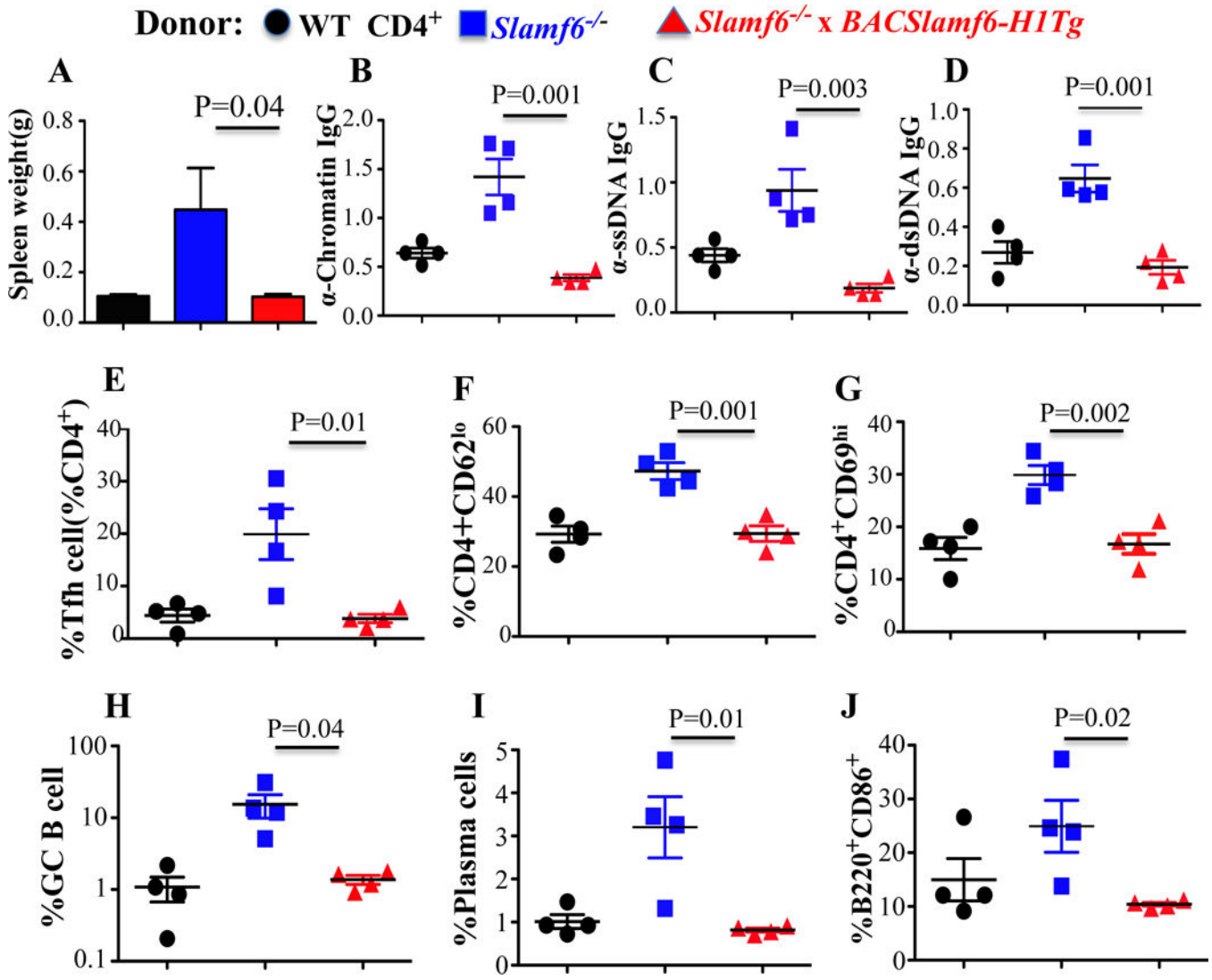

Figure 2. Expression of one copy of tgBAC-*Slamf6-H1* in *Slamf6*$^{-/-}$ CD4^{+} T cells prevents the autoimmunity phenotype observed after the transfer of *Slamf6*$^{-/-}$ CD4 T cells

3×10^{6} CD4^{+} T cells from *Slamf6*$^{-/-}$, *Slamf6*$^{-/-}$ × *tgBACSlamf6-H1* or *WT* female mice were *i.p.* injected into *bm12* female recipients. After 4 weeks, the indicated recipient mice were scarified and their spleens and serum were analyzed.

A. Spleen weights.

B–D. Anti-chromatin, -ssDNA and -dsDNA IgG titers in the serum were determined by ELISA and expressed as ELISA units (EU).

E–G. Percentages of CD4^{+}PD-1^{+}CXCR5^{+} Tfh cells, CD4^{+}CD44^{hi}CD62^{lo} memory T cells and CD4^{+}CD44^{hi}CD69^{+} activated cells.

H–J. Percentages of B220^{+}GL-7^{+}FAS^{+}GC B cells, B220^{+}IgD^{-}CD138^{+} plasma cells and B220^{+}CD86^{+} activated B cells.

Data are representative of at lest three independent experiments.

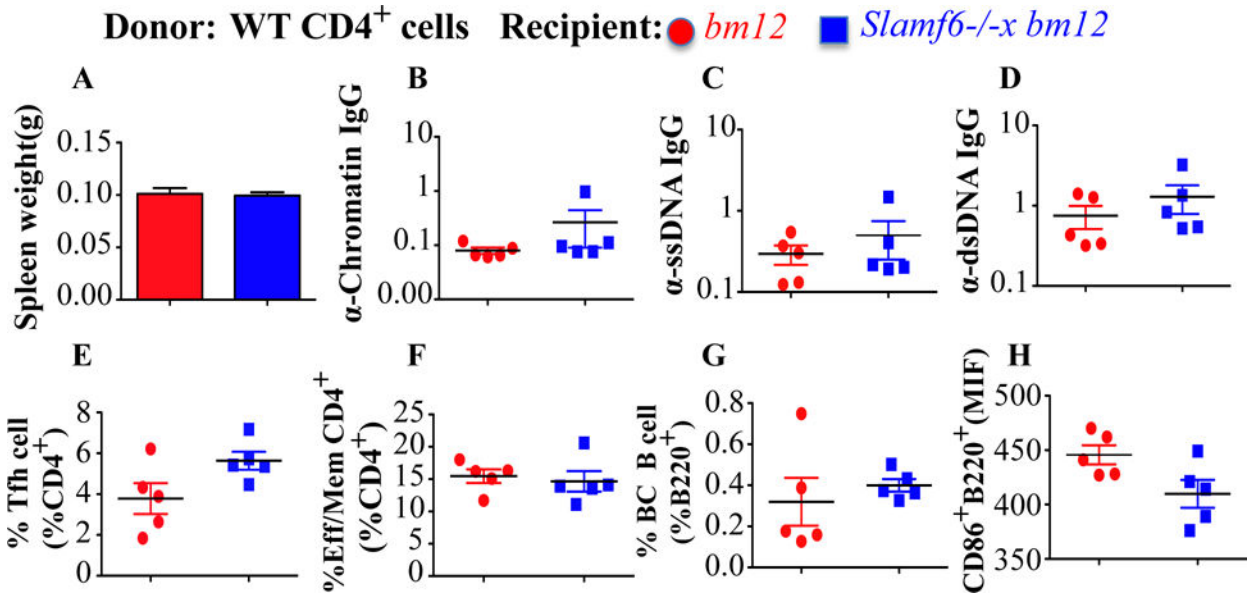

Figure 3. *Slamf6*−/− × *bm12* mice are protected from the development of higher autoantibody responses after the transfer of *bm12* CD4$^+$ T cells

WT CD4 cells were isolated from spleens and transferred into Bm12 and *Slamf6*$^{-/-}$ × *bm12* mice by i.p. injection. After 4 weeks, the indicated recipients mice were scarified and their spleens and serum were analyzed.

A. Spleen weight

B–D. Anti-chromatin antibody titer, anti-ssDAN, and anti-ds-DNA in sera of Bm12 and Slamf6−/−xBm12 recipients.

E. Percentages of CD4+PD-1$^+$CXCR5$^+$ Tfh cells in spleens of recipients.

F. Percentages of CD4+ effector and memory cells in spleens of recipients.

G. Percentages of B220$^+$GL-7$^+$FAS$^+$ GC B cells in spleens of recipients.

H. Percentages of B220$^+$CD86$^+$ activated B cells in spleens of recipients.

Data are representative of three independent experiments.

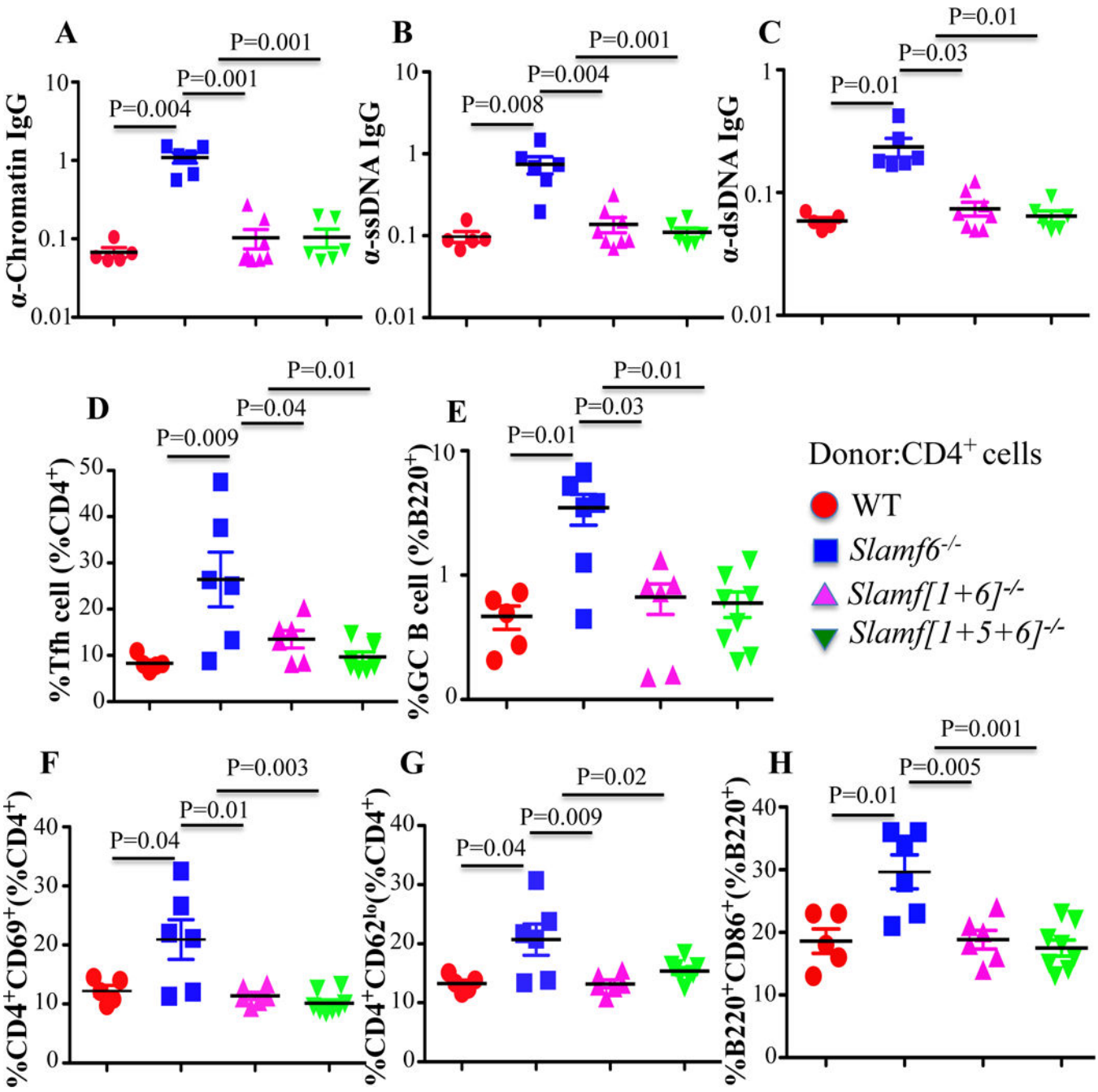

Figure 4. Expression of Slamf1 is requisite for the increased GC responses and autoantibody production induced by the transfer of *Slamf6*$^{-/-}$ CD4 T cells

3×10^6 CD4+ T cells from *Slamf6*$^{-/-}$, *Slamf[1+6]*$^{-/-}$, *Slamf[1+5+6]*$^{-/-}$ or *WT* female mice were *i.p.* injected into *bm12* female recipients. After 4 weeks, the indicated recipients mice were scarified and their spleens and serum were analyzed.

A–C. Anti-chromatin, Anti-ssDNA and Anti-dsDNA antibody titers in the serum were determined as in Fig. 1

A. Percentages of $CD4^+PD\text{-}1^+CXCR5^+$ Tfh cells in $CD4^+$ T cells

B. Percentages of $B220^+GL\text{-}7^+FAS^+$ GC B cells in $B220^+$ B cells

F–H. Percentages of $CD4^+CD44^{hi}CD69^+$ activated cells, $CD4^+CD44^{hi}CD62^{lo}$ memory T cells and $B220^+CD86^+$ activated B cells.

Data are representative of at lest three independent experiments

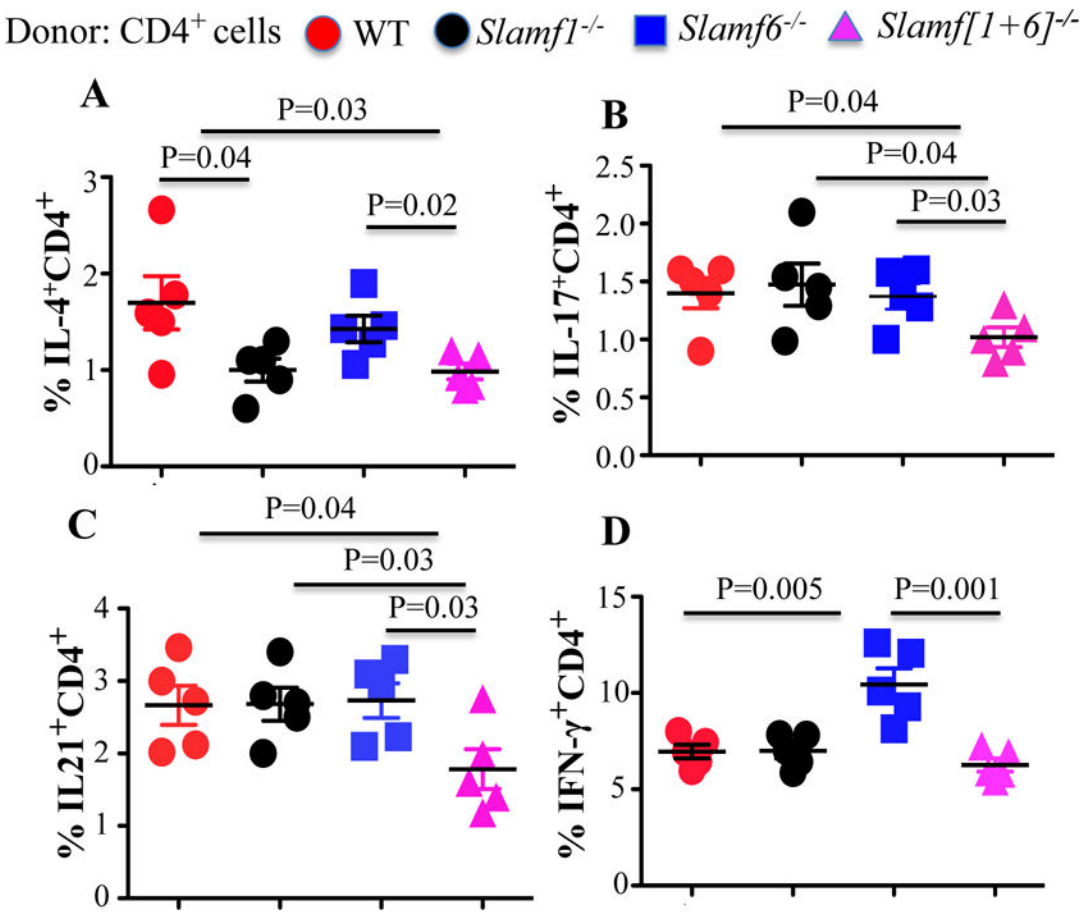

Figure 5. Defective cytokine production in recipient of *Slamf[1+6]*$^{-/-}$ CD4^{+} cells

WT, Slamf1$^{-/-}$, *Slamf6*$^{-/-}$ and *Slamf[1+6]*$^{-/-}$ CD4^{+} T cells were transferred into bm12 recipients for four weeks. Single-cell suspensions from spleens of these recipients were stimulated with PAM and ionomycin for 4h. Cells were surface stained for CD4 and permeabilized and stained for IL-4, IL-17, IL-21 and INF-γ.

Data are representative of three independent experiments

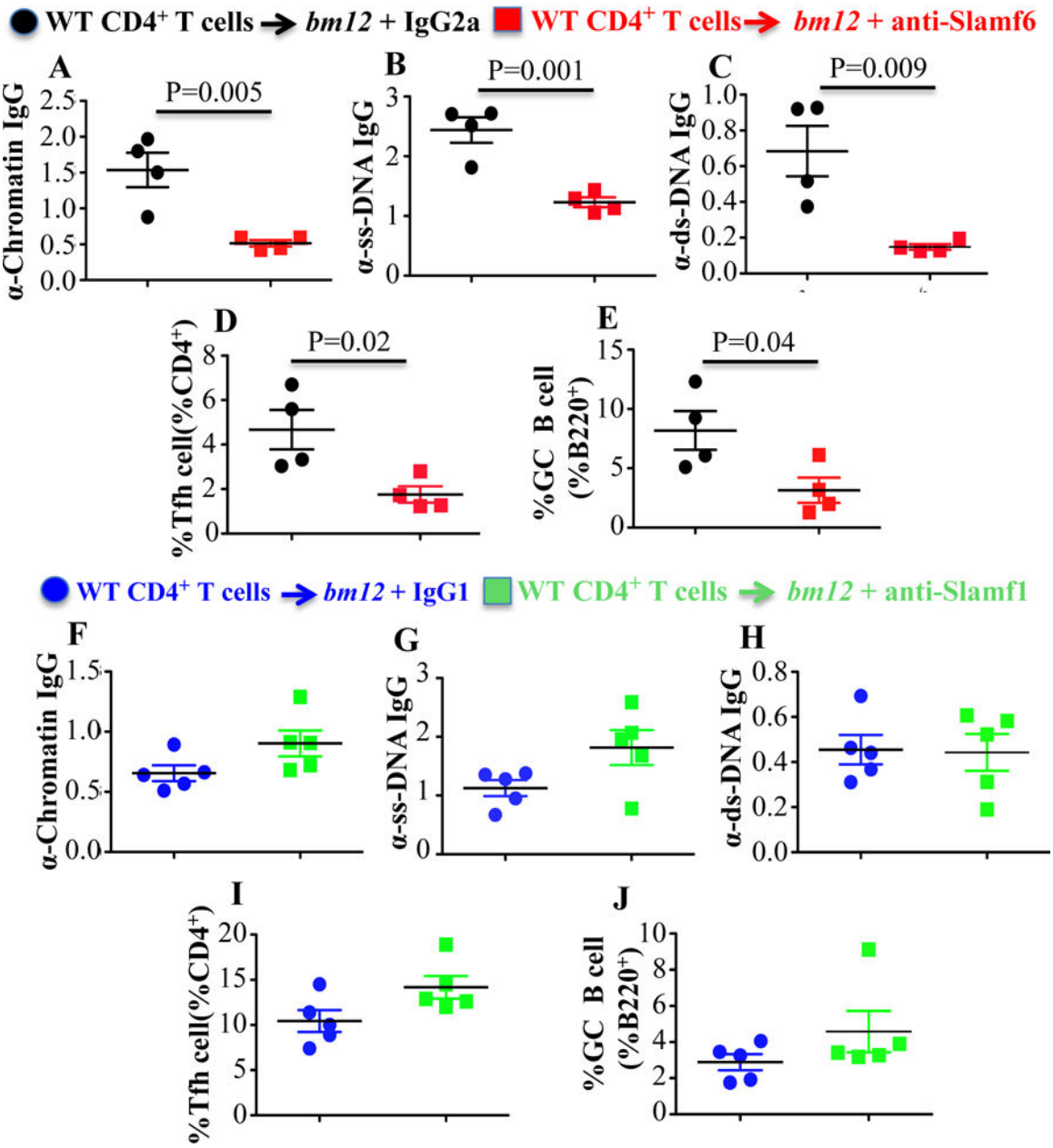

Figure 6. Administration of anti-Slamf6 antibody, not anti-Slamf1 antibody, protects *bm12* recipients of B6 CD4$^+$ T transfer from autoimmune responses and inhibits Nox2 activity in B cells

A–E, the female bm12, which were recipient of WT CD4$^+$ T cells were injected with anti-slamf6 (13G3) or Ig isotype control. The mice were sacrificed on day 28 and were analyzed.

A–C, Sera were collected to measure anti-chromatin antibody, anti-ss-DNA antibody and anti-ds-DNA antibody in anti-Slamf6 treated recipients

D. Percentages of CD4$^+$PD-1$^+$CXCR5$^+$ Tfh cells in the spleens of anti-Slamf6 treated *bm12* recipients

E. Percentages of B220$^+$GL-7$^+$FAS$^+$ GC B cells in anti-Slamf6 treated recipients

F–J, the bm12 recipient mice of WT CD4+ T cells were injected with anti-slamf1 (9D1) or Ig isotype control. The mice were sacrificed on day 28 and were analyzed.

F–H, Sera were collected to measure anti-chromatin antibody, anti-ds-DNA antibody and Anti-ss-DNA antibody in anti-Slamf1 treated recipients

I. Percentages of $CD4^{+}PD\text{-}1^{+}CXCR5^{+}$ Tfh cells in the spleens of anti-Slamf1 treated *bm12* recipients

J. Percentages of $B220^{+}GL\text{-}7^{+}FAS^{+}$ GC B cells in anti-Slamf1 treated recipients

Data are representative of at lest three independent experiment.