Activation of Signaling Lymphocytic Activation Molecule Triggers a Signaling Cascade That Enhances Th1 Responses in Human Intracellular Infection[1]

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T cell production of IFN-γ contributes to host defense against infection by intracellular pathogens, including mycobacteria. Lepromatous leprosy, the disseminated form of infection caused by *Mycobacterium leprae*, is characterized by loss of cellular response against the pathogen and diminished Th1 cytokine production. Relieving bacterial burden in Ag-unresponsive patients might be achieved through alternative receptors that stimulate IFN-γ production. We have previously shown that ligation of signaling lymphocytic activation molecule (SLAM) enhances IFN-γ in mycobacterial infection; therefore, we investigated molecular pathways leading from SLAM activation to IFN-γ production in human leprosy. The expression of the SLAM-associated protein (an inhibitory factor for IFN-γ induction) on *M. leprae*-stimulated cells from leprosy patients was inversely correlated to IFN-γ production. However, SLAM ligation or exposure of cells from lepromatous patients to a proinflammatory microenvironment down-regulated SLAM-associated protein expression. Moreover, SLAM activation induced a sequence of signaling proteins, including activation of the NF-κB complex, phosphorylation of Stat1, and induction of T-bet expression, resulting in the promotion of IFN-γ production, a pathway that remains quiescent in response to Ag in lepromatous patients. Therefore, our findings reveal a cascade of molecular events during signaling through SLAM in leprosy that cooperate to induce IFN-γ production and strongly suggest that SLAM might be a focal point for therapeutic modulation of T cell cytokine responses in diseases characterized by dysfunctional Th2 responses. ***The Journal of Immunology*****, 2004, 173: 4120–4129.**

Cytokines produced during the immune response against an intracellular pathogen such as *Mycobacterium leprae* play an important role in host defense. Leprosy is a dynamic infectious disease in which distinct *M. leprae*-responsive T cell subsets appear to control the clinical and immunologic spectrum. Tuberculoid leprosy (T-Lep)[3] 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 (1, 2). In contrast, lepromatous leprosy (L-Lep) patients manifest disseminated infection, their T cells weakly respond to *M. leprae*, and their lesions express Th2 cytokines, typical of humoral responses and suppression of cell-mediated immunity (CMI). Thus, understanding the regulation of immunity against mycobacteria requires elucidating how the amounts of effector cytokines released in response to Ag are controlled; for example, at which differentiation step Th precursor cells activate previously silent cytokine genes and become committed to a new state, such as the Th1 phenotype (3, 4). Several signaling molecules, such as the signaling lymphocytic activation molecule (SLAM) and the SLAM-associated protein (SAP), have been shown to modulate the level and pattern of cytokines produced by naive T cells. SLAM, a homophilic receptor expressed on lymphocytes, immature thymocytes, and activated dendritic cells (DC) (5, 6), functions through bidirectional signaling after SLAM-SLAM associations. In T cells, ligation of SLAM with mAbs heightens the proliferation and/or secretion of IFN-γ (5, 7), suggesting that SLAM might mediate context-dependent functions in lymphocytes. In contrast, SAP, an Src homology 2 (SH2) domain-containing protein of T and NK cells that interacts with SLAM, was shown to participate in the differentiation process that leads T cells to the commitment of producing a specific pattern of cytokines (8–10). The SAP SH2 domain binds to the SH3 domain of FynT kinase and directly couples FynT to SLAM (11). Studies in SAP-deficient humans (X-linked lymphoproliferative (XLP) syndrome) and mice revealed abnormalities in cytokine secretion that might result from defects in the propagation of SLAM-induced signals (12, 13) and suggested that the lack of SAP expression results in skewing toward a Th1 phenotype (10). Then, restoring IFN-γ production in leprosy patients to reverse disseminated mycobacterial infection might be

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[3] Abbreviations used in this paper: T-Lep, tuberculoid leprosy; CMI, cell-mediated immunity; DC, dendritic cell; L-Lep, lepromatous leprosy; SAP, SLAM-associated protein; SH2, Src homology 2; SLAM, signaling lymphocytic activation molecule; XLP, X-linked lymphoproliferative disease.

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achieved through ligation of pathways that stimulate IFN-γ production. Engagement of SLAM enhances IFN-γ production in mycobacterial infection, even in Ag-unresponsive patients (14, 15), whereas SAP expression in cells from tuberculosis patients is inversely correlated with IFN-γ production (15). Therefore, in this study we investigated molecular pathways leading from SLAM ligation to IFN-γ production in human leprosy. We found that high expression of SAP in cells from lepromatous patients abolished *M. leprae*-induced IFN-γ, whereas cells from tuberculoid patients that expressed SLAM (and almost undetectable levels of SAP) were able to produce high levels of IFN-γ against the pathogen. However, during SLAM signaling in unresponsive patients, IFN-γ was increased in parallel with a striking down-regulation of SAP expression and a notable impairment of FynT binding to SLAM. Moreover, SAP expression in lepromatous patients could be modulated by a proinflammatory microenvironment during *M. leprae* stimulation, in direct correlation with the regulation of IFN-γ production in these individuals. By analyzing inducible gene products implicated in regulating Th1 responses, we observed that signaling through SLAM induced NF-κB activation in *M. leprae*-stimulated cells containing SLAM and barely detectable SAP levels (T-Lep patients) as well as in cells expressing SLAM and high levels of SAP (L-Lep patients). Because NF-κB induction in T cells regulates Th1 differentiation and IFN-γ production (16), our findings suggest that SLAM ligation during *M. leprae* stimulation triggers a signaling pathway leading to NF-κB activation, contributing to avoid the interference of SAP expression with Th1 responses. Moreover, *M. leprae* stimulation induced phosphorylation of Stat1 and T-bet expression only in responsive tuberculoid patients, but engagement of SLAM led to Stat1 activation and expression of T-bet in both tuberculoid and lepromatous patients, in direct correlation with our results on NF-κB activation in these individuals. Taken together, our findings suggest the existence of a cascade of molecular events during signaling through SLAM in leprosy where signaling molecules and transcription factors participate in the induction or reinforcement of IFN-γ production, promoting CMI responses to mycobacterial infection.

Materials and Methods

Patients

Patients with leprosy were evaluated at the Hospital de Infecciosas F. J. Muñiz (Buenos Aires, Argentina) and were classified according to the criteria of Ridley and Jopling (17). Peripheral blood was collected in heparinized tubes from patients with T-Lep and L-Lep leprosy. Bacillus Calmette-Guérin-vaccinated healthy control individuals from the community participated in this study. Two individuals with XLP diagnosis confirmed at the International XLP Registry Headquarters (NE) (18) were also included in this study. Peripheral blood was collected from all individuals participating in the study after receiving informed consent.

M. leprae

M. leprae was provided by Dr. P. Brennan (Colorado State University, Ft. Collins, CO) and prepared by probe sonication (19). In vitro stimulation of cells throughout the present study was performed with this sonicated preparation of mycobacteria.

Cell preparations and culture conditions

PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and cultured (1×10^6/ml) with sonicated *M. leprae* Ag (10 μg/ml) in 24- or 96-well plates with RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY) supplemented with glutamine (2 mM; Sigma-Aldrich, St. Louis, MO), streptomycin, penicillin, and 10% human serum. In different experiments, sonicated *M. leprae* Ag-stimulated cells were cultured at various times in the presence or the absence of anti-SLAM mAb (A12; 10 μg/ml; eBioscience, San Diego, CA). Human rIL-12 (500 pg/ml; Endogen, Cambridge, MA), human rIFN-γ (7.5 ng/ml; Endogen), human rIL-4 (150 IU/ml; Endogen), neutralizing anti-human IL-10 Ab (5 μg/ml; BD Pharmingen, Franklin Lakes, NJ), and sulfasalazine (0.5 mM; Sigma-Aldrich) were added to some cultures at final concentrations. After stimulation, SLAM, SAP, FynT, phosphorylated Stat1, total Stat1, IκB, and T-bet expression were determined by Western blot (see below). IFN-γ production was measured by ELISA (Endogen).

Western blot and immunoprecipitation

PMBCs were stimulated as described above; thereafter, cells were washed and solubilized in lysis 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 as previously described (15). Immunoprecipitation experiments were performed as described previously (20). Briefly, cells were incubated for 15 min with *M. leprae* Ag in the presence or the absence of anti-SLAM mAb, and extracts were obtained by lysing cells with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and Protease Inhibitor Cocktail (Sigma-Aldrich)). Extracts were incubated for 2 h with anti-SLAM Ab (SLAM (N-19); Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. After incubation, protein G-agarose (Santa Cruz Biotechnology) was added, and immunoprecipitates were obtained by centrifugation at 2500 rpm for 30 s. Equivalent amounts of protein from whole-cell lysates or immunoprecipitates were analyzed by 12% SDS-PAGE, transferred to nitrocellulose (Hybond ECL nitrocellulose membrane; Amersham Biosciences), and incubated with anti-SLAM (1/500; SLAM (N-19); Santa Cruz Biotechnology), anti-SAP (1/500; SAP (FL-128); Santa Cruz Biotechnology), anti-I-κB (1/1000; Iκ-Bα (C-21); Santa Cruz Biotechnology), anti-phospho-Stat1 (1/1000; phospho-Stat1 (Tyr701); Cell Signaling Technology, Beverly, MA), anti-Stat1 (1/1000; Stat1 p84/p91 (E-23); Santa Cruz Biotechnology), anti-FynT (1/200; FynT (15); Santa Cruz Biotechnology), or anti-T-bet (1/400; T-bet (39D); Santa Cruz Biotechnology) Abs. Bound Abs were revealed with HRP-conjugated anti-rabbit Ab (1/3000; Bio-Rad, Hercules, CA); HRP-conjugated, affinity-purified anti-goat Ab (1/2500; Chemicon International, Temecula, CA); or HRP-conjugated, affinity-purified anti-mouse Ab (1/7000; Santa Cruz Biotechnology) using ECL (Amersham Biosciences) and BioMax films (Eastman Kodak, Rochester, NY). Films were analyzed with Image Analysis software (Scion, Frederick, MD), and the intensity of each band was recorded and expressed as arbitrary units.

EMSA

EMSA was performed as described previously (21). Briefly, after 45 min of Ag stimulation in the presence or the absence of anti-SLAM mAb, PBMCs were collected and washed with PBS, and nuclear extracts were prepared (21). Double-stranded oligonucleotides encoding the consensus NF-κB binding site (5′-TCGAAATGTGGGATTTTCCCATGAGT-3′) were end-labeled using γ-^{32}P and T_4 polynucleotide kinase. Equal amounts of each sample (5–10 μg) were incubated in a total volume of 20 μl of buffer containing 600 ng of poly(dI-dC)·(dI-dC). After incubation on ice for 10 min, end-labeled oligonucleotides were added, and the incubation was continued for 20 min at 25°C. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels. Gels were dried under vacuum and autoradiographed at −70°C. A 50- to 100-fold excess of unlabeled NF-κB probe was included in the competition reaction.

Statistical analysis

Statistical analysis was performed using the nonparametric Wilcoxon signed rank test for paired samples. Values of $p < 0.05$ were considered significant.

Results

Expression of SAP in leprosy patients correlates with increased disease severity

We previously showed that *M. leprae* stimulation significantly increases the expression of SLAM in cells from Ag-responsive T-Lep patients, but not in Ag-unresponsive L-Lep patients and that SLAM up-regulates IFN-γ production in leprosy (14). In contrast, SAP interrupts IFN-γ production in tuberculosis (15). Therefore, we investigated at the molecular level the pathways leading from SLAM ligation to IFN-γ production during *M. leprae* infection. First, we analyzed SAP expression in PBMCs from leprosy patients after specific Ag stimulation and correlated the protein expression with IFN-γ production from the same individuals. Patients who displayed basal levels of SLAM (14) and produced low levels of IFN-γ upon *M. leprae* stimulation (L-Lep patients; Fig.

1B) showed substantial levels of SAP (Fig. 1*A*). In contrast, in T-Lep patients, who showed higher levels of SLAM upon Ag stimulation (14) and produced elevated levels of IFN-γ against the bacteria (Fig. 1*B*), SAP was undetectable (Fig. 1*A*). These data, indicating that the expression of SAP is lowest in the group of patients who displayed high immune responses to *M. leprae*, that is Th1-type immune responses, are in accord with our findings in tuberculosis (15) and suggest that the expression of SAP interferes with Th1 responses during mycobacterial infection.

Effect of SLAM engagement on SAP expression and Stat1 activation

To investigate the modulatory role of the SLAM-SAP signaling pathway on IFN-γ production by T cells in leprosy, we studied the regulation of the expression of SAP. PBMCs from leprosy patients were stimulated with *M. leprae* Ag and an agonistic anti-SLAM mAb, and SAP expression was determined by Western blot. As shown in Fig. 2*A*, engagement of SLAM induced a striking down-regulation of SAP expression in Ag-stimulated cells from L-Lep individuals. As expected, in T-Lep patients, SAP was undetectable or barely expressed under all conditions tested (Fig. 2*A*). These results indicate that the increase in IFN-γ production induced by ligation of SLAM in L-Lep patients (Fig. 2*B*) parallels a decrease in SAP expression (Fig. 2*A*), reinforcing the concept that the presence of SAP negates IFN-γ production.

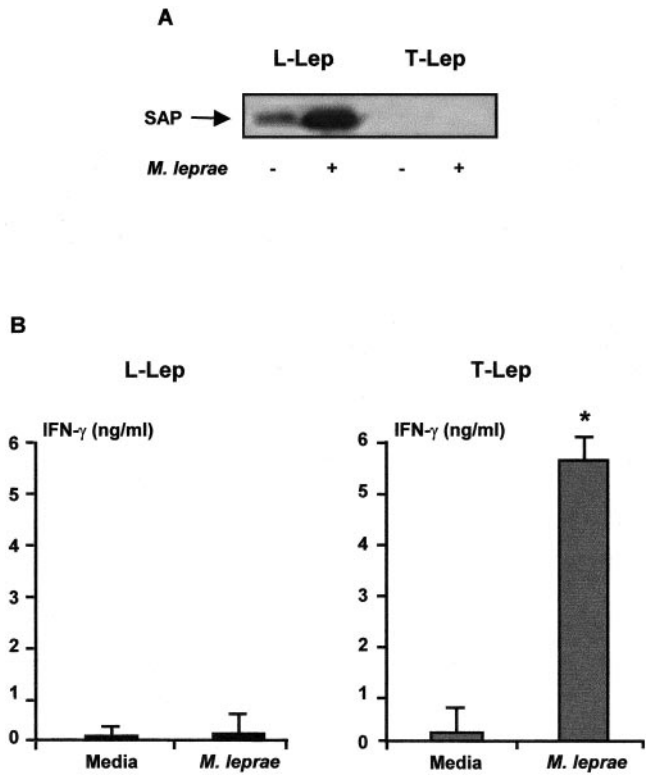

FIGURE 1. Effect of *M. leprae* Ag stimulation on the expression of SAP in leprosy patients. PBMCs from T-Lep and L-Lep patients were stimulated with sonicated *M. leprae* Ag, and SAP expression (*A*) and IFN-γ production (*B*) were determined. *A*, After 48 h of Ag stimulation, total cell extracts were prepared and assayed for SAP expression by Western blot. A representative patient of five is shown for each group. *B*, 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 for 10 L-Lep and 10 T-Lep patients. The *p* values were calculated using the signed rank test. ∗, $p < 0.001$.

Continuing our studies on the molecular pathways leading from SLAM activation to IFN-γ production, and because Stat1 was shown to be necessary for the IFN-γ-mediated control of intracellular infection (22), we investigated whether the observed effect of SLAM engagement on IFN-γ production by T cells in leprosy patients could be explained at the molecular level on the basis of Stat1 activation. JAK-Stat proteins are a family of signal transduction molecules that have critical signaling roles for IFN-γ (23). Moreover, binding sites for Stat1, -4, -5, and -6 in the first intron of the human IFN-γ gene have been detected (23). Therefore, we next investigated whether Stat1 (a molecule that exists in two isoforms, Stat1α and Stat1β), participated in the regulation of IFN-γ production by T cells after SLAM engagement. PBMCs from leprosy patients were cultured with *M. leprae* Ag in the presence or the absence of anti-SLAM mAb, and Stat1 activation was determined by Western blot. Fig. 2*C* shows that *M. leprae* induced phosphorylation of Stat1 in tuberculoid patients, whereas in cells cultured with medium alone, activation of the protein was not detected. Moreover, SLAM ligation in Ag-stimulated cells also induced phosphorylation of Stat1 in tuberculoid patients (Fig. 2*C*). In contrast, in unresponsive L-Lep patients, *M. leprae* stimulation did not induce phosphorylation of Stat1 (Fig. 2*C*). However, SLAM engagement led to Stat1 activation in these individuals (Fig. 2*C*). Although a variable pattern of phosphorylation of the two isoforms of Stat1 (Stat1α and Stat1β) was found among leprosy patients (Fig. 2*C*), Stat1 phosphorylation was always directly correlated with IFN-γ production (data not shown). Interestingly, treatment of Ag-stimulated cells from leprosy patients with anti-SLAM mAb in the presence of neutralizing anti-IFN-γ mAb resulted in reduced (but not absent) phosphorylation of Stat1 (data not shown). These results suggest that besides IFN-γ, SLAM signaling might act together with TCR triggering to activate Stat1.

Taken together, our results show that SAP and Stat1, two signaling molecules that regulate cytokine production by T cells, participate in SLAM activation in leprosy.

Regulation of SAP expression by cytokines of the microenvironment

The relative amounts of SAP and SLAM expression may vary during lymphocyte activation (24). In addition, it has been shown that some proinflammatory cytokines can augment *M. leprae*-specific T cell IFN-γ production in L-Lep patients (25). Therefore, we analyzed the regulation of the expression of SAP by the cytokine microenvironment during *M. leprae* infection. PBMCs from leprosy patients were stimulated with *M. leprae* Ag in the presence of cytokines or anti-cytokine mAbs, and the expression of SAP was determined by Western blot. As shown in Fig. 3*A*, *M. leprae* stimulation strikingly up-regulated the expression of SAP in L-Lep patients. However, the presence of either rIL-12 or rIFN-γ in the microenvironment during Ag stimulation induced a significant down-regulation of SAP expression in unresponsive individuals (Fig. 3*A*; $p < 0.05$, by signed rank test). Moreover, treatment of cells with *M. leprae* Ag in the presence of anti-IL-10 mAb significantly reduced the expression of SAP (Fig. 3*A*; $p < 0.05$, by signed rank test). SAP expression in L-Lep patients was correlated with IFN-γ production in the same individuals. As shown in Fig. 3*B*, modulation of SAP expression by proinflammatory conditions paralleled IFN-γ production in unresponsive patients (Fig. 3*B*; $p < 0.05$, by signed rank test). The effect of IL-4 or IL-10 treatment on the regulation of SAP expression in *M. leprae*-stimulated cells from leprosy patients was also investigated. However, the levels of SAP in Ag-stimulated T cells were not modified by IL-4 or IL-10 (data not shown), indicating that Th2 cytokine signaling does not

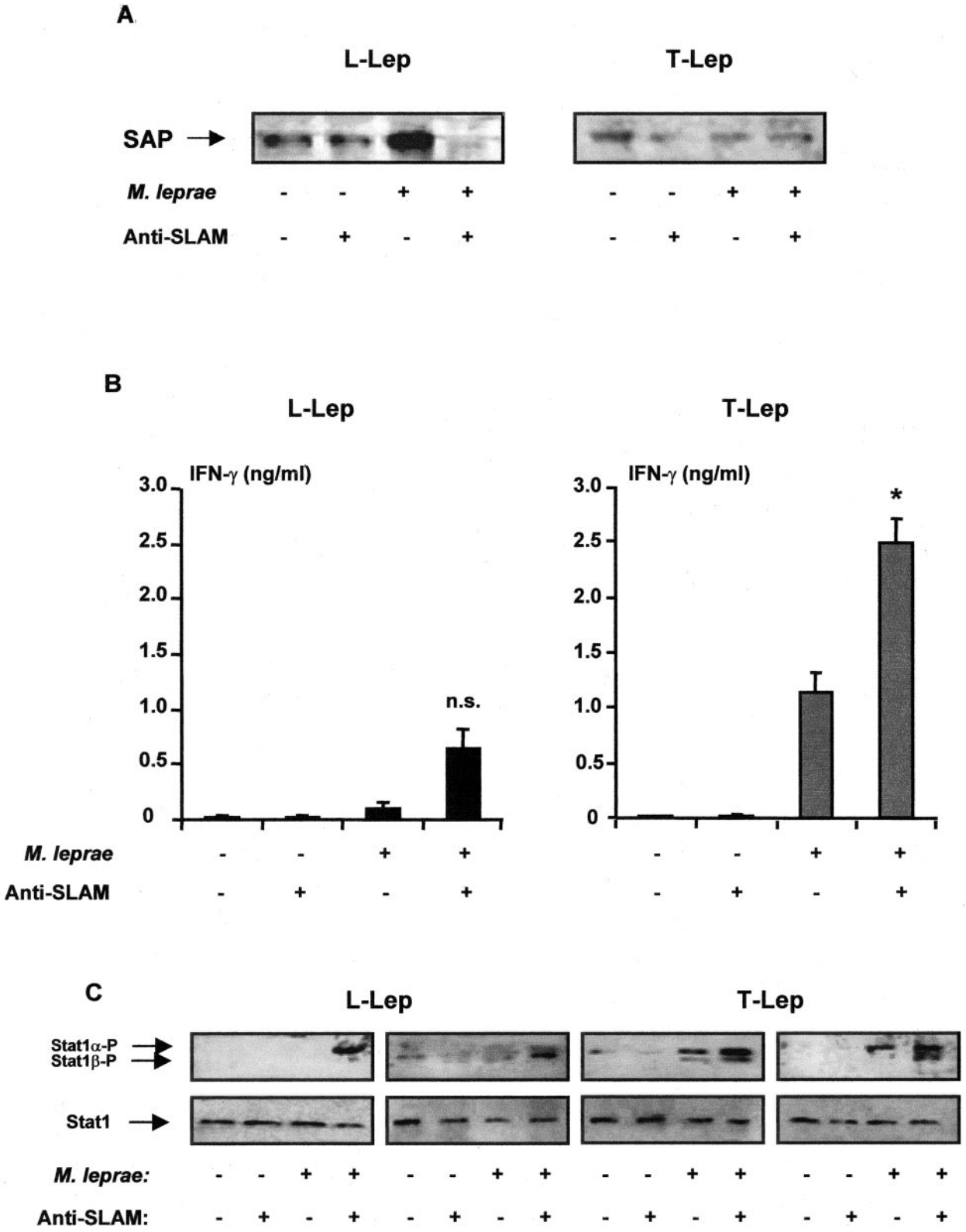

FIGURE 2. Effect of anti-SLAM mAb on SAP expression in leprosy patients. *A*, PBMCs from leprosy patients were cultured with sonicated *M. leprae* Ag in the presence or the absence of anti-SLAM mAb, and after 48 h total cell extracts were prepared and assayed for SAP expression by Western blot. A representative patient of four is shown for each group. *B*, PBMCs from leprosy patients (eight T-Lep and eight L-Lep patients) were stimulated with *M. leprae* Ag, and after 5 days, cells were cultured in the presence or the absence of anti-SLAM mAb. Cell-free supernatants were collected after 48 h and assayed for IFN-γ by ELISA. Values are expressed as the mean of triplicate determinations. The *p* values were calculated using the signed rank test, comparing IFN-γ production from cells cultured with medium after *M. leprae* stimulation vs cells cultured with anti-SLAM mAb after *M. leprae* stimulation. ∗, $p < 0.001$; n.s., differences not significant. *C*, Effect of SLAM ligation on Stat1 activation in leprosy patients. PBMCs from leprosy patients were cultured with sonicated *M. leprae* Ag in the presence or the absence of anti-SLAM mAb for 16 h. Total extracts were then prepared and assayed for phosphorylated Stat1 by Western blot (*upper*). Total Stat1 (*lower*) was measured by Western blot as a control. Two representative T-Lep patients of six and two representative L-Lep patients of five are shown.

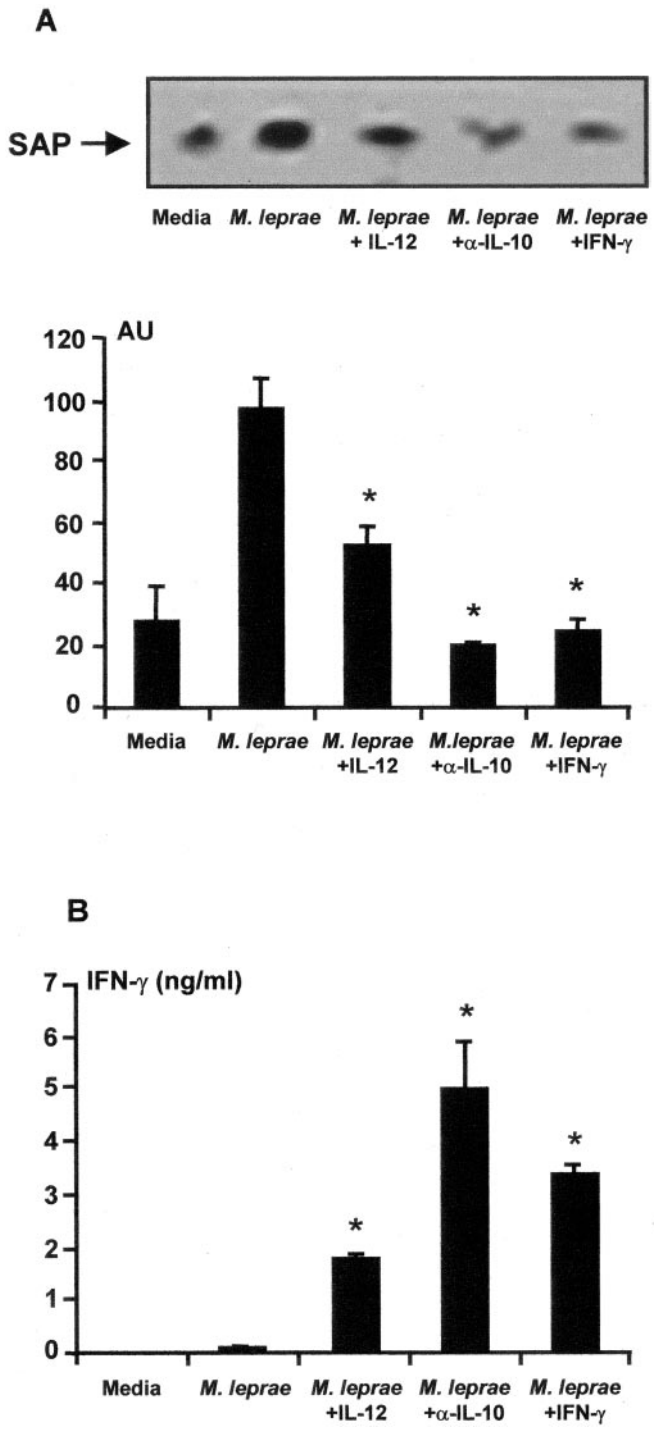

FIGURE 3. Modulation of the expression of SAP by cytokines from the microenvironment. *A*, PBMCs from L-Lep patients were stimulated with sonicated *M. leprae* Ag in the presence or the absence of rIL-12, rIFN-γ, or neutralizing anti-human IL-10 mAb for 48 h. Total cell extracts were then prepared and assayed for SAP expression by Western blot. A representative L-Lep patient of six is shown (*upper*). Polyacrylamide gels from L-Lep patients were scanned, densitometry was performed, and the results were expressed as arbitrary units (AU; *lower*). The *p* values were calculated using the signed rank test, comparing SAP expression in cells cultured with *M. leprae* vs *M. leprae* plus rIL-12, rIFN-γ, or anti-IL-10. ∗, $p < 0.05$. *B*, After 48 h of Ag stimulation in the presence of rIL-12, rIFN-γ, or anti-IL-10, cell-free supernatants were recovered, and IFN-γ production was determined by ELISA. Values are expressed as the mean of triplicate determinations for six L-Lep. The *p* values were calculated using the signed rank test, comparing IFN-γ production from cells cultured with *M. leprae* vs *M. leprae* plus rIL-12, rIFN-γ, or anti-IL-10. ∗, $p < 0.05$.

induce cell activation, and therefore, it cannot modulate SAP expression. Thus, our data indicate that the expression of SAP can be modulated by a proinflammatory cytokine microenvironment during *M. leprae* stimulation.

SAP mediates recruitment of FynT to SLAM in leprosy

In mice, FynT combines with SAP to form a complex with SLAM that inhibits IFN-γ production (7, 11, 26). Therefore, we analyzed whether FynT participated in the SLAM-SAP pathway during human *M. leprae* infection. Ag-stimulated cells from L-Lep patients cultured with anti-SLAM mAb were immunoprecipitated with SLAM, and Western blot for FynT was performed. We found FynT expression in *M. leprae*-stimulated cells from unresponsive patients (Fig. 4), individuals who expressed high levels of SAP after Ag stimulation (Fig. 1). However, engagement of SLAM induced a striking down-regulation of FynT binding to SLAM in both *M. leprae*-stimulated cells and cells cultured with medium (Fig. 4). Our results in Ag-stimulated cells suggest that T cell activation induced during TCR signaling and SLAM costimulation down-regulates SAP expression (Fig. 2*A*), preventing the SAP-mediated recruitment of FynT to SLAM and thus allowing IFN-γ production (Fig. 2*B*). Although FynT binding to SLAM was decreased in cells cultured with medium after SLAM ligation, the production of IFN-γ is not induced unless specific TCR stimulation occurs (Fig. 2*B*). In contrast, in T-Lep patients, no FynT expression was detected under the same experimental conditions (data not shown). Together, our present results indicate that the SLAM-anti-SLAM interactions in Ag-stimulated cells from unresponsive patients might impair the binding of SAP to FynT and, in turn, to SLAM, possibly because of the lower number of SAP molecules available for that interaction.

Because it was proposed that in mice, SAP-mediated recruitment of FynT is a key step in the immune function of SLAM (26), it was hypothesized that a defect in this signaling mechanism might be involved in the abnormal immune function of XLP patients (26). Therefore, the ability of SLAM to associate with FynT in XLP patients was next analyzed. Cells from XLP patients were cultured with or without anti-SLAM mAb, and thereafter immunoprecipitation with SLAM and Western blot for FynT was performed. However, SLAM failed to associate with FynT, even though T cells from these individuals clearly expressed FynT (data not shown), suggesting that SLAM was not tyrosine phosphorylated and confirming SAP participation in the interaction of FynT with SLAM in humans.

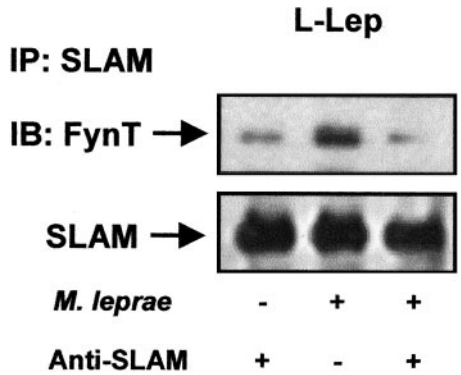

FIGURE 4. Effect of anti-SLAM mAb on FynT binding to SLAM in L-Lep patients. PBMCs from L-Lep patients were cultured in the presence or the absence of anti-SLAM mAb together with sonicated *M. leprae* Ag for 15 min. Immunoprecipitates were obtained as described in *Materials and Methods*, and FynT and SLAM expression were determined by Western blot. A representative L-Lep patient of five is shown.

NF-κB activation during signaling through SLAM

Our results suggested that SAP might participate in the differentiation process that allows activated T cells to produce different patterns of cytokines during *M. leprae* infection. NF-κB induction in T cells regulates efficient Th1 clonal expansion and controls the amount of IFN-γ produced from a differentiated Th1 population (16). Moreover, NF-κB activation could be linked to XLP disease (27). Therefore, to investigate the role of NF-κB in signaling through SLAM, we initially studied NF-κB activation in SAP-deficient humans. Treatment of *M. leprae*-stimulated PBMCs from XLP individuals with sulfasalazine, an anti-inflammatory agent that inhibits NF-κB signaling (28), induced a marked decrease in IFN-γ even after SLAM ligation (Fig. 5*A*). XLP patients probably responded to *M. leprae* Ag because these individuals were vaccinated with bacillus Calmette-Guérin and contained *M. tuberculosis*-reactive T cells that cross-reacted with the leprosy bacillus. Similar to our results in XLP patients, in responsive T-Lep patients, sulfasalazine inhibited IFN-γ production by *M. leprae*-stimulated cells (Fig. 5*A*; $p < 0.05$, by signed rank test). Moreover, a significant decrease in IFN-γ levels was induced during SLAM engagement (Fig. 5*A*; $p < 0.05$, by signed rank test). Together, our results indicate that NF-κB participates in the control of IFN-γ production by T cells against *M. leprae* during signaling through SLAM.

Physiologic induction of the NF-κB/Rel pathway is regulated by the controlled release of dimeric complexes from cytosolic retention molecules termed IκBs (29, 30). Cell surface receptor engagement activates an enzymatic complex, the IκB kinases, leading to regulated serine phosphorylation, ubiquitination, and degradation of IκBs such as IκBα (29–31). Therefore, to investigate NF-κB activation after SLAM triggering in leprosy, we examined IκB expression in cells from leprosy patients stimulated with Ag in the presence or the absence of anti-SLAM mAb. As shown in Fig. 5*B*, in T-Lep patients, *M. leprae* induced a significant degradation of IκB after 45 min of stimulation (Fig. 5*B*; $p < 0.05$, by signed rank test). Moreover, after signaling through SLAM, IκB expression was undetectable in these individuals (Fig. 5*B*). In contrast, in unresponsive L-Lep patients, a striking expression of IκB was detected in Ag-stimulated cells. However, engagement of SLAM in *M. leprae*-stimulated cells induced a significant degradation of IκB (Fig. 5*B*; $p < 0.05$, by signed rank test), suggesting that in lepromatous patients, NF-κB activation requires both TCR and SLAM

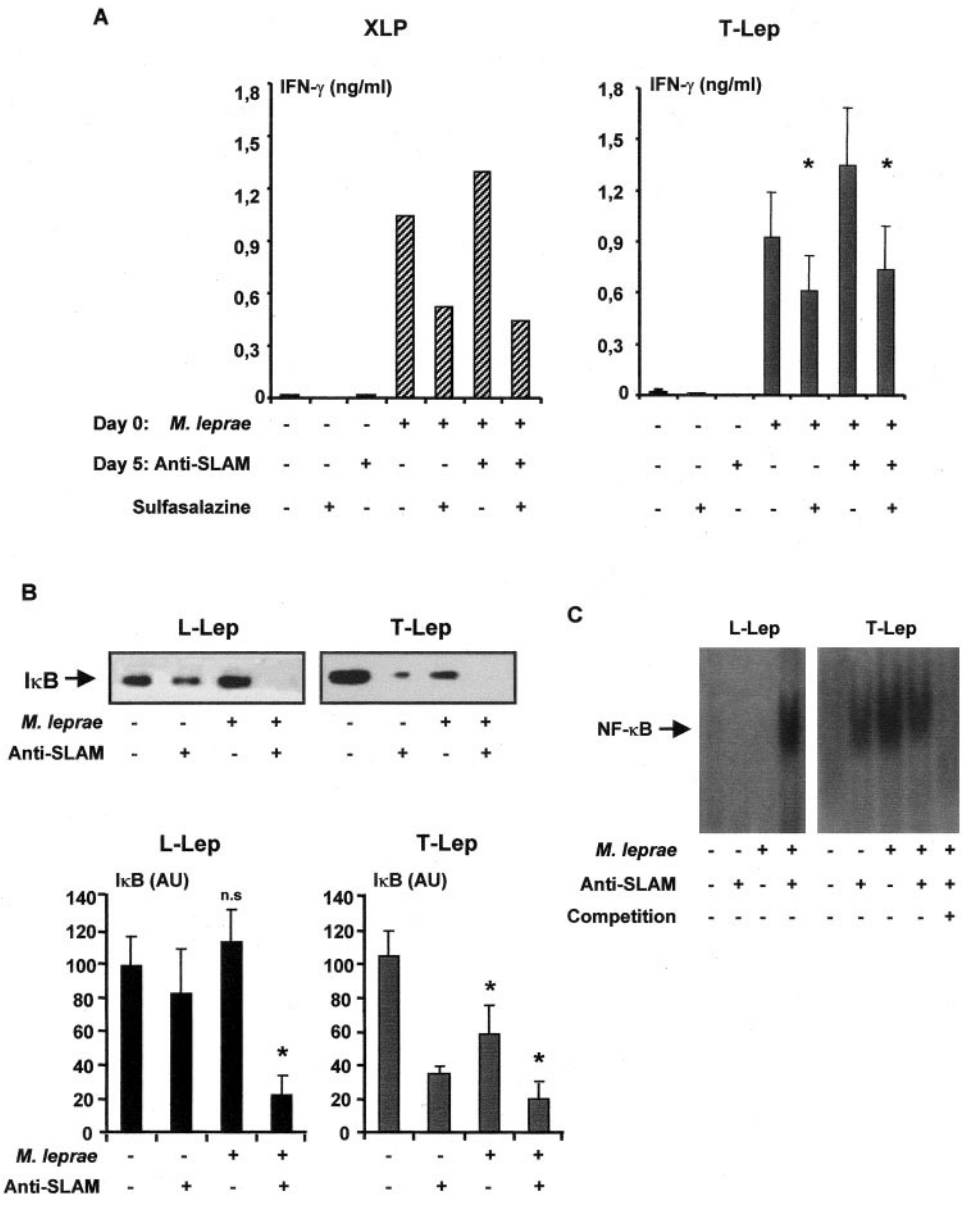

FIGURE 5. Effect of SLAM ligation on NF-κB activation. *A*, PBMCs from two XLP patients and seven responder T-Lep patients were stimulated with *M. leprae* Ag, and after 5 days cells were cultured in the presence or the absence of anti-SLAM mAb plus sulfasalazine. Cell-free supernatants were collected at 48 h and assayed for IFN-γ by ELISA. Values are expressed as the mean of triplicate determinations. Each bar represents the mean ± SEM. For T-Lep individuals, *p* values were calculated using the signed rank test, comparing cells treated with *M. leprae* Ag vs *M. leprae* Ag plus sulfasalazine and *M. leprae* Ag plus anti-SLAM mAb vs cells cultured with *M. leprae* Ag plus anti-SLAM mAb plus sulfasalazine. *B*, PBMCs from T-Lep and L-Lep patients were stimulated with *M. leprae* in the presence or the absence of anti-SLAM mAb, and after 45 min, whole-cell extracts were prepared and assayed for IκB protein expression by Western blot. A representative patient of five from each group is shown (*upper*). Polyacrylamide gels from leprosy patients were scanned, densitometry was performed, and the results were expressed as arbitrary units (AU; *lower*). The *p* values were calculated using the signed rank test, comparing IκB expression in cells from leprosy patients cultured with medium vs cells cultured with *M. leprae* Ag, and cells cultured with *M. leprae* Ag vs *M. leprae* Ag plus anti-SLAM mAb. *, $p < 0.05$; n.s., differences not significant. *C*, PBMCs from leprosy patients were stimulated with sonicated *M. leprae* Ag in the presence or the absence of an anti-SLAM mAb for 45 min. Nuclear extracts were prepared, and EMSA was performed as described in *Materials and Methods.* Competition assay was performed using a 50- to 100-fold excess of unlabeled NF-κB probe. A representative patient of five from each group is shown.

signaling. These results on IκB degradation during signaling of SLAM in leprosy patients were corroborated by performing EMSAs. As shown in Fig. 5*C*, in tuberculoid patients, both *M. leprae* and SLAM stimulation induced a distinct binding complex compared with medium alone, indicating binding of NF-κB to a radiolabeled oligonucleotide probe encoding the consensus NF-κB binding site (Fig. 5*C*, *right panel*, *lanes 2–4*). In contrast, in lepromatous patients, NF-κB binding to its consensus site was only detected after TCR signaling and SLAM costimulation (Fig. 5*C*, *left panel*, *lane 4*). Together, our data indicate that engagement of SLAM in *M. leprae*-stimulated cells from leprosy patients induces NF-κB activation.

Effect of SLAM engagement on T-bet expression

To investigate inducible gene products implicated in regulation of the molecular pathways that control the levels of IFN-γ produced after SLAM ligation, we analyzed T-bet expression. T-bet was proposed to be the master switch for Th1 development based on its IFN-γ induction and its direct activation of IFN-γ reporter activity (32). Then, we investigated whether this transcription factor participated in signaling through SLAM in leprosy. As shown in Fig. 6*A*, in T-Lep patients *M. leprae* stimulation induced T-bet expression. Moreover, signaling through SLAM induced the expression of T-bet as well, and this expression was enhanced after Ag stimulation. On the contrary, in L-Lep patients, T-bet expression was not detected after *M. leprae* Ag stimulation (Fig. 6*A*). However, signaling through SLAM augmented the levels of T-bet in both unstimulated and Ag-stimulated cells (Fig. 6*A*). Although T-bet expression was detected after SLAM ligation in the absence of Ag stimulation, IFN-γ was not produced by leprosy patients under those conditions (Fig. 6*B*). These results suggest that the sole presence of T-bet in the cell may not be enough to induce IFN-γ secretion. Because different cytokine receptors and pathways have been proposed to potentially target the transcription of T-bet (33), our present data suggest that SLAM activation could be a new regulator of this transcription factor in intracellular infection, directly inducing T-bet, but requiring additional signaling through the TCR to completely activate the cell and trigger IFN-γ synthesis against the pathogen.

Discussion

Protective immunity against mycobacterial infection requires the generation of Th1 cytokine responses (34). In lepromatous leprosy patients, the specific unresponsiveness of their T cells to *M. leprae* impairs IFN-γ production (a macrophage-activating cytokine required to eliminate the bacteria), leading to disseminated disease. However, signaling through SLAM promotes cell-mediated immune responses to the pathogen in these patients (14). Therefore, in an attempt to gain insight into the mechanisms involved in the enhancement of CMI responses to mycobacterial infection, we investigated signaling pathways leading from SLAM ligation to IFN-γ production in human leprosy. We found an inverse correlation between the expression of SAP on *M. leprae* Ag-stimulated T cells from leprosy patients and the production of IFN-γ by these individuals. However, either SLAM engagement or exposure of lepromatous patients' cells in vitro to a proinflammatory microenvironment induced down-regulation of SAP expression in unresponsive patients, in parallel with an up-regulation of IFN-γ production. Furthermore, in addition to SAP, we demonstrated that other signaling proteins, including FynT, NF-κB, Stat1, and T-bet, participate in SLAM signaling in leprosy, contributing to regulate IFN-γ production through a pathway that remains untriggered by Ag in lepromatous patients. Together, our data suggest that a cascade of molecular events controls IFN-γ production during signaling through SLAM in leprosy, contributing to modulate the amounts of this effector cytokine that participate in the eradication of mycobacterial infection.

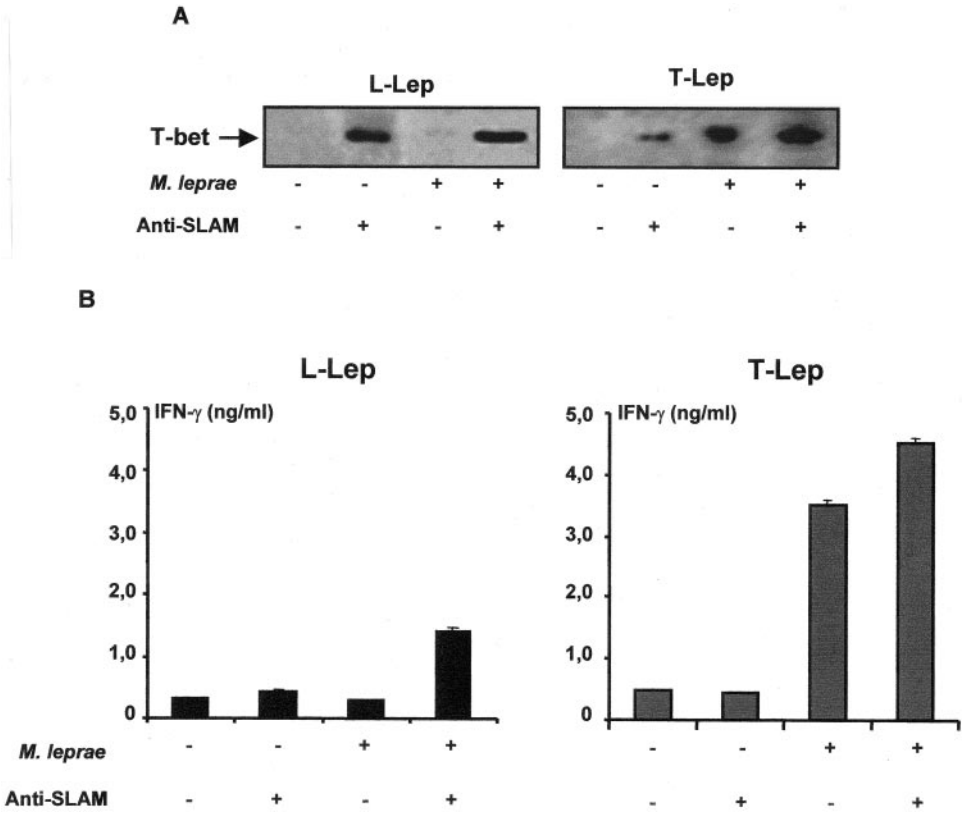

FIGURE 6. Effect of SLAM signaling on T-bet expression in leprosy patients. *A*, PBMCs from leprosy patients were cultured with sonicated *M. leprae* Ag, and after 5 days cells were cultured in the presence or the absence of anti-SLAM mAb for 48 h. Total extracts were then prepared and assayed for T-bet expression by Western blot. A representative patient of four is shown for each group. *B*, PBMCs from the two representative leprosy patients shown in *A* were stimulated with *M. leprae* Ag, and after 5 days cells were cultured in the presence or the 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 ± SD of triplicate determinations.

It has been hypothesized that in the anergic form of leprosy, the interaction between the MHC class II/Ag complex on APC and the TCR on T cells is not strong enough to induce T cell activation, suggesting that a second signal delivered through costimulatory molecules such as CD28 would be essential for T cell proliferation against the pathogen (35). Because signaling through the costimulatory molecule SLAM enhanced IFN-γ production in leprosy patients (14), we investigated at the molecular level the alternative pathway triggered by SLAM that leads to IFN-γ production in human leprosy. The existence of an inverse relationship between SAP expression and IFN-γ production by Ag-stimulated T cells in tuberculosis and XLP patients (15) indicates that SAP attenuates Th1 immune responses (7, 15). In fact, we showed that SAP turns off IFN-γ production in L-Lep patients, but stimulation with SLAM and TCR together decreased SAP expression in unresponsive patients. Given the differences in SAP expression we found in Ag-stimulated cells from the polar forms of leprosy, we ascertained whether SAP could be modulated in vitro by cytokines present in the local microenvironment. *M. leprae*-stimulated cells from unresponsive patients cultivated under proinflammatory conditions strikingly down-regulated SAP. Because both IL-12 and IFN-γ augment the expression of SLAM in *M. leprae*-stimulated cells from leprosy patients (14), whereas IL-10 down-regulates SLAM expression (36), the regulation of SAP expression in a proinflammatory microenvironment may be associated with changes in the activation status of the cell due to cytokine signaling, augmenting SLAM levels (14) and allowing SLAM-SLAM interactions from T cells interacting with other activated T cells or with DC (6). Moreover, because SLAM ligation in human DC augments IL-12 release (6), induction of SLAM signaling might create a positive feedback loop of proinflammatory cytokines, leading to a decrease in SAP. Conversely, SAP expression was not modified by IL-4 or IL-10, indicating that Th2 cytokine signaling does not induce cell activation during Ag stimulation, and therefore, SAP expression is not modulated. This could be related to the fact that in responsive tuberculoid patients, who express high levels of SLAM and low levels of SAP and produce high levels of IFN-γ after *M. leprae* stimulation, IL-10 would reduce (but not abolish) the IFN-γ produced against the bacteria. Moreover, although SLAM expression might be decreased by IL-10, the remaining levels of SLAM and IFN-γ might be sufficient to allow activation of the cell, preventing the increase in SAP levels. Taken together, our data are in agreement with previous results showing that SLAM is up-regulated early during mouse T cell activation, whereas SAP is rapidly down-regulated (37). Thus, proinflammatory cytokines present in the microenvironment during Ag stimulation would induce T cell activation, leading to an increase in the SLAM:SAP ratio, which, in turn, would give rise to IFN-γ production and, finally, would promote elimination of bacteria.

SAP couples FynT to SLAM, and the SAP-FynT interaction is required for modulation of cytokine production in mouse T cells (11, 26). Thus, we analyzed how SAP recruited FynT to SLAM and regulated IFN-γ production during *M. leprae* infection. By examining the ability of SLAM to bind FynT, we found a clear association of SLAM and FynT in SAP-containing cells, suggesting that this pathway might be critical for normal immune function in humans, as shown in mice (11, 26). SLAM ligation markedly down-regulated FynT binding to SLAM in unresponsive patients, suggesting that SAP might be degraded within the cell. In fact, upon T cell activation, SAP is quickly degraded by up-regulated mRNA degradation protein(s) (37). Moreover, SLAM failed to associate with FynT in T cells from SAP-deficient humans, although their T cells clearly expressed FynT (data not shown), confirming the role of SAP in the interaction of SLAM with FynT. Together, our data allowed us to evaluate the biological significance of the interaction among SLAM, SAP, and FynT during human bacterial intracellular infection.

To investigate the regulation of the amounts of IFN-γ released in response to SLAM ligation in cells from leprosy patients, we studied the activation of transcription factors that contribute to the regulated differentiation of Th1 cells (38–40). Members of the NF-κB/Rel family are activated after TCR ligation (41), and the promoters of IFN-γ and IL-4 (both crucial cytokines in the immune response to *M. leprae*) may be regulated by NF-κB binding (42, 43). Our results showed that in responsive leprosy patients, Ag stimulation led to IκB degradation, indicating NF-κB activation. Even though in unresponsive patients, *M. leprae* stimulation failed to induce IκB degradation, engagement of TCR and SLAM together led to degradation of the protein. IκB degradation is a key regulatory target in determining the characteristics of a T cell response to TCR engagement, and T cells from unresponsive lepromatous patients are specifically unresponsiveness to *M. leprae*. Our data might then suggest an impaired NF-κB signaling pathway in unresponsive patients' T cells upon Ag stimulation, leading to interference with efficient induction of effector cytokines such as IFN-γ (42, 43). In fact, alterations in the expression of signal transduction molecules have been demonstrated in T cells from lepromatous patients, like the absence of nuclear NF-κB p65 and c-Rel (44). However, SLAM ligation in Ag-stimulated cells from unresponsive patients reversed the impairment in NF-κB signaling, probably by modulating the altered expression of nuclear transcription factors, allowing signal transduction, regulation of Th1 differentiation, and IFN-γ production by NF-κB (16). These data showed for the first time that SLAM signaling induced NF-κB activation in Ag-stimulated T cells from leprosy patients, contributing to the Th1 immune response necessary to eliminate the bacteria.

Continuing our studies of inducible gene products implicated in regulation of the molecular pathways leading from SLAM ligation to IFN-γ production, we analyzed Stat1 activation and T-bet expression during signaling through SLAM in leprosy. Binding of IFN-γ to cell surface receptors results in activation of JAKs and phosphorylation of cytoplasmic Stat1, which translocates to the nucleus and activates transcription of specific genes (45). Stat1 exists in two isoforms as the result of alternative RNA splicing, Stat1α (p91) and Stat1β (p84). It has been proposed that only Stat1α is able to activate the transcription of IFN-γ-responsive genes (46–48). However, it has been shown that both Stat1α and Stat1β bind DNA, stimulating transcription of naked DNA and revealing a formerly unrecognized transcriptional activation function common to Stat1α and Stat1β (49). In leprosy patients, *M. leprae* stimulation activated Stat1 only in responsive tuberculoid individuals, whereas no phosphorylation of the protein was detected in unresponsive lepromatous patients, in accord with our results for NF-κB activation. However, engagement of SLAM led to Stat1 activation in both types of patients, indicating that Stat1 participated in signaling through SLAM in leprosy. We observed that the pattern of phosphorylation of the two isoforms of Stat1 could vary among leprosy patients, independently of whether they were tuberculoid or lepromatous. However, regardless of the intensity of phosphorylation of each isoform, Stat1 phosphorylation was directly correlated with IFN-γ production by the individuals (data not shown). Although Stat1β phosphorylation was stronger in some individuals, Stat1α was also phosphorylated, allowing transcription of the IFN-γ gene. Therefore, in our experimental system either Stat1β would not be acting as an inhibitor of IFN-γ, as suggested in macrophages (50), or the levels of Stat1α phosphorylation would be compensating Stat1β inhibition to allow

IFN-γ production after Ag or Ag plus anti-SLAM signaling in cells from leprosy patients.

Cross-linking of CD40 on B cells induced activation of Stat proteins (51–53). Moreover, stimulation of CD2 in primary T lymphocytes leads to a delayed and prolonged activation of Stat1 and Stat1-dependent gene expression (52). Therefore, both cytokine receptors and molecules such as CD2 might use the Stat pathway, allowing integration of diverse external signals. Moreover, a similar phenomenon was observed in B lymphocytes upon Ag receptor stimulation (53), suggesting that this might represent a unique mechanism to activate Stat transcription factors during lymphocyte activation (52, 53). Because SLAM is a transmembrane receptor belonging to the CD2 family (5), our results suggest that ligation through SLAM could lead to Stat1 activation in T cells from leprosy patients.

Finally, we analyzed whether T-bet, the master switch transcription factor for Th1 development (32), was involved in SLAM signaling. Similar to our data on NF-κB and Stat1 activation, *M. leprae* stimulation induced T-bet expression in responsive, but not in unresponsive, lepromatous patients. However, after signaling through SLAM, we detected T-bet expression in both groups of leprosy patients, although IFN-γ production was induced only after TCR signaling, indicating that the sole presence of T-bet is not enough to induce IFN-γ secretion. In accord with our present results, transfection of mouse T cells with a retroviral vector containing T-bet did not lead to IFN-γ production unless polyclonal or Ag-specific stimulation was performed, suggesting that TCR stimulation is required to induce IFN-γ secretion (32). Moreover, it has been recently demonstrated that T-bet is either absent or expressed at low levels by naive CD4 T cells, but when these cells recognize the Ag in the presence of IFN-γ, coordinated signaling is activated through TCR and Stat1, respectively, resulting in increased expression of T-bet (54). These results are in agreement with our data showing that signaling through TCR in tuberculoid patients might directly induce T-bet and lead to downstream activation of IFN-γ, a process that would be amplified by Stat1. It has been proposed that during pathogenic infections, activated cells from the innate immune system stimulate naive T cells, leading to T-bet expression in an Ag-independent manner, although the nature of the molecules involved in this mechanism is currently unknown (55). These data are in accord with our results showing T-bet expression (and no IFN-γ production) in both types of leprosy patients after anti-SLAM stimulation in the absence of signaling through the TCR. Nevertheless, SLAM ligation in *M. leprae*-stimulated cells led to T-bet expression in both T-Lep and L-Lep patients, demonstrating T-bet participation in signaling through SLAM during *M. leprae* infection. Thus, our present results together with previous published data (54, 55) suggest that SLAM-SLAM interactions could directly induce T-bet, but signaling through the TCR and amplification of T-bet expression by Stat1 would be required to induce IFN-γ production in leprosy patients. Furthermore, because ligation of SLAM by anti-SLAM mAbs redirects Th2 responses of human Ag-specific clones (56) and allergen-specific Th2 cell lines (36) to a Th1/Th0 phenotype and promotes Th1 cytokine production in leprosy (14), the observed up-regulation of T-bet expression in *M. leprae*-stimulated T cells from lepromatous patients after SLAM ligation would indicate a reversion of the phenotype of those cells to a Th1 phenotype. Because very little information regarding T-bet expression in human infection is available, our results might indicate a novel aspect of T-bet regulation during human intracellular infection.

We propose that several signaling proteins sequentially participate in the control of IFN-γ production during SLAM ligation in leprosy. The regulation of IFN-γ production would be essentially dependent on T cell recognition of Ag. T cells responding to *M. leprae* are rapidly activated, up-regulating SLAM and transiently down-regulating SAP, which, in turn, impairs SAP binding of FynT to SLAM, triggering inducible gene products implicated in the regulation of Th1 responses. In this way, SLAM activation releases a series of signaling molecules that combine to promote IFN-γ production against the bacteria. In contrast, the lack of T cell responsiveness to Ag prevents up-regulation of SLAM, the existing SAP couples FynT to SLAM, and inducible gene products implicated in Th1 regulation are not induced, leading to inhibition of IFN-γ. If SAP is down-regulated by either SLAM ligation or cell exposure to a proinflammatory microenvironment, this signaling cascade is unlocked, allowing IFN-γ production.

Overall, our results indicate the existence of a cascade of molecular events during signaling through SLAM in human leprosy, where different signaling molecules cooperate to regulate IFN-γ production. These findings provide new insights into the immunopathology of the disease and possibly into the mechanisms leading to anergy in L-Lep patients. Moreover, our study strongly suggests that the SLAM signaling pathway might be a focal point for therapeutic modulation of T cell cytokine responses in diseases characterized by dysfunctional Th2 responses, such as allergy (36) or leishmaniasis (57), because SLAM activation may promote CMI to intracellular pathogens.

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