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Inducible Costimulator: A Modulator of IFN-γ Production in Human Tuberculosis[1]

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Effective host defense against *Mycobacterium tuberculosis* requires the induction of Th1 cytokine responses. We investigated the regulated expression and functional role of the inducible costimulator (ICOS), a receptor known to regulate Th cytokine production, in the context of human tuberculosis. Patients with active disease, classified as high responder (HR) or low responder (LR) patients according to their in vitro T cell responses against the Ag, were evaluated for T cell expression of ICOS after *M. tuberculosis*-stimulation. We found that ICOS expression significantly correlated with IFN-γ production by tuberculosis patients. ICOS expression levels were regulated in HR patients by Th cytokines: Th1 cytokines increased ICOS levels, whereas Th2-polarizing conditions down-regulated ICOS in these individuals. Besides, in human polarized Th cells, engagement of ICOS increased *M. tuberculosis* IFN-γ production with a magnitude proportional to ICOS levels on those cells. Moreover, ICOS ligation augmented Ag-specific secretion of the Th1 cytokine IFN-γ from responsive individuals. In contrast, neither Th1 nor Th2 cytokines dramatically affected ICOS levels on Ag-stimulated T cells from LR patients, and ICOS activation did not enhance IFN-γ production. However, simultaneous activation of ICOS and CD3 slightly augmented IFN-γ secretion by LR patients. Together, our data suggest that the regulation of ICOS expression depends primarily on the response of T cells from tuberculosis patients to the specific Ag. IFN-γ released by *M. tuberculosis*-specific T cells modulates ICOS levels, and accordingly, ICOS ligation induces IFN-γ secretion. Thus, ICOS activation may promote the induction of protective Th1 cytokine responses to intracellular bacterial pathogens. ***The Journal of Immunology,*** **2006, 176: 5965–5974.**

Protective immunity against *Mycobacterium tuberculosis* requires the generation of cell-mediated immunity (CMI).[4] Therefore, the production of cytokines during the immune response to tuberculosis plays an important role in host defense (1). It has been widely demonstrated that IFN-γ, a macrophage-activating cytokine produced by T cells, is a crucial mediator of the protection against *M. tuberculosis* (2–4). Most *M. tuberculosis*-infected persons are healthy tuberculin reactors with protective immunity against infection, their PBMC produce high concentrations of IFN-γ in response to the bacteria and they do not develop active disease, indicating that CMI controls the infection in most (5). In contrast, reduced IFN-γ production by PBMC is a marker of severe disease (6), confirming the role of CMI in protection against tuberculosis.

Several signal transduction molecules have been shown to modulate the level and pattern of cytokines produced by T cells. Previously we demonstrated that the signaling lymphocytic activation molecule (SLAM), a receptor that influences the pattern of cytokines produced by activated T cells (7–9), enhances CMI to *M. tuberculosis* infection (10), whereas the SLAM-associated protein, an Src homology 2 domain-containing protein of T and NK cells that interacts with SLAM (11–13), interferes with IFN-γ production during mycobacterial infection (10). Another regulator of T lymphocyte function and differentiation is the inducible costimulator ICOS, the third member of the CD28/CTLA-4 family (14). ICOS is not expressed by naive Th cells, but induced after T cell activation (14). Interaction of ICOS with its ligand, a member of the B7 family, induces T cell proliferation and production of various cytokines, including IFN-γ, IL-10, and IL-4 (14–17). The important role of ICOS in various immune responses has been well documented (18). In particular, much attention has been drawn to the function of ICOS in Th differentiation. Blocking of ICOS-mediated costimulation with Abs or with an ICOS-Ig fusion protein, resulted in the preferential inhibition of Th2-mediated immune responses (19–21), although some Th1 responses were found to be affected (22, 23).

In infectious diseases, the role of ICOS as a positive regulator for both Th1 and Th2 responses has been deeply examined using mouse models (24–29). However, to date, the function of ICOS in human intracellular infection has not been analyzed. To further

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[4] Abbreviations used in this paper: CMI, cell-mediated immunity; ICOS, inducible costimulator; SLAM, signaling lymphocytic activation molecule; HR, high responder; LR, low responder.

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explore the relationship between costimulation and Th1 cytokine responses, we investigated the role of ICOS in the host response to tuberculosis infection.

Materials and Methods

Study subjects

Patients with active tuberculosis were evaluated at the Department of Immunology, Instituto de Tisioneumonología Prof. Dr. R. Vaccarezza, University of Buenos Aires, School of Medicine (Buenos Aires, Argentina), and at the División Tisioneumonología, Hospital F. J. Muñiz (Buenos Aires, Argentina). The diagnosis of tuberculosis was established based on clinical and radiological data together with the identification of acid-fast bacilli in sputum. All patients had received less than 1 wk of antituberculosis therapy at the time that blood samples were obtained. Tuberculosis patients were classified according to the criteria used by Pasquinelli et al. (10). Briefly, two groups of patients were distinguished based on in vitro T cell responses to an extract of virulent *M. tuberculosis* Ag: high responder (HR) tuberculosis patients are individuals that displayed significant proliferative responses (proliferation index ≥ 4), IFN-γ production (fold stimulation ≥ 34), and an increase ≥ 8 in the percentage of SLAM-positive cells in response to the Ag; and low responder (LR) tuberculosis patients who exhibited low proliferative responses (proliferation index < 4), IFN-γ secretion (fold-stimulation < 34), and an increase < 8 in the levels of SLAM. If a patient fulfilled two of three of these criteria, the patient was assigned to that group. Proliferation and IFN-γ production paralleled common clinical parameters analyzed in patients with active tuberculosis in Argentina (10). Bacillus Calmette-Guérin-vaccinated healthy control individuals from the community participated in this study. Peripheral blood was collected in heparinized tubes from all individuals after receiving informed consent.

Ag stimulation

In vitro stimulation of cells throughout the present study was performed with an extract from the virulent *M. tuberculosis* strain H37Rv, gently provided by Dr. P. Brennan (Colorado State University, Fort Collins, CO) prepared by probe sonication (sonicated *M. tuberculosis* Ag) (30).

Cell preparations and culture conditions

PBMC were isolated from heparinized blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences) and cultured (1×10^6/ml) with sonicated *M. tuberculosis* Ag (10 μg/ml) or with PHA-M (2.5 μg/ml; Sigma-Aldrich) in 24- or 96-well plates with RPMI 1640 (Invitrogen Life Technologies) supplemented with L-glutamine (2 mM; Sigma-Aldrich), streptomycin, penicillin, and 10% human serum. After 5 days, cells were pulsed with [^{3}H]TdR (1 μCi/well) and harvested 16 h later, and [^{3}H]TdR incorporation was measured in a liquid scintillation counter. In different experiments, sonicated *M. tuberculosis* Ag-stimulated cells were washed and cultured in the presence of anti-ICOS mAb (clone ISA3, 12.5 μg/ml; eBioscience), or with magnetic beads coated with a constant, suboptimal amount of OKT3 plus anti-ICOS mAb clone JMAb52 (gently provided by Dr. J. Riley, University of Pennsylvania, Philadelphia, PA) as previously described (17). Human rIL-12 (1 ng/ml; PeproTech), human rIFN-γ (7.5 ng/ml; Endogen), human rIL-4 (5 ng/ml; Endogen), human rIL-10 (1 ng/ml; Endogen), neutralizing anti-human IL-12 Ab (0.9 mg/ml; Endogen), anti-IL-10 (5 μg/ml; BD Pharmingen), anti-IL-4 (1 μg/ml; Endogen), anti-IFN-γ (10 μg/ml; Endogen), anti-ICOS ligand (clone MIH12, 5 μg/ml; eBioscience), stimulatory anti-CD28 (clone CD28.2, 100 ng/ml; eBioscience), or blocking anti-CD28 (clone CD28.6, 15 μg/ml; eBioscience) were added to some cultures at final concentrations. After stimulation, ICOS, T-bet, and/or GATA-3 expression were determined by Western blot or flow cytometry. IFN-γ and IL-10 production was measured by ELISA (15 pg/ml detection limits of both ELISA; Endogen), or by intracellular cytokine staining (31).

Generation of polarized Th1 and Th2 cells

Polarized Th1 and Th2 cell lines and unpolarized Th0 cell lines were generated from PBMC as previously described, with minor modifications (32). Briefly, PBMC from healthy donors were stimulated with PHA-M (2.5 mg/ml), and cultured in the absence (for Th0 cell lines) or in the presence of human rIL-12 (1 ng/ml; PeproTech) plus neutralizing anti-IL-4 mAb (1 mg/ml; Endogen) (for Th1 cell lines), or in the presence of human rIL-4 (5 ng/ml; Endogen) plus neutralizing anti-IL-12 (0.9 mg/ml; Endogen) (for Th2 cell lines). At different time points, IFN-γ production was measured by ELISA or by intracellular cytokine staining as previously described (31). ICOS levels were determined by flow cytometry (see below) and T-bet and GATA-3 expression were analyzed by Western blot (see below). T cell lines were then stimulated with sonicated *M. tuberculosis* Ag and anti-ICOS mAb plus 5000-rad irradiated feeder cells (autologous PBMC) and IFN-γ production was measured by ELISA.

Western blot

PBMC were stimulated as described and thereafter cells were washed and solubilized in lysis buffer (50 mM Tris (pH 8), 1% CHAPS, and protease inhibitor mixture; Sigma-Aldrich) to prepare whole cell extracts as described (10). Equivalent amounts of protein were analyzed by 12% SDS-PAGE, transferred to nitrocellulose (Hybond ECL Nitrocellulose membrane; Amersham Biosciences), and incubated with anti-ICOS (1/400, ICOS (W18); Santa Cruz Biotechnology), anti-T-bet (1/400, T-bet (39D); Santa Cruz Biotechnology), or anti-GATA-3 (1/100, GATA-3 (H-48); Santa Cruz Biotechnology). For comparison of protein levels among different samples, protein concentrations were normalized to yield equivalent β-actin products (1/1000, β-actin (H-300); Santa Cruz Biotechnology). Bound Abs were revealed with HRP-conjugated affinity purified anti-goat Ab (1/2500; Chemicon International), HRP-conjugated affinity-purified anti-mouse Ab (1/2500; Bio-Rad) or anti-rabbit Ab (1/3000; Bio-Rad) using ECL (Amersham Biosciences) and Kodak BioMax films.

Flow cytometry

To determine the level of ICOS expression on T cells, PBMC cultured with sonicated *M. tuberculosis* Ag together with recombinant cytokines or neutralizing or costimulatory Abs, were stained for surface expression with mAbs specific for CD3 (OKT3; eBioscience) and ICOS (ISA-3; eBioscience) before and after culture. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Intracellular cytokine staining was used to determine IFN-γ and IL-10 production at the single cell level as previously described (31). Briefly, *M. tuberculosis*-stimulated cells were cultured with brefeldin A (10 mg/ml; Fluka) for the final 6 h to induce the intracellular accumulation of newly synthesized proteins. Cells were then harvested, stained for surface expression with mAbs specific for CD3 (eBioscience) and SLAM (clone A12) (7) or ICOS, and washed with PBS-2% FCS. Intracellular cytokine staining was performed with PE-conjugated anti-IFN-γ (IQ Products) or PE-conjugated anti-IL-10 (eBioscience), using the Cytodetect kit (IQ Products), which uses saponin as a permeabilizing agent, according to the manufacturer's instructions. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Negative control samples were incubated with irrelevant, isotype-matched Abs in parallel with all experimental samples.

Statistical analysis

Statistical analysis was performed using the nonparametric Wilcoxon matched-pairs signed rank test for paired samples and the Mann-Whitney *U* test for unpaired samples. Simple regression analysis and the Spearman's correlation coefficients were adopted to study the relationship between variables. Values of $p < 0.05$ were considered significant.

Results

Expression of ICOS is associated with IFN-γ production in patients with active tuberculosis

To evaluate the regulated expression and functional role of ICOS in human tuberculosis, we initially analyzed cytokine production induced by *M. tuberculosis* by two groups of patients with active disease. Because T cell responsiveness is critical for immunity against the pathogen, the tuberculosis patients were classified as HR or LR based on in vitro T cell responses to Ag, as described in *Materials and Methods* and Ref. 10. HR patients produced significantly higher levels of Ag-induced IFN-γ in comparison to LR patients (mean ratio of IFN-γ production: 373.87 $\pm$ 181.85 ng/ml; HR group 5.45 $\pm$ 2.08 ng/ml, LR group $p < 0.0001$, Mann-Whitney *U* test) (Fig. 1*A*, *left*). Although Ag-induced IFN-γ production was distinct between the two groups of individuals, both HR and LR patients made comparable levels of IL-10 against *M. tuberculosis* (Fig. 1*A*, *right*; p = not significant). Cytokine production by tuberculosis patients were routinely analyzed at 48 h (Fig. 1*A*) and 5 days after *M. tuberculosis* stimulation, but no significant differences were found at the two time points (mean IFN-γ production, HR: 2 ng/ml at 2 days vs 2.26 ng/ml at 5 days; LR: 0.12 ng/ml at

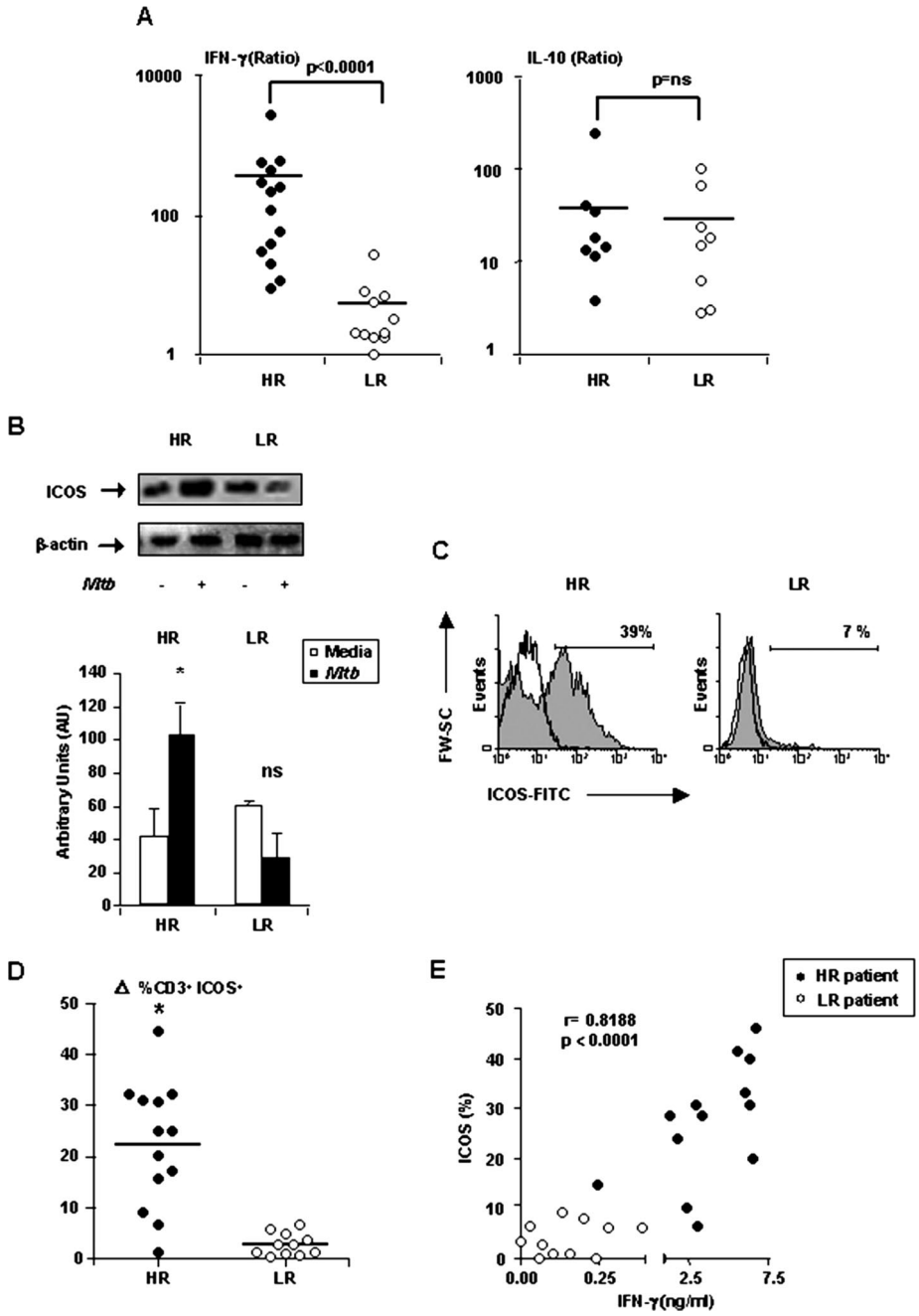

FIGURE 1. ICOS expression is associated with IFN-γ production in patients with active tuberculosis. *A*, Tuberculosis patients' cytokine response to *M. tuberculosis*. PBMC from HR and LR tuberculosis patients were cultured in the presence or absence of sonicated *M. tuberculosis* Ag for 48 h, and IFN-γ and IL-10 production were measured by ELISA. Each symbol represents the ratio of cytokine production for an individual patient (nanograms per milliliter cytokine produced by *M. tuberculosis*-stimulated cells per nanogram per milliliter cytokine secreted by cells cultured with medium). Thick horizontal line represents the mean of the ratio of cytokine production by each group of tuberculosis patients. Values for p were calculated using the Mann-Whitney U test by comparing the mean of the ratio of cytokine production by HR patients' cells vs the mean of the ratio of cytokine production by LR patients' cells. Significant difference ($p < 0.0001$) or none (ns, differences not significant) is indicated. *B–D*, Effect of sonicated *M. tuberculosis* (*Mtb*) Ag on ICOS expression in tuberculosis patients. PBMC from HR and LR tuberculosis patients were cultured in the presence or absence of Ag. *B*, After 5 days of Ag stimulation, total cell extracts were prepared and assayed for ICOS protein expression by Western blot. For comparison of ICOS levels among different samples, protein concentrations were normalized to yield equivalent β-actin products. One representative patient of five is shown for each group. Polyacrylamide gels were scanned, densitometry was performed, and the results were expressed as arbitrary units (AU). Data represent the mean ± SEM of arbitrary units in each group of patients. ∗, $p < 0.05$ by Wilcoxon matched-pairs signed rank test. ICOS surface expression to *Mtb* was determined in representative HR and LR tuberculosis patients (*C*) or in multiple HR and LR tuberculosis patients using two-color flow cytometry (*D*). ICOS expression on T cells was determined by first gating on $CD3^+$ cells, then evaluating ICOS expression. *C*, Histograms of ICOS (gray histogram) vs the isotype control (open histogram) are shown in a representative individual of each group. *D*, Each symbol represents an individual patient, and the value was calculated as: Percentage of $CD3^+$ $ICOS^+$ T cells after *M. tuberculosis* stimulation − percentage of $CD3^+$ $ICOS^+$ T cells after culture with medium. Thick horizontal line represents the mean of the increase in $CD3^+ICOS^+$ T cells after *M. tuberculosis* stimulation in each group of tuberculosis patients. Values for p were calculated using the Mann-Whitney U test by comparing the mean of the increase in $CD3^+$ $ICOS^+$ T cells after *M. tuberculosis* stimulation in HR vs LR patient's cells. ∗, $p < 0.0001$. *E*, Direct correlation between ICOS expression and IFN-γ production in tuberculosis patients. To determine the association between ICOS levels and IFN-γ, statistical analysis was performed using the Spearman test. Each symbol represents the percentage of $ICOS^+$ $CD3^+$ T cells and the IFN-γ secretion in *M. tuberculosis*-stimulated cells for each tuberculosis patient. $\alpha = 0.05$, Spearman coefficient, $r = 0.8188$, $p < 0.0001$.

2 days vs 0.17 ng/ml at 5 days). Moreover, *M. tuberculosis* increased T-bet expression and strikingly decreased GATA-3 levels in HR tuberculosis patients, whereas LR patients displayed a marked increase in GATA-3 expression and no detectable levels of T-bet after Ag stimulation (our unpublished observations). Therefore, our results suggest to us that HR patients that secreted IL-10, but produced high levels of IFN-γ in response to *M. tuberculosis* might be creating a Th1-like microenvironment. In contrast, in LR patients, *M. tuberculosis* induced IL-10 production but very low levels of IFN-γ, generating a predominantly Th2-like environment.

Because ICOS-mediated signaling contributes to the responses of Th1 and Th2 effector cells (33), we investigated Ag-induced ICOS expression in T cells of tuberculosis patients. In HR patients and healthy donors, individuals who secreted high levels of IFN-γ to the pathogen (Fig. 1*A* and Ref. 10), ICOS expression was significantly increased in total cell extracts (Fig. 1*B*, $p < 0.05$, Wilcoxon signed rank test). In contrast, no significant differences in the protein expression were found in LR patients (Fig. 1*B*), individuals that produced very low levels of IFN-γ to *M. tuberculosis* (Fig. 1*A*). Moreover, in correlation with the results on total ICOS expression, *M. tuberculosis* stimulation increased ICOS levels on the surface of T cells in HR patients (Fig. 1*C*, *left* and *D*, $p < 0.0001$, Wilcoxon signed rank test), whereas the receptor expression showed no changes after Ag stimulation in LR individuals (Fig. 1*C*, *right*, and *D*).

To further investigate the association between ICOS expression and IFN-γ production in tuberculosis patients, we performed statistical analysis, which indicated a significant correlation (Spearman coefficient, $r = 0.8188$, $p < 0.0001$) (Fig. 1*E*), and a significant linear regression ($r^2 = 0.7116$; $p < 0.0001$) between both parameters. Thus, our present results formally demonstrated a direct correlation between the levels of ICOS and IFN-γ secretion in tuberculosis patients.

We reasoned that the inability of LR patients to increase ICOS expression might be due to a specific unresponsiveness to *M. tuberculosis* rather than a general inability to express ICOS. Thus, cells from tuberculosis patients and healthy donors were stimulated with PHA, and ICOS expression was determined. In the three groups of individuals, ICOS levels were increased after PHA stimulation (Fig. 2), demonstrating that there was no global defect in ICOS expression in T cells from LR tuberculosis patients. Therefore, we speculate that the variations detected in ICOS expression in HR and LR tuberculosis patients might be related primarily to the recognition of the specific Ag by T cells from these individuals, suggesting a lack or weak *M. tuberculosis* TCR signaling in LR patients.

To further evaluate the relationship between ICOS expression and cytokine production by T cells from tuberculosis patients, we simultaneously measured ICOS and cytokine expression after *M. tuberculosis* stimulation using flow cytometry. In HR patients, the majority of the IFN-γ-producing cells expressed either ICOS or SLAM, a receptor that enhances secretion of IFN-γ in T cells (7, 34) (Fig. 3*A*). Moreover, we found a significantly higher percentage of IFN-γ^+ ICOS$^+$ T cells in HR patients compared with LR individuals (Fig. 3*B*, $p < 0.01$, Mann-Whitney *U* test). Similarly, a significant high number of IFN-γ^+ SLAM$^+$ T cells were detected in responsive individuals in comparison to unresponsive patients (Fig. 3*B*, $p < 0.05$, Mann-Whitney *U* test), suggesting that ICOS$^+$ IFN-γ^+ T cells might also express SLAM. In fact, we measured a high number of T cells (87%) coexpressing ICOS and SLAM in HR patients (Fig. 3*C*, *top panels*). In contrast, in LR patients, we found <5% of either IFN-γ^+ ICOS$^+$ or IFN-γ^+ SLAM$^+$ T cells (Fig. 3, *A* and *B*). Accordingly, low levels of T cells from unresponsive patients coexpressed SLAM and ICOS on their surface (Fig. 3*C*, *bottom panels*).

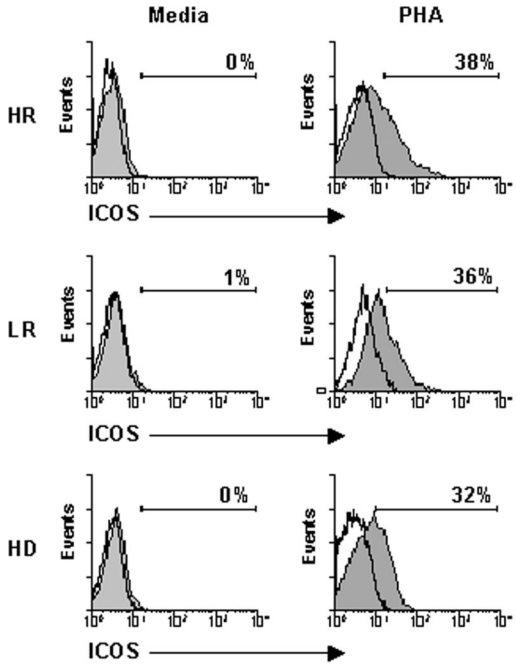

FIGURE 2. Polyclonal stimulation increased ICOS levels in HR and LR tuberculosis patients and in healthy donors (HD). To determine the expression of ICOS on T cells, PBMC stimulated with media or with PHA for 5 days were stained for surface expression with mAbs specific for CD3 and ICOS. Samples were analyzed on a FACSCalibur flow cytometer. ICOS expression on T cells was determined by first gating on CD3$^+$ cells, then evaluating ICOS expression. ICOS (gray histogram) vs the isotype control (open histogram) are shown in one representative individual of five in each group.

We found similar low levels of both ICOS$^+$ IL-10$^+$ and SLAM$^+$ IL-10$^+$ T cells in the two groups of patients (<5%, data not shown). Because during *M. tuberculosis* infection macrophages elicit IL-10 (35), we speculate that these cells might be also secreting IL-10, contributing to the IL-10 levels detected in tuberculosis patients in response to the Ag (Fig. 1*A*). Together, and in agreement with our previous results on SLAM (10, 36), the present data suggest that ICOS could be either modulated by Th1 cytokines or implicated in the generation of type 1 immune responses against *M. tuberculosis*.

Regulation of ICOS expression during active tuberculosis

To identify regulatory mechanisms for ICOS expression, we analyzed the modulation of *M. tuberculosis*-induced ICOS levels by the cytokine microenvironment. Given that CD28 stimulation was shown to be an important inducer of ICOS expression on polyclonal-stimulated mouse T cells (37), we investigated whether signaling through CD28 participated in the regulation of ICOS expression in tuberculosis. Similar levels of CD28 were detected on T cells from both groups of patients after *M. tuberculosis* stimulation (CD28$^+$CD3$^+$ T cells, HR 78%; LR 80%). However, engagement of CD28 markedly increased ICOS expression on Ag-stimulated cells from HR patients (Fig. 4*Ac*), whereas it did not noticeably modify ICOS expression in LR individuals (data not shown). Moreover, blocking anti-CD28 mAb diminished ICOS expression on *M. tuberculosis*-stimulated T cells from HR individuals (Fig. 4, *Ad* and *B*), demonstrating that signaling through CD28 participated in the regulation of ICOS expression. Furthermore, the simultaneous neutralization of endogenous IFN-γ and blockage of

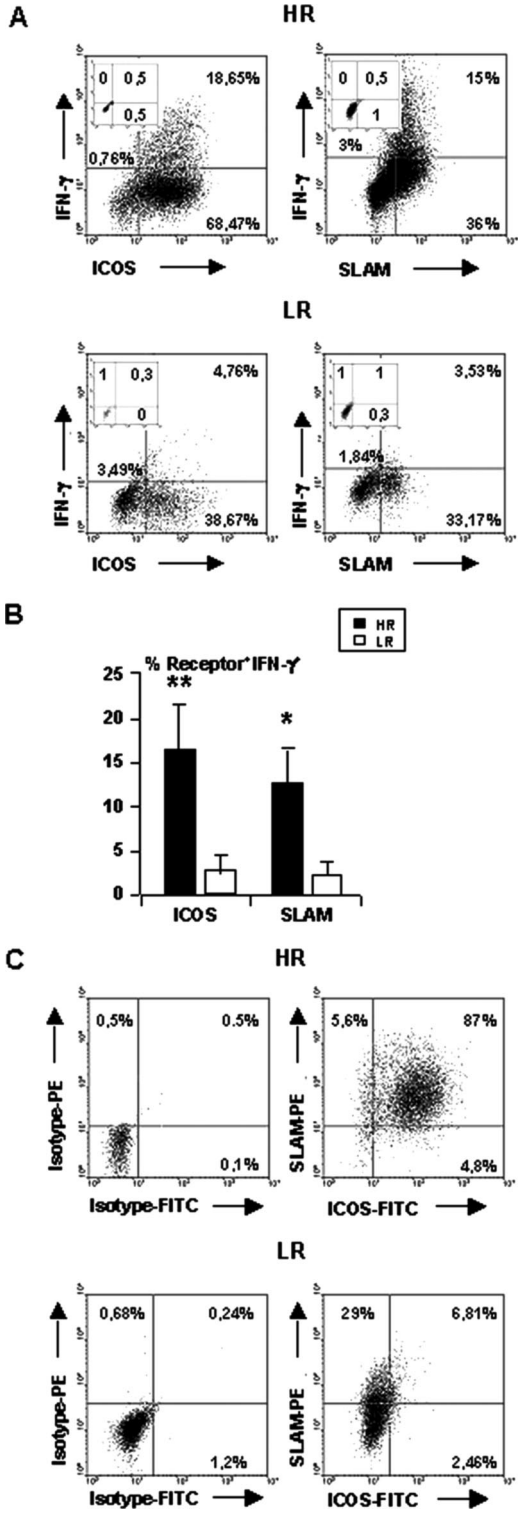

FIGURE 3. Effect of *M. tuberculosis* on ICOS, SLAM, and IFN-γ expression in patients with active tuberculosis. *A*, PBMC were stimulated with the Ag for 4 days and the expression of ICOS or SLAM on *M. tuberculosis*-reactive IFN-γ-producing T cells was examined by flow cytometry. T cells were identified by first gating on blast lymphocytes by light scatter properties, then by gating on $CD3^+$ cells. One representative HR and LR tuberculosis patient of five is shown for each group. The percentage of $ICOS^+$ or $SLAM^+$ IFN-γ^+ cells after culturing with *M. tuberculosis* Ag (each quadrant) is expressed in the right portion of the panels. Isotype controls (*inset*) are shown. *B*, ICOS or SLAM expression on *M. tuberculosis*-reactive IFN-γ-producing T cells as determined by flow cytometry. Data represent the mean of the percentage of Receptor-positive (ICOS or SLAM) IFN-γ^+ T cells stimulated with the Ag ± SEM in each group of tuberculosis patients. Values for *p* were calculated using the Mann-Whitney *U* test by comparing the mean of the percentage of receptor-positive (ICOS or SLAM) IFN-γ^+ T cells in HR patient cells vs the mean of the percentage of Receptor-positive (ICOS or SLAM) IFN-γ^+ T cells in LR patient cells. *, $p < 0.05$; **, $p < 0.01$. *C*, Simultaneous expression of SLAM and ICOS on *M. tuberculosis*-stimulated cells from HR and LR tuberculosis patients. PBMC were stimulated with the Ag for 5 days and the expression of ICOS and SLAM on *M. tuberculosis*-stimulated T cells was examined by flow cytometry. T cells were identified first gating on blast lymphocytes by light scatter properties, then by gating on $CD3^+$ cells, and finally evaluating ICOS and SLAM expression. The percentage of $ICOS^+$ $SLAM^+$ T cells after culturing with *M. tuberculosis* Ag (each quadrant) is shown in a representative HR (*top panels*) and LR (*bottom panels*) tuberculosis patient out of five in each group.

the CD28 pathway in Ag-stimulated cells from HR patients significantly reduced the expression of ICOS on these cells (Fig. 4, *Ae* and *B*).

Although *M. tuberculosis* strikingly up-regulated ICOS expression in HR patients (Fig. 1, *B–D*), Th2 polarizing conditions significantly reduced the levels of ICOS (Fig. 4, *Af* and *C*), in correlation with a striking decrease in IFN-γ production in all the conditions (data not shown). In contrast, even though T cells from LR tuberculosis patients displayed characteristics of Th2 cells (Fig. 1*A*) and expressed low ICOS levels in response to *M. tuberculosis* (Fig. 1, *B–D*), Th2 polarizing conditions barely modified ICOS expression (our unpublished observation). Furthermore, culture of Ag-stimulated cells from HR patients with rIFN-γ significantly increased ICOS expression (Fig. 4, *Ag* and *D*), confirming the role of this cytokine in the modulation of ICOS. On the contrary, Th1 polarizing conditions had only a slight effect on ICOS levels in *M. tuberculosis*-stimulated cells form LR patients (our unpublished observation). Together, our results suggest that the regulation of ICOS expression could be dependent on T cell responsiveness to *M. tuberculosis*, which would determine the subsequent regulation of ICOS. Moreover, we demonstrate for the first time that ICOS expression can be regulated by the cytokine microenvironment and signaling through CD28 during an intracellular human infection.

Expression and activation of ICOS in polarized T cell lines

It has been reported that ICOS levels on polarized Th cells are not fixed and that the Th1 cytokine IL-12, would have an important role on ICOS expression in human activated Th cell lines (32). Accordingly, we found that ICOS expression might be regulated by the cytokine microenvironment during *M. tuberculosis* infection. To further explore the regulation and role of ICOS in Th cells, we used PBMC from healthy donors stimulated under Th0, Th1, and Th2 polarizing conditions. We found that Th1 cells, which secreted high levels of IFN-γ (Fig. 5*A*) and displayed elevated amounts of T-bet (Fig. 5*B*), expressed high levels of ICOS (Fig. 5*C*). Similarly, Th0 cells, that also produced considerable levels of IFN-γ and strong T-bet expression (Fig. 5, *A* and *B*), showed high ICOS expression as well (Fig. 5*C*), although lower compared with ICOS expression on Th1 cells (Fig. 5*C*). Moreover, the lowest expression of ICOS was detected on cells stimulated under Th2 polarizing conditions, which produced very low (or undetectable) levels of IFN-γ and T-bet, but showed elevated expression of GATA-3 (Fig. 5). These results indicated that according to their polarization state, human Th cells express differential levels of ICOS, which increase in correlation to IFN-γ production by those cells.

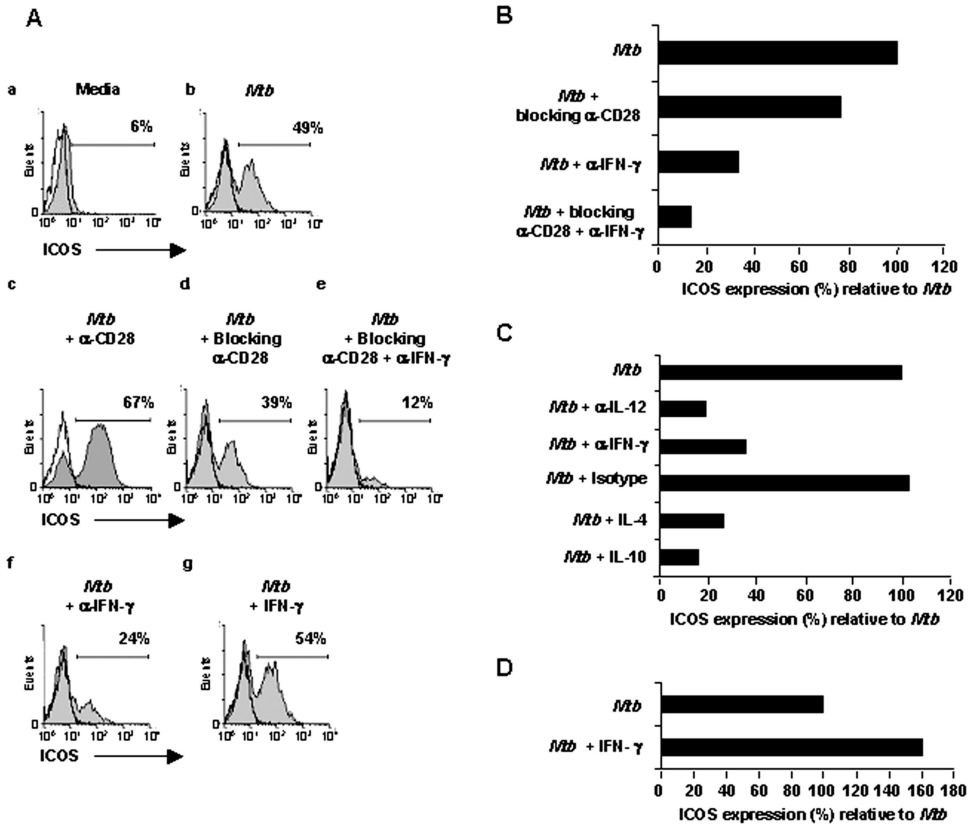

FIGURE 4. Modulation of ICOS expression by CD28 signaling or by cytokines from the local microenvironment. PBMC from HR tuberculosis patients were cultured with medium or with *M. tuberculosis* (*Mtb*) for 5 days in the presence or absence of stimulating mAbs, neutralizing mAbs, and/or recombinant cytokines, and the levels of ICOS expression were determined by flow cytometry. Cells were gated based on CD3 expression. *Aa–g*, ICOS expression (gray histogram) vs the isotype control (open histogram) in one representative HR patient of seven in each condition. *B–D*, Values are expressed as the percentage of ICOS expression in seven HR tuberculosis patients relative to cells cultured with *Mtb* alone. Values were calculated as follows: 100 − [(expression of ICOS on *Mtb*-stimulated cells − ICOS expression on *Mtb* + recombinant cytokine and/or neutralizing mAb-stimulated cells)/(ICOS expression on *Mtb*-stimulated cells − ICOS expression in medium) × 100]. *B*, Values for *p* were calculated by comparing the mean of the percentage of ICOS of cells cultured with *Mtb*) vs the mean of the percentage of ICOS of cells cultured with *Mtb* + anti-IFN-γ or *Mtb* + blocking anti-CD28 + anti-IFN-γ. ∗, $p < 0.05$, Wilcoxon matched-pairs signed rank test. *C*, Values for *p* were calculated using the Wilcoxon matched-pairs signed rank test by comparing the mean of the percentage of ICOS of cells cultured with *Mtb* vs the mean of the percentage of ICOS of cells cultured with *Mtb* + anti-IL-12, *Mtb* + anti-IFN-γ, *Mtb* + IL-4, and *Mtb* + IL-10. ∗, $p < 0.05$. *D*, Values for *p* were calculated by comparing the mean of the percentage of ICOS of cells cultured with *Mtb* vs the mean of the percentage of ICOS of cells cultured with *Mtb* vs the mean of the percentage of ICOS of cells cultured with *Mtb* + IFN-γ. ∗, $p < 0.005$, Wilcoxon matched-pairs signed rank test.

ICOS ligation can stimulate production of both Th1 and Th2 cytokines during initial priming and during effector T cell responses (14, 37). Therefore, we next investigated the effect of ICOS ligation on the modulation of *M. tuberculosis*-induced cytokine responses in polarized Th cell lines. Cells were stimulated with the Ag together with anti-ICOS mAb, and IFN-γ production was determined. *M. tuberculosis* increased IFN-γ production by cells stimulated under Th0 and Th1 conditions and those levels were even augmented after ICOS ligation (Fig. 5*D*). In contrast, neither Ag stimulation nor ICOS costimulation modified the IFN-γ levels secreted by Th2 cells (Fig. 5*D*). These data indicate that ICOS ligation on *M. tuberculosis*-stimulated polarized Th cells increases IFN-γ production with a magnitude proportional to their level of ICOS expression. Thus, these findings strongly support our results showing the effect of the cytokine microenvironment on ICOS expression in tuberculosis patients.

Effect of ICOS ligation on cytokine responses by patients with active tuberculosis

Considering our results on the expression and function of ICOS in polarized human Th cell lines, and given that the role of ICOS in modulating cytokine levels in human intracellular infection remains unknown, we investigated whether engagement of ICOS could modulate *M. tuberculosis*-induced T cell cytokine responses. PBMC from tuberculosis patients were stimulated with *M. tuberculosis* (5 days) and then with an agonistic anti-ICOS mAb. ICOS engagement significantly augmented IFN-γ production from HR patients (Fig. 6*A*, $p < 0.0001$, Wilcoxon signed rank test). Moreover, by analyzing the percentage of IFN-γ-positive cells by intracellular flow cytometry, we found that the increase in IFN-γ production detected after ICOS ligation by *M. tuberculosis*-stimulated cells was due to enhanced secretion of this cytokine from previously activated T cells. We measured similar percentages of IFN-γ^+ T cells after *M. tuberculosis* and *M. tuberculosis* plus ICOS stimulation, but higher median fluorescence intensity in cells stimulated through ICOS (Fig. 6*B*). No effect of anti-ICOS mAb on IFN-γ production was observed if cells were cultured in the absence of Ag. Furthermore, the enhancement in IFN-γ production after ICOS activation correlated with an increase in T-bet expression but no detection of GATA-3 protein (our unpublished observation), suggesting that ICOS activation in HR patients led to the generation of a Th1-like microenvironment. In contrast, ligation of

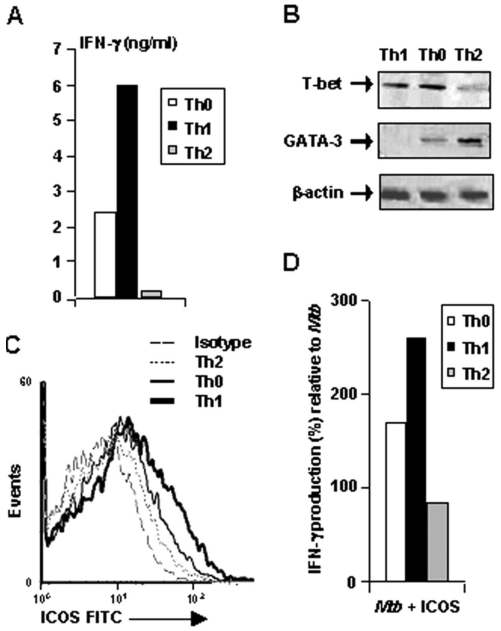

FIGURE 5. Expression and function of ICOS in short-term polarized human cell lines. PBMC from healthy donors were stimulated under polarizing conditions as described in *Materials and Methods*. After polarization, the production of IFN-γ by ELISA (*A*) and the expression of T-bet and GATA-3 by Western blot (*B*) were determined in cells from Th0, Th1, and Th2 cell lines. For comparison of T-bet and GATA-3 levels among different samples, protein concentrations were normalized to yield equivalent β-actin products. *C*, The expression of ICOS on cells from Th0, Th1, and Th2 cell lines was analyzed by flow cytometry. *D*, Effect of ICOS ligation on IFN-γ production by Th0, Th1, and Th2 cell lines in response to *M. tuberculosis*. T cells from the polarized lines were stimulated with *M. tuberculosis* Ag as described in *Materials and Methods* and anti-ICOS mAb for 48 h and IFN-γ production was determined by ELISA. Values are expressed as the percentage of IFN-γ production relative to cells cultured with *M. tuberculosis* (*Mtb*) alone. Values were calculated as follows: 100 − ((production of IFN-γ by *Mtb*-stimulated cells − production of IFN-γ by *Mtb* + anti-ICOS mAb-stimulated cells)/(production of IFN-γ by *Mtb*-stimulated cells − production of IFN-γ by cells cultured in medium) × 100). One representative experiment of five is shown in *A–D*.

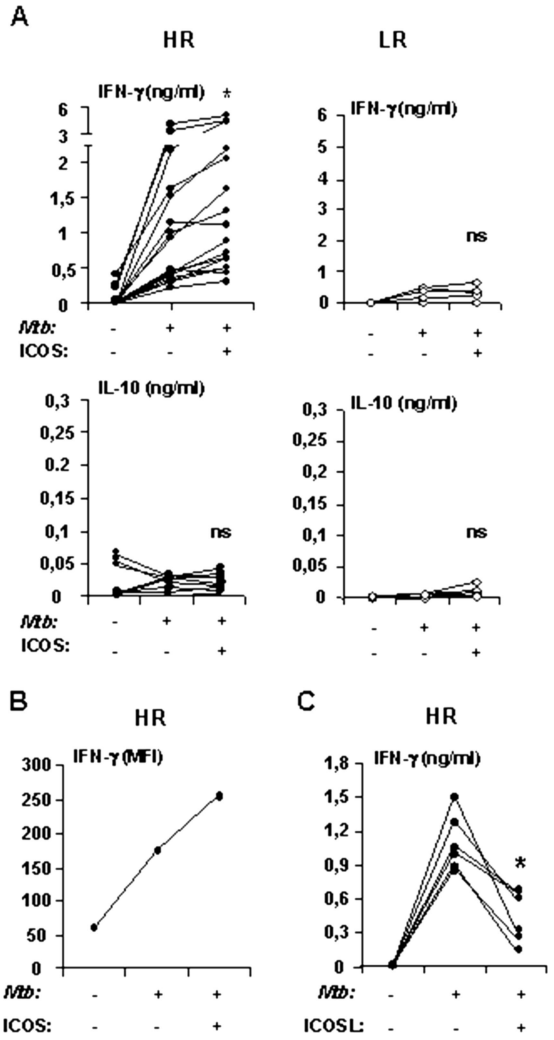

FIGURE 6. Effect of ICOS ligation on cytokine responses to *M. tuberculosis* (*Mtb*). *A* and *B*, Effect of anti-ICOS mAb on the production of IFN-γ and IL-10 by patients with active tuberculosis after stimulation with *M. tuberculosis* Ag. *A*, PBMC from HR and LR tuberculosis patients were stimulated with sonicated *M. tuberculosis* Ag and after 5 days cells were cultured in the presence or absence of anti-ICOS mAb. Cell-free supernatants were collected at 48 h and assayed for IFN-γ and IL-10 by ELISA. Values are expressed as the mean of triplicate determinations. Each line connects the data for an individual patient. ∗, $p < 0.0001$, Wilcoxon matched-pairs signed rank test comparing the cytokine production from cells cultured with *M. tuberculosis* then medium vs cells cultured with *M. tuberculosis* then anti-ICOS; ns, differences not significant. *B*, Intracellular IFN-γ production by cells stimulated with *M. tuberculosis* or *M. tuberculosis* + ICOS mAb. Each symbol represents the median of fluorescent intensity (MFI) for IFN-γ in intracellular stained T cells. One representative HR tuberculosis patient of five is shown. *C*, Effect of ICOS blockade by an anti-ICOS ligand (ICOSL) mAb on *M. tuberculosis* Ag-induced IFN-γ production. PBMC from HR tuberculosis patients were cultured with *M. tuberculosis* Ag in the presence or absence of anti-ICOS ligand mAb and after 48 h, IFN-γ was measured by ELISA. Each line connects the data for an individual patient. ∗, $p < 0.05$ using the Wilcoxon matched-pairs signed rank test comparing the production of IFN-γ by cells cultured with *M. tuberculosis* Ag vs *M. tuberculosis* Ag + anti-ICOS ligand.

ICOS did not modify the levels of Ag-induced IFN-γ produced by LR patients (Fig. 6*A*), likely because ICOS was not considerably up-regulated on the surface of their T cells in response to *M. tuberculosis* (Fig. 1). Moreover, even after addition of rIFN-γ to the culture, ICOS levels were not significantly up-regulated on T cells from LR patients and therefore, signaling through ICOS did not increase IFN-γ production in those individuals (data not shown). Surprisingly, engagement of ICOS had no effect on IL-10 (Fig. 6*A*) or IL-4 secretion (data not shown) from both groups of tuberculosis patients. Finally, we observed that ICOS blockade induced a striking decrease in IFN-γ production by Ag-stimulated cells from HR individuals (Fig. 6*C*, $p < 0.05$, Wilcoxon signed rank test), with no changes in IL-10 secretion (data not shown), confirming that ICOS participates in the generation of Th1 responses against *M. tuberculosis*.

To further investigate whether signaling through ICOS could induce IL-10 production in *M. tuberculosis*-stimulated cells, we used magnetic beads coated with suboptimal amounts of anti-CD3 plus anti-ICOS (ICOS beads). Simultaneous signaling through ICOS and CD3 significantly increased IL-10 production from HR patients and healthy donors, either in the presence (HR: $p < 0.01$; healthy donors: $p < 0.05$, Wilcoxon signed rank test) or absence (HR: $p < 0.01$; healthy donors: $p < 0.05$, Wilcoxon signed rank test) of Ag (Fig. 7). Moreover, costimulation through ICOS beads also induced a significant augment of IFN-γ from HR patients and healthy donors (Fig. 7), but in contrast to IL-10, the increase in IFN-γ production induced by ICOS beads was higher in *M. tuberculosis*-stimulated cells (HR: $p < 0.005$; healthy donors: $p < 0.05$,

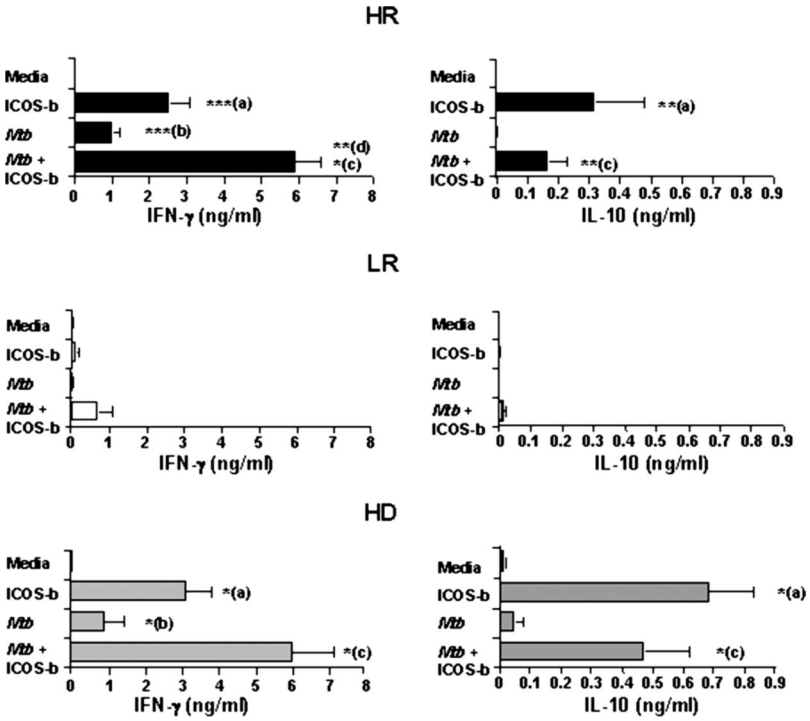

FIGURE 7. Effect of ICOS engagement on cytokine production against *M. tuberculosis* (*Mtb*) using beads coated with anti-ICOS mAb and suboptimal amounts of anti-CD3 (ICOS beads). PBMC from tuberculosis patients and healthy donors (HD) were stimulated with sonicated *M. tuberculosis* Ag. After 5 days, the cells were cultured in the presence or absence of ICOS beads (ICOS-b). Cell-free supernatants were collected at 48 h and assayed for IFN-γ and IL-10 by ELISA. Data represent the mean ± SEM of cytokine production by each group (seven individuals per group). Values for *p* were calculated using the Wilcoxon matched-pairs signed rank test comparing cytokine production from cells cultured in medium vs cells cultured with ICOS beads (a); cytokine production from cells cultured in medium vs cells cultured with *M. tuberculosis* Ag (b); cytokine production from cells cultured with *M. tuberculosis* then medium vs cells cultured with *M. tuberculosis* then ICOS beads (c); and cytokine production from cells cultured with ICOS beads vs cells cultured with *M. tuberculosis* then ICOS beads (d). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Wilcoxon signed rank test), confirming that signaling through ICOS induced a specific IFN-γ response against *M. tuberculosis* (Fig. 7). On the contrary, in LR tuberculosis patients, ICOS beads engagement did not induce IL-10, either in the presence or absence of Ag, but slightly increased IFN-γ production from *M. tuberculosis*-stimulated cells (Fig. 7), suggesting that CD3 stimulation might moderately augment the strength of the TCR signal in unresponsive individuals. Together, our data indicate that anti-CD3 stimulation may induce IL-10 production by T cells from HR tuberculosis patients and healthy donors during ICOS signaling, but the presence of *M. tuberculosis* Ag in the culture down-regulates those levels of IL-10 produced, probably because of the high IFN-γ secreted under those stimulation conditions.

Discussion

The role of ICOS, a key regulator of T cell cytokine patterns (14, 17, 38), was investigated using tuberculosis as a model. ICOS expression and IFN-γ production were directly correlated in patients with tuberculosis. Patients with robust Th1 cytokine production in response to *M. tuberculosis* (HR patients) express high levels of ICOS in contrast to low levels of both IFN-γ and ICOS in LR patients. In fact, ICOS and IFN-γ exhibited a causal relationship in our experimental system given that T cells that produced IFN-γ almost always expressed ICOS. Interestingly, SLAM, another Th1-regulating cell surface molecule, showed differential regulation relative to ICOS, an observation that may be important in understanding how costimulation affects Th1 cytokine production against tuberculosis infection.

An important observation in these studies was the association of ICOS levels with Th1 cytokine production during a human intracellular infection. In fact, ICOS expression in HR patients was inhibited by neutralizing anti-IFN-γ Abs. Moreover, short-term human Th1 cell lines expressed higher levels of ICOS than Th2 cells lines. Accordingly, it was demonstrated that differentiation into Th1 and Th2 cells precedes the difference in ICOS expression (37). Furthermore, and in line with our findings, it was shown that the strength of the effector response of human Th cells is regulated by the control of ICOS expression by locally produced cytokines in peripheral tissues (32). In mouse, ICOS expression was shown to be much higher in Th2 cells that in Th1 cells (19, 37, 39), in correlation with IL-4 production (40), but a critical role of ICOS in the resistance to certain infections was also demonstrated in in vivo infectious Th1 mouse models (24, 26). Thus, our data in tuberculosis extend earlier findings regarding the relationship of ICOS expression and Th cytokine production.

We observed that ICOS levels also correlated with the expression of SLAM, in sharp contrast to a previous report in mouse polarized Th lines, indicating that the pattern of ICOS expression is in contrast to SLAM expression (37). However, we demonstrated that both SLAM and ICOS are required in the human immune response against *M. tuberculosis*. In HR patients, ICOS and SLAM were coordinately up-regulated after Ag stimulation, suggesting that expression of both costimulators in tuberculosis correlated with reduced disease severity. Furthermore, the majority of IFN-γ-secreting T cells expressed either ICOS and/or SLAM in HR patients, whereas few IFN-γ-producing T cells expressed SLAM or ICOS in LR individuals, suggesting that both receptors can be either modulated by Th1 cytokines or implicated in the generation of type 1 immune responses against *M. tuberculosis*. However, although SLAM engagement significantly enhanced IFN-γ production from both HR and LR tuberculosis patients (10), ICOS signaling enhanced IFN-γ secretion only in HR individuals. Nevertheless, simultaneous activation of SLAM and ICOS synergistically increased IFN-γ production by responsive individuals (our unpublished observation). Besides, and also in contrast to our previous results on SLAM (36), addition of IFN-γ to the local cell microenvironment did not significantly increase ICOS expression on T cells from LR individuals, and accordingly, subsequent ICOS ligation did not augment IFN-γ production (data not shown). Thus, the two cytokine modulators SLAM and ICOS involved in the generation of Th1 responses during *M. tuberculosis* infection, might be regulated in a distinct way and/or might function during different steps of the host immune response. In fact, although SLAM is rapidly up-regulated after T cell activation (7), ICOS

would play an active role during the effector function of activated human Th cells in inflamed peripheral tissues (41, 42). In addition, mouse ICOS$^+$ Th cells expressed strikingly different cytokines depending on the type of infection encountered and the cell anatomical localization, indicating that ICOS might costimulate distinct effector functions in different immune responses, depending on the nature of the Ag encountered and localization and chronicity of the infection (42). Thus, our findings suggest that ICOS, together with other costimulatory molecules such as SLAM, may promote the CMI response to intracellular bacterial pathogens.

Our observations regarding ICOS expression in tuberculosis patients lead us to speculate that ICOS expression is dependent on T cell responsiveness. Whereas HR patients generate a Th1-like microenvironment that led to heightened ICOS expression, LR patients create a predominant Th2-like environment that impairs ICOS increase. Unresponsiveness of LR individuals' T cells to *M. tuberculosis* impaired the production of enough IFN-γ, contributing to reduced ICOS levels and failure of costimulation through the receptor. In contrast, Ag recognition together with cytokine production to *M. tuberculosis*, allowed the expression of cytokine modulators like ICOS in HR patients. As a result, ICOS ligation enhanced IFN-γ secretion by Ag-activated T cells, leading to up-regulation of ICOS levels, creating a positive feedback loop. However, although *M. tuberculosis* did not modify ICOS expression in LR individuals, PHA stimulation did augment ICOS in these individuals, suggesting that the lack of up-regulation of the receptor in LR patients would be associated with an Ag unresponsiveness or weak TCR signaling against the specific Ag, in line with previous reports showing that TCR-mediated stimulation of PBMC from anergic tuberculosis patients led to defective phosphorylation of TCR-ς and defective activation of Zap70 and MAPK (43). Moreover, although it was proposed that local regulation of ICOS expression on polyclonal-activated Th cells by proinflammatory cytokines is independent of their polarization state (32), we demonstrated that Th2-like cells from LR patients could not respond to the specific antigenic stimulus, which impaired the increase in ICOS levels, even by effect of exogenous Th1 cytokines. Furthermore, neither *M. tuberculosis* stimulation nor ICOS activation modified the IFN-γ secreted by Th2-polarized cells, which expressed very low ICOS levels. In contrast, ICOS ligation significantly augmented IFN-γ production by *M. tuberculosis*-stimulated human Th1 and Th0 cells, in direct correlation to their high levels of ICOS expression. Thus, during the immune response against *M. tuberculosis*, the regulation of ICOS expression on Ag-stimulated T cells might depend primarily on the strength of TCR signaling together with the polarization state of Th cells.

Based on our results, we hypothesize that regulation of IFN-γ production by signaling molecules in tuberculosis would be primarily dependent on T cell recognition of Ag. T cells responding to *M. tuberculosis* rapidly up-regulate SLAM and these two signals might combine to promote IFN-γ production. Activated T lymphocytes would be recruited to the site of infection with increased SLAM levels on their surface. Then, Ag recognition would induce the local release of proinflammatory cytokines, leading to up-regulation of SLAM and ICOS. Subsequent signaling through those receptors would augment IFN-γ in the local microenvironment, creating a positive feedback loop. Furthermore, ICOS levels would stay high on memory T cells from HR tuberculosis patients, facilitating their migration to the site of infection and inducing beneficial Th1 responses. Failure of T cells from LR patients to respond to *M. tuberculosis* impairs SLAM up-regulation and IFN-γ production, contributing to reduced expression of ICOS.

Together, our present findings suggest that the regulation of ICOS levels in tuberculosis patients is primarily dependent on the specific response of their T cells to *M. tuberculosis*. Considering that reduced IFN-γ production is a marker of severe tuberculosis (6), following Ag recognition different amounts of IFN-γ would be secreted by tuberculosis patients, accordingly to their immune response to the pathogen. Effectively Ag-activated T cells would up-regulate ICOS and consequently, ICOS ligation would increase IFN-γ secretion, while *M. tuberculosis* weak-responsive T cells would fail to produce IFN-γ against the bacteria, leading to reduced ICOS expression. Although in intracellular mouse infections ICOS was identified as a key regulator of Th1 cytokine responses (24, 26), our findings identify for the first time that ICOS activation is associated with Th1 cell responses in human intracellular infection and may contribute to the amplification of those responses. Therapeutic regulation of ICOS and SLAM may ultimately alter the T cell response in tuberculosis and promote more effective immunity against tuberculosis infection.

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Disclosures

The authors have no financial conflict of interest.

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