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Signaling Lymphocytic Activation Molecule Expression and Regulation in Human Intracellular Infection Correlate with Th1 Cytokine Patterns

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Induction of Th1 cytokines, those associated with cell-mediated immunity, is critical for host defense against infection by intracellular pathogens, including mycobacteria. Signaling lymphocytic activation molecule (SLAM, CD150) is a transmembrane protein expressed on lymphocytes that promotes T cell proliferation and IFN-γ production. The expression and role of SLAM in human infectious disease was investigated using leprosy as a model. We found that SLAM mRNA and protein were more strongly expressed in skin lesions of tuberculoid patients, those with measurable CMI to the pathogen, Mycobacterium leprae, compared with lepromatous patients, who have weak CMI against M. leprae. Peripheral blood T cells from tuberculoid patients showed a striking increase in the level of SLAM expression after stimulation with M. leprae, whereas the expression of SLAM on T cells from lepromatous patients showed little change by M. leprae stimulation. Engagement of SLAM by an agonistic mAb up-regulated IFN-γ production from tuberculoid patients and slightly increased the levels of IFN-γ in lepromatous patients. In addition, IFN-γ augmented SLAM expression on M. leprae-stimulated peripheral blood T cells from lepromyop patients. Signaling through SLAM after IFN-γ treatment of Ag-stimulated cells enhanced IFN-γ production in lepromatous patients to the levels of tuberculoid patients. Our data suggest that the local release of IFN-γ by M. leprae-activated T cells in tuberculoid leprosy lesions leads to up-regulation of SLAM expression. Ligation of SLAM augments IFN-γ production in the local microenvironment, creating a positive feedback loop. Failure of T cells from lepromatous leprosy patients to produce IFN-γ in response to M. leprae contributes to reduced expression of SLAM. Therefore, the activation of SLAM may promote the cell-mediated immune response to intracellular bacterial pathogens. The Journal of Immunology, 2001, 167: 5719–5724.

Immunity to intracellular pathogens such as mycobacteria requires IFN-γ (1), a macrophage-activating cytokine produced by T cells. Leprosy is a dynamic infectious disease in which distinct Mycobacterium leprae-responsive T cell subsets appear to control the clinical and immunologic spectrum. For example, tuberculoid leprosy patients, those able to restrict the growth of the pathogen and mount strong T cell responses to M. leprae, locally produce the Th1 cytokine pattern, including IFN-γ (2, 3). In contrast, lepromatous patients manifest disseminated infection, their T cells weakly respond to M. leprae, and their lesions express the Th2 cytokines, typical of humoral responses and suppression of cell-mediated immunity (CMI).3

Although Th cells go through a differentiation process that “programs” their cytokine production pattern upon TCR stimulation, additional factors can influence the level and pattern of cytokines produced by activated T cells. One of these factors is signaling lymphocytic activation molecule (SLAM, CD150), a transmembrane type I glycoprotein of the CD2 subfamily expressed on lymphocytes and immature thymocytes that boosts IFN-γ production and proliferation (4). The expression of SLAM is rapidly induced on naive T cells after activation and ligation of SLAM redirects Th2 responses to a Th1 or Th0 phenotype (5).

We wished to ascertain whether signaling through SLAM enhances T cell production of IFN-γ in intracellular infection, therefore, we investigated the role of SLAM in leprosy. We found that SLAM expression in leprosy correlates with Th1 responses in vitro and in vivo. SLAM ligation enhanced IFN-γ production and SLAM expression was up-regulated by IFN-γ, suggesting that a positive feedback loop exists whereby SLAM and IFN-γ promote CMI responses to mycobacterial infection.

Materials and Methods

Patients

Patients with leprosy were evaluated at the Hospital de Clínicas José de San Martín (University of Buenos Aires School of Medicine, Buenos Aires, Argentina) and at the Hospital de Infecciosas F.J. Muñiz (Buenos Aires School of Medicine, Buenos Aires, Argentina; † Division of Dermatology and ‡ Division of Dermatology, Hospital de Clínicas José de San Martín, University of Buenos Aires, Buenos Aires, Argentina; † Division of Dermatology and Department of Microbiology and Immunology, University of California, Los Angeles, CA 90095; † Division of Dermatology, Leprosy Section, Hospital de Infecciosas F.J. Muñiz, Buenos Aires, Argentina; and **Autoimmune Diseases Unit, Novartis Forschungsinstitut, Vienna, Austria

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3 Abbreviations used in this paper: CMI, cell-mediated immunity; SLAM, signaling lymphocytic activation molecule; T-Lep, tuberculoid leprosy; L-Lep, lepromatous leprosy.
Aires, Argentina) and were classified according to the criteria of Ridley and Jopling (6). Peripheral blood was collected in heparinized tubes from patients with tuberculous (T-Lep) and lepromatous (L-Lep) leprosy. After receiving informed consent, skin biopsies from leprosy patients were obtained, embedded in OCT medium (Ames, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70°C.

M. lepraem.

M. lepraewas provided by Dr. P. Brennan (Colorado State University, Ft. Collins, CO, National Institutes of Health contract N01-AI-75320) and was prepared by probe sonication (7). A similar extract was prepared from the virulentM. tuberculosisstrain H37Rv. In vitro stimulation of cells throughout the present study was performed with these sonicated preparations of mycobacteria.

Cell preparations and culture conditions

PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) and were cultured (1 × 10⁶/ml) with sonicated M. lepraerecombinant M. lepraee (10 µg/ml) in 24- or 96-well plates with RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with glutamine (2 mM, Sigma-Aldrich, St. Louis, MO), streptomycin, and human serum (10%). After 5 days, cells were washed and examined for SLAM expression by flow cytometry (below) or they were cultured in the presence of anti-SLAM mAb (A12) and examined for IFN-γ production by ELISA (Endogen, Woburn, MA). For studies involving SLAM expression, neutralizing IFN-γ Abs or isotype control Abs were added together with M. lepraecells in some cases. For IFN-γ production studies, human rIL-12 (100 nM) or human rIFN-γ (7.5 ng/ml; Endogen) were added to some cultures at final concentrations. The anti-SLAM mAb A12 has previously been described (4).

SLAM mRNA detection

RT-PCR for SLAM mRNA in leprosy lesions was performed using specific primers, as described (4). Briefly, total RNA was isolated using TRIzol reagent (Life Technologies). First-strand cDNA was synthesized using SuperScript reverse transcriptase (Life Technologies, Rockville, MD) and oligo dT priming (Amersham Pharmacia Biotech). cDNA samples were amplified with SLAM-specific PCR primers and Taq polymerase (PerkinElmer/Cetus, Norwalk, CT) in a DNA Thermocycler (PerkinElmer/Cetus). For comparison of SLAM mRNA levels among different samples, cDNA concentrations were normalized to yield equivalent β-actin PCR products. PCR products were electrophoresed on 1.5% agarose gels, transferred to nylon membranes (Amersham, Arlington Heights, IL), probed with a 32P-labeled SLAM oligonucleotide internal to the PCR primers, and visualized by autoradiography. The sequences of primer pairs, 5’ and 3’, were as follows: 5’-ATCTAGTTGAGAACGTG3’ and 3’-CCGTTACATGCCCC. The SLAM probe sequence was as follows: 5’-GACCCTGCACCTTGATCTGG-3’. PCR for IFN-γ mRNA was performed as described using specific primers (3). The IFN-γ probe sequence was as follows: 5’-TTCGAGACCCAAATTTGGTCTCC-3’.

SLAM protein detection

SLAM expression in leprosy lesions was determined by immunoperoxidase labeling of cryostat sections of biopsy samples (8) using anti-SLAM mAb (IPO-3; Kamiya Biomedical, Seattle, WA) or an isotype-matched control mAb. Briefly, cryostat sections (3–4 µm) were aceton fixed and blocked with normal horse serum before incubation with the mAbs for 60 min, followed by biotinylated horse anti-mouse IgG for 30 min. Slides were then incubated with phosphate buffer between incubations. Primary Abs were visualized with the ABC Elite system (Vector Laboratories, Burlingame, CA), which uses avidin and biotin-peroxidase conjugate for signal amplification. ABC was incubated for 30 min, followed by the addition of substrate (3-amin-9-ethylcarbazole) for 10 min. Slides were counterstained with hematoxylin and were mounted in aqueous dry mounting medium (Crystal Mount; Biomedica, Foster City, CA). The level of SLAM-positive cells in dermal granulomas was quantitated by calculating the percentage of positive cells based on the total number of cells within the granuloma as described (8).

To determine the level of SLAM expression on T cells, PBMCs cultured withM. lepraewere stained for surface expression with mAbs specific for CD3 (BD Biosciences, Mountain View, CA) and SLAM (A12) (4) before and after culture. Samples were analyzed on a FACScan flow cytometer (BD Biosciences). SLAM expression on cells cultured in media alone varied from patient to patient, but this variability was within the same range for both tuberculous and lepromatous patients.

Intracellular analysis ofIFN-γ production

Intracellular cytokine staining was used to determine the IFN-γ production at the single-cell level as previously described (9). Briefly, M. lepraestimulated cells were cultured with monensin (2 mM, Calbiochem, La Jolla, CA) for the final 2 h to induce the intracellular accumulation of newly synthesized proteins. Cells were then harvested, stained for surface expression with mAbs specific for CD3 (BD Biosciences) and SLAM (A12-4), and washed with PBS-2% FCS. Intracellular IFN-γ staining was performed with PE-conjugated anti-IFN-γ (BD Biosciences), using the Fix & Perm kit (Caltag Laboratories, San Francisco, CA) according to the manufacturer’s instructions. Samples were analyzed on a FACScan flow cytometer (BD Biosciences). Negative control samples were incubated with irrelevant, isotype-matched Abs in parallel with all experimental samples.

Statistical analysis

Statistical analysis was performed using nonparametric methods including the Wilcoxon signed rank test for paired samples or the Mann Whitney U rank sum test for independent samples. Values of p < 0.05 were considered significant.

Results

SLAM expression in leprosy lesions correlates with reduced disease severity

To investigate the role of SLAM in the Th1 response to M. lepraefs, we examined the pattern of SLAM mRNA expression in cells from leprosy lesions using RT-PCR. Our results showed that SLAM mRNA was strongly expressed in cells from tuberculoid lesions, whereas it was absent in most of the lepromatous patients (Fig. 1A). Furthermore, we determined the expression of IFN-γ mRNA in the same skin lesions. As expected, IFN-γ mRNAs were markedly higher in tuberculoid lesions than in lepromatous lesions (3). Interestingly, the only lepromatous patient that expressed weak levels of SLAM mRNA also expressed IFN-γ mRNA (Fig. 1A). The altered levels of SLAM in cells from leprosy patients suggest that differential expression of this activation molecule may contribute to the outcome of leprosy infection.

Next, we investigated the distribution of SLAM protein in leprosy using immunohistochemistry. SLAM protein expression in leprosy lesions correlated with mRNA levels. Fig. 1B shows numerous SLAM-positive cells with strong staining in a tuberculoid granuloma. In contrast, SLAM-positive cells were rare in lepromatous lesions. Isotype-matched control mAb staining was negative (data not shown). Quantification of SLAM-positive cells in leprosy lesions revealed that tuberculoid lesions contained 12.3 ± 3.8% (mean ± SEM) SLAM-positive cells, whereas lepromatous lesions contained 1.7 ± 0.8% (p < 0.001, rank sum test; Fig. 1C). These data indicate that local expression of SLAM is greatest in the group of patients with effective CMI, suggesting that the distribution of SLAM in leprosy parallels the level of CMI toM. lepraee. In contrast to our findings in the active lesions of leprosy patients, the levels of SLAM on resting peripheral blood T cells from tuberculoid and lepromatous patients were similar (Fig. 1D), supporting the hypothesis that the different T lymphocyte subsets in skin biopsies from leprosy patients are more closely related to the immune status of these individuals than those in peripheral blood (10). However, we found higher SLAM expression on CD3+ T cells from leprosy patients compared with healthy donors (data not shown), suggesting that, systemically, T cells in leprosy patients exist in an activated state, although not necessarily responsive to the pathogen.
T cells up-regulate SLAM after Ag stimulation in responsive, but not unresponsive, patients

Because SLAM was predominantly expressed in the lesions of patients characterized by enhanced CMI against *M. leprae*, we hypothesized that SLAM expression correlated with T cell activity against the pathogen. To investigate this possibility, PBMCs from leprosy patients were stimulated with *M. leprae*, and the expression of SLAM was determined. Fig. 2A shows the increase in SLAM expression in a tuberculoid patient after *M. leprae* stimulation, whereas *M. leprae* did not modify the percentage of SLAM-expressing T cells in a lepromatous patient (Fig. 2B). These results were confirmed in a large group of leprosy patients. Fig. 2C illustrates that *M. leprae* stimulation significantly increased the expression of SLAM in tuberculoid patients (*p* < 0.001, signed rank test), whereas the expression of the receptor was not modified by specific *M. leprae* stimulation in lepromatous patients (Fig. 2D). Cells from healthy donors cultured with *M. leprae* also up-regulated SLAM expression (data not shown). Healthy donors likely responded to *M. leprae* because the donors were bacillus Calmette-Guérin vaccinated and contained *M. tuberculosis*-reactive T cells that cross-reacted with the leprosy bacillus. Thus, SLAM expression correlates with T cell responsiveness to *M. leprae*.

To investigate whether the lack of up-regulation of SLAM expression in lepromatous patients in response to *M. leprae* stimulation was due to a general inability to express SLAM, we stimulated PBMCs from leprosy patients with *M. tuberculosis* or with *M. leprae* and the expression of SLAM was determined. Lepromatous leprosy patients are specifically unresponsive to *M. leprae*, whereas they are capable of responding to *M. tuberculosis* Ags (11, 12). SLAM expression was up-regulated either after *M. leprae* stimulation or after *M. tuberculosis* stimulation in tuberculoid patients (Fig. 2E). In contrast, in lepromatous patients, *M. leprae* did not modify the percentage of SLAM expression, whereas *M. tuberculosis* strongly increased the levels of SLAM on CD3+ T cells from these patients (Fig. 2F). Our results indicate that the lack of up-regulation of SLAM after specific Ag stimulation in unresponsive lepromatous patients is not related to a global defect in the expression of the protein, but is associated with the specific unresponsiveness of T cells from these individuals to *M. leprae*.

**Regulation of SLAM expression by IFN-γ**

Data from Fig. 1 demonstrate that SLAM expression in leprosy lesions correlates with IFN-γ expression and that SLAM is expressed in the group of patients who demonstrate CMI against the pathogen. To further define the relationship between SLAM and IFN-γ production, we measured SLAM expression and IFN-γ production by flow cytometry after *M. leprae* stimulation. We found that virtually all (93%) of the IFN-γ-producing T cells expressed SLAM (Fig. 3A). T cells that expressed SLAM but did not express...
IFN-γ may have taken up IFN-γ from neighboring T cells. Together, these data suggested to us that SLAM could be regulated by Th1 cytokines.

To confirm that SLAM expression was regulated by IFN-γ, we studied the effect of neutralizing anti-IFN-γ mAb on M. leprae-induced SLAM expression. PBMCs from responder tuberculoid patients were stimulated with M. leprae Ag in the presence or absence of neutralizing anti-IFN-γ mAb or an isotype control mAb, and the expression of SLAM was determined after five days. As shown in Fig. 3, B (one donor) and C (multiple donors), the

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levels of SLAM induced in response to Ag were markedly inhibited by anti-IFN-γ, suggesting that *M. leprae*-induced IFN-γ participates in the up-regulation of SLAM expression. Up-regulation of SLAM in healthy donors after stimulation with *M. leprae* was also IFN-γ dependent (data not shown).

We next examined whether SLAM expression could be enhanced in *M. leprae*-unresponsive patients through the addition of proinflammatory cytokines. PBMCs from leprosy patients were stimulated with *M. leprae* in the presence or absence of IFN-γ or IL-12, and after 5 days the cells were stained for SLAM expression. When added alone, cytokines did not alter the expression of SLAM (data not shown). However, when the cells were stimulated with *M. leprae*, IFN-γ significantly increased the expression of SLAM on T cells from lepromatous patients when compared with cells stimulated with *M. leprae* alone (Fig. 3D). As expected, IFN-γ treatment also increased SLAM expression on *M. leprae*-stimulated T cells from tuberculoid patients (Fig. 3D).

Enhancement of *M. leprae*-activated SLAM expression by rIFN-γ appears modest relative to the ability of IFN-γ Abs to reduce SLAM expression. Neutralization of IFN-γ by Ab reduces the ability of *M. leprae*-triggered IFN-γ as well as endogenous IFN-γ to enhance SLAM expression, thus the effect of the Ab can be expected to be more striking. rIL-12 enhanced the expression of SLAM on *M. leprae*-stimulated T cells, however, this effect was not as striking as the effect of IFN-γ (data not shown). These results, together with our observation that anti-IFN-γ mAb blocked the ability of Ag to up-regulate SLAM, indicate that the up-regulation of SLAM expression in tuberculoid patients in response to *M. leprae* is mediated by IFN-γ.

**Effect of SLAM ligation on Th1 responses to *M. leprae***

Because specific unresponsiveness of T cells to *M. leprae* is the primary immunologic characteristic of lepromatous leprosy patients and because engagement of SLAM was demonstrated to induce up-regulation of IFN-γ production by T cell clones (13), even Th2 clones, we investigated whether ligation of SLAM could enhance *M. leprae*-induced Th1 responses in leprosy. To address this question, PBMCs from a large group of tuberculoid and lepromatous patients were stimulated for 5 days with *M. leprae* and then with an agonistic anti-SLAM mAb. Fig. 4A shows that engagement through SLAM significantly increased IFN-γ production from tuberculoid patients (*p* < 0.01, signed rank test). No effect of anti-SLAM mAb on IFN-γ production was observed if cells were cultured in the absence of *M. leprae*. We also investigated whether engagement through SLAM could enhance *M. leprae*-induced T cell responses in unresponsive lepromatous patients. Although stimulation with anti-SLAM mAb slightly augmented the levels of Ag-induced IFN-γ produced by L-Lep patients (Fig. 4B), the levels of IFN-γ produced by lepromatous patients did not reach those produced by tuberculoid patients after ligation through SLAM (Fig. 4A), likely because SLAM is not induced dramatically on the T cells of lepromatous patients in response to *M. leprae* (Fig. 2). Addition of anti-SLAM mAb had no effect on IL-4 production, either from tuberculoid or from lepromatous patients.

**Effect of SLAM engagement on IFN-γ production to *M. leprae***

We wished to determine whether IFN-γ production from unresponsive patients could be enhanced by ligation of SLAM. To investigate this possibility, we stimulated PBMCs from lepromatous patients with *M. leprae* in the presence or absence of IFN-γ or IL-12. After 5 days, stimulated cells were washed to remove exogenously added cytokines and were cultured in the presence or absence of anti-SLAM mAb. In tuberculoid patients, culture with *M. leprae* alone caused T cells to be responsive to SLAM ligation as measured by IFN-γ production (Fig. 4A). In contrast, when lepromatous patient PBMCs were cultured with *M. leprae* alone, SLAM ligation induced IFN-γ only slightly above that of cells cultured without Ag. However, signaling through SLAM after treatment with rIFN-γ plus *M. leprae* enhanced IFN-γ production in unresponsive lepromatous patients up to the levels of responsive tuberculoid patients (Fig. 4, A and B). Treatment with rIL-12 plus *M. leprae* caused a small increase in the levels of IFN-γ produced by L-Lep patients after SLAM ligation (Fig. 4B). Our data indicate that the ability of T cells to respond to SLAM ligation can be enhanced by culture with IFN-γ.

**Discussion**

Effective host defense against intracellular infection requires the generation of Th1 immune responses. We investigated the role of one key regulator of T cell cytokine patterns, SLAM (4, 13), in the context of leprosy, an infectious disease in which a Th1-like response is correlated with the self-limiting form of the disease. We found that local expression of SLAM was higher in lesions from patients with lepromatous disease than from those with tuberculoid disease. We also found that local expression of SLAM was higher in lesions from patients with lepromatous disease than from those with tuberculoid disease. We also found that local expression of SLAM was higher in lesions from patients with lepromatous disease than from those with tuberculoid disease. We also found that local expression of SLAM was higher in lesions from patients with lepromatous disease than from those with tuberculoid disease.
An important aspect of our study was the ability to investigate the expression of SLAM at the site of a human infection. We found that SLAM mRNA and protein expression correlated with the self-limiting form of leprosy and with IFN-γ expression in vivo, providing evidence that SLAM signaling promotes Th1 responses at the site of infection. The activation of SLAM on T cells not only promotes Th1 responses in human disease, but also has the capacity to reverse existing Th2 responses toward the Th1 pole in HIV disease (14) and allergy (13). Further evidence implicating SLAM in directing Th1 cytokine responses in human disease has recently emerged from the study of X-linked lymphoproliferative disease, a disease resulting from a deficiency in a protein termed SLAM-associated protein (15), resulting in disregulated SLAM signaling. These patients are characterized by T cells that, in response to virus, produce high levels of IFN-γ (16), an inability to differentiate into Th2 cells (16), and deficient T and B cell interactions (15).

Differences in SLAM expression in the polar forms of leprosy led us to examine the regulation of SLAM expression in vitro. Our data suggest that two factors may be involved in regulating SLAM expression: TCR activation and IFN-γ production. Tuberculoid leprosy patients, whose T cells proliferate (10) and make IFN-γ (17) in response to M. leprae, up-regulated SLAM in response to the pathogen; in contrast, lepromatous patients, whose T cells show only weak proliferative and Th1 responses to M. leprae (10, 17), did not up-regulate SLAM under the same conditions. However, coculture of PBMCs with IFN-γ and M. leprae did up-regulate SLAM expression in lepromatous patients to the level of tuberculoid patients, suggesting that IFN-γ production is critical to induction of SLAM expression. Our finding that neutralizing IFN-γ strongly inhibited the M. leprae-stimulated up-regulation of SLAM in tuberculoid patients further supported this hypothesis. Furthermore, murine studies indicate that Th1 cells up-regulate SLAM when stimulated through the TCR, whereas Th2 cells did not (5). We found that IFN-γ alone did not up-regulate SLAM, indicating that at least two signals are required for the up-regulation of SLAM. Therefore, the polar lesions of leprosy patients are a snapshot of the extremes of SLAM expression. Tuberculoid lesions contain Ag-responsive, IFN-γ-producing T cells resulting in SLAM expression; in contrast, lepromatous lesions contain M. leprae-unresponsive T cells and lack IFN-γ production, but express Th2 cytokines, including IL-10 (3), a cytokine shown to inhibit SLAM expression (18).

Our study suggests that SLAM might be a focal point for therapeutic modulation of T cell cytokine responses in diseases characterized by dysfunctional Th2 responses, as has been suggested in allergy (13) and HIV disease (14). However, caution must be used in designing ways to manipulate SLAM to promote Th1 responses because inappropriate expression of SLAM can result in overproduction of IFN-γ and autoimmune pathology. For example, when T cells recognize self-Ag such as in rheumatoid arthritis and multiple sclerosis, SLAM expression and IFN-γ production are up-regulated (5, 19). Thus, through the induction of IFN-γ, SLAM can either enhance antimicrobial immunity or promote autoimmunity, depending on the context of the immune response, making it critical to understand the regulation of SLAM expression and function in greater detail.

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