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Cutting Edge: An NK Cell-Independent Role for Slamf4 in Controlling Humoral Autoimmunity

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Several genes within a syntenic region of human and mouse chromosome 1 are associated with predisposition to systemic lupus erythematosus. Analyses of lupus-prone congenic mice have pointed to an important role for the signaling lymphocyte activation molecule family (slamf6) surface receptor in lupus pathogenesis. In this paper, we demonstrate that a second member of the Slamf gene family, Slamf4 (Cd244), contributes to lupus-related autoimmunity. B6.Slamf4−/− mice spontaneously develop activated CD4 T cells and B cells and increased numbers of T follicular helper cells and a proportion develop autoantibodies to nuclear Ags. B6.Slamf4−/− mice also exhibit markedly increased autoantibody production in the B6.C-H-2bm12/KhEg → B6 transfer model of lupus. Although slamf4 function is best characterized in NK cells, the enhanced humoral autoimmunity of B6.Slamf4−/− mice is NK cell independent, as judged by depletion studies. Taken together, our findings reveal that slamf4 has an NK cell-independent negative regulatory role in the pathogenesis of lupus a normally non-autoimmune prone genetic background. The Journal of Immunology, 2011, 187: 000–000.

Systemic lupus erythematosus (SLE) is an autoimmune disease resulting from the production of multiple autoantibodies. Virtually all patients with SLE develop anti-nuclear Abs, and many develop Abs to dsDNA, which serves as a specific marker of disease activity. Multisystem organ dysfunction results from the direct effect of autoantibodies and deposition of immune complexes in capillaries with subsequent activation of innate immune responses. The mechanisms behind the humoral autoimmunity are complex, involving a "network" of immune cells (T, B, and dendritic cells and macrophages) and a combination of factors resulting in systemic inflammation.

Genetic linkage studies are revealing some of the molecules involved in the pathogenesis of SLE (1, 2). In mice with spontaneous SLE-like disease, autoantibody production has been linked to a small region of mouse chromosome 1 (sle1) (3, 4). Genome-wide association studies have implicated the orthologous region of human chromosome 1 in SLE susceptibility (5). Further analyses revealed that sle1 contains three subloci that each contribute to autoimmunity (sle1a–1c) with sle1b having the largest contribution to autoantibody production (6–8). When the sle1b region from lupus-susceptible NZM2410 mice is bred onto the normally resistant C57BL/6 (B6) background, the resulting B6.sle1b strain develops a mild autoimmune phenotype, with activated T and B cells and Abs to nucleosomes (4).

Among the 24 genes encoded in the sle1b locus, seven genes of the signaling lymphocyte activation molecule family (Slamf) are the only genes with known immunologic function. Slamf genes encode cell surface receptors capable of homophilic and heterophilic interactions that regulate T cell and B cell responses as well as NK cell, macrophage, dendritic cell, neutrophil, and platelet functions, making them attractive candidates for controlling SLE-relevant cellular and signal transduction pathways (9, 10). Slamf molecules from SLE-resistant and -susceptible mouse strains show sequence polymorphisms, splice variation, and expression differences (8).

Slamf6 (Ly108) has an important role in tolerance to chromatin and susceptibility to lupus (7). The functional diversity and overlapping signaling of other slamf receptors and their isoforms suggests that multiple Slamf genes may contribute to the role of sle1b in tolerance. Slamf4 (Cd244, 2B4) is of particular interest because of extensive polymorphisms in its putative extracellular ligand binding domain; furthermore, the broad expression profile of its receptor, slamf2 (Cd48), on all hematopoietic cells would allow slamf4 to influence a multitude of cellular immune functions (8). Although slamf4 is expressed on NK cells, intraepithelial CD8 cells, γδ T cells, myeloid precursors, granulocytes, and monocyte-derived cells, most work has focused on its function in regulating NK responses. Slamf4 on NK cells regulates killing of tumor targets and isotype switching of B cells (11–13).

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Abbreviations used in this article: B6, C57BL/6; B6.Slamf4−−; Slamf4-deficient; bm12, B6.C-H-2bm12/KhEg; GVH, graft-versus-host; slamf, signaling lymphocyte activation molecule family; SLE, systemic lupus erythematosus; WT, wild-type.

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In this study, we use Slamf4-deficient mice generated using B6-derived ES cells (B6.Slamf4−/− mice) to interrogate the role of slamf4 in lupus. This strain circumvents potentially confounding issues of mixed genetic background in our analyses (14). B6.Slamf4−/− mice spontaneously develop increased immune activation and autoantibodies and exhibit dramatically enhanced autoantibody production compared with wild-type (WT) B6 mice in a well-characterized graft-versus-host (GVH) disease model of SLE (15). Ab depletion experiments demonstrate an NK cell-independent role for slamf4 in regulating tolerance to chromatin. Thus, our studies identify a novel inhibitory function for slamf4 in humoral autoimmunity.

Materials and Methods

Mice

WT B6 and B6.C-H-2bm12/KhEg (bm12) mice were purchased from The Jackson Laboratory or bred in our animal facility. The generation of B6.Slamf4−/− mice by targeted deletion of exons II and III using Brucex B6 ES cells is described elsewhere (S. Calpe, manuscript in preparation). Slamf4−/− mice were initially backcrossed with WT B6 mice or B6 Thy1 congenic mice before intercrossing. Targeted disruption of the Slamf4 gene did not significantly alter the expression of the neighboring Slamf genes (data not shown). All mice were maintained in a pathogen-free facility and used according to institutional and National Institutes of Health guidelines. Harvard Medical School and Beth Israel Deaconess Medical Center are accredited by the American Association of Accreditation of Laboratory Animal Care.

Flow cytometry

Single-cell suspensions of spleen and thymus were prepared by mechanical dissociation and stained for expression of indicated molecules as described previously (16). All Abs for flow cytometry were purchased from BD Biosciences, BioLegend, or eBioscience. Intracellular staining was performed after 5 h of stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) in the presence of brefeldin A using reagents from eBioscience. Samples were collected on a FACS Calibur and analyzed using FlowJo software (Tree Star).

Autoantibody analysis

ELISAs were performed to quantify levels of anti-dsDNA Abs using a 1:10 dilution of serum as described previously (16). Raw OD values were compared with an 11-point standard curve prepared by serial 1:2 dilutions of aged MRL/lpr serum defining a 1:100 dilution as 100 reference units. The activated lymphocytes together with elevated basal serum Ig levels in B6.Slamf4−/− mice suggested a breach in self-tolerance and led us to assess a cohort of aged B6.Slamf4−/− mice and WT littermate controls for development of spontaneous autoantibodies. Although titers were low in comparison with MLR/lpr mice, 32% of 12-mo-old female B6.Slamf4−/− mice developed a significant increase in anti-dsDNA Abs (Fig. 1C). Thus, B6.Slamf4−/− mice can spontaneously develop humoral autoimmunity with age.

Results and Discussion

B6.Slamf4−/− mice develop mild spontaneous autoimmunity

To assess the role of slamf4 in autoimmune disease without the confounding influence of mixed genetic background, we generated B6.Slamf4−/− mice. T cell maturation in the thymus and levels of mature lymphocyte subsets (CD4, CD8, and B cell) in the spleens of B6.Slamf4−/− mice are comparable to WT mice (data not shown), consistent with published reports of other B6.Slamf4−/− mice (17). To determine whether B6.Slamf4−/− mice developed spontaneous immune activation, we first compared percentages of activated lymphocytes in spleens of 10- to 22-wk-old WT and B6.Slamf4−/− mice. Compared with WT controls, B6.Slamf4−/− mice showed significantly higher percentages of CD69+, CD44hi, and CD62lo CD4 cells (Fig. 1A) but a similar proportion of CD69+ CD8 cells (data not shown) and a modest increase in splenocyte numbers compared with WT mice (~2- to 3-fold more activated CD4 cells and B cells per spleen). In addition, B6.Slamf4−/− mice had a significantly greater percentage of CXCR5+CD4+ T follicular helper cells, a greater proportion of GC cells (GL-7+), and fewer marginal zone B cells (AA4.1+, CD21+, and CD23−), reminiscent of autoimmune-prone mouse strains (Supplemental Fig. 1A) (18, 19). These data suggest slamf4 plays an inhibitory role in regulating T cell-dependent B cell responses.

We next compared serum Ig levels in naive and immunized WT and B6.Slamf4−/− mice. Naive 8- to 12-wk-old B6.Slamf4−/− mice exhibited a trend toward higher basal levels of IgM compared with WT mice, but this did not reach statistical significance. There was, however, a 2-fold increase in total serum IgG (Fig. 1B) with a relative decrease in IgG1 and an increase in IgG2c in B6.Slamf4−/− compared with WT mice (data not shown). Despite the increased basal serum IgG, B6.Slamf4−/− mice developed similar levels of Ag-specific Ab responses as WT mice to both T-dependent and T-independent Ags (Supplemental Fig. 1B). These findings indicate that the Slamf4 deficiency does not alter overall Ab responses.

The activated lymphocytes together with elevated basal serum Ig levels in B6.Slamf4−/− mice suggested a breach in self-tolerance and led us to assess a cohort of aged B6.Slamf4−/− mice and WT littermate controls for development of spontaneous autoantibodies. Although titers were low in comparison with MLR/lpr mice, 32% of 12-mo-old female B6.Slamf4−/− mice developed a significant increase in anti-dsDNA Abs (Fig. 1C). Thus, B6.Slamf4−/− mice can spontaneously develop humoral autoimmunity with age.

B6.Slamf4−/− mice exhibit significantly enhanced responses in an induced model of SLE

To further investigate the role of slamf4 in humoral autoimmunity, we used a well-characterized induced GVH model of autoimmunity. B6 and bm12 mice share MHC class I haplotypes but differ in their MHC class II alleles by 3 aa; therefore, transfer of bm12 splenocytes into B6 mice results in polyclonal activation of donor CD4 cells that mediate autoimmunity, providing help to recipient B cells and driving high titers of anti-dsDNA Abs (Fig. 2A) (15). This model has the advantage of providing a defined cell population to break tolerance in recipient mice and enables measurement of anti-dsDNA Abs in as little as 2 wk.
Transfer of bm12 splenocytes into WT or B6.Slamf4^{2/2} mice resulted in a marked increase in anti-dsDNA production in B6.Slamf4^{2/2} recipients 2–4 wk posttransfer (Fig. 2B).

Transfer of purified CD4 cells from bm12 mice into WT or B6.Slamf4^{2/2} mice led to a similar 5-fold increase in levels of anti-dsDNA in B6.Slamf4^{2/2} mice (Fig. 2B). In addition, B6.Slamf4^{2/2} mice exhibited a striking increase in spleen size and splenocyte numbers compared with WT recipients (Fig. 2C). Spleens from B6.Slamf4^{2/2} recipients had a marked elevation in plasma cells compared with WT recipients (Fig. 3A) and increased percentages of GC cells, IgM^{+}/IgD^{+} cells, and CD27^{+} B cells 2 wk after bm12 splenocyte transfer (data not shown). Thus, B6.Slamf4^{2/2} mice exhibit significantly increased autoreactive responses to a GVH stimulus compared with WT mice.

The enhanced humoral autoimmunity observed in the B6. Slamf4^{2/2} recipients could either be due to a failure to restrain autoreactive B cells or secondary to dysregulated CD4 help for autoreactive B cells. To begin to investigate this issue, we compared CD4 T cells responses in WT thy1.1 versus B6.Slamf4^{2/2} thy1.1 recipients of bm12 splenocytes, which express the thy1.2 allele on their T cells. There was a significantly larger population of donor CD4 cells in B6.Slamf4^{2/2} compared with WT recipients 2 wk after transfer (Fig. 3B). Intracellular cytokine staining of splenocytes stimulated directly ex vivo revealed a greater proportion of donor cells producing IL-10 and fewer donor cells producing IL-2 in B6.Slamf4^{2/2} compared with WT recipients (Fig. 3C). Transfer of CFSE-labeled donor cells failed to show any difference in the initial proliferative response of bm12 CD4 cells in WT versus B6.Slamf4^{2/2} recipients (Supplemental Fig. 2), suggesting a role for slamf4 in controlling T cell survival. Thus, despite receiving an identical population of splenocytes, the presence or absence of slamf4 in the recipient altered the function of donor CD4 T cells. These data point to a key role for slamf4 in limiting CD4 T cell responses.
depletion of NK cells (Fig. 4A), *B6.Slamf4*−/− recipients exhibited augmented splenomegaly with increased spleen cellularity (Fig. 4B, 4C), increased plasma cell numbers (Fig. 4D), a greater number of donor CD4 cells (Fig. 4E), and elevated anti-dsDNA Abs (Fig. 4F). Taken together, our data demonstrate a novel NK-independent role for slamf4 in regulating humoral autoimmunity.

The absence of slamf4 may impact the balance between T cell activation and tolerance in a number of ways. Our NK depletion studies point to a role for slamf4 on a non-NK cell in limiting CD4 T cell responses and give impetus to further study of slamf4 function on other cells such as APCs, CD8 T cells, γδ T cells, and granulocytes. Slamf4 on APCs may engage slamf2 on CD4 cells and thereby regulate CD4 T cell responses and T cell-dependent B cell responses. However, it is also possible that slamf4 on γδ T cells, CD8 cells, or APCs may alter the B response, and these altered B cells subsequently drive the differences in CD4 cell function. In addition, because slamf2 binds CD2 as well as slamf4, disruption of slamf4–slamf2 interactions may have indirect effects by allowing an increase in slamf2–CD2 interactions. Finally, because slamf molecules share common signaling pathways, it is possible that in the absence of slamf4, these signaling molecules would be free to augment responses through other slamf receptors such as slamf6.

The data presented in this article shed light on new functions for slamf4 outside of its role on NK cells. Our results clearly show slamf4 has an NK-independent negative regulatory function in vivo and can contribute to tolerance to chromatin in murine models of SLE. In addition, the phenotype of *B6.Slamf4*−/− mice supports roles for multiple slamf members in

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**FIGURE 3.** Slamf4 deficiency alters B cell and CD4 cell responses in a GVH model of SLE. A. Two weeks after transfer of *bm12* splenocytes, spleen cells from WT or *B6.Slamf4*−/− (KO) recipients were stained for CD138 to enumerate plasma cells by flow cytometry. Representative flow cytometry plots are shown (left panels) with symbols depicting the percentage of CD138+ cells of a live gate (right panel) from one of three representative experiments using a total of 15 mice/group. B. Splenocytes from *bm12* mice (bearing Thy1.2 allele) were transferred into WT Thy1.1 or *B6.Slamf4*−/− Thy1.1 mice. Blood was serially sampled from recipients (left panel), and spleens were harvested after 2 wk (right panel) for analysis of donor origin cells by staining for CD4 and Thy1.2. Symbols depict the mean with error bars showing SEM with representative data from two (blood) or five (spleen) experiments using a total of 17 or 32 mice/group, respectively. C. Splenocytes were harvested 2 wk after adoptive transfer of *bm12* cells and stimulated directly ex vivo for intracellular cytokine staining. Representative FACS plots of Thy1.2-gated donor CD4 cells are shown (left panel); symbols depict the percentage of donor cells positive for indicated cytokines (right panel). Data are from one of two experiments using a total of 10 mice/group.

**Enhanced autoimmune response of B6.Slamf4−/− mice is NK independent**

Most functional studies of slamf4 have focused on its role in modulating NK cell function. Recent work indicates that slamf4 on NK cells can affect the adaptive immune response by altering B cell and CD8 function (11, 20). Thus, it is possible that perturbed NK function in *B6.Slamf4*−/− mice might lead to enhanced CD4 and/or B cell responses. To address this issue, we examined production of anti-dsDNA and splenocyte phenotype in NK-depleted WT and *B6.Slamf4*−/− recipients following transfer of *bm12* splenocytes. Despite a significant

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**FIGURE 4.** Enhanced autoimmunity of *B6.Slamf4*−/− mice in chronic GVH is NK independent. A. Splenocytes from *bm12* mice were injected into WT and *B6.Slamf4*−/− (KO) mice given control Ig (ctrl Ig) or anti-NK1.1 (aNK) to deplete NK cells. Mice were analyzed after 2 wk to assess the efficacy of NK depletion by staining spleenocytes for NK1.1 and DX-5. Spleens were examined grossly (B), and live cells enumerated using light scatter properties (C). D. Percentages of plasma cells in the live cell gate were determined by staining for CD138. E. Percentages of donor origin cells in the CD4 gate were determined by staining for Thy1.2. F. Anti-dsDNA was measured by ELISA. Data are from a single experiment using five mice per group.
contributing to the Sle1b phenotype and regulating lupus susceptibility. Further investigation will be necessary to elucidate the precise mechanisms by which slamf4 protects from autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplement Figure Legends

**Suppl. Figure 1S.** *B6.Slamf4/-* mice have increased T follicular helper cells and altered B cell populations but mount antigen-specific antibody responses comparable to wild-type mice. (A) Splenocytes from WT (black circles) or *B6.Slamf4/-* (KO) (white circles) mice were stained for CXCR5 as a marker of T follicular helper cells, GL-7 as a marker for germinal center B cells, or CD21 and CD23 to determine the percent of marginal zone B cells. Representative flow cytometry plots of the indicated gate are shown (left panels) as well as the percentage of positive cells for individual mice (right panels). Data shown are from 1 of 3 representative experiments using a total of 32 mice between 10 and 22 weeks old. (B) WT or *B6.Slamf4/-* mice were immunized with TNP-Ovalbumin (T-dependent) or NP-Ficoll (T-independent) emulsified in a 1:1 mixture of PBS with Complete Freund’s Adjuvant. For primary immune responses, mice were bled 2 weeks later and antigen-specific IgM and IgG were measured by ELISA. For T-dependent immune responses, a secondary challenge with TNP-Ovalbumin emulsified in 1:1 PBS with Incomplete Freund’s Adjuvant was performed. Secondary antibody responses were measured 13 days after the rechallenge. Data for T-independent responses are representative of 2 experiments using a total of 15 mice per group and data for T-dependent responses are from a single experiment using 8 mice per group. Symbols depict the mean O.D. values from a representative experiment using 8 mice per group with error bars showing SEM.
Suppl. Figure 2S. Initial proliferation of \textit{bm12} CD4 cells during GVH induction is comparable between WT and \textit{B6.Slamf4-/-} recipients. Splenocytes from \textit{bm12} mice were labeled with 1 µM CFSE before adoptive transfer into WT Thy1.1 (black line) or \textit{B6.Slamf4-/-} Thy1.1 (green line) mice. Splenocytes were harvested from recipients 3 or 5 days later and analyzed by flow cytometry for the dilution of CFSE in donor (Thy1.2+), CD4 gated cells. Histograms show the percentage of cells with a given CFSE fluorescence on the x-axis summed from 3 WT and 2 \textit{B6.Slamf4-/-} (Day 3) or 5 WT and 5 \textit{B6.Slamf4-/-} (Day 5) independently analyzed mice.
Day 3

Day 5