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Expression of Signaling Lymphocytic Activation Molecule-Associated Protein Interrupts IFN-γ Production in Human Tuberculosis

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Production of the Th1 cytokine IFN-γ by T cells is considered crucial for immunity against Mycobacterium tuberculosis infection. We evaluated IFN-γ production in tuberculosis in the context of signaling molecules known to regulate Th1 cytokines. Two populations of patients who have active tuberculosis were identified, based on their T cell responses to the bacterium. High responder tuberculosis patients displayed significant M. tuberculosis-dependent T cell proliferation and IFN-γ production, whereas low responder tuberculosis patients displayed weak or no T cell responses to M. tuberculosis. The expression of the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) on cells from tuberculosis patients was inversely correlated with IFN-γ production in those individuals. Moreover, patients with a nonfunctional SAP gene displayed immune responses to M. tuberculosis similar to those of high responder tuberculosis patients. In contrast to SAP, T cell expression of SLAM was directly correlated with responsiveness to M. tuberculosis Ag. Our data suggest that expression of SAP interferes with Th1 responses whereas SLAM expression contributes to Th1 cytokine responses in tuberculosis. The study further suggests that SAP and SLAM might be focal points for therapeutic modulation of T cell cytokine responses in tuberculosis. 

In contrast, reduced IFN-γ production by PBMCs is a marker of severe disease (3), confirming the role of CMI in protection against tuberculosis.

Several signal transduction molecules have been shown to modulate the level and pattern of cytokines produced by naive T cells. For example, the adaptor-like signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), an SH2 domain-containing protein of T and NK cells that interacts with SLAM (4–6), was shown to participate in the differentiation process that leads T cells to the commitment of producing a specific pattern of cytokines. SAP operates as a natural inhibitor of the networking between signal transduction pathways and the cytoplasmic tail of members of the SLAM family (7). SAP SH2 domain binds to the SH3 domain of FynT and directly couples FynT to SLAM (8). Studies in SAP-deficient mice revealed increased IFN-γ secretion responses and deficient IgE production, either at baseline or after infection with various agents (7, 9), suggesting that the lack of SAP expression results in skewing toward a Th1 phenotype. Moreover, in SAP-deficient humans (X-linked lymphoproliferative (XLP) syndrome), concentrations of IFN-γ are elevated during primary EBV infection, suggesting that a bias toward the production of Th1 cytokines may contribute to the progressive immunopathology observed in affected patients (6). Therefore, the abnormalities in cytokine secretion that occur both in SAP-deficient humans and mice, might result from defects in the propagation of SLAM-induced signals (9). In fact, the lymphocyte receptor SLAM is another factor that can influence the pattern of cytokines produced by activated T cells (10–12). SLAM is homophilic and functions through bidirectional signaling after SLAM-SLAM associations. In T cells, ligation of SLAM with mAbs heightens proliferation and/or secretion of IFN-γ (10, 13), suggesting that SLAM might mediate context-dependent functions in lymphocytes.

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We investigated the role of SAP and SLAM regulation on the pattern of cytokines produced during human tuberculosis. We found that \textit{M. tuberculosis}-induced IFN-\( \gamma \) was abolished in cells from tuberculosis patients expressing SAP, whereas cells that expressed SLAM alone or contained SLAM and nonfunctional SAP were able to produce high levels of IFN-\( \gamma \) in response to the pathogen. In contrast, SLAM up-regulation in tuberculosis correlated with TH1 responses in vitro. Moreover, SLAM ligation significantly enhanced IFN-\( \gamma \) production in tuberculosis patients with high CMI to the bacteria (high responder [HR] tuberculosis patients) and slightly augmented the levels of the cytokine in individuals with weak CMI against \textit{M. tuberculosis} (low responder [LR] tuberculosis patients). Interestingly, analyses of SAP expression in those LR tuberculosis patients showed a striking down-regulation of SAP after ligation of SLAM. Finally, signaling through SLAM after IFN-\( \gamma \) or IL-12 treatment of Ag-stimulated cells enhanced IFN-\( \gamma \) production in LR tuberculosis patients up to the levels of responder tuberculosis patients. Therefore, our results suggest that SAP interferes with IFN-\( \gamma \) production during mycobacterial infection, whereas SLAM activation enhances CMI to \textit{M. tuberculosis} infection.

**Materials and Methods**

**Study subjects**

Patients who had active tuberculosis were evaluated at the Department of Immunology, Instituto de Tisoneumonología Prof. Dr. Raúl Vaccarezza, University of Buenos Aires, School of Medicine, and at the División Tisoneumonología, Hospital FJ Mafiz (Buenos Aires, Argentina). The diagnosis of tuberculosis was established based on clinical and radiological data together with the identification of acid-fast bacilli in sputum. Bacillus Calmette-Guérin (BCG)-vaccinated healthy control individuals from the community, purified protein derivative (PPD) (induration >10 mm) and PPD \( \gamma \) (no induration) participated in this study. Two individuals diagnosed with XLP confirmed at the International XLP Registry Headquarters, University of Nebraska Medical Center (Omaha, NE) (14) were included in this study. Peripheral blood was collected in heparinized tubes from all individuals participating in the study after receiving informed consent.

**Antigen**

In vitro stimulation of cells throughout the present study was performed with an extract from the virulent \textit{M. tuberculosis} strain H37Rv prepared by probe sonication (sonicated \textit{M. tuberculosis} Ag) (15).

**Cell preparations and culture conditions**

PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Picataway, NJ) and cultured \( (1 \times 10^6/ml) \) with sonicated \textit{M. tuberculosis} Ag (5 \( \mu \)g/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, penicillin, and human serum (10%). After 5 days, cells were pulsed with \( [[H]^{3}TdT} (1 \mu Ci/well) \) and harvested 16 h later, and \( [[H]^{3}TdT} incorporation was measured in a liquid scintillation counter. In different experiments, sonicated \textit{M. tuberculosis} Ag-stimulated cells were washed and examined for SLAM expression by flow cytometry described below, or cultured in the presence of anti-SLAM mAb (A12, 10 \( \mu \)g/ml; eBioscience, San Diego, CA) examined for IFN-\( \gamma \) production by ELISA (Endogen, Cambridge, MA), or by intracellular cytokine staining (16), or solubilized in lysate buffer (50 mM Tris, pH 8, 1% Nonidet P-40, 200 mM NaCl, 10% glycerol, 0.5 mM EDTA and Protease Inhibitor Cocktail (Sigma-Aldrich) to prepare whole cell extracts. For IFN-\( \gamma \) production studies, human rIL-12 (500 pg/ml) or human rIFN-\( \gamma \) (7.5 ng/ml) (Endogen) were added to some cultures at final concentrations. The anti-SLAM mAb A12 has previously been described (10).

**SLAM and SAP detection**

SLAM and SAP expression in PBMCs from the individuals participating in the study was determined by Western blot. Briefly, lymphoblasts were stimulated with sonicated \textit{M. tuberculosis} Ag for 5 days. Whole cell extracts were then prepared and equivalent amounts of protein were analyzed by 12–15% SDS-PAGE, transferred to nitrocellulose (Hybond ECL Nitrocellulose Membrane; Amersham Biosciences) for immunoblotting and incubated with anti-SLAM (1/500, SLAM (N-19) sc-1334; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-SAP (1/1000, SAP (FL-128) sc-8333; Santa Cruz Biotechnology) polyclonal Abs. Bound Ab was revealed with HRP-conjugated anti-rabbit Ab (1/3000; Bio-Rad, Hercules, CA) and HRP-conjugated affinity-purified anti-goat Ab (1/2500; Chemicon International, Temecula, CA) respectively, using ECL (Amersham Biosciences) and Kodak BioMax films. Films were analyzed with the Scion Image Analysis software (Scion, Frederick, MD), and the intensity of each band was recorded and expressed as arbitrary units (AU).

To determine the level of SLAM expression on T cells, PBMCs cultured with sonicated \textit{M. tuberculosis} Ag were stained for surface expression with mAbs specific for CD3 (BD Biosciences, San Jose, CA) and SLAM (A12) (10) before and after culture. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Statistical analysis**

Statistical analysis was performed using nonparametric methods including the Wilcoxon signed rank test and the Student \( t \) test. Values of \( p < 0.05 \) were considered significant.

**Results**

Characterization of immunological status of two populations of tuberculosis patients

To investigate the response to \textit{M. tuberculosis} in a population of patients who had active tuberculosis in Argentina, we examined proliferative responses and IFN-\( \gamma \) production of PBMCs from patients to an extract of virulent \textit{M. tuberculosis}. Our results revealed two groups of patients based on in vitro T cell responses to sonicated \textit{M. tuberculosis} Ag. HR tuberculosis patients are those individuals that displayed significant proliferative responses to the Ag (mean proliferation index: 11.49 \pm 1.43), and LR tuberculosis patients (mean proliferation index: 2.21 \pm 0.66), who exhibited low responses to \textit{M. tuberculosis} (Fig. 1A, \( p < 0.001 \), the Student \( t \) test). Proliferative results correlated with IFN-\( \gamma \) production in response to Ag: stimulation with sonicated \textit{M. tuberculosis} H37Rv significantly increased IFN-\( \gamma \) production from HR tuberculosis patients (Fig. 1B, \( p < 0.01 \), the Student \( t \) test), but only slightly increased the levels of IFN-\( \gamma \) in LR tuberculosis patients. Moreover, proliferative results and IFN-\( \gamma \) production in response to Ag in tuberculosis patients were also in correlation with the expression of SLAM against sonicated \textit{M. tuberculosis} Ag-stimulation. Therefore, to assign patients to HR and LR groups, three criteria were used. First, the primary criterion was proliferation index. The proliferation index was calculated as: (cpm after sonicated \textit{M. tuberculosis} Ag-stimulation)/(cpm after culturing with medium). If this number was \( \geq 4 \), the individual was classified HR, and if the proliferation index was \( \leq 4 \), the individual was classified as LR. Second, the level of IFN-\( \gamma \) produced in response to \textit{M. tuberculosis} was considered. Again if the fold-stimulation ((nanograms per milliliter after sonicated \textit{M. tuberculosis} Ag-stimulation)/(nanograms per milliliter after culturing with medium)) was \( > 34 \), the patient was considered HR, and for a value \( < 34 \), the individual was considered LR. Third, the increase in the percentage of SLAM-positive cells in response to sonicated \textit{M. tuberculosis} Ag-stimulation was considered. An increase in percentage of SLAM-positive cells of \( \geq 8 \) assigned the patient to the HR group and \( < 8 \) was considered a LR. If a patient fulfilled two of these criteria, the patient was assigned to that group.

Interestingly, immunological parameters paralleled common clinical parameters analyzed in patients with active tuberculosis in Argentina. HR patients had significantly higher levels of the percentage of total lymphocytes compared with LR patients (26.1 \pm 2.7% vs 14.4 \pm 1.6%, \( p < 0.001 \), the Student \( t \) test); HR patients had higher PPD diameters than LR patients (8.43 \pm 1.4 mm vs 3.62 \pm 0.956 mm, \( p < 0.001 \), the Student \( t \) test); LR individuals had severe pulmonary lesions, a striking loss of weight, and had been ill longer (days since symptoms begin until patient hospital...
cytokine patterns in *M. tuberculosis*.

To investigate the role of the SAP pathway on the regulation of the SAP expression in tuberculosis correlates with disease severity, PBMCs from tuberculosis patients and healthy donors were stimulated with sonicated *M. tuberculosis* Ag. SAP expression and IFN-γ production were investigated. After 5 days of Ag stimulation, total cell extracts were prepared and assayed for SAP protein expression by Western blot. Representative HR and LR patients and healthy donors (HD) are shown. B. Polyacrylamide gels were scanned, densitometry was performed, and the results were expressed as arbitrary units. C. After 48 h of Ag stimulation, cell-free supernatants were recovered and IFN-γ production was determined by ELISA. Values are expressed as the mean of triplicate determinations. Each line connects the data for an individual patient. Values for *p* were calculated using the Wilcoxon signed rank test; *, *p* < 0.001. ○, LR tuberculosis patients; ●, HR tuberculosis patients.

SAP expression in tuberculosis correlates with disease severity

To investigate the role of the SAP pathway on the regulation of the cytokine patterns in *M. tuberculosis* infection, we first analyzed SAP expression in PBMCs from infected patients and healthy donors after stimulation with sonicated *M. tuberculosis* Ag. SAP expression was compared with IFN-γ production from the same individuals. Therefore, cells were cultured with *M. tuberculosis* Ag, IFN-γ production was tested after 48 h of culture, and the expression of SAP was analyzed by Western blot after 5 days of Ag stimulation. In those individuals who produced low levels of IFN-γ upon *M. tuberculosis* Ag-stimulation (LR tuberculosis patients, Fig. 1B and Fig. 2C), substantial levels of SAP protein were detected by Western blot (Fig. 2, A and B). In contrast, in patients who produced high levels of IFN-γ against *M. tuberculosis* Ag (HR tuberculosis patients, Fig. 1B and Fig. 2C), SAP protein was undetectable (Fig. 2, A and B). Moreover, similar to our findings in HR patients, in cells from healthy donors, individuals who produced considerable levels of IFN-γ to the Ag (Fig. 2C), *M. tuberculosis* Ag-stimulation did not induce expression of SAP (Fig. 2, A and B). These data indicate that expression of SAP is greatest in the group of patients that displayed low immune responses to *M. tuberculosis*, suggesting that differential expression of this signaling molecule may be involved in the outcome of tuberculosis infection.

SLAM expression in tuberculosis correlates with reduced disease severity

SLAM expression is regulated by TCR signaling and modulates the production of IFN-γ from T cells (10, 11). We have previously...
demonstrated that *M. leprae* stimulation significantly increases the expression of SLAM in responsive tuberculoid leprosy patients but not in susceptible lepromatous leprosy individuals (17). Therefore, we hypothesized that SLAM expression might correlate with T cell activity against *M. tuberculosis*. To investigate this possibility, PBMCs from tuberculosis patients were stimulated with sonicated *M. tuberculosis* Ag and SLAM expression was determined by flow cytometry. SLAM expression increased in a HR patient after *M. tuberculosis* Ag-stimulation (Fig. 3A, lower left panel), whereas *M. tuberculosis* Ag did not modify significantly the percentage of SLAM expressing T cells in a LR patient (Fig. 3A, upper left panel). Moreover, cells from BCG-vaccinated healthy control individuals (PPD panel). SLAM up-regulation on T cells from tuberculosis patients and healthy donors after stimulation with sonicated *M. tuberculosis* Ag is shown. A, The percentage of SLAM⁺ T cells after culturing with medium (inset) or *M. tuberculosis* Ag (full panel) is expressed in the upper right of each density plot. B, Values for *p* are expressed using the Student *t* test comparing SLAM expression on cells cultured with medium vs *M. tuberculosis*; *, *p* < 0.01. Each line connects the data for an individual patient; n.s., differences not significant. ○, HR tuberculosis patients; ●, LR tuberculosis patients; □, Healthy donors.

FIGURE 3. SLAM up-regulation on T cells from tuberculosis patients and healthy donors after stimulation with sonicated *M. tuberculosis* Ag. PBMCs were cultured in the presence or absence of Ag for 5 days and the expression of SLAM in representative HR and LR tuberculosis patients and PPD⁺ and PPD⁻ healthy donors (A) or in multiple tuberculosis HR and LR tuberculosis patients (B) was evaluated using two-color flow cytometry. SLAM expression on T cells was determined by first gating on CD3⁺ cells, then evaluating SLAM expression. A. The percentage of SLAM⁺ cells after culturing with medium (inset) or *M. tuberculosis* Ag (full panel) is expressed in the upper right of each density plot. B. Values for *p* are expressed using the Student *t* test comparing SLAM expression on cells cultured with medium vs *M. tuberculosis*; *, *p* < 0.01. Each line connects the data for an individual patient; n.s., differences not significant. ○, HR tuberculosis patients; ●, LR tuberculosis patients; □, Healthy donors.

Because SAP interacts with the cytoplasmic tail of SLAM (4–6) and signaling via the SLAM-SAP pathway can alter the profile of cytokine production during T cell activation (13), we next investigated the role of SAP and SLAM expression on the regulation of the pattern of cytokines produced during active tuberculosis. *M. tuberculosis* Ag-stimulated cells from HR and LR tuberculosis patients were assayed for IFN-γ production by ELISA, and SLAM and SAP expression by Western blot. As shown in Fig. 4, we detected expression of SLAM protein both in tuberculosis patients that produced high levels of IFN-γ (HR tuberculosis patients) and no expression of SAP, and in those individuals that produced low levels of IFN-γ upon *M. tuberculosis* Ag-stimulation (LR tuberculosis patients) and substantial levels of SAP protein (Fig. 4, A and B). Moreover, similar to our findings in HR patients, SLAM protein was detected in cells from healthy donors, individuals that produced IFN-γ to the Ag and displayed no SAP expression (Fig. 4, A and B). Interestingly, our results are in agreement with a mouse model recently proposed where SAP is required to turn off expression of SLAM in HR tuberculosis patients (Fig. 3B, *p* < 0.01, the Student *t* test), whereas the expression of the receptor was not modified by specific *M. tuberculosis* Ag-stimulation in LR tuberculosis patients (Fig. 3B). Moreover, in accord to our findings in leprosy demonstrating that SLAM expression correlated with IFN-γ expression (17), when we simultaneously measured the expression of SLAM and IFN-γ production after *M. tuberculosis* Ag-stimulation using flow cytometry, we found that virtually all of the IFN-γ-producing T cells in HR individuals expressed SLAM (data not shown), suggesting that SLAM could be regulated by Th1 cytokines. Therefore, our present results indicate that SLAM expression correlates with T cell responsiveness to *M. tuberculosis*.

Expression of SAP and SLAM in tuberculosis patients according to IFN-γ response to the bacteria
IFN-γ production (13). In the mouse T cell line BI-14, the SLAM-SAP interaction was accompanied by selective inhibition of IFN-γ release during T cell activation (13). However, no impact on IFN-γ release was observed in cells that contained SLAM or SAP alone (13). Thus, these published results on mice together with our present data showing expression of SLAM but distinct expression of SAP levels in tuberculosis patients further support the hypothesis that differential expression of both activation molecules might participate in the modulation of the pattern of cytokines produced during tuberculosis infection. Furthermore, our data demonstrate for the first time the role of SAP in human intracellular infection.

**SLAM expression and IFN-γ production in individuals with defective SAP gene**

Because SAP was absent in cells of tuberculosis patients that produced high levels of IFN-γ against *M. tuberculosis* Ag, and it was expressed in the cells of patients characterized by the lowest IFN-γ production, we hypothesized that SAP might participate in the differentiation process that allows activated T cells to produce different patterns of cytokines during *M. tuberculosis* infection. Therefore, to further investigate the role of SAP in regulating cytokine responses to *M. tuberculosis* through SLAM, we performed experiments using cells from patients with XLP, individuals with nonfunctional SAP. SAP is the principal gene affected in XLP, and it has been suggested that some of the disease states that XLP patients undergo could be caused by abnormal regulation of the SAP gene and/or of the T cell subsets that express SAP (4). Thus, to demonstrate whether *M. tuberculosis* might modify the SLAM/SAP pathway in cells with nonfunctional SAP, we studied the response of SAP-deficient humans to sonicated *M. tuberculosis* Ag-stimulation. We first analyzed the expression of SLAM on resting peripheral blood T cells from healthy donors, patients who had

**FIGURE 5.** SAP and SLAM expression and IFN-γ production in XLP patients. A, Determination of the levels of surface SLAM on resting peripheral blood T cells from representative HR and LR tuberculosis patients, an XLP patient, and a healthy donor using two-color flow cytometry. SLAM expression on T cells was determined by first gating on CD3⁺ cells, then evaluating SLAM expression. The percentage of SLAM⁺ cells shows isotype control for SLAM (inset) or SLAM⁺ cells (full panel) is expressed in the upper right of each density plot. B, Each bar represents the mean of the percentage of the expression of SLAM⁺ T cells in each group of individuals ± SEM. Values for *p* using the Student t test comparing the percentage of SLAM⁺ cells vs SLAM⁻ cells; *, *p* < 0.01. C, Effect of *M. tuberculosis* Ag on SLAM and SAP expression in XLP patients. PBMCs from two XLP individuals were stimulated with sonicated *M. tuberculosis* Ag for 5 days and SLAM and SAP expression was investigated by Western blot. Jurkat cells were used as positive control. D, IFN-γ production in healthy donors, HR and LR tuberculosis patients, and XLP patients is shown. Each bar represents the mean of the levels of IFN-γ produced by each group of individuals ± SEM.
active tuberculosis, and XLP patients. In contrast to our findings with PBMCs from tuberculosis patients in response to Ag stimulation, the levels of SLAM on resting peripheral blood T cells from HR and LR tuberculosis patients were similar (Fig. 5, A and B), in accord with our previous finding in leprosy patients (17). CD3+ T cells from XLP patients displayed levels of SLAM surface expression comparable to those expressed by tuberculosis patients (Fig. 5, A and B). However, we found higher SLAM expression on CD3+ T cells from tuberculosis and XLP patients as compared with healthy donors (Fig. 5, A and B), suggesting that systemically T cells in tuberculosis and in XLP patients exist in an activated state although not necessarily responsive to the pathogen. Moreover, similar to our findings in HR tuberculosis patients, M. tuberculosis Ag-stimulation increased the levels of SLAM expression in XLP patients (Fig. 5C), whereas no SAP protein was detected (Fig. 5C). Therefore, we next investigated IFN-γ production of cells from XLP patients to sonicated M. tuberculosis Ag and compared that production to the levels of IFN-γ produced by healthy donors and tuberculosis patients. As shown in Fig. 5D, XLP patients produced lower levels of IFN-γ against M. tuberculosis Ag compared with HD and HR tuberculosis patients, but higher levels of the cytokine compared with LR tuberculosis patients, indicating that cells from XLP patients can respond to the bacteria. It is not surprising the relative high basal levels of IFN-γ produced by XLP patients (Fig. 5D) because the defect in XLP individuals results from their inability to mount an appropriate Th2 response after EBV infection, which leads to an uncontrolled Th1 response, with increases IFN-γ production causing parenchymal damage (7). To rule out the possibility of a nonspecific response of XLP patients to M. tuberculosis Ag, related to the lack of SAP, we stimulated PBMCs from XLP individuals with recombinant cytoplasmic Brucella Ags (gently provided by Dr. Guillermo Giambar-tolomei, University of Buenos Aires School of Biochemistry), and IFN-γ production was measured 48 h later. Our results showed no response from the two XLP patients to Brucella Ags (data not shown). Therefore, XLP patients responded to M. tuberculosis Ag-stimulation because they were BCG-vaccinated (like tuberculosis patients and healthy donors) and thus contained M. tuberculosis-reactive T cells, suggesting a specific immune response. In conclusion, our results in XLP patients further support the hypothesis that differential expression of SLAM and SAP proteins might modulate the pattern of cytokines during the immune response against M. tuberculosis.

FIGURE 6. Effect of anti-SLAM mAb on M. tuberculosis Ag-induced IFN-γ production in tuberculosis patients and XLP patients, and on SAP expression in LR tuberculosis patients. A, PBMCs from HR (left) and LR (right) tuberculosis patients were stimulated with sonicated M. tuberculosis Ag, and after 5 days cells were cultured in the presence or absence of anti-SLAM mAb. Cell-free supernatants were collected at 48 h and assayed for IFN-γ by ELISA. Values are expressed as the mean of triplicate determinations. Each line connects the data for an individual patient. Value for p were calculated using the Wilcoxon signed rank test comparing IFN-γ production from cells cultured with M. tuberculosis then medium vs cells cultured with M. tuberculosis then SLAM; *, p < 0.001. B, PBMCs from LR tuberculosis patients were cultured with sonicated M. tuberculosis Ag in the presence or absence of anti-SLAM mAb and after 5 days total cell extracts were prepared and assayed for SAP expression by Western blot. C, PBMCs from XLP patients were stimulated with M. tuberculosis Ag for 5 days. Thereafter, cells were cultured with anti-SLAM mAb, and IFN-γ was measured by ELISA. Each line connects the data for an individual patient. A representative experiment of three is shown.
Effect of SLAM engagement on cytokine responses to M. tuberculosis

SLAM activation induces up-regulation of IFN-γ production by T cell clones (18), including Th2 clones. Moreover, we have demonstrated that engagement of SLAM significantly enhances M. leprae-induced IFN-γ in responsive tuberculoid patients (17). We therefore sought to determine the effect of SLAM ligation on IFN-γ production by tuberculosis patients. Sonicated M. tuberculosis Ag-stimulated PBMCs were cultured with an agonistic anti-SLAM mAb. As shown in Fig. 6A, engagement through SLAM significantly increased M. tuberculosis-induced IFN-γ production from HR patients (Fig. 6A, left panel, p < 0.01, the Student t test). SLAM ligation augmented the levels of the cytokine produced by several LR patients (Fig. 6A, right panel, p < 0.05, the Student t test), although they did not reach the levels produced by HR patients. Addition of anti-SLAM mAb had no effect on IL-4 production from HR and LR tuberculosis patients (data not shown). These results suggest that engagement of SLAM could induce type 1 responses in tuberculosis patients in response to Ag.

The increase in IFN-γ production induced in several LR patients upon ligation of SLAM, caused us to question whether SAP expression is modulated in LR patients. Interestingly, as it is shown in Fig. 6B in Ag-stimulated cells from a representative LR patient, a striking down-regulation of SAP expression was induced upon engagement of SLAM. Thus these results, showing that the increase in IFN-γ production parallels a decrease in SAP expression, reinforce our data suggesting that the presence of SAP negates IFN-γ production.

To investigate the effect of the SAP defect on cytokine production after M. tuberculosis stimulation, PBMCs from XLP patients were stimulated with sonicated M. tuberculosis Ag and after 5 days cells were cultured with anti-SLAM mAb, and IFN-γ was assayed by ELISA. Fig. 6C shows that, similar to our results with HR tuberculosis patients, engagement through SLAM strikingly increased IFN-γ production from XLP patients. Therefore, individuals with a nonfunctional SAP gene who display SLAM surface expression are able to produce IFN-γ in response to M. tuberculosis Ag-stimulation and this production of IFN-γ can be markedly enhanced through engagement of SLAM. In contrast, patients with active tuberculosis that do not express SAP but show surface expression of SLAM, produce significant levels of IFN-γ in response to the specific Ag, whereas patients with active disease that display SAP and SLAM expression are not able to produce IFN-γ in response to M. tuberculosis Ag. Taken together, our data indicate that SAP might be required to turn off IFN-γ production.

Effect of SLAM engagement on IFN-γ production to M. tuberculosis after treatment with proinflammatory cytokines

We have previously demonstrated that the local release of IFN-γ by M. leprae-activated T cells in tuberculoid leprosy lesions leads to up-regulation of SLAM expression and that signaling through SLAM after IFN-γ treatment of Ag-stimulated cells enhanced IFN-γ production in unresponsive lepromatous patients (17). We therefore investigated whether IFN-γ production from LR tuberculosis patients could be enhanced through SLAM ligation up to the levels produced by HR individuals. PBMCs from HR and LR patients were stimulated with sonicated M. tuberculosis Ag in the presence or absence of anti-SLAM mAb. In HR tuberculosis patients, culture with M. tuberculosis Ag alone for 5 days caused T cells to be responsive to SLAM ligation as measured by IFN-γ production (Fig. 7). In contrast, LR patients produced lower levels of IFN-γ in response to the Ag (Fig. 7), and although engagement of SLAM increased IFN-γ production, the levels of the cytokine did not reach those produced by HR patients (Fig. 7). However, signaling through SLAM after treatment with recombinant IFN-γ plus M. tuberculosis Ag significantly enhanced IFN-γ production in LR patients (Fig. 7, left panel).

Following phagocytosis of M. tuberculosis bacilli by macrophages and dendritic cells, IL-12 production is induced, driving development of a Th1 response with IFN-γ production (19, 20). Therefore, because IL-12 is a crucial cytokine in controlling M. tuberculosis infection, we next performed experiments stimulating cells from tuberculosis patients with IL-12 plus Ag followed by anti-SLAM stimulation. PBMCs from HR and LR patients were stimulated with sonicated M. tuberculosis Ag in the presence or absence of rIL-12. After 5 days, stimulated cells were washed to remove exogenously added cytokines and cultured in the presence or absence of anti-SLAM mAb. In HR tuberculosis patients, culture with M. tuberculosis Ag alone for 5 days caused T cells to be responsive to SLAM ligation as measured by IFN-γ production (Fig. 7). In contrast, LR patients produced lower levels of IFN-γ in response to the Ag (Fig. 7), and although engagement of SLAM increased IFN-γ production, the levels of the cytokine did not reach those produced by HR patients (Fig. 7). However, signaling through SLAM after treatment with recombinant IFN-γ plus M. tuberculosis Ag significantly enhanced IFN-γ production in LR patients (Fig. 7, right panel). These results suggest that T
cell ability to respond to SLAM ligation can be enhanced by culture with proinflammatory cytokines, further supporting the hypothesis that decreased IFN-γ production is dependent on inability of T cells to respond to Ag.

Discussion
Protective immunity against mycobacterial infection requires CMI (21, 22) and the generation of Th1 cytokine responses (23). In this report, we investigated the role of two signaling molecules that regulate T cell cytokine patterns in the context of human tuberculosis. We found that reduced expression of SAP protein correlated with strong IFN-γ production from M. tuberculosis Ag-stimulated T cells. Moreover, XLP patients, deficient in SAP expression, produced elevated levels of IFN-γ spontaneously and in response to M. tuberculosis. In contrast to SAP, T cell expression of SLAM was directly correlated with responsiveness to M. tuberculosis Ag. Engagement of SLAM up-regulated IFN-γ production in HR tuberculosis patients and slightly increased IFN-γ production from several LR patients. Furthermore, analyses of SAP expression in those LR tuberculosis individuals showed a striking down-regulation of SAP after ligation of SLAM. Finally, signaling through SLAM after IL-12 or IFN-γ treatment of Ag-stimulated cells enhanced IFN-γ production in all LR patients up to the levels of HR patients. Our data suggest that expression of SAP might deliver an inhibitory signal associated with the selective attenuation of IFN-γ by T lymphocytes during M. tuberculosis infection, whereas expression of SLAM contributes to Th1 cytokine responses in tuberculosis.

An important aspect of our study was the ability to study the SLAM-SAP interactions during human bacterial intracellular infection. We found an inverse relationship between SAP protein expression and IFN-γ production by sonicated M. tuberculosis Ag-stimulated T cells in tuberculosis and XLP patients. Our data corroborate studies in SAP-deficient mice showing that the absence of SAP caused an excessive IFN-γ secretion by T cells (7). Moreover, activated T cells from mice over-expressing wild-type SAP displayed an increase in IL-4 production as well as a decrease in IFN-γ secretion (24). Our findings, together with the studies in mice, strengthen the hypothesis that SAP attenuates Th1-type immune responses (13).

Our results showed that SAP expression was down-regulated in LR patients when stimulated with SLAM and TCR together. It has been reported that the relative amounts of SAP and SLAM expression may vary during lymphocyte activation and in some autoimmune disorders (25), and that the ratio of SLAM to SAP abundance could have a role in the context-dependent functions of SLAM (9). In fact, the differential expression of SLAM and SAP was proposed to be related to the activation state of the cells (26). In mice, SAP expression is rapidly down-regulated in vitro activated T cells, whereas SLAM is up-regulated early in T cell activation. We propose that regulation of IFN-γ production by signaling molecules in tuberculosis is primarily dependent on T cell recognition of Ag. T cells responding to M. tuberculosis Ag rapidly up-regulate SLAM and these two signals combine to promote IFN-γ production. At the same time, SAP is transiently down-regulated in response to T cell activation. This cascade of signaling is stalled in Ag unresponsive donors because lack of T cell responsiveness prevents up-regulation of SLAM and the existing SAP prevents IFN-γ production. If SLAM ligation is restored as described in our experimental system, IFN-γ levels are significantly increased.

Our results showed that SAP expression decreased in LR patients when SLAM and TCR were ligated in tandem. These data suggest a costimulatory effect of SLAM and TCR for IFN-γ production as has been shown for CD4 T cells (10). Although SLAM is inhibited by high-affinity interactions with SAP, the synergism of SLAM and TCR in the context of tuberculosis infection overcomes to some extent, the negative influence of SAP. SLAM-SAP interactions might arise from T cells interacting with other activated T cells, or by dendritic cells (27). The synergism of SLAM and TCR signaling overcoming the regulatory effect of SAP on IFN-γ production is also suggested by the studies of Howie et al. (28). They reported that T cells from SAP−/− mice produced more IFN-γ than wild-type littermates upon anti-CD3 stimulation, but on stimulation with anti-CD3 and anti-SAP Abs both wild-type and SAP−/− T cells produced significantly higher amounts of IFN-γ (28). Through a mechanism whereby SAP recruits FynT to SLAM and regulates IFN-γ (8), SHP-2 binding to SLAM and signaling for IFN-γ is prevented.

We further found that the presence of IL-12 during TCR signal, followed by SLAM engagement, promotes IFN-γ responses in LR tuberculosis patients. One explanation of these findings is that proinflammatory cytokines enhances the expression of SLAM on mycobacteria-stimulated T cells (17). Overall, our results suggest that regulation of SAP signaling during tuberculosis disease treatment, making it critical to understand the regulation of SAP expression and function in greater detail. The results presented in this investigation together with our earlier studies in leprosy (17) suggest that intervention in the signaling pathways of IFN-γ might promote protective immune responsiveness against intracellular infection.

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References


