IFN-γ Production during Active Tuberculosis Is Regulated by Mechanisms That Involve IL-17, SLAM, and CREB

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Interferon-γ (IFN–γ) is crucial for protection against *Mycobacterium tuberculosis,* and the transcription factor cAMP response element binding protein (CREB) increases IFN-γ transcription. We determined whether the transmembrane receptor signaling lymphocyte activation molecule (SLAM) and interleukin-17 (IL-17) affect CREB phosphorylation and IFN-γ production in persons with tuberculosis. When T cells from patients with tuberculosis were activated with *M. tuberculosis,* 80% of SLAM$^+$ T cells expressed phosphorylated CREB, and SLAM activation increased CREB phosphorylation and IFN-γ production. In contrast, IL-17 down-regulated SLAM expression, CREB phosphorylation, and IFN-γ production. Therefore, IL-17 and SLAM have opposing effects on IFN-γ production through CREB activation in persons with tuberculosis.

Tuberculosis remains an enormous global health problem despite currently available drug treatments. There are nearly 9 million new cases of and 1.7 million deaths due to tuberculosis annually, and it is one of the most common causes of morbidity and mortality among patients with HIV infection. BCG, the only available vaccine, is of variable efficacy, especially in tuberculosis-endemic regions. Development of a more effective vaccine depends on a better understanding of the human immune response to this pathogen.

Protective immunity against mycobacterial infection requires the generation of IFN-γ, a macrophage-activating cytokine produced by T cells that is crucial in immunity to *Mycobacterium tuberculosis* infection [1]. The degree of reduction in IFN-γ production by peripheral blood mononuclear cells (PBMCs) is a marker of disease severity in patients with tuberculosis [2]. Thus, elucidation of the mechanisms for reduced IFN-γ production in individuals that develop the disease will enhance our knowledge of the pathogenesis of tuberculosis. To understand these mechanisms, it is important to delineate how T helper precursor cells activate the gene encoding IFN-γ and become committed to the Th1 phenotype [3]. Therefore, to gain insight into the mechanisms that enhance cell-mediated immune responses to *M. tuberculosis,* we studied proteins that might regulate transcription of IFN-γ. Previously, we showed that the transcription factor cAMP response element binding protein (CREB) increased *M. tuberculosis*–stimulated IFN-γ secretion by binding to the IFN-γ proximal promoter [4]. Furthermore, we demonstrated that T cell expression of signaling lymphocytic activation molecule (SLAM) was directly correlated with responsiveness to *M. tuberculosis* antigen and that SLAM engagement up-regulated IFN-γ production in patients with tuberculosis [5]. In addition, IFN-γ inhibits IL-17 expression, but the effects of IL-17 on IFN-γ production are unclear [6]. Thus, here we analyzed the role of SLAM, CREB, and IL-17 as regulators of the molecular pathway that leads to IFN-γ production during active tuberculosis.

Subjects, materials, and methods. We evaluated 35 patients with tuberculosis diagnosed at Hospital Muñiz (Buenos Aires, Argentina) on the basis of clinical and radiological data, together with identification of acid-fast bacilli in sputum. All patients had received antituberculosis therapy for <1 week. We also studied 30 BCG-vaccinated healthy donors. After informed consent was obtained, we collected peripheral blood in heparinized tubes. The local ethics committee approved all studies.

Cells were stimulated in vitro with an extract from the virulent *M. tuberculosis* strain H37Rv, generously provided by Mycobacteria Research Laboratories (Colorado State University [Fort Collins, CO]), that was prepared by probe sonication. PBMCs were isolated by density gradient centrifugation with Ficoll-Paque (Amersham Biosciences) and were cultured in RPMI

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1640 medium (Gibco) supplemented with L-glutamine (2 mmol/L [Sigma-Aldrich]), streptomycin, penicillin, and 10% human serum. Cells were stimulated with *M. tuberculosis* antigen (10 μg/mL), SLAM monoclonal antibody (10 μg/mL; A12 [eBioscience]), or recombinant IL-17 (5 ng/mL [eBioscience]). After stimulation, IFN-γ production was determined in cell-free supernatants by ELISA (eBioscience).

Expression of phosphorylated CREB (pCREB) was determined on CD3$^+$ cells, following the manufacturer's instructions (BD Biosciences). Negative control samples were incubated with irrelevant, isotype-matched monoclonal antibodies in parallel with experimental samples. Samples were analyzed on a flow cytometer (FACScalibur [BD Biosciences]).

The DNA template for transcription was prepared as recommended by Elbashir et al. [7]: forward T7-tagged primer, 5'-GCGTAATACGACTCACTATAGGGAGAGAGCATGAACAAAAGCATCCAC-3'; reverse T7-tagged primer, 5'-GCGTAATACGACTCACTATAGGGAGACTTCTGAAGAGGACCTGGTTTC-3' (Integrated DNA Technologies). The PCR product was sequenced (Northwood DNA), and DNA purification was performed using the QIAquick Gel Extraction Kit Protocol (Qiagen). iRNA was generated using the Dicer siRNA generation kit (Genlantis), and transfection was performed according to the manufacturer's instructions (Genlantis).

Western blotting for SLAM was performed to determine the efficiency of iRNA inhibition [5]. Equivalent amounts of protein were analyzed using pCREB and β-actin–specific antibodies (Epitomics [BD Biosciences]), as previously described [4].

Statistical analysis was performed using the nonparametric Wilcoxon rank sum test for paired samples and the Mann Whitney test for unpaired samples. *P* values of $<.05$ were considered statistically significant.

Results. We have previously shown that SLAM expression contributed to Th1 cytokine responses in tuberculosis [5] and that CREB positively regulated IFN-γ production [4]. Because the level of *M. tuberculosis*–induced IFN-γ in patients with tuberculosis is lower than that in healthy donors [8], we investigated the role of SLAM and CREB during IFN-γ production in patients with tuberculosis. Thus, we initially analyzed the coexpression of SLAM and pCREB in T cells from patients with active disease. As shown in figure 1*A*, a mean percentage ($\pm$SEM) of 83% $\pm$ 3.5% of SLAM$^+$ cells were also pCREB$^+$. However, 66% $\pm$ 11% of pCREB$^+$ T cells did not express SLAM. In contrast, in 8 healthy BCG-vaccinated donors, only 2.1% $\pm$ 1.7% of pCREB$^+$ cells failed to express SLAM. Ten healthy donors also produced significantly higher amounts of IFN-γ in response to *M. tuberculosis,* compared with 14 patients with tuberculosis (54 $\pm$ 13 ng/mL vs. 13.8 $\pm$ 3.7 ng/mL; $P < .05$ by the Mann-Whitney *U* test), suggesting that reduced IFN-γ production in patients with tuberculosis could be mediated by a decrease in antigen-activated pCREB$^+$ SLAM$^+$ T cells.

To further investigate the relationship between SLAM and pCREB, we blocked the expression of SLAM and measured pCREB expression by Western blotting. Using SLAM siRNA, we efficiently inhibited expression of SLAM, as measured by Western blot (data not shown). Furthermore, SLAM siRNA strongly inhibited the *M. tuberculosis*–induced expression of pCREB and reduced IFN-γ production by T cells from patients with tuberculosis (figure 1*B*).

Because our present and published results indicated that both SLAM and CREB contribute to *M. tuberculosis*–induced IFN-γ production, we next determined whether the effects of SLAM ligation might be mediated in part through CREB. Thus, we evaluated the effect of SLAM activation on phosphorylation of CREB. PBMCs from patients with tuberculosis were stimulated for 24 h with *M. tuberculosis* antigen in the presence or absence of an agonistic SLAM antibody, and phosphorylation of CREB was determined by Western blotting. SLAM activation enhanced phosphorylation of CREB (figure 1*C*), and this was confirmed in a larger number of patients (figure 1*C*), indicating that it is a potential mechanism by which SLAM induces IFN-γ secretion. Increased pCREB expression induced after SLAM ligation directly correlated with higher levels of IFN-γ produced against the antigen by PBMCs from patients with tuberculosis (figure 1*C*). Together, our data provide the first evidence suggesting that SLAM ligation activates CREB, which in turn induces IFN-γ secretion by T cells during a physiologic response to *M. tuberculosis*.

IL-17 has recently been shown to contribute to immune responses to *M. tuberculosis* in mice [9] and humans [10]. Although IFN-γ inhibits development of Th17 cells [13], the effects of IL-17 on IFN-γ production are unclear. To investigate this question, we added rhIL-17 to *M. tuberculosis*–stimulated PBMCs from patients with tuberculosis. As shown in figure 2*A*, IL-17 markedly reduced the levels of SLAM on T cells from patients with tuberculosis that were stimulated in vitro with *M. tuberculosis*. Because SLAM expression is directly correlated with IFN-γ production in tuberculosis [5] and our present data indicated that IL-17 diminished the levels of SLAM (figure 2*A* and 2*B*), we next investigated the effect of IL-17 on IFN-γ production. We observed that IL-17 inhibited IFN-γ secretion by antigen-stimulated cells (figure 2*B*). Because *M. tuberculosis* stimulation induced 80% of SLAM$^+$ T cells to express pCREB (figure 1*A*), we then investigated whether IL-17 would affect this population. IL-17 significantly decreased the number of SLAM$^+$pCREB$^+$ T cells by $>$50% (figure 2*C*).

To determine whether our findings were relevant in vivo, we compared IL-17 and IFN-γ production by PBMCs in healthy donors and patients with tuberculosis. After antigen stimulation, the percentage of IL-17$^+$ T cells was 10% $\pm$ 3% in 10 patients with tuberculosis, compared with 4.0% $\pm$ 0.7% in 10 healthy donors ($P < .05$ by the Mann-Whitney *U* test). These results correlated with levels of IFN-γ produced by *M. tubercu-*

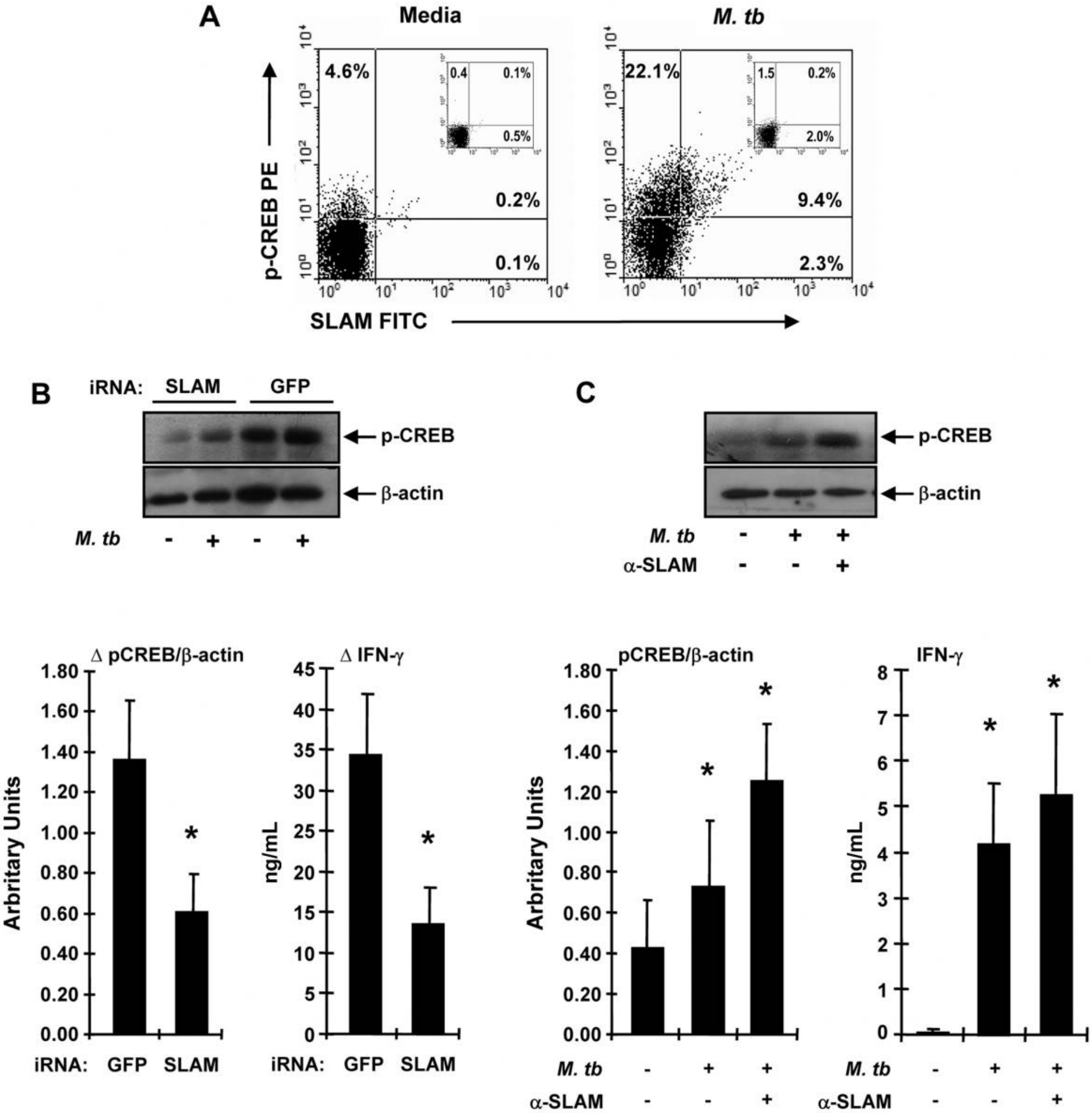

Figure 1. *A,* Peripheral blood mononuclear cells (PBMCs) from 10 patients with tuberculosis were stimulated with *Mycobacterium tuberculosis (M. tb)* antigen (Ag) for 5 days. Then, phosphorylated cAMP response element binding protein (pCREB) and signaling lymphocyte activation molecule (SLAM) expression were measured by flow cytometry, first gating on lymphocytes by light scatter and then on $CD3^+$ T cells. The percentages of $pCREB^+$ and/or $SLAM^+$ cells after culturing with media and *M. tuberculosis* are shown in the *large quadrants*. The *insets* show results with isotype controls. A representative result is shown. *B,* SLAM or GFP control siRNA and transfection reagent were incubated with PBMCs from patients with tuberculosis for 48 h. The cells were then collected, washed, and stimulated with *M. tuberculosis* Ag for an additional 48 h. Cell extracts were then prepared and assayed for pCREB and β-actin expression by Western blotting *(upper panel)*. Results for 1 representative patient of 5 are shown. Polyacrylamide gels were scanned, densitometry was performed, and the results were expressed as arbitrary units. pCREB levels in different samples were normalized to yield equivalent β-actin products. Values are expressed as the mean ± SEM of pCREB expression, relative to β-actin on *M. tuberculosis*–stimulated cells, minus pCREB expression in media *(lower left panel)*. IFN-γ production was also determined by ELISA *(lower right panel)*. Values are expressed as IFN-γ levels of *M. tuberculosis*–stimulated cells minus those of cells in media. $^*P < .05$ by the Wilcoxon signed rank test. *C,* PBMCs from patients with tuberculosis were stimulated with *M. tuberculosis,* with or without agonistic SLAM monoclonal antibody. After 24 h, pCREB expression and IFN-γ production were determined by Western blot and ELISA, respectively, as in *B*. The *upper panel* shows a Western blot from 1 representative patient of 6. The *lower left panel* shows mean ± SEM of pCREB expression, relative to β-actin, as measured by densitometry of Western blots. The *lower right panel* shows IFN-γ levels produced by cells from the 6 patients. $^*P < .05$ by the Wilcoxon signed rank test, compared with unstimulated samples.

losis–stimulated PBMCs (13.8 ± 3.7 ng/mL in patients with tuberculosis, compared with 54 ± 13 ng/mL in healthy donors; $P < .05$ by the Mann-Whitney *U* test). Together, our data indicate that IL-17 inhibits IFN-γ production induced by *M. tuberculosis* during active tuberculosis, in part through down-regulation of SLAM and pCREB expression.

Discussion. IFN-γ is pivotal for human defenses against *M. tuberculosis* [11]. In this work, we demonstrated that down-

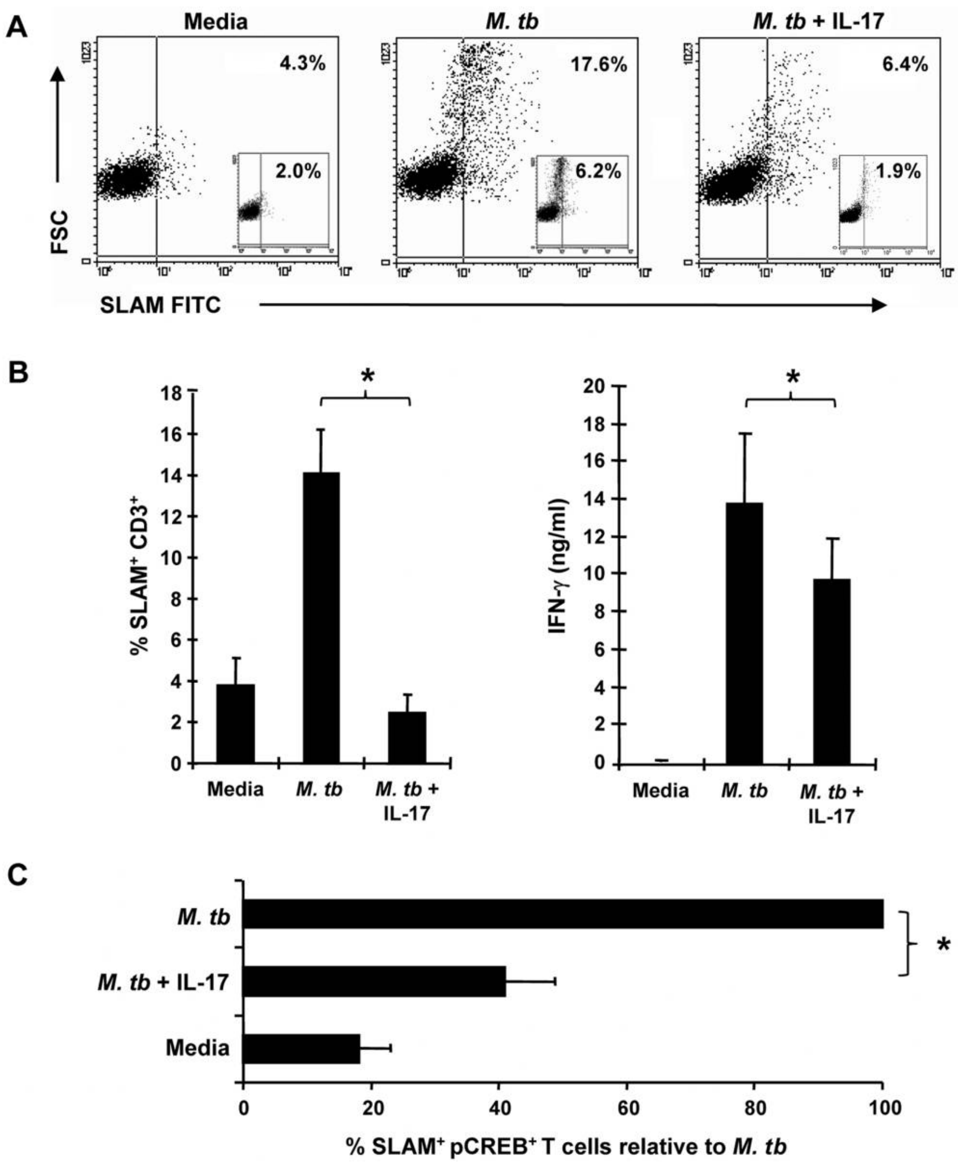

Figure 2. Peripheral blood mononuclear cells (PBMCs) from 7 patients with tuberculosis were stimulated with *Mycobacterium tuberculosis* antigen (Ag), with or without IL-17, for 5 days. Signaling lymphocyte activation molecule (SLAM) expression was then determined by flow cytometry, first gating on lymphocytes by light scatter then on $CD3^+$ T cells. *A,* Results for 1 representative patient are shown. The percentage of $SLAM^+$ cells is shown in the *upper right portions* of the *large quadrants*. The *insets* show corresponding values for the isotype controls. *B,* For 7 patients with tuberculosis, SLAM expression was determined by flow cytometry, as in *A (left panel),* and IFN-γ production was measured by ELISA *(right panel).* Mean values ± SEM are shown. *C,* SLAM and phosphorylated cAMP response element binding protein (pCREB) expression were determined by flow cytometry in PBMCs from 8 patients with tuberculosis (figure 1*A*), and the percentage of $SLAM^+pCREB^+$ T cells was determined. The percentage of $SLAM^+pCREB^+$ T cells after stimulation with *M. tuberculosis* was arbitrarily designated as 100%. $^*P < .05$, compared with *M. tuberculosis*–stimulated cells.

regulation of SLAM and pCREB expression participated in IFN-γ inhibition in persons with tuberculosis. However, IFN-γ gene regulation is complex, and production of IFN-γ is controlled by several signaling proteins, as well as by multiple transcription factors that bind to different promoter elements upon antigen stimulation, indicating that additional factors besides SLAM and CREB control IFN-γ transcription. For example, we recently demonstrated that ATF-2 binds as part of a complex with CREB to the IFN-γ proximal promoter during T cell activation and positively regulates IFN-γ production [12]. Stimulation of PBMCs with *M. tuberculosis* also increases production of IL-12 and IL-18. The former signals through STAT4, which binds to a site 236 bp upstream of the IFN-γ start codon [13], and the combination of IL-12 and IL-18 up-regulates binding of AP-1 transcription factors to the −196 to −183 bp region [13]. In addition, we previously reported that SLAM, a transmembrane signaling receptor that influences cytokine production by activated T cells, enhanced IFN-γ secretion in response to mycobacteria through a signaling cascade that increased activation of NFκB and T-bet [14]. In the current study, we demonstrated that ~80% of antigen-induced SLAM$^+$ T cells expressed pCREB, that blocking SLAM with siRNA markedly reduced pCREB, and that engaging SLAM with agonistic monoclonal antibody stimulated pCREB expression. In both cases, pCREB expression paralleled IFN-γ production by patients with tuberculosis. Moreover, we showed that pCREB$^+$SLAM$^+$ T cells were markedly decreased in patients with tuberculosis, compared with healthy donors, which correlated with the reduction in IFN-γ production by PBMCs from individuals with active tuberculosis. Together, these findings demonstrate for the first time that the physiologic mechanism by which SLAM activation leads to the production of IFN-γ against *M. tuberculosis* involves activation of the transcription factor CREB.

T cells that produce IL-17 have recently been found to contribute to pulmonary immune responses to *M. tuberculosis* and *M. bovis* BCG, in part by inducing chemokines that recruit CD4$^+$ cells that produce IFN-γ [9, 15]. IFN-γ inhibits the expansion of IL-17–producing cells during murine mycobacterial infection by reducing IL-23 production [13], and IL-17–producing T cells constitute a significant proportion of *M. tuberculosis*–activated cells in PBMCs of healthy tuberculin reactors [10]. However, the effect of IL-17 on IFN-γ production in persons with tuberculosis remains unclear. In the current study, we observed that IL-17 decreased SLAM expression and the frequency of SLAM$^+$pCREB$^+$ cells in *M. tuberculosis*–activated T cells from patients with tuberculosis, and these decreases directly correlated with reduced IFN-γ production (figure 2). These findings demonstrate that IL-17 inhibits IFN-γ production during tuberculosis by decreasing the expression and function of stimulatory signaling proteins in this pathway. We also found that the numbers of Th17 cells were significantly increased in patients with tuberculosis, compared with BCG-vaccinated healthy donors. We speculate that increased IL-17 production may regulate the capacity of T cells from patients with tuberculosis to produce IFN-γ in response to mycobacterial antigens, consistent with the known antagonistic effects of IFN-γ and Th17 cells [13].

In summary, our results provide new information linking the costimulatory molecule SLAM to the transcription factor CREB and the proinflammatory cytokine IL-17. Additional work is needed to elucidate the pathways that participate in the control of IFN-γ secretion in persons with tuberculosis.

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