MAJOR ARTICLE

Cross-Talk between CD31 and the Signaling Lymphocytic Activation Molecule–Associated Protein during Interferon-γ Production against *Mycobacterium tuberculosis*

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Effective host defense against tuberculosis requires Th1 cytokine responses. We studied the regulation of interferon (IFN)-γ production during tuberculosis by investigating the role of CD31, a receptor that attenuates T cell receptor signals. After antigen stimulation, $CD3^+CD31^+$ blood lymphocytes decreased in healthy donors and in tuberculosis patients with robust Th1 responses to *Mycobacterium tuberculosis* and IFN-γ was secreted only by $CD31^-$ T cells. In contrast, in patients with weak Th1 cytokine responses to *M. tuberculosis,* the level of $CD3^+CD31^+$ lymphocytes was increased and IFN-γ production was low. Furthermore, the inverse relationship between CD31 expression and IFN-γ production was in contrast to signaling lymphocytic activation molecule (SLAM) expression, an IFN-γ inducer in tuberculosis. Interestingly, CD31 bound to SLAM-associated protein (SAP), an IFN-γ inhibitor in tuberculosis, and when CD31 and SAP were coexpressed in lymphocytes, their association inhibited the IFN-γ response to *M. tuberculosis.* Thus, CD31, when binding to SAP, interferes with Th1 responses, suggesting that CD31 has a key regulatory role in the signaling pathway(s) leading to the IFN-γ response to *M. tuberculosis.*

Protective immunity against *Mycobacterium tuberculosis* requires the generation of cell-mediated immunity (CMI). The secretion of Th1 cytokines by antigen-specific T cells plays an important role in protective granuloma formation and stimulates the antimicrobial activity of infected macrophages [1]. Thus, impaired immunity in tuberculosis infection is associated with a depressed Th1 cytokine response and reduced production of interferon (IFN)-γ [2, 3].

The strength of the signal transduced by the T cell receptor (TCR) is influenced by the cellular context of antigen recognition. One variable that provides context for antigen recognition is the expression of activating and inhibitory receptors on T cells [4]. Thus, active mechanisms that prevent or terminate T lymphocyte responses include pathways that counterregulate the initial phase of T cell activation and regulatory feedback

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systems whose primary function is to control the late stages of T cell proliferation and differentiation [5]. Several signaling proteins contribute to active up- or down-regulation during the priming of a T cell [6, 7], thus modulating the level and pattern of cytokines produced by T cells after antigen-stimulation. Accordingly, we previously reported that signaling lymphocytic activation molecule (SLAM) and inducible costimulator (ICOS), 2 receptors that influence cytokine production by activated T cells [8–14], enhanced IFN-γ secretion against *M. tuberculosis* [15, 16], whereas the association of SLAM with the adaptor protein SLAM-associated protein (SAP) [9, 17] inhibited IFN-γ production in patients with tuberculosis [15]. Moreover, we demonstrated the existence of a cascade of molecular events during signaling through SLAM in mycobacterial infection in which signaling molecules participate in the induction of IFN-γ production [18].

CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), a transmembrane glycoprotein member of the immunoglobulin (Ig) superfamily, is expressed on monocytes, granulocytes, platelets, endothelial cells, and certain T cell subsets [19]. CD31 expression on the surface of T lymphocytes decreases after T cell activation, thus establishing a correlation between the loss of this molecule and the functional maturity of the cell [20]. Moreover, CD31 attenuates the signals induced after TCR engagement [21]. Actually, although CD31 is a cell-adhesion molecule, its function is not restricted to its adhesive properties. CD31 also mediates signal transduction by a series of interactions with adaptor molecules, mainly through phosphorylation of specific tyrosine residues located in an immunoregulatory tyrosine-based activation motif (ITAM) domain in the CD31 cytoplasmic tail. Furthermore, CD31 encodes an immunoregulatory tyrosine-based inhibition motif (ITIM), which is able, upon the recruitment of SH2-containing adaptor molecules, to affect a wide range of cellular events [22]. Because the C-terminal ITIM of CD31 contains a domain similar to the one present on the cytoplasmic tail of SLAM, it was suggested that SAP might interact with CD31, regulating its function [19].

Because CD31 is involved in mediating signal transduction by its interactions with adaptor molecules and might bind to the inhibitory protein SAP, we investigated the role of this receptor in the regulation of T cell signaling during tuberculosis infection. Our specific aim was to elucidate the molecular basis and biological role of CD31 in the regulation of the IFN-γ response to *M. tuberculosis,* which is critical for host defense against this pathogen.

MATERIALS AND METHODS

Patients. Patients with active tuberculosis were evaluated at the Hospital Muñiz, Buenos Aires, Argentina. The diagnosis of tuberculosis was established on the basis of clinical and radiological data together with the identification of acid-fast bacilli in sputum. All participating patients had received less than 1 week of antituberculosis therapy. Tuberculosis patients were characterized on the basis of in vitro T cell responses to an extract of virulent *M. tuberculosis* antigen (Ag). "Responder tuberculosis patients" were individuals who displayed significant proliferative responses (ie, proliferation index $\geqslant$4), significant IFN-γ production (ie, fold-stimulation $\geqslant$34), and an increase of $\geqslant$8 percent in SLAM-positive T cells in response to the Ag. "Low-responder tuberculosis patients" were individuals who exhibited low proliferation (proliferation index $<$4), low IFN-γ secretion (fold-stimulation $<$34), and an increase of $<$8 percent in SLAM-positive T cells. If a patient fulfilled 2 of 3 of these criteria, the patient was assigned to that group [15]. Bacillus Calmette-Guerin (BCG)–vaccinated healthy control donors (hereafter, "healthy donors") from the community participated in this study. Two individuals with X-linked lymphoproliferative (XLP) disease, diagnosis confirmed at the International XLP Registry Headquarters, Omaha, Nebraska [23], were also included. After participants received informed consent, peripheral blood was collected from all individuals in heparinized tubes. The local ethics committee approved all studies performed.

Antigen. In vitro stimulation of cells throughout the present study was performed with an extract from the virulent *M. tuberculosis* strain H37Rv (generously provided by Dr. Brennan, Colorado State University), prepared by probe sonication [15, 24].

Cell preparations and culture conditions. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Paque (Amersham Biosciences) and cultured (1×10^6/mL) with *M. tuberculosis* Ag (10 μg/mL) in 24- or 96-well plates with RPMI 1640 medium (Gibco BRL) supplemented with l-glutamine (2mM; Sigma-Aldrich), streptomycin, penicillin, and 10% human serum. Anti-CD31 monoclonal antibody (mAb) (clone PECAM 1.2 [5 μg/mL]; Caltag Laboratories), recombinant human CD31 (rCD31[100 μg/mL]; R&D Systems), or isotype control were added at the same time as or after the Ag for 5 days and IFN-γ, and interleukin (IL)-10 production was measured by ELISA (Pierce Endogen) or intracellular staining (see below).

Western blot analysis. PBMCs from tuberculosis patients and healthy donors were stimulated for 15 min or 5 days with *M. tuberculosis* and afterward cells were washed and solubilized in lysis buffer to prepare whole-cell extracts, as described elsewhere [18]. Equivalent amounts of protein were analyzed by 12% or 7.5% SDS-PAGE, transferred to nitrocellulose (Hybond ECL Nitrocellulose Membrane; Amersham Biosciences) and incubated with anti-SAP, anti-CD31, or anti-β-actin (Santa Cruz Biotechnology) antibodies. Bound antibodies were revealed

with horseradish peroxidase (HRP)-conjugated affinity purified anti-goat antibody (Chemicon International) or HRP-conjugated affinity purified anti-rabbit antibody (BioRad), by use of enhanced chemiluminescence (ECL)(Amersham Biosciences) and Kodak BioMax films.

Immunoprecipitation. Cell extracts were prepared as previously described [18]. Immunoprecipitation assays were then performed using specific antibodies: anti-SAP (3 μg/mL), anti-CD31 (3 μg/mL), or irrelevant mAbs in accordance with the manufacturer's instructions (Santa Cruz Biotechnology). Briefly, cell extracts from stimulated cells were incubated with mAbs plus TrueBlot anti-rabbit Ig IP Beads (eBioscience) or protein G Plus-Agarose (Santa Cruz Biotechnology), and immunoprecipitates were obtained. Next, CD31 or SAP expression in the immunoprecipitates was assessed by Western blot analysis.

Purification of CD31 cells. PBMCs were incubated for 16 h in complete media to allow adherence of monocytes to plastic. Nonadherent cells were recovered and $CD31^+$ and $CD31^-$ cells were then obtained by 2 rounds of positive selection by use of magnetic beads (Dynal beads; Dynal Biotech) and anti-CD31 (mAb 2B3, IgG1; generously provided by Dr. Villela, Servei d'Immunologia, Hospital Clinic, Barcelona). The purity of the cells was evaluated by flow cytometry (90% for both fractions). $CD31^+$ and $CD31^-$ T cells were subsequently cultured with sonicated *M. tuberculosis* plus feeder cells, as previously described [25] and IFN-γ was measured by ELISA.

Flow cytometry. PBMCs were cultured with *M. tuberculosis* and stained for CD3, CD31, and SLAM expression using specific mAbs (CD3, UCHT1 [eBioscience]; CD31, mAb 5H2 [26]; SLAM, clone A12 [eBioscience]), before and after culture with the Ag. Intracellular cytokine staining was performed to determine IFN-γ production at the single-cell level as previously described [27]. Negative control samples were incubated with irrelevant, isotype-matched mAbs in parallel with experimental samples. Samples were analyzed on a flow cytometer (FACScalibur; BD Biosciences).

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

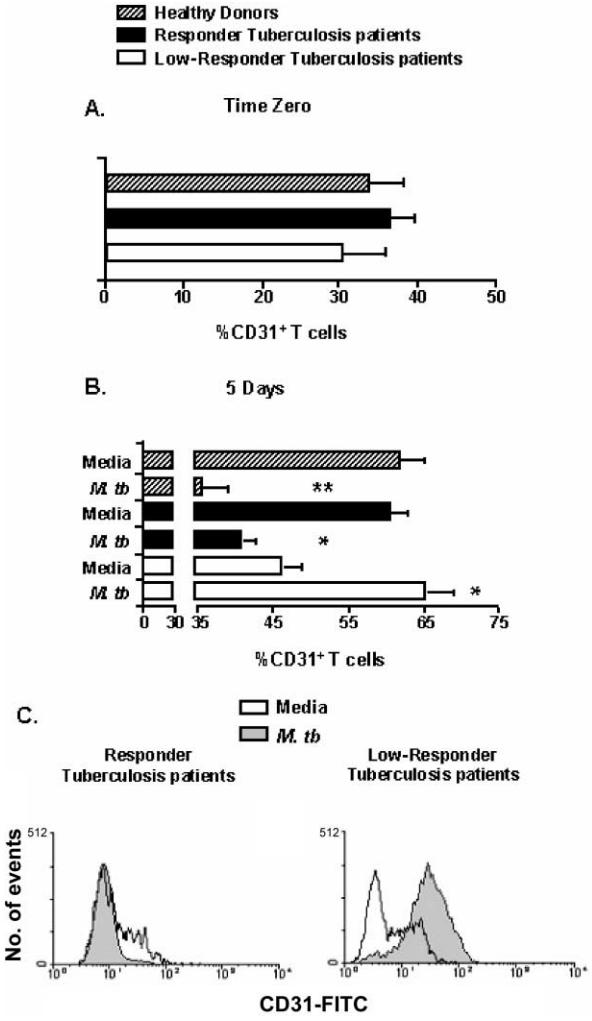

Figure 1. CD31 expression in tuberculosis patients and healthy control donors. *A,* Basal expression on peripheral blood mononuclear cells (PBMCs) from tuberculosis patients and healthy control donors . Each bar represents the mean value $\pm$ standard error of the mean (SEM) from each group (10 individuals per group). *B,* Expression in response to 5 days of stimulation with *Mycobacterium tuberculosis (M. tb)* antigen, comparing stimulated T cells and cells cultured with media alone. Each bar represents the mean value $\pm$ SEM from each group (10 individuals per group). $*P < .01$; $**P < .001$, Wilcoxon rank sum test. *C,* Histograms of expression on stimulated T cells *(shaded histogram)* and cells cultured with media *(unshaded histogram)* from representative patients with strong (responder) and weak (low responder) T cell responses to the bacteria.

RESULTS

CD31 expression in tuberculosis patients and healthy donors. We investigated the expression of CD31 in T cells from tuberculosis patients and bacillus Calmette-Guerin–vaccinated healthy donors. Basal expression of CD31 was similar in tuberculosis patients with strong immunity against *M. tuberculosis* (responder patients) [15], in low-responder patients [15], and in healthy donors (figure 1*A*). Moreover, we observed similar values for the mean intensity fluorescence of CD31 for the 3 groups of individuals (data not shown). We examined CD31 expression in response to *M. tuberculosis* Ag stimulation and found a significant decrease in the percentage of $CD3^+CD31^+$ T lymphocytes from responder individuals (ie, responder patients and healthy donors), compared with the expression of the receptor on cells cultured in media alone (figure 1*B*). In striking contrast, for low-responder patients we detected lower levels of CD31 in media, compared with responder individuals, but we also detected a significant increase in the percentage of $CD3^+CD31^+$ T cells after *M. tuberculosis* stimulation, compared

with cells cultured in media alone. Therefore, our data suggest that the expression of CD31 is regulated by *M. tuberculosis* in association with the maturation into Th effector cells.

Effect of CD31 signaling on cytokine production during* M. tuberculosis *stimulation. A role for CD31 signaling after Ag-stimulation in human infection has not been demonstrated. Therefore, PBMCs from responder individuals were cultured with the Ag together with immobilized anti-CD31 mAb or soluble rCD31 and IFN-γ and IL-10 production was determined. Simultaneous *M. tuberculosis* stimulation and ligation of CD31 significantly inhibited IFN-γ production (figure 2). However, signaling through CD31 did not modify IL-10 secretion for either tuberculosis patients or healthy donors (figure 2), suggesting that CD31 signaling specifically inhibited Th1 responses.

***Role of CD31 subpopulations during the IFN-γ pathway against* M. tuberculosis.** Given that CD31 signaling inhibited *M. tuberculosis*–induced IFN-γ production, we next studied the CD31 T cell subpopulations that might be involved in this inhibitory effect. After stimulating $CD31^-$ and $CD31^+$ T cells from responder individuals with *M. tuberculosis*, $CD31^-$ T lymphocytes produced large amounts of IFN-γ, similar to the levels of IFN-γ secreted by PBMCs from the same individuals (figure 3*A*). In contrast, $CD31^+$ T cells did not produce IFN-γ following *M. tuberculosis* stimulation (figure 3*A*). Moreover, by staining PBMCs from tuberculosis patients for CD3, CD31 and intracellular IFN-γ, we observed that in responder patients, the majority of IFN-γ–producing cells were $CD31^-$, and the total percentage of $CD31^+$ T cells decreased by 50% after Ag stimulation, whereas in low-responder patients, *M. tuberculosis* stimulation increased the $CD31^+$ T cell subpopulation by almost 100%, and no IFN-γ^+ T cells were detected (figure 3*B*). Therefore, we demonstrated that in responder tuberculosis patients, $CD31^-$ T lymphocytes are the cells that produce IFN-γ in response to the pathogen, whereas in low-responder patients, the percentage of $CD31^+$ lymphocytes increased (figure 1*B* and figure 3*B*), the $CD31^-$ T cell subset decreased, and no IFN-γ against the bacteria was produced (figure 3*B*).

Role of CD31 on the modulation of the expression and function of SLAM in tuberculosis. To further analyze the phenotype of IFN-γ producing T cells induced by *M. tuberculosis* in tuberculosis patients, we measured SLAM, CD31, and IFN-γ. More than 80% of $CD31^-$ T cells from responder patients expressed SLAM , whereas fewer than 20% of $CD31^-$ T cells from low-responder patients expressed SLAM (figure 4*A* and 4*B*). Furthermore, in responder patients, both IFN-γ^+CD31^- and IFN-γ^+SLAM^+ lymphocytes accounted for more than 80% of IFN-γ secreting cells in response to *M. tuberculosis* (figure 4*C*). Moreover, CD31 signaling decreased the levels of $SLAM^+$ T lymphocytes in responder patients by more than 50% (figure

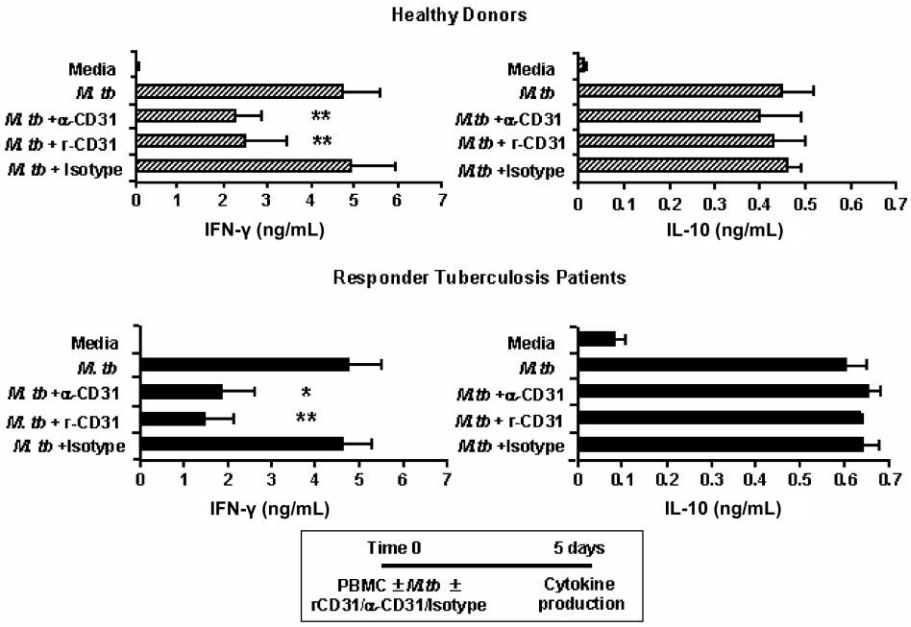

Figure 2. Effect of CD31 signaling on cytokine production during Mycobacterium tuberculosis (M. tb) stimulation. Peripheral blood mononuclear cells from responder tuberculosis patients and healthy control donors were stimulated with *M. tuberculosis* plus anti-CD31 monoclonal antibody (mAb) (platelet endothelial cell adhesion molecule–1.2), recombinant human CD31 (rCD31), or control isotype. After 5 days, interferon (IFN)-γ or interleukin (IL)-10 production was assayed by ELISA. Each bar represents the mean value ± standard error of the mean of IFN-γ or IL-10 produced by each group (11 individuals per group). *P* values were calculated by comparing the mean of the cytokine produced by *M. tuberculosis*–stimulated cells with the mean of the cytokine produced by cells cultured with *M. tuberculosis* and anti-CD31 mAb or *M. tuberculosis* and rCD31. $*P < .05$, $**P < .01$, Wilcoxon rank sum test.

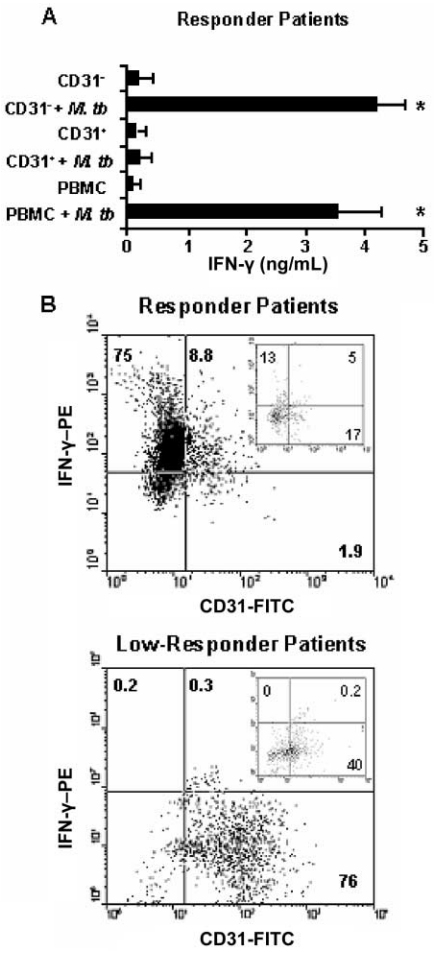

Figure 3. CD31 subpopulations that participate in interferon (IFN)-γ production in response to *Mycobacterium tuberculosis (M. tb). A,* Mean value $\pm$ standard error of the mean of IFN-γ production for each group (10 individuals per group). CD31 populations and peripheral blood mononuclear cells (PBMCs) were stimulated with *M. tuberculosis* for 5 days, and IFN-γ was assayed by ELISA. *P* values were calculated by comparing the mean of IFN-γ produced by *M. tuberculosis*–stimulated PBMCs or CD31$^+$ or CD31$^-$ T cells with the mean of IFN-γ produced by PBMCs or CD31$^+$ or CD31$^-$ T cells cultured with media. *$P < .05$, Wilcoxon rank sum test. *B,* CD31 expression and IFN-γ production by CD3$^+$ T cells for 1 representative patient of 7 for each group. PBMCs from tuberculosis patients were stimulated with *M. tuberculosis* for 4 days *(full panel)* or with media *(inset)* and examined by flow cytometry.

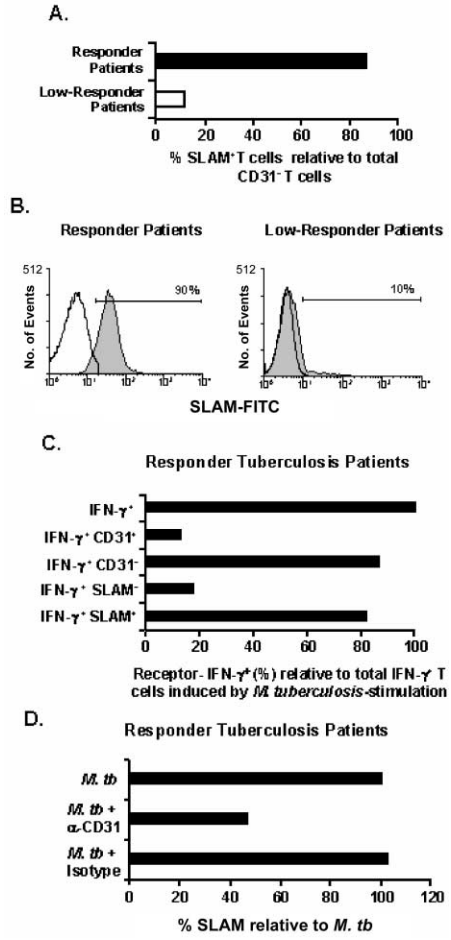

Figure 4. Role of CD31 in the modulation of the function and expression of signaling lymphocytic activation molecule (SLAM) in response to *Mycobacterium tuberculosis (M. tb). A,* mean percentage of SLAM$^+$ T cells relative to total stimulated CD31$^-$ lymphocytes (mean for 7 individuals per patient group). Peripheral blood mononuclear cells (PBMCs) from tuberculosis patients were stimulated with *M. tuberculosis* for 5 days, and SLAM and CD31 expression was examined by flow cytometry. *B,* Histograms of SLAM expression on CD31$^-$ cells *(shaded histogram)* versus the isotype control *(unshaded histogram)* are shown from a representative individual of each group. PBMCs from tuberculosis patients were stimulated and stained as described in *A.* T cells were identified by first gating on blast lymphocytes according to light scatter properties, then by gating on CD3$^+$ cells, and finally SLAM expression was evaluated on CD31$^-$ cells. *C,* Mean of the percentages of CD31 or SLAM–interferon (IFN)-γ^+ cells relative to total *M. tuberculosis*–induced IFN-γ^+ lymphocytes (8 individuals). PBMCs from responder tuberculosis patients were stimulated with *M. tuberculosis* for 4 days. CD31 or SLAM expression on *M. tuberculosis*–reactive IFN-γ–producing T cells was examined by flow cytometry. $P < .005$, for comparison of the percentage of *M. tuberculosis*–induced-IFN-γ^+SLAM$^+$ T cells with the percentage of *M. tuberculosis*–induced IFN-γ^+SLAM$^-$ T cells; $P < .05$, for comparison of the percentage of *M. tuberculosis*–induced IFN-γ^+CD31$^+$ T cells with the percentage of *M. tuberculosis*–induced IFN-γ^+CD31$^-$ T cells, Wilcoxon rank sum test. *D,* Percentage of the SLAM expression on cells cultured with α-CD31 plus antigen relative to SLAM expression in *M. tuberculosis*–stimulated cells (7 individuals). PBMCs from responder tuberculosis patients were stimulated with antigen (5 days) plus anti-CD31 mAb (platelet endothelial cell adhesion molecule–1.2) or control isotype and SLAM was determined by flow cytometry.

4*D*), strongly suggesting that CD31 participates in the regulation of Th1 lymphocyte activation after Ag stimulation.

***Interaction of CD31 with SAP, a signaling molecule that modulates IFN-γ production to* M. tuberculosis.** To investigate whether CD31 and SAP might participate in the regulation of the signaling pathway that controls IFN-γ secretion during tuberculosis infection by a direct association, as has been suggested [19], we first analyzed the expression of CD31 and SAP in PBMCs from tuberculosis patients and healthy donors at different time points. Similar expression of both proteins

was observed at basal levels and after 15 min of Ag stimulation in all the groups (figures 5*A* and 6*A*). However, at 5 days of *M. tuberculosis* stimulation, a marked decrease in total CD31 and SAP expression was observed in responder patients and healthy donors (figures 5*A* and 6*A*), whereas an increase of both proteins was detected in low-responder patients (figure 6*A*). Nevertheless, when SAP basal expression was analyzed in $CD31^-$ and $CD31^+$ lymphocytes from responder individuals, we found high constitutive levels of SAP in $CD31^+$ T lymphocytes and markedly lower expression of the protein in the $CD31^-$ fraction of T cells (figure 5*B*). Moreover, when PBMCs from healthy donors were stimulated with *M. tuberculosis* for 15 min, and immunoprecipitation of SAP or CD31 was performed, the 2 proteins coprecipitated with each other, both in Ag-stimulated T cells and in cells cultured with media (figure 5*C*). Similar results were observed in tuberculosis patients (figure 6*B*; other data not shown), demonstrating a direct association between the 2 inhibitory proteins SAP and CD31 during the early stages of Ag stimulation. However, after 5 days of *M. tuberculosis* stimulation, no association was observed in responder individuals (figures 5*D* and 6*C*). In contrast, the increased CD31 and SAP levels induced after 5 days of Ag stimulation in low-responder patients (figures 1*B* and 6*A*) led to a clear association of these molecules (figure 6*C*). Thus, these results suggest that both molecules might participate in the regulation of T cell effector functions in tuberculosis.

To further investigate the role of CD31 and SAP during signaling for IFN-γ production, we investigated CD31 signaling in *M. tuberculosis*–activated T cells. PBMCs from responder individuals and healthy donors were stimulated for 5 days with *M. tuberculosis,* washed, and cultured in the presence or absence of anti-CD31 mAb. Under these conditions, signaling through CD31 was unable to decrease IFN-γ levels (figure 6*D*). Thus, although T cells express residual levels of CD31 upon *M. tuberculosis* activation, the Ag-reactive lymphocytes become functionally unresponsive to CD31 ligation.

Expression and role of CD31 in individuals with defective SAP gene. To definitively confirm that binding between CD31 and SAP interfered with IFN-γ production in response to *M. tuberculosis,* we performed experiments using cells from patients with defective SAP gene (XLP). As shown in figure 7*A,* we detected similar levels of surface CD31 in cells from patients with XLP disease, compared with cells from healthy donors and tuberculosis patients (figure 1*A*), at both time zero and after *M. tuberculosis* stimulation. Moreover, and as expected, when PBMCs from patients with XLP disease were stimulated with *M. tuberculosis* for 15 min, and co-immunoprecipitation with CD31 was performed, no immunoprecipitation with SAP was observed (data not shown). Interestingly, culture of PBMCs from patients with XLP disease in which the Ag was combined with immobilized anti-CD31 mAb did not modify IFN-γ secretion (figure 7*B*), further confirming that the association of CD31 with SAP interferes with IFN-γ production in response to *M. tuberculosis.*

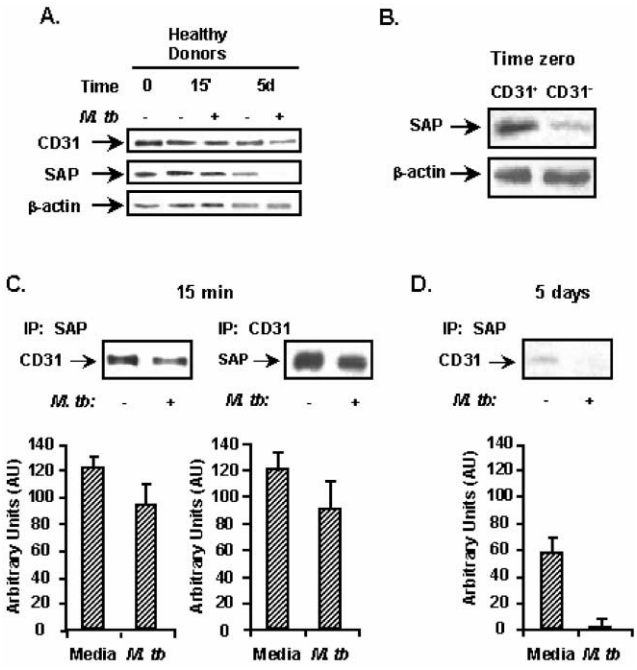

Figure 5. Analysis of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP)-CD31 association in healthy control donors after *Mycobacterium tuberculosis* stimulation. *A,* Western blot analysis of SAP, CD31 or β-actin protein expression. Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with *M. tuberculosis* antigen for 15 min or 5 days, and total cell extracts were prepared and assayed. One representative donor (out of 7) is shown. *B,* Western blot analysis of SAP or β-actin basal expression in $CD31^-$ and $CD31^+$ subpopulations. One representative donor (out of 6) is shown. For *A* and *B,* so that SAP or CD31 levels could be compared among different samples, protein concentrations were normalized to yield equivalent β-actin products. *C,*Expression of CD31 or SAP by Western blot analysis after immunoprecipitation. PBMCs from healthy donors were stimulated with *M. tuberculosis* for 15 min and immunoprecipitation assays against SAP or CD31 were performed. One representative healthy donor (out of 8) is shown. *D,* Western blot analysis of CD31 expression after immunoprecipitation against SAP. PBMCs from healthy donors were stimulated with *M. tuberculosis* for 5 days and immunoprecipitation assays against SAP were performed. One representative donor (out of 8) is shown. For *C* and *D,* polyacrylamide gels were scanned, densitometry was performed, and the results were expressed as arbitrary units (AU). Each bar represents the mean ± standard error of the mean of AU.

DISCUSSION

Previously, we had demonstrated that SLAM, a receptor that influences the pattern of cytokines produced by activated T cells [8–10], enhances cell-mediated immunity to *M. tuberculosis* infection [15], whereas SAP interacts with SLAM [17, 28, 29] and interferes with IFN-γ production during mycobacterial

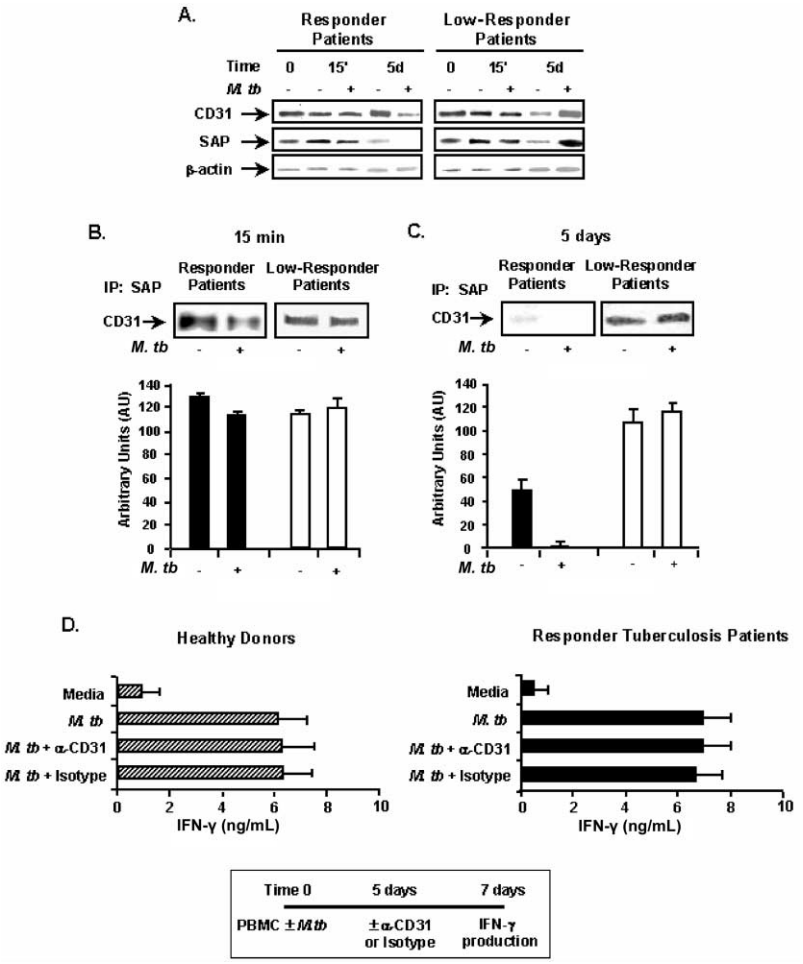

Figure 6. Analysis of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP)-CD31 association in tuberculosis patients after *Mycobacterium tuberculosis* stimulation. *A,* Western blot analysis of total cell extracts for SAP, CD31, or β-actin expression. Peripheral blood mononuclear cells (PBMCs) from tuberculosis patients were stimulated with *M. tuberculosis* antigen for 15 min (15′) or 5 days (5d). One representative tuberculosis patient (out of 8) is shown for each group. *B,* Western blot analysis of CD31 expression after immunoprecipitation against SAP. PBMCs from tuberculosis patients were stimulated with *M. tuberculosis* for 15 min and immunoprecipitation assays against SAP were performed. One representative patient (out of 9) is shown for each group. *C,* Western blot analysis of CD31 expression after immunoprecipitation against SAP. PBMCs from tuberculosis patients were stimulated with *M. tuberculosis* for 5 days and immunoprecipitation assays against SAP were performed. One representative patient (out of 8) is shown for each group. For *B* and *C,* the results were expressed as arbitrary units (AU). Each bar represents the mean ± standard error of the mean (SEM) of AU from each group. *D,* Effect of CD31 signaling on interferon (IFN)-γ production from *M. tuberculosis*–stimulated cells. PBMCs from responder patients and healthy control donors were stimulated with *M. tuberculosis* for 5 days. Afterwards, cells were washed, cultured with anti-CD31 mAb (platelet endothelial cell adhesion molecule–1.2) or control isotype for 48 h and IFN-γ production was determined by ELISA. Each bar represents the mean ± SEM of IFN-γ produced by each group (10 individuals per group).

infection [15, 18]. Since CD31 is down-regulated during human $CD4^+$ T cell maturation [30] and plays a role as an inhibitory receptor interfering with TCR-mediated signal transduction [21], in this study we investigated signaling through CD31 in T cells during *M. tuberculosis* infection. We found that after Ag stimulation, CD31 expression decreased on T cells which were activated by *M. tuberculosis*. In striking contrast, CD31 increased on T cells from patients who had a weak response to the antigen, demonstrating that antigens from a human intracellular pathogen like *M. tuberculosis* might modulate CD31 expression during T cell activation. Moreover, by investigating the effect of coligation of CD31 simultaneously with TCR signaling in cells from responder individuals, we observed a significant decrease in IFN-γ secretion in direct association with a strong reduction of $SLAM^+$ T cells. However, CD31 signaling did not affect IL-10 production, suggesting that the activation of this receptor's signaling pathway participates in the regulation of Th1, but not Th2, cytokine patterns in tuberculosis.

We demonstrated for the first time, to our knowledge, that CD31 associates with SAP during signaling events that lead to a decrease in $SLAM^+$ T lymphocytes and inhibition of signaling for IFN-γ production in response to *M. tuberculosis*. It was

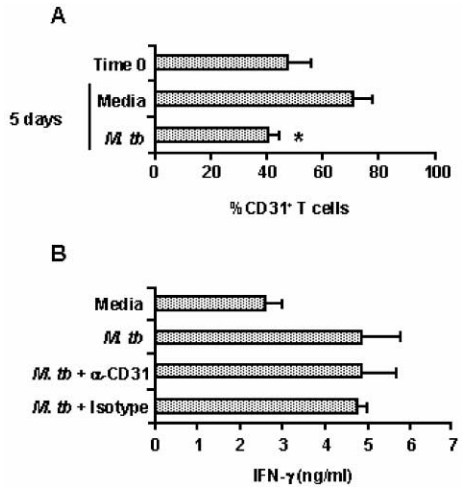

Figure 7. Expression and role of CD31 in patients with X-linked lymphoproliferative (XLP) disease. *A,* Mean value ± standard error of the mean (SEM) of CD31 expression under each condition (6 experiments for each bar). CD31 expression on T cells from patients with XLP disease was determined by flow cytometry. *$P < .05$, Wilcoxon rank sum test. *B,* Mean value ± SEM of IFN-γ produced under each condition (6 experiments for each bar). Peripheral blood mononuclear cells from patients with XLP disease were stimulated with *Mycobacterium tuberculosis* plus anti-CD31 mAb (platelet endothelial cell adhesion molecule–1.2). After 5 days, interferon (IFN)-γ production was assayed by ELISA.

suggested that SAP might interact with CD31, regulating its function [19]. SAP, a protein present in T cells [17, 28, 29], NK cells [31], and in a subpopulation of tonsillar B cells [32], might function as a signaling inhibitor by blocking and/or regulating the binding of signaling molecules to SH2 docking sites. In T lymphocytes, SAP binds to the SH3 domain of FynT and directly couples FynT to SLAM [33]. In B cells, SAP may block recruitment of SHP-2 to SLAM [9, 17], a mechanism that, it has been suggested, could be based on SAP's higher affinity for SLAM than SHP-2 [32]. We speculate that a similar mechanism may be at work in the binding of SAP to CD31 during *M. tuberculosis* stimulation of T lymphocytes, that is, when SAP is present, it associates with CD31.

We investigated the relationship between CD31 and SAP by studying the role of CD31 in T cells from SAP-deficient humans. In patients with XLP disease, concentrations of IFN-γ are elevated during primary Epstein-Barr virus infection, suggesting that a bias towards the production of Th1 cytokines may contribute to the progressive immunopathology [29]. Previously we showed that *M. tuberculosis* stimulation increased IFN-γ and SLAM expression in patients with XLP disease, whereas no SAP protein was detected [15]. Our present results show that *M. tuberculosis* decreased CD31 expression in T cells from patients with XLP disease, similar to the result shown by our data for healthy donors and responder tuberculosis patients. Moreover, we observed that *M. tuberculosis*–induced IFN-γ was not inhibited in T cells from SAP-deficient humans after signaling through CD31 by antibody ligation. These data clearly confirm that the association of CD31 with SAP participates in the regulation of IFN-γ production in response to *M. tuberculosis.* Furthermore, given that our results show that CD31 signaling decreased IFN-γ secretion induced by a human pathogen in individuals with SAP, our findings in patients with XLP disease might explain, in part, the suggested bias towards the production of Th1 cytokines in these individuals.

Given that *M. tuberculosis* modulated CD31 expression on T lymphocytes, that signaling through CD31 decreased IFN-γ production, and that CD31 associated with SAP, which is a recognized inhibitor of IFN-γ production in tuberculosis, we analyzed the phenotype and function of CD31 T cells that might be modulating the IFN-γ response to *M. tuberculosis.* We found that $CD31^-$ lymphocytes comprised the largest population of IFN-γ–producing T cells induced in response to this pathogen. Furthermore, in responder patients, more than 80% of $CD31^-$ lymphocytes expressed SLAM, indicating that IFN-γ–secreting T cells induced by Ag stimulation are of the $CD31^-SLAM^+$ phenotype. In keeping with our findings, it has been demonstrated that the majority of helper activity for B cell IgG synthesis and memory function to recall antigens, such as tetanus toxoid, is provided by $CD31^-$ CD4 T cells [34] and that CD31-deficient mice show elevated levels of serum IFN-γ in response to systemic lipopolysaccharide stimulation [35].

Interestingly, when an agonistic CD31 signal was induced in T lymphocytes from healthy donors and responder patients previously exposed to *M. tuberculosis,* no inhibition of IFN-γ secretion was detected. Furthermore, the inhibitory effect of CD31 engagement was observed by a direct association in cells expressing SAP , but not in *M. tuberculosis*–stimulated cells from responder individuals or in T lymphocytes from SAP-deficient humans (both T cells lacking the protein). Thus, our results suggest that the cross-talk between SAP and CD31 might be involved in the modulation of Th1 cytokine responses in tuberculosis. Therefore, in responder tuberculosis patients, *M. tuberculosis* Ag recognition by T cells down-regulates SAP, decreases CD31 expression, and augments SLAM expression. Then, in turn, $SLAM^+$ $CD31^-$ T lymphocytes become activated via SLAM for production of IFN-γ. However, if anti-CD31 mAb or rCD31 are added to the culture of cells with *M. tuberculosis,* the Ag engagement does not decrease CD31 expression and the interaction of CD31-CD31 or CD31-ligand suppresses signaling through the TCR, and because SAP is still present in the cell, no IFN-γ is produced. Moreover, because CD31 engagement decreases the levels of $SLAM^+$ T cells, some SAP might remain bound to CD31 and/or might recruit FynT [18] and bind to the remaining SLAM, resulting in few $CD31^-$ $SLAM^+$ T cells producing IFN-γ. On the other hand, in low-responder patients, *M. tuberculosis* induces a weak signal through the TCR,

and these patients' T cells are therefore unable to increase SLAM levels; however, the inhibitory proteins SAP and CD31 are strikingly up-regulated, and very few $CD31^-$ T cells are generated, resulting in very little IFN-γ secretion. Thus, the high levels of SAP and CD31 present might allow these 2 proteins to continue to associate, and/or SAP might recruit FynT to associate with SLAM, either of which would prevent IFN-γ secretion.

In summary, we have demonstrated for the first time, to our knowledge, that CD31 participates in the signaling pathway that inhibits IFN-γ production against a human pathogen. We showed that CD31 and SAP expression were inversely associated with IFN-γ production in a time-dependent manner during *M. tuberculosis* stimulation. Moreover, we demonstrated that, as a result of the host's T cell Ag response, the cross-talk between the inhibitory signaling proteins CD31 and SAP differentially regulates the signaling pathway that controls IFN-γ production in response to *M. tuberculosis,* and that *M. tuberculosis* induces $SLAM^+$ lymphocytes lacking surface CD31, which produce the protective Th1 response in responder individuals. Overall, the present results significantly contribute to elucidation of the role of the signaling proteins that modulate the critical IFN-γ response to a major human intracellular pathogen, thus furthering our understanding of the human immune response to *M. tuberculosis.*

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