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Increased Expression of SLAM Receptors SLAMF3 and SLAMF6 in Systemic Lupus Erythematosus T Lymphocytes Promotes Th17 Differentiation

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Altered T cell function in systemic lupus erythematosus (SLE) is determined by various molecular and cellular abnormalities, including increased IL-17 production. Recent evidence suggests a crucial role for signaling lymphocyte activation molecules (SLAMs) in the expression of autoimmunity. In this study, we demonstrate that SLAMF3 and SLAMF6 expression is increased on the surface of SLE T cells compared with normal cells. SLAM coengagement with CD3 under Th17 polarizing conditions results in increased IL-17 production. SLAMF3 and SLAMF6 T cell surface expression and IL-17 levels significantly correlate with disease activity in SLE patients. Both naive and memory CD4+ T cells produce more IL-17 in response to SLAM costimulation as compared with CD28 costimulation. In naive CD4+ cells, IL-17 production after CD28 costimulation peaks on day 3, whereas costimulation with anti-SLAMF3 and anti-SLAMF6 Abs results in a prolonged and yet increasing production during 6 d. Unlike costimulation with anti-CD28, SLAM costimulation requires the presence of the adaptor molecule SLAM-associated protein. Thus, engagement of SLAMF3 and SLAMF6 along with Ag-mediated CD3/TCR stimulation represents an important source of IL-17 production, and disruption of this interaction with decoy receptors or blocking Abs should mitigate disease expression in SLE and other autoimmune conditions. The Journal of Immunology, 2012, 188: 1206–1212.

For optimal T cell activation, recognition of the Ag/MHC complex by the TCR is accompanied by signals mediated through costimulatory pathways (1, 2). CD28 costimulation is best characterized for T cell activation (3), but there is evidence for other costimulatory molecules, including signaling lymphocyte activation molecule (SLAM) receptor family members (4, 5). Recently, the SLAM family of type I transmembrane receptors has been reported to mediate important regulatory signals between immune cells through their homophilic or heterophilic interactions. SLAM receptors are expressed on hematopoietic cells, including cells of the innate immune system, as well as T and B cells. By virtue of their ability to transduce tyrosine phosphorylation signals through immunoreceptor tyrosine-based switch motif sequences, SLAM receptors play an important role in regulating both innate and adaptive immune responses. Upon activation, SLAM molecules associate with intracellular adaptor proteins, for example, those of the SLAM-associated protein family (6–11). SLAM-associated proteins (SAPs) contribute to SLAM receptor activation as they mediate dimerization of SLAM receptors and compete with SLAM-induced signals. SAP deficiency is associated with severe NK, T, and B cell abnormalities and reduced Ab production (12).

Recent evidence indicates that SLAM signaling is also involved in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE) (13, 14). SLE is a chronic autoimmune inflammatory disease that is characterized by improper regulation of B and T cell function (15). The SLAM gene cluster encodes several costimulatory receptors, including SLAMF3 and SLAMF6. It is located within a genomic region, which entails genes with key immunological functions, including the Fc receptor cluster, the SLAM cluster, CD58, IL23R, TLR5, and complement receptor CR1 (16, 17). Polymorphisms in the SLAM cluster have been associated with autoimmune diseases in mice and humans for which this region was designated Sle1b locus and is considered as a genetic susceptibility region for the development of SLE (16, 18, 19). Polymorphisms in the Ly108 gene, one of the members of the SLAM family receptors (corresponding to SLAMF6 in humans), result in the generation of an Ly108 splice variant in lupus-prone mice that is involved in the pathogenesis of SLE (16, 20).

Differentiation of CD4+ Th cells into distinct effector populations is one of the hallmarks of adaptive immune responses. Previous reports suggest that costimulation of Th cells through SLAMF6 promotes a Th1 phenotype under polarizing and nonpolarizing conditions and, furthermore, SLAMF6 appears to have superior costimulatory capacities when compared with CD28, especially on CD8+ and CD4/CD8 double-negative T cells (14, 21). Another member of the SLAM family, SLAMF3 (also known as CD229/Ly9 in mice), which is expressed on T and B cells, has been reported to promote Th2 differentiation (22). The most recently discovered Th cell subset, denoted Th17 cells, is characterized by abundant production of IL-17A (referred to in this article as IL-17), IL-21, and IL-22 and plays a major role in host
responses against bacterial infections and the development of autoimmune diseases, including SLE (23, 24). Indeed, higher serum concentrations of IL-17 have been reported in SLE patients (25, 26), and studies in lupus-prone mice provide evidence for IL-17 as a crucial mediator of disease pathology in SLE (27–29).

Because SLAMF6 and SLAMF3 have been shown to be engaged in Th cell differentiation, we asked whether SLAM receptors play a role in the pathogenesis of SLE, and whether they contribute to Th17 differentiation. We report that surface expression of SLAMF6 and SLAMF3 is increased in SLE T cells and mirrors disease activity. Our results indicate that coactivation of SLAMF6 and SLAMF3 receptors along with CD3/TCR stimulation potently induces IL-17 production and thus accounts for Th17 generation in T cells from both healthy controls and SLE patients.

Materials and Methods

Study subjects and T cell culture

All SLE patients included in our studies were recruited from the Division of Rheumatology at Beth Israel Deaconess Medical Center (Boston, MA) and diagnosed according to the revised SLE classification criteria of the American College of Rheumatology (30). SLE disease activity index (SLEDAI) scores ranged from 0 to 10. Written informed consent was obtained from all patients. Control blood samples were obtained from healthy platelet donors at the Kraft Family Blood Donor Center (Dana-Farber Cancer Institute, Boston, MA). Primary total T cells were isolated from peripheral venous blood by negative selection as described previously (14). All primary T cells were kept in RPMI 1640 medium supplemented with 10% FBS. Naive (CD4+CD45RA+) and memory (CD4+CD45RO+) T cells were purified using T cell isolation kits from Miltenyi Biotec according to the manufacturer’s instructions.

T cell stimulation, Th17 differentiation assays, and ELISAs

Cell culture plates were precoated overnight with 0.5 μg/ml monoclonal anti-CD3 (clone OKT3; BioXCell), 0.5 μg/ml anti-CD28 (BioLegend), 0.5 μg/ml anti-SLAMF6 (clone 24D8.1H5.1F5; Genentech), 0.5 μg/ml anti-SLAMF3 Abs (clone HLy-9.1.25; BioLegend), or 0.5 μg/ml control IgG1 as indicated. Naive and memory CD4+ T cells (1 × 10^6/well) were differentiated into Th17 cells in serum-free X-VIVO 10 medium (Bio-Whittaker) by the addition of IL-6 (25 ng/ml), TGF-β1 (5 ng/ml), IL-1β (12.5 ng/ml), IL-21 (25 ng/ml), and IL-23 (25 ng/ml) for the indicated times. IL-6, IL-1β, IL-23, and TGF-β1 were obtained from R&D Systems. IL-21 was purchased from Cell Sciences. Supernatants were collected at different time points and tested for IFN-γ (Endogen) and IL-17 (eBioscience) by ELISA.
Flow cytometry

Intracellular T cell staining for IL-17 and IFN-γ was performed at the indicated time points using the BD Cytofix/Cytoperm kit and Alexa 647-labeled anti-IL-17 (BD Biosciences) and PE-labeled anti–IFN-γ (BioLegend) Abs. Cells were stimulated with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for a total period of 6 h. GolgiStop (monensin) was added 30 min after T cell stimulation was initiated. Surface staining was performed using a Pacific Blue-labeled anti-CD4 Ab (BioLegend) for 20 min on ice. Samples were analyzed on an LSRII flow cytometer (BD Biosciences) and analysis was performed with FlowJo software version 8.3.3 (Tree Star).

Small interfering RNA experiments

Naive (CD4+CD45RA+) T cells were purified from healthy donors and transfected with different concentrations of small interfering RNA (siRNA) oligonucleotides specific for SLAMF3, SLAMF6, SAP, or a nonspecific control siRNA (all from Applied Biosystems) using the Amaxa transfection system (Lonza). SAP siRNA transfection efficiency was confirmed by immunoblotting of cytosolic protein lysates using anti-SAP (Cell Signal Technology), anti–β-actin (Sigma-Aldrich), and suitable HRP-linked secondary Abs. SLAMF3 and SLAMF6 siRNA transfection efficiency was confirmed by analyzing their surface expression by flow cytometry using anti-SLAMF3 and anti-SLAMF6 Abs (both from BioLegend). All subsequent assays were performed 4 d after transfection at the point of maximal knockdown.

Statistical analyses

The paired two-tailed Student t test and the Pearson product moment correlation coefficient (r) were used for statistical analyses.

FIGURE 3. SLAM costimulation promotes Th17 differentiation in naive CD4+ T cells. A–C, Naive (CD45RA+CD62Lhi) CD4+ T cells were stimulated with anti-CD3 and costimulatory anti-CD28, anti-SLAMF3, SLAMF6, and control IgG under Th17 conditions for 7 d. IL-17 and IFN-γ production was analyzed by intracellular staining and ELISA. A, Secretion of IL-17 by naive CD4+ T cells on day 7 was measured by ELISA. B, Percentages of IL-17-producing cells. The relative number of IL-17–producing cells is given as a percentage of naive CD4+ T cells. C, One representative experiment is displayed (from day 7). D, Kinetics of IL-17 secretion by naive CD4+ T cells in response to costimulation through CD28, SLAMF3, and SLAMF6. On days 3, 5, and 7 the IL-17 levels were monitored by ELISA. E, Naive CD4+ T cells were labeled with CFSE, then stimulated and differentiated until day 7. Staining on day 7 was as above. No differences in CFSE staining were detected between the different groups, indicating comparable proliferation rates.
Results

SLAMF3 and SLAMF6 are upregulated on SLE T cells and serve as major costimuli for Th17 differentiation

To assess the involvement of SLAM costimulation in SLE pathogenesis, we performed flow cytometric analysis of SLAM receptor surface expression on CD4+ T cells obtained from 11 SLE patients at different levels of disease activity as measured by the SLEDAI and from 11 healthy control subjects. We observed a significantly increased surface expression of both SLAMF3 and SLAMF6 on CD4+ T cells from SLE patients in comparison with the healthy individuals (Fig. 1A,1B). Of note, there was a robust correlation between the individual cell surface expression of SLAMF6 on SLE CD4+ T cells and the corresponding SLEDAI indices (r = 0.9178; Fig. 1D); however, we did not find a significant correlation in the case of SLAMF3 (Fig. 1C).

Because it has been reported that SLAM signaling is involved in Th cell differentiation and that SLE patients display increased serum levels of IL-17, we hypothesized that the observed upregulation of SLAMF3/SLAMF6 expression in SLE CD4+ T cells contributes to an augmented production of IL-17. Thus, we stimulated T cells from SLE patients and healthy controls with anti-CD3 and costimulatory anti-CD28, anti-SLAMF3, or anti-SLAMF6 Abs in the presence of Th17 polarizing stimuli (i.e., IL-6, IL-1β, IL-21, IL-23, and TGF-β1). On day 6 after stimulation and differentiation had been initiated, we measured IL-17 levels in cell culture supernatants (Fig. 2A) and the percentage of IL-17–producing CD4+ T cells (Fig. 2B, Supplemental Fig. 1). Overall, SLE CD4+ T cells were found to produce more IL-17 under these conditions. Intriguingly, costimulation through both SLAM receptors was more potent in the induction of IL-17 production when compared with CD28 costimulation. We observed a strong correlation between the clinical disease activity and the corresponding percentage of IL-17–producing CD4+ T cells as induced through costimulation with either anti-SLAMF3 or anti-SLAMF6 Abs (r = 0.9361 and 0.9701, respectively; Fig. 2C, 2D). To determine whether Th17 polarizing cytokines were necessary for the production of IL-17 following engagement of SLAMF3 and SLAMF6 molecules on the surface of SLE T cells, T cells from SLE patients and healthy controls were stimulated with anti-CD3 and anti-CD28, anti-SLAMF3, or anti-SLAMF6 Abs in the absence of Th17 polarizing cytokines. We observed an increase in IL-17A production (Supplemental Fig. 2A, 2B) following SLAM costimulation compared with stimulation with CD28, suggesting that SLAMF3/6 costimulation can cause IL-17 production in SLE T cells in the absence of Th17 polarizing cytokines.

Taken together, our data suggest that T cells from active SLE patients are characterized by an increased SLAM receptor expression, which is linked to increased IL-17 production.

SLAM-mediated costimulation promotes Th17 differentiation in human T cells

Next, we studied the involvement of SLAM receptors in human naive (CD45RA+) and memory (CD45RO+) CD4+ T cells. Thus, we stimulated naive CD4+ T cells with plate-bound anti-CD3 and costimulatory anti-CD28, anti-SLAMF3, or anti-SLAMF6 Abs under Th17 differentiation conditions for 7 d and subsequently analyzed IL-17 production by flow cytometry and ELISA. In line with our observations in total CD4+ T cells, Th17-differentiated naive CD4+ T cells produced significantly more IL-17 in response to SLAM costimulation when compared with CD28 costimulation (Fig. 3A–C). Interestingly, time kinetics of CD28 and SLAM costimulation were profoundly different. Whereas IL-17 protein expression peaked on day 3 following T cell activation through CD3/CD28 costimulation and gradually decreased over time until day 7, it further increased in response to either SLAMF3 or SLAMF6 costimulation throughout the observation period (Fig. 3D). To exclude the possibility that IL-17 production is an effect of increased proliferation versus Th17 differentiation in response...
to SLAM costimulation, we performed comparative proliferation analysis of SLAM and CD28 costimulated naive CD4+ T cells. Naive CD4+ T lymphocytes exhibited comparable rates of cell division in response to CD28 and SLAM costimulation as assessed by CSFE staining, whereas IL-17 production was increased upon SLAM costimulation (Fig. 3E). This suggests that activated SLAM signaling transfers enhanced differentiation capacities to naive CD4+ T cells toward a Th17 phenotype under polarizing conditions, rather than solely exerting proliferative effects on IL-17–producing cells.

Next, we performed similar studies in memory CD4+ T cells. As expected, CD28 costimulation yielded higher IL-17 concentrations when compared with naive CD4+ T cells. CD3/SLAM-mediated costimulation of memory T cells induced levels of IL-17 comparable to CD3/CD28 costimulation (Fig. 4A–C); however, time kinetics revealed an increase in IL-17 production by memory CD4+ T cells from day 3 to 6 in response to SLAM costimulation, whereas CD28 costimulation led to a mild decrease during the observed time period (Fig. 4D).

SLAM depletion reduces IL-17 production in response to T cell stimulation

To investigate the specificity of SLAM-promoted Th17 differentiation, we silenced SLAMF3 and SLAMF6 expression in naive CD4+ T cells with siRNA. Efficiency of SLAM knockdown was assessed by surface staining (Supplemental Fig. 3A, 3C). Four days after siRNA transfection, cells were stimulated with anti-CD3 and anti-SLAM Abs under Th17 polarizing conditions. Naive CD4+ T cells that had been transfected with SLAM siRNA were costimulated with anti-CD3/anti-SLAM Abs, which resulted in a severely impaired IL-17 production as determined by ELISA analyses (Supplemental Fig. 3B, 3D).

SLAM-promoted Th17 differentiation in human T cells involves SAP-dependent pathways

Next, we sought to analyze whether the adaptor protein SAP is involved in SLAM-mediated Th17 differentiation. Therefore, we knocked down endogenous SAP protein using siRNA transfections in naive CD4+ T cells (Fig. 5A). After SAP depletion, we observed a severely impaired IL-17 production following anti-CD3/anti-SLAM but not following anti-CD3/anti-CD28 stimulation under Th17 polarizing conditions (Fig. 5B, 5C). This suggests that SAP is engaged in SLAM-mediated Th17 differentiation of human naive CD4+ T cells.

**Discussion**

In the present study, we investigated classical/canonical and noncanonical TCR costimulatory molecules and their contribution to the differentiation of total, naïve, and memory CD4+ T cells toward a Th17 phenotype. We document that the noncanonical costimulatory molecules SLAMF3 and SLAMF6 exhibit more potent costimulatory capacities in Th17 generation in both control and SLE T cells. This is of special interest, as SLE T cells display increased surface expression of SLAM receptor proteins. The degree of SLAMF3 and SLAMF6 expression correlated with disease severity as assessed by individual SLEDAI scores.

In the presence of Th17 polarizing cytokines, costimulation through SLAMF3 or SLAMF6 results in increased production of IL-17 in naive CD4+ T cells and follows different time kinetics than those elicited by CD28. IL-17 production following CD28 costimulation peaks at day 3 and may relate to a normal immune response elicited by the Ag involving the CD3/TCR complex. In contrast, engagement of SLAMF3 and SLAMF6 after exposure to Ag may relate to a prolonged inflammatory response, which may cause organ damage. In SLE patients the CD3/TCR complex may be engaged by autoantigens or circulating anti-CD3/TCR autoantibodies (31).

It is well documented that effector memory CD4+ T cells are principal producers of IL-17 in vivo (32, 33). Our observations provide evidence that under Th17 polarizing conditions IL-17 production by memory CD4+ T cells is significantly increased upon TCR costimulation through SLAMF3 when compared with canonical TCR costimulation by CD28. This is of special interest because pathogenic memory CD4+ T cells are expanded and naive CD4+ T cells are decreased in SLE patients (34–37). This suggests that in addition to increased Th17 differentiation from naive CD4+ T cells, SLAM signaling contributes to increased IL-17 expression from pathologically expanded memory CD4+ T cells in SLE patients.

SLAM molecules associate with intracellular adaptor proteins, such as SAP family members. SAP proteins contribute to SLAM receptor activation as they mediate dimerization of SLAM receptors. SAP proteins have been reported to promote Th1 and Th2 differentiation (38–42). Our observation that SAP blockade with SAP siRNA results in significantly reduced IL-17 expression from T cells suggests a key role for SAP as a common proximal signal in SLAM-promoted IL-17 production. As for the aforementioned SLAM
receptors, SAP proteins may be promising targets for pharmacological blockade. Unlike further downstream molecules, such as MAPK, SAP proteins are limited to SLAM-associated pathways, which contribute to (pathological) Th17 generation and IL-17 expression in SLE and presumably other autoimmune diseases (43). However, farther downstream molecules through which SLAM molecules promote Th17 differentiation remain to be elucidated, and future studies are warranted to unravel mechanisms how SLAM receptors promote and sustain an increased IL-17 production.

Our observations that TCR costimulation with SLAMF3 and SLAMF6 promotes Th17 differentiation, and that SLAMF3 and SLAMF6 surface expression on SLE T cells is significantly increased when compared with healthy controls and reflects disease activity, may also link the genomic location of SLAM family genes within the lupus susceptibility locus Sle1b to reported SLAM polymorphisms in lupus-prone mice and SLE patients (13, 16, 18, 20). Previous studies indicated that SLAM polymorphisms contribute to SLE pathogenesis through increased gene expression (16). Polymorphisms in the Ly108 (SLAMF6) gene result in overexpression of an Ly108 splice variant in lupus-prone mice. This is in agreement with our previous report that SLAMF6 co-stimulation of human SLE T cells results in reduced secretion of IL-2 and Th1 cytokines resembling a SLE phenotype (14).

To our knowledge, this is the first report to demonstrate that the noncanonical costimulatory molecules SLAMF3 and SLAMF6 promote Th17 differentiation. Both of these molecules are more potent inducers of Th17 differentiation when compared with the canonical costimulatory molecule CD28. Furthermore, SLAMF3 and SLAMF6 surface expression is increased on SLE T cells in a disease activity-dependent manner. The superior capacities of SLAM molecules in Th17 generation may be attributed to yet unidentified downstream signal transducers. We think that our studies hold the promise to create a new platform to study Th17 cells from a different perspective, and the SLAM family of costimulators could be chosen as therapeutic targets to control SLE and autoimmune disorders. Future studies are needed to 1) investigate SLAM receptor expression in different autoimmune diseases, 2) dissect SLAM propensities in Th cell differentiation under polarizing and nonpolarizing conditions, and 3) analyze the involvement of downstream factors that account for SLAM-promoted IL-17 gene expression.

The significance of our findings is manifold. The strong correlation between SLE disease activity and SLAMF3 and SLAMF6 expression on the surface of SLE T cells suggests that their expression levels may gain biomarker value. The fact that SLAM engagement leads to increased and prolonged production of IL-17 strongly suggests that blockade of their engagement either with blocking Abs or decoy receptors may suppress the inflammatory response without affecting IL-17 production through the canonical engagement of CD28.

Disclosures

The authors have no financial conflicts of interest.

References


Suppl. figure 1: IL-17 production in CD28-, SLAMF3- and SLAMF6-co-stimulated CD4⁺ T cells from a healthy control (CON) and a SLE patient. Total T cells obtained from healthy controls (CON) and SLE patients were cultured under Th17 differentiation conditions along with anti-CD3 antibodies and co-stimulatory anti-CD28, anti-SLAMF3 or anti-SLAMF6 antibodies as indicated for a total period of 6 days. Subsequently, intracellular IL-17 staining was performed gating on CD4⁺ T cells. Representative flow cytometry pictures from one control and one SLE T cell sample is displayed.

Suppl. figure 2: SLAMF3 and SLAMF6 co-stimulation produce IL-17A in SLE patients even under non Th17 polarizing conditions. Total T cells obtained from 12 healthy controls (CON) and SLE patients were cultured in complete RPMI along with anti-CD3 antibodies and co-stimulatory anti-CD28, anti-SLAMF3 or anti-SLAMF6 antibodies as indicated for a total period of 6 days without any Th17 polarizing cytokines. Subsequently, intracellular IL-17 staining was performed gating on CD4⁺ T cells (A) and the supernatants were analysed for ELISA (B).

Suppl. figure 3: Effects of SLAM depletion on IL-17 production. Naïve CD4⁺ T cells were transfected with siRNA targeting SLAMF3 (A, B), SLAMF6 (C, D), and control siRNA. Efficacy of siRNA transfection was determined by investigating SLAM surface expression (A, C). Then, cells were co-stimulated with SLAMF3 (B) and SLAMF6 (D) in addition to CD3 under Th17 differentiation conditions (3 days). In response to SLAM knock-down, IL-17 expression was significantly reduced.
Suppl. figure 1
Suppl. figure 2