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Early Secreted Antigenic Target of 6-kDa Protein of *Mycobacterium tuberculosis* Primes Dendritic Cells To Stimulate Th17 and Inhibit Th1 Immune Responses

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Early secreted antigenic target of 6 kDa (ESAT-6) of *Mycobacterium tuberculosis* is a T cell Ag that is a potential vaccine candidate, but it is also a virulence factor that mediates pathogenicity. To better understand the effects of ESAT-6 on the immune response, we studied the effect of ESAT-6 on human dendritic cells (DCs). Peripheral blood monocytes were treated with GM-CSF and IL-4 to yield immature DCs, which were matured by addition of LPS and CD40 ligand (CD40L), with or without ESAT-6. ESAT-6 inhibited LPS/CD40L-induced DC expression of costimulatory molecules, reduced DC-stimulated allogeneic T cell proliferation and IL-2 and IFN-γ production, and enhanced IL-17 production. ESAT-6–treated DCs also increased IL-17 and reduced IFN-γ production by *M. tuberculosis*-specific autologous T cells. ESAT-6 inhibited LPS/CD40L-induced DC production of IL-12 and enhanced that of IL-23 and IL-1β, without affecting secretion of TNF-α, IL-6, or IL-8 through specific interaction with immature DCs. The effects of ESAT-6 were not mediated through cAMP or p38 MAPK. Medium from ESAT-6–conditioned DCs increased IL-17 and reduced IFN-γ production by T cells stimulated with anti-CD3 plus anti-CD28, and ESAT-6–induced IL-17 production was blocked by neutralizing both IL-23 and IL-1β. ESAT-6 reduced LPS/CD40L-stimulated transcription of IL-12p35 and enhanced that of IL-23p19 through inhibition of IFN regulatory factor-1 and upregulation of activating transcription factor-2 and c-Jun, transcriptional regulators of IL-12p35 and IL-23p19, respectively. We conclude that ESAT-6 increases DC production of IL-23 and IL-1β while inhibiting that of IL-12, thus enhancing Th17 at the expense of protective Th1 responses. ***The Journal of Immunology*****, 2012, 189: 3092–3103.**

Mycobacterium tuberculosis infects more than a third of the world's population, causing an estimated 1.8 million deaths in 2009 worldwide (1), accompanied by a staggering economic burden, especially in developing countries. Vaccination is the most cost-effective strategy for control and eventual elimination of tuberculosis. However, the most widely used tuberculosis vaccine, attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), provides some protection against the most severe forms of childhood tuberculosis but does not prevent disease in adults, who comprise most tuberculosis cases (2, 3). Therefore, development of an effective vaccine is essential for tuberculosis control, which depends in part on a better understanding of host–pathogen interactions.

Early secreted antigenic target of 6 kDa (ESAT-6) is a potent T cell Ag identified in the short-term culture filtrate of *M. tuberculosis* (4, 5). ESAT-6–based vaccines confer protection against tuberculosis in animal models (6–9), and several such vaccines are either in clinical trials or undergoing preclinical development (10, 11). However, substantial evidence also indicates that ESAT-6 is a virulence factor. The gene encoding ESAT-6, Rv3875 (12), is in the region of difference 1, which is present in many pathogenic mycobacteria, including *M. tuberculosis* and *M. bovis*, but not in attenuated BCG (13). ESAT-6 lyses alveolar epithelial cells and macrophages (14, 15), favoring intercellular spread of *M. tuberculosis* (15, 16), and it can destabilize phagolysosomes, perhaps allowing *M. tuberculosis* and its products to escape the phagosome (17). Therefore, delineating the role of ESAT-6 in the immunopathogenesis of tuberculosis is important for optimizing ESAT-6–based vaccines.

Previously, we demonstrated that ESAT-6 directly inhibits human T cell IFN-γ production (18) through a process that requires activation of p38 MAPK (19). However, the effect of ESAT-6 on human dendritic cells (DCs) has not been investigated. DCs are crucial in bridging innate and adaptive immunity, and they play an essential role in initiation and maintenance of balanced T cell responses to infection (20). Upon encounter with pathogens, immature DCs (iDCs) in the local tissue take up the pathogen and mature after recognizing pathogen-associated molecular patterns through their pattern recognition receptors, such as TLRs, which induce increased expression of costimulatory molecules, such as

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Abbreviations used in this article: ATF, activating transcription factor; BCG, bacillus Calmette-Guérin; DC, dendritic cell; ESAT-6, early secreted antigenic target of 6 kDa; iDC, immature dendritic cell; IRF, IFN regulatory factor; LT, ligand trimer; mDC, mature dendritic cell.

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CD80 and CD86, and production of cytokines, including IL-12, IL-23, and IL-β. Pathogen-experienced mature DCs (mDCs) then migrate to the local draining lymph nodes and initiate T cell responses by presenting microbial Ags in the context of costimulatory molecules and cytokines. IL-12 favors expansion of Th1 cells that produce IFN-γ, and IL-23 and IL-1β induce development of Th17 cells that produce IL-17 (21). Studies in gene-deleted mice and in humans have clearly demonstrated that the IL-12/Th1 pathway is essential for immunity against tuberculosis (22–26). In contrast, the role of IL-23 and Th17 cells in protection against tuberculosis is more complex and controversial. One study found that the absence of IL-17 did not increase susceptibility to tuberculosis (27), whereas another showed that a gene deletion of IL-17A markedly increased bacillary burdens and impaired granuloma formation (28). IL-17 contributed significantly to vaccine-induced protection against challenge with *M. tuberculosis* (29), but it also mediated tissue damage after repeated BCG vaccination in *M. tuberculosis*-infected mice (30) and may provide minimal protection during chronic infection (31).

M. tuberculosis infection can alter the normal process of DC maturation (32, 33), which is crucial for priming Ag-specific T cells. In this study, we examined the immune regulatory effects of ESAT-6 on human DCs. We found that ESAT-6 inhibits production of IL-12 but promotes production of IL-23 and IL-1β through inhibition of IFN regulatory factor (IRF)-1 and enhancement of AP-1 transcription factors. ESAT-6–treated DCs favor T cell production of IL-17 over IFN-γ, providing a potentially novel mechanism for modulation of host immune responses by *M. tuberculosis* through its secreted proteins.

Materials and Methods

Human subjects

Blood samples were obtained from 21 healthy donors without prior *M. tuberculosis* infection and 8 donors with latent tuberculosis infection, based on QuantiFERON-TB Gold test results. All studies were approved by the Institutional Review Board of The University of Texas Health Science Center at Tyler, and signed consent forms from all study subjects were obtained before collection of blood samples.

M. tuberculosis *culture and cell stimulation*

Heat-killed *M. tuberculosis* Erdman (provided by Dr. Patrick Brennan, Colorado State University, Fort Collins, CO) and live *M. tuberculosis* H37Rv and its *esat-6* (Rv3875) deletion mutant H37RvΔ3875 (provided by Dr. David Sherman, University of Washington, Seattle, WA) were used. H37Rv and H37RvΔ3875 were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol and 10% ADC enrichment (Remel). Logarithmically growing cultures were sonicated briefly and centrifuged at 800 rpm for 10 min to eliminate clumped mycobacteria. The upper part of the culture was collected and the bacterial concentration was determined by measuring OD using the formula: 1 $OD_{600} = 3 \times 10^8$ CFU/ml. The concentration was confirmed by plating serially diluted bacterial suspensions on 7H10 agar and counting CFUs after 3 wk.

Preparation of rESAT-6

The recombinant plasmid containing *Rv3875*, encoding ESAT-6, was obtained through the TB Vaccine Testing and Research Materials Contract (Colorado State University), and rESAT-6 was prepared, as described previously (18). Analysis of rESAT-6 preparations with gel filtration chromatography by fast protein liquid chromatography showed no protein aggregates, and >95% of the protein formed a peak of ~24 kDa, probably representing ESAT-6 homodimers (results not shown).

Generation and culture of DCs and detection of cytokines

PBMCs were isolated by differential centrifugation of heparinized blood over Ficoll-Paque (GE Healthcare Life Sciences). $CD14^+$ and $CD4^+$ cells were purified from PBMCs by positive immunomagnetic selection, and $CD3^+$ cells were purified by negative selection (human pan T cell isolation kit) (all from Miltenyi Biotec). Cell purity was >98% as measured by immunolabeling and flow cytometry analysis with a FACSCalibur (BD Biosciences). To generate iDCs, $CD14^+$ cells were cultured at 10^6 cells/ml in RPMI 1640 (Invitrogen), supplemented with 10% heat-inactivated pooled human serum (Atlanta Biologicals), 100 U or 100 μg/ml penicillin and streptomycin, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids (all from Invitrogen), and 25 μg/ml human GM-CSF and IL-4 (both from R&D Systems) for 3–5 d. The iDCs were further stimulated with human CD40 ligand trimer (CD40LT; Immunex, Seattle, WA) at 2.5 μg/ml plus LPS (Sigma-Aldrich) at 1 μg/ml, with or without ESAT-6, for 24 h. In some experiments, $CD3^+$ cells were cultured at 2×10^6/ml with autologous or allogeneic DCs in 96-well flat-bottom plates for 3–5 d. Cell-free supernatants were collected and cytokine concentrations were measured by ELISA, using capture and detection Abs for IFN-γ and TNF-α (BD Biosciences), and ELISA kits for IL-2, IL-12p70 (both from BD Biosciences), IL-17, IL-8 (both from R&D Systems), IL-1β, IL-23 (both from Mabtech), and IL-6 (BioLegend). The detection limits of these kits are 15–25 pg/ml.

In some experiments, iDCs were infected with live H37Rv and H37RvΔ3875 at a multiplicity of infection of 10 for 4 h, and free bacteria were washed off with prewarmed culture media before stimulation with LPS plus CD40LT. The concentrations of IL-12p70, IL-23 and IL-1β in 24-h culture supernatants were measured by ELISA, as described above.

Flow cytometry

Expression of CD80, CD86, HLA-DR, and CD83 was measured by incubating DCs with FITC-anti-CD80 (clone 2D10), PE-anti-CD86 (clone IT2.2), FITC-anti–HLA-DR (clone L243) (all from BioLegend) or FITC-anti-CD83 (clone HB15) (eBioscience) on ice for 30 min. Interaction of ESAT-6 with DCs was examined by incubation of iDCs with Alexa Fluor 488-labeled ESAT-6 after confirming that labeled ESAT-6 inhibits T cell IFN-γ production with the same potency as unlabeled ESAT-6. The specificity of interaction was examined by blocking experiments by incubating iDCs with increasing concentrations of unlabeled ESAT-6 or BSA as control prior to incubation with 20 μg/ml labeled ESAT-6. Flow cytometry analysis was performed with a FACSCalibur, using FlowJo software (Tree Star).

Cell viability and proliferation assays

Cell viability was measured by the MTT cleavage assay (USB). Proliferation of $CD4^+$ cells in response to allogeneic stimulation was measured by the CFSE dilution assay. Briefly, purified cells were labeled with CFSE (Invitrogen) as described (18) and incubated with allogeneic DCs in a 96-well flat-bottom plate. After 4 d, the cells were collected and proliferation was analyzed by flow cytometry.

Cytokine mRNA quantification by real-time PCR

Total RNA was extracted from 2.5×10^5 DCs with TRIzol reagent (Invitrogen), cDNA was synthesized, and IL-12p35 (p35), IL-23p19 (p19), and IL-12/23p40 mRNA were quantified by minor modifications of our published methods (18). The relative quantity of each mRNA was calculated by the $\Delta\Delta C_t$ method (34).

Depletion of ESAT-6

ESAT-6 was removed from the recombinant protein preparations, as described previously (18). Briefly, 500 μg rESAT-6 was incubated with 250 μl activated nickel resin in a 1.5-ml Eppendorf tube at room temperature. For sham depletion, ESAT-6 was incubated with unactivated nickel resin. After 30 min incubation, samples were centrifuged to pellet the resin, and the resin-free supernatants were collected as ESAT-6–depleted samples. The protein content of the samples was measured by the bicinchoninic acid assay. Samples were also subjected to SDS-PAGE, followed by Coomassie blue staining and Western blotting with anti–ESAT-6 (HYB 76-8; provided by Dr. Peter Andersen, Statens Seruminstitut, Copenhagen, Denmark).

ESAT-6–conditioned DC medium and T cell cytokine production

iDCs were stimulated with LPS plus CD40LT, with or without ESAT-6, for maturation. Twenty-four hours later, the cell-free supernatants were harvested and stored at −20°C. Negatively selected $CD3^+$ T cells from PBMCs of healthy donors were resuspended in RPMI 1640 with 10% heat-inactivated pooled human AB serum and plated at 4×10^5 cells in 100 μl/well in a 96-well flat-bottom culture plate precoated with anti-CD3 and anti-CD28. Another 100 μl conditioned medium was added to each well. The cells were incubated at 37°C and 5% CO_2. Forty-eight hours later, cell-free supernatants were harvested and IFN-γ and IL-17 levels were measured by ELISA. For cytokine neutralization experiments, the $CD3^+$

cells with conditioned medium were incubated with anti–IL-23 or anti–IL-1β at 10 μg/ml for 1 h before being cultured in a 96-well plate precoated with anti-CD3 plus anti-CD28.

Preparation of DC nuclear protein extracts

DCs were incubated in a 12-well plate at 1×10^6 cells/ml, with or without 20 μg/ml ESAT-6 for 1 h, followed by stimulation with LPS plus CD40LT for 4 h. DCs were then collected by scraping with rubber policemen, washed twice with prechilled PBS, and cytosolic and nuclear protein extracts were prepared, as described (35). The protein concentration was measured by the bicinchoninic acid assay, and the extracts were kept at –70°C in aliquots until use.

Detection of transcription factors by Western blotting

Twenty-five micrograms of nuclear and cytosolic protein extracts of DCs was resolved by 10% SDS-PAGE in reducing conditions, electroblotted to a nitrocellulose membrane, and expression and phosphorylation of the transcription factors IRF-1, activating transcription factor (ATF)-2, and c-Jun AP-1 were evaluated by Western blotting, as described previously (18), using Abs against IRF-1 (H-205), ATF-2 (c-19), and c-Jun AP-1 (H79), all from Santa Cruz Biotechnology. To control for protein loading, the blot was stripped and expression of GAPDH was evaluated by immunoblotting (FL-335; Santa Cruz Biotechnology).

Detection of DNA bindng activities of AP-1 transcription factors by EMSA

The promoter binding activity of AP-1 transcription factors in DCs was evaluated by EMSAs, using our published methods (35). DC nuclear protein extracts were used as transcription factor sources and the probe was a radiolabeled AP-1 binding site of the human p19 promoter, which corresponds to the nucleotides from −228 to −198, relative to the p19 transcription start site. To characterize the binding specificity, we used unlabeled oligonucleotide DNAs: consensus AP-1 binding site, human p19 AP-1 binding site, and NF-κB consensus binding site for competition assays. To identify proteins in DNA binding complexes, we used Abs against ATF-2 (polyclonal C-19 and monoclonal F2BR-1), anti–c-Jun (H79), anti-CREB mAb, and control IgG, all from Santa Cruz Biotechnology.

Detection of recruitment of IRF-1 to the IL-12p35 promoter in live DC by chromatin immunoprecipitation

iDCs were stimulated with LPS plus CD40LT, with or without ESAT-6, for 6 h and then treated with 1% formaldehyde at room temperature with shaking. After 10 min, the cells were treated with 2 M glycine to neutralize free formaldehyde. The preparation of DC chromatin and the chromatin immunoprecipitation assay was performed as described previously (36) using anti–IRF-1 (H-205) for immunoprecipitation and a chromatin immunoprecipitation assay kit (Millipore). The amount of IRF-1–bound IL-12p35 promoter in the samples was determined by PCR, using specific primer sets: forward, 5′-GAACATTTCGCTTTCATTTTGGG-3′; reverse, 5′-ACTCTGGTCTCTTGCTTTCT-3′. This yields a 187-bp fragment of the p35 promoter containing the IRF-1 binding site. To control for equal sample loading, the same DNA fragment of p35 was amplified by PCR from an equal amount of chromatin supernatants, prior to immunoprecipitation.

Statistical analysis

A Student *t* test was used to evaluate differences between groups. A p value of <0.05 was considered to be statistically significant.

Results

ESAT-6 inhibits DC maturation

Because DCs are essential for eliciting protective immunity against *M. tuberculosis* (37), and this bacterium targets DC functions (38, 39), we hypothesized that ESAT-6 may play a role in this process. To test this hypothesis, we first generated iDCs by culturing $CD14^+$ monocytes with GM-CSF and IL-4 for 5 d (40). We stimulated iDCs through TLR4 and CD40 with their respective ligands, LPS and CD40LT, which induce DC maturation (41). LPS/CD40LT treatment yielded mDCs, which showed upregulation of the costimulatory molecules CD86 and CD80, MHC class II molecule HLA-DR, and the DC maturation marker CD83 (Fig. 1). The presence of ESAT-6 during maturation diminished expression of these surface markers (Fig. 1). When human DCs were purified directly from PBMCs by positive selection, using magnetic beads conjugated with anti-CD304 (BDCA4/neuropilin; Miltenyi Biotec) and treated with LPS/CD40LT, with or without ESAT-6, ESAT-6 inhibited upregulation of CD86, CD80, and HLA-DR (data not shown). Because of the extremely low numbers of primary DCs, further experiments were performed with monocyte-derived DCs.

ESAT-6 inhibits Th1 and enhances Th17 responses induced by DCs

To evaluate the functional effects of ESAT-6 on DC maturation, we examined the allostimulatory capacity of DCs matured in the presence of ESAT-6 by coculturing with allogeneic T cells. iDCs from four donors were stimulated with LPS/CD40LT, with or without ESAT-6, and cultured with CFSE-labeled allogeneic $CD4^+$ T cells. ESAT-6 reduced the capacity of DCs to stimulate proliferation by allogeneic T cells, particularly at low DC/T cell ratios (Fig. 2A, 2B). ESAT-6 also markedly inhibited T cell production of IFN-γ by five donors (Fig. 2C) and had similar effects on IL-2 production (Fig. 2D). DCs matured in the presence of ESAT-6 increased T cell IL-17 production, with marked differences at higher DC/T cell ratios (Fig. 2E). Thus, iDCs matured in the presence of ESAT-6 reduced proliferation and Th1 cytokine production by allogeneic T cells, but enhanced production of IL-17. To determine whether ESAT-6 had similar effects on T cells stimulated with mycobacterial Ags, we prepared iDCs and autologous $CD4^+$ T cells from four donors with latent tuberculosis infection. ESAT-6–treated DCs induced less IFN-γ and more IL-17 production by T cells stimulated with heat-killed *M. tuberculosis* or infected with live bacilli (Fig. 3). These results together suggested that DCs matured with ESAT-6 support Th17 cells at the expense of Th1 cells.

ESAT-6 differentially regulates DC cytokine production

Because DCs matured in the presence of ESAT-6 increased IL-17 and reduced IFN-γ production by T cells, and cytokines produced

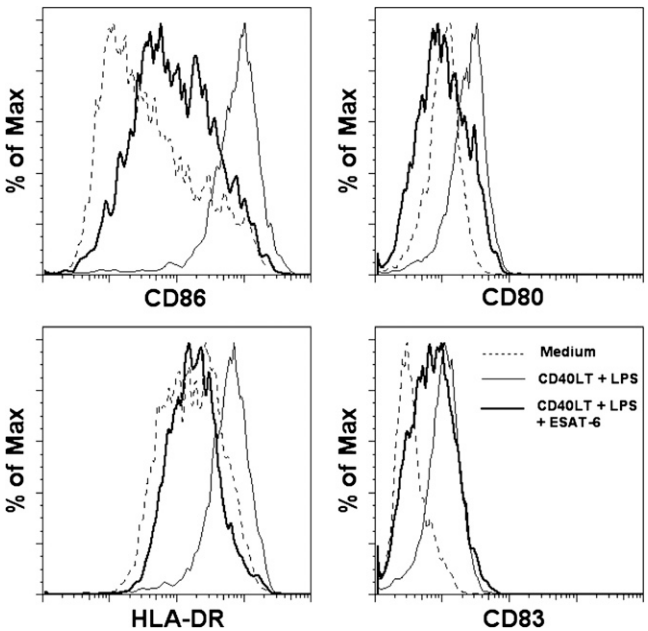

FIGURE 1. ESAT-6 inhibits DC maturation. iDCs from four donors were stimulated with LPS/CD40LT for maturation with or without 20 μg/ml ESAT-6. Twenty-four hours after stimulation, cells were stained with different Abs to costimulatory molecules and maturation markers and analyzed by flow cytometry. One representative result from four different experiments is shown for each molecule.

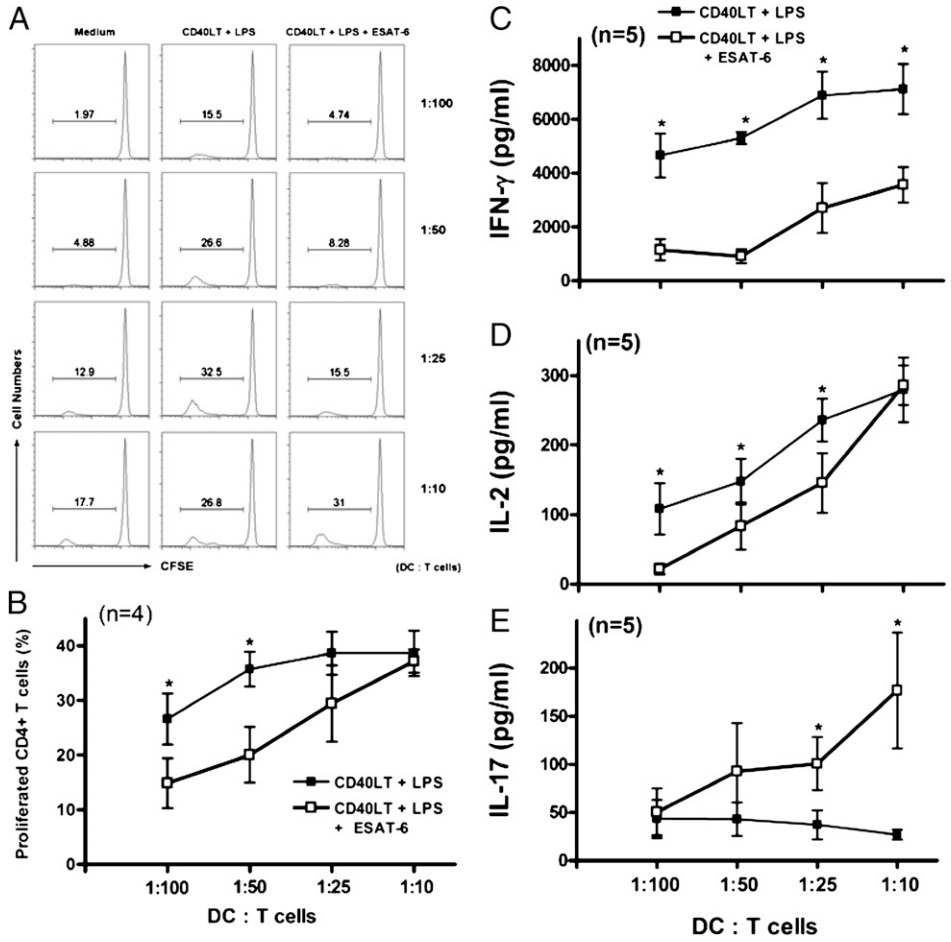

FIGURE 2. DCs matured in the presence of ESAT-6 inhibit production of Th1 cytokines and enhance production of IL-17 by allogeneic T cells. iDCs generated from four healthy donors were matured with LPS/CD40LT with or without 20 μg/ml ESAT-6. After extensive washing to remove free ESAT-6 and LPS/CD40LT, varying numbers of mDCs, ranging from 4×10^3 to 4×10^4, were incubated with 4×10^5 CFSE-labeled, allogeneic $CD4^+$ T cells at the indicated ratios. (**A**) After 4 d, the percentages of proliferating cells were determined by flow cytometry. A representative result is shown. (**B**) The means and SEMs of the percentage of proliferated $CD4^+$ cells are shown from four experiments. (**C**–**E**) The supernatants from cocultures of mDCs and allogeneic $CD4^+$ T cells from five donors were collected after 24 h for measurement of IL-2 (D) and after 96 h for measurement of IFN-γ (C) and IL-17 (E) by ELISA. Means and SEMs are shown. $*p < 0.05$ (B–E) compared with cells stimulated with LPS/CD40LT at the same DC/T cell ratio.

by DCs strongly affect T cell differentiation (42), we examined the effect of ESAT-6 on DC cytokine production. Stimulation of iDCs with LPS/CD40LT induced robust production of IL-12, IL-23, TNF-α, IL-6, and IL-8 but minimal amounts of IL-1β (Fig. 4A–F). ESAT-6 inhibited DC maturation-induced IL-12 production and induced production of IL-1β in a dose-dependent manner (Fig. 4A, 4C) and increased IL-23 production (Fig. 4B). However, ESAT-6 did not affect secretion of TNF-α, IL-6, or IL-8 (Fig. 4D–F). ESAT-6 alone, in the absence of LPS/CD40LT, did not stimulate production of any cytokines except for minimal levels of IL-6 and IL-8. Because ESAT-6 activates the inflammasome and caspase-1–dependent production of IL-1β in monocytes (43), we

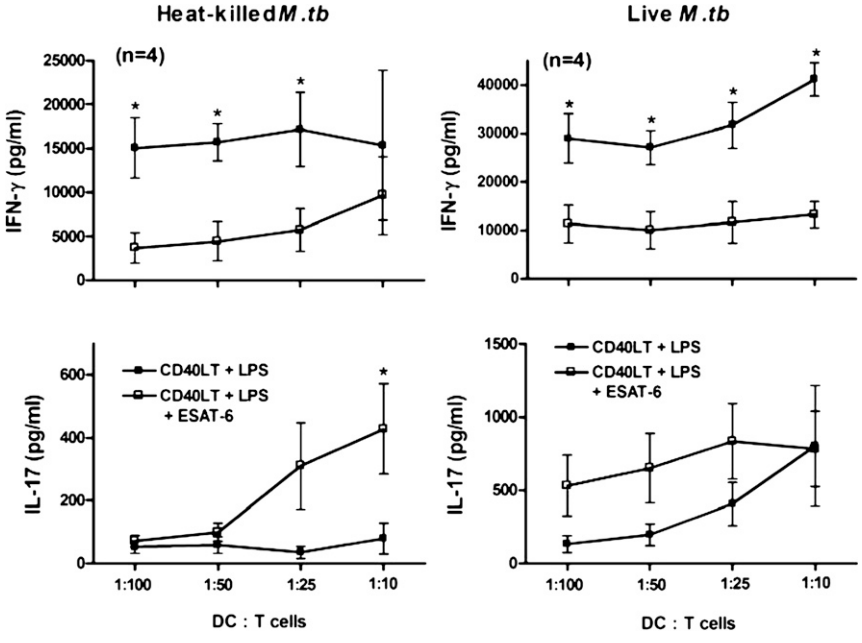

FIGURE 3. DCs matured with ESAT-6 stimulate IL-17 and inhibit IFN-γ production by *M. tuberculosis*-responsive T cells. iDCs from four donors with latent tuberculosis infection were matured with LPS/CD40LT, with or without ESAT-6, for 24 h. The cells were then washed and incubated with autologous $CD3^+$ cells at different ratios, and stimulated with heat-killed *M. tuberculosis* at 2.5 μg/ml (*left panels*) or infected with H37Rv at a multiplicity of infection of 20 (*right panels*). Forty-eight hours later, the supernatants were harvested and IFN-γ and IL-17 levels were measured by ELISA. Means and SEMs are shown. $*p < 0.05$ compared with cells matured with LPS/CD40LT at the same DC/T cell ratio.

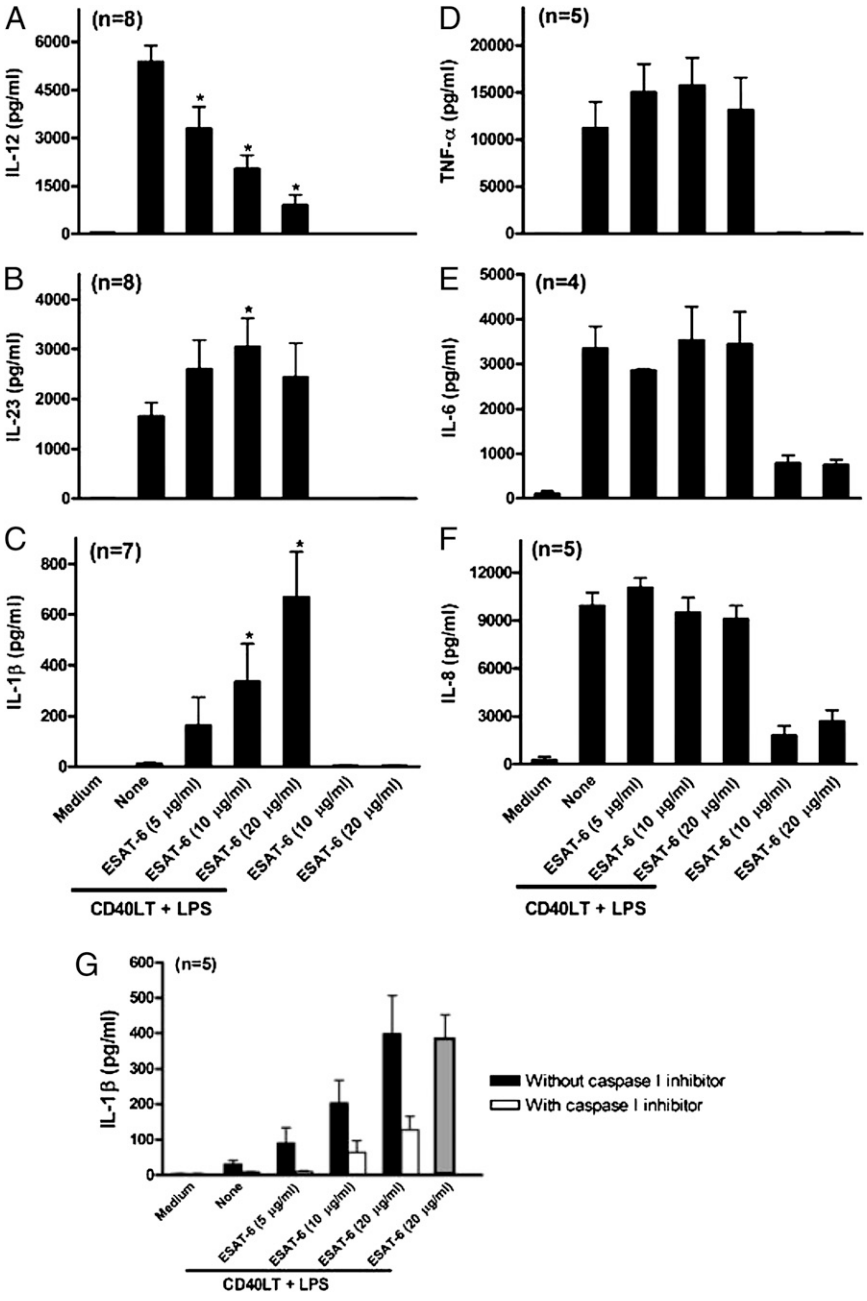

FIGURE 4. DCs matured in the presence of ESAT-6 produce less IL-12 and more IL-23 and IL-1β. iDCs were stimulated with LPS/CD40LT, with or without ESAT-6, and some cells were incubated with ESAT-6 only. Twenty-four hours after stimulation, the supernatants were collected and levels of IL-12p70 (**A**), IL-23 (**B**), IL-1β (**C**), TNF-α (**D**), IL-6 (**E**), and IL-8 (**F**) were measured by ELISA. (**G**) Some cells were treated with a caspase-1 inhibitor (20 μM) prior to stimulation with LPS/CD40LT and ESAT-6. Twenty-four hours later, supernatants were collected and IL-1β levels were measured. The *far right gray bar* represents cells treated with DMSO only as a vehicle control. For all panels, means and SEMs are shown. $*p < 0.05$ compared with cells stimulated with LPS/CD40LT only.

incubated iDCs from four donors with a caspase-1 inhibitor before treatment with LPS/CD40LT and ESAT-6. ESAT-6 increased IL-1β production in a dose-dependent manner, but this was markedly reduced by caspase-1 inhibition (Fig. 4G), demonstrating that this effect requires caspase-1.

The effects of ESAT-6 on DC cytokine production are not mimicked by other M. tuberculosis *Ags and are not due to contaminants in recombinant ESAT-6*

To determine whether the effects of ESAT-6 on DC cytokine production were specific for this mycobacterial protein, we tested Ag85A, an immunogenic secreted protein of *M. tuberculosis* (44). Using iDCs from four donors, ESAT-6 reduced IL-12 secretion and increased IL-23 and IL-1β production, but Ag85A did not (Fig. 5A). To further test whether the effect of ESAT-6 on DC cytokine production is not due to nonspecific effects of ESAT-6 aggregates, we dissolved both rESAT-6 and Ag85A with organic solvent DMSO and tested the effects of solubilized proteins on cytokine production by iDCs from four donors. DMSO-treated ESAT-6, but not Ag85A, inhibited IL-12 production by iDCs in response to maturation stimulation in the same manner as non-DMSO–treated ESAT-6, further suggesting the specific effect of ESAT-6 on DC cytokine production (data not shown).

To confirm that the effects of ESAT-6 were not due to contaminants, we depleted ESAT-6 from recombinant protein preparations using a nickel resin, as outlined in *Materials and Methods*. The depletion process removed 99% of total protein, as measured by the bicinchoninic acid protein assay and confirmed by Western blot for ESAT-6 (data not shown). Depletion of ESAT-6 restored IL-12 production by iDCs, but sham depletion had no effect (Fig. 5B), indicating that IL-12 inhibition was not due to contaminants. Because ESAT-6 lyses human lung epithelial cells and monocytic cells, probably through apoptosis (14, 45), and apoptotic cells inhibit DC IL-12 production (46), we determined whether ESAT-6

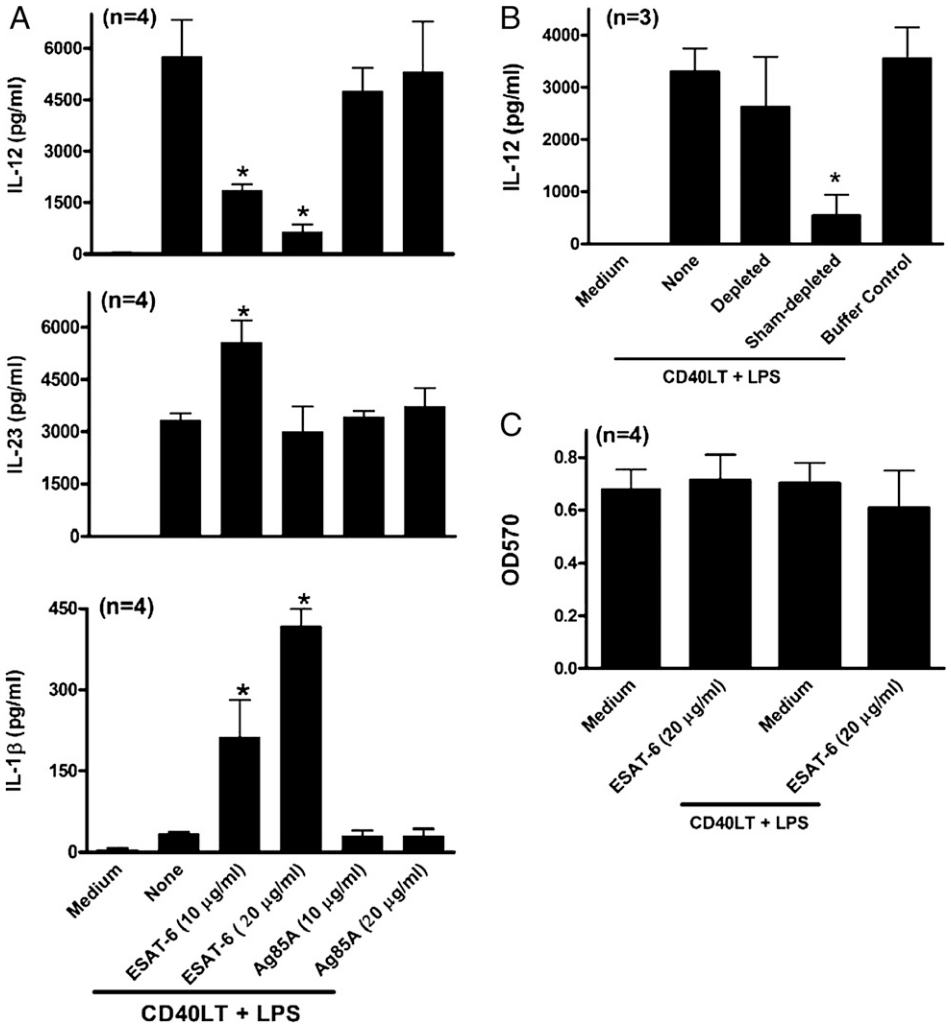

FIGURE 5. The effects of ESAT-6 on DC cytokine production are not mimicked by Ag85A and are not due to contaminants in recombinant ESAT-6 or cell death. (**A**) iDCs were matured by stimulation with LPS/CD40LT with or without ESAT-6 or Ag85A at different concentrations as indicated. Twenty-four hours later, culture supernatants were collected and IL-12p70, IL-23, and IL-1β levels were measured by ELISA. (**B**) iDCs were stimulated with LPS/CD40LT, in the presence of ESAT-6 preparations, depleted or sham-depleted of ESAT-6 with a nickel column, or with a depletion buffer without ESAT-6. Twenty-four hours later, supernatants were harvested and IL-12 levels were measured. (**C**) iDCs were stimulated with LPS/CD40LT, with or without ESAT-6, for 24 h, and cell viability was evaluated with an MTT assay. For all panels, means and SEMs are shown. *$p < 0.05$ compared with cells stimulated with LPS/CD40LT only.

affects DC viability. The highest concentration of ESAT-6 used in our experiments (20 μg/ml), either alone or with LPS/CD40LT, did not reduce viability of iDCs after 24–96 h (Fig. 5C and data not shown), based on the MTT assay, indicating that ESAT-6 was not cytotoxic in our experimental system.

ESAT-6 does not affect DC cytokine production through cAMP or p38 MAPK

We considered the possibility that ESAT-6 reduced DC IL-12 production through increasing intracellular cAMP, as other bacterial toxins, such as adenylate cyclase toxin of *Bordetella pertusis*, act through this mechanism (47, 48). However, blocking cAMP with the cAMP-specific chemical inhibitor Rp-cAMP did not affect ESAT-6–induced differential regulation of DC cytokine production (Supplemental Fig. 1), and treatment with ESAT-6 did not affect cAMP levels in iDCs (data not shown), indicating that cAMP does not mediate the effects of ESAT-6 on DC cytokine production.

Next, we determined whether p38 MAPK mediates the effects of ESAT-6 on DC cytokine production, because ESAT-6 inhibits T cell IFN-γ production through this signaling pathway (19). Consistent with previous reports (49, 50), SB203580, a specific p38 MAPK inhibitor, reduced LPS/CD40LT-stimulated DC IL-12 production. SB203580 further reduced ESAT-6 inhibition of IL-12 production (Supplemental Fig. 2), making it unlikely that ESAT-6 acted through p38 MAPK. The results were more definitive for IL-23 and IL-1β. In both cases, SB203580 did not affect cytokine production and did not abrogate the effects of ESAT-6 (Supplemental Fig. 2). Thus, p38 MAPK does not contribute to ESAT-6–mediated differential regulation of DC cytokine production.

ESAT-6 binds to DCs with specificity

Although DCs are known to take up non-self components, such as bacterial proteins, for Ag presentation, we tested whether ESAT-6 interacts with DCs specifically by incubating iDCs from three different donors with Alexa Fluor 488-labeled ESAT-6. The results demonstrated that ESAT-6 binds to iDCs (Fig. 6A), and this binding is saturable (Fig. 6B), as successive doublings of ESAT-6 concentrations led to proportionately smaller increases in mean fluorescence intensities of DC-bound labeled ESAT-6. Prior incubation of iDCs with increased concentrations of unlabeled ESAT-6 (Fig. 6C) but not BSA (Fig. 6D) progressively reduced binding of labeled ESAT-6 to iDCs with significant reduction at 160 μg/ml (from a mean fluorescence intensity of 55 to 22), suggesting that interaction of ESAT-6 with iDCs is specific and reversible. We could not achieve full blocking in binding since ESAT-6 at ≥320 μg/ml was toxic. In conclusion, ESAT-6 interacts with human iDCs specifically, providing mechanistic clues for specific effects of ESAT-6 on DC maturation and cytokine production.

ESAT-6–treated DCs favor T cell production of IL-17 through IL-23 and IL-1β

The data above (Figs. 2–4) show that ESAT-6–treated DCs enhanced T cell IL-17 and reduced IFN-γ production, and that ESAT-6 increased DC production of IL-23 and IL-1β and reduced

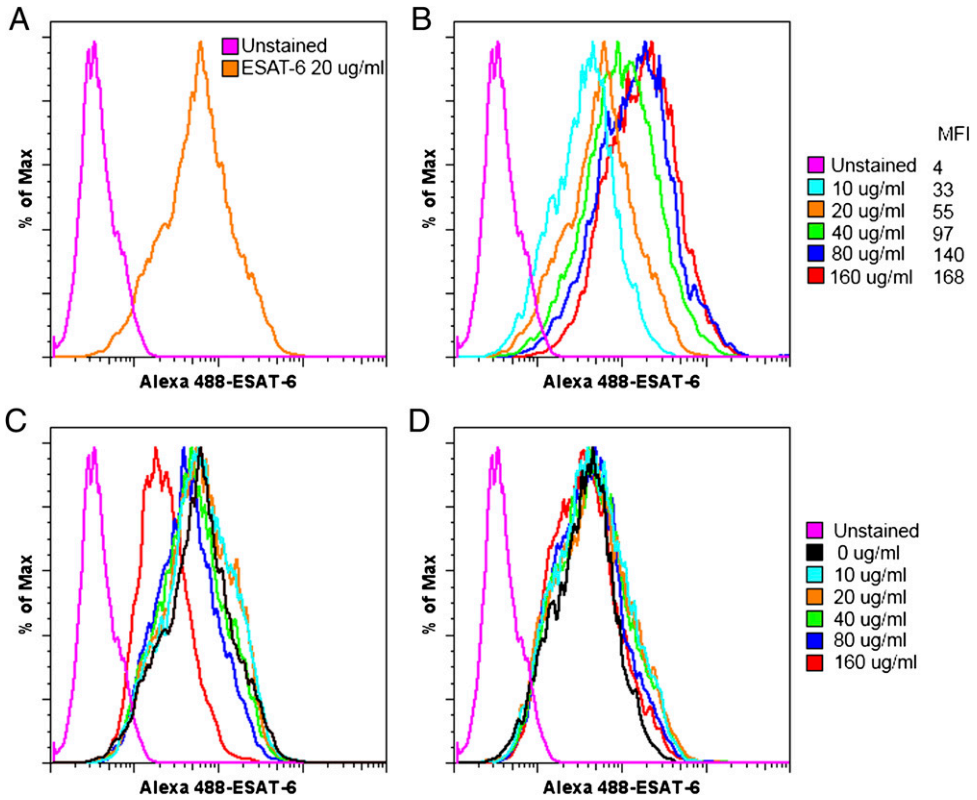

FIGURE 6. ESAT-6 interacts DCs with specificity. iDCs from three healthy donors were incubated with 20 μg/ml (**A**) or different concentrations of Alexa Fluor 488-labeled ESAT-6 as indicated (**B**). One hour later, the cells were washed and examined by flow cytometry. MFI, mean fluorescence intensities for corresponding concentrations of labeled ESAT-6. For blocking experiments, iDCs were incubated with increasing concentrations of unlabeled ESAT-6 (**C**) or BSA (**D**) as indicated for 30 min prior to incubation with 20 μg/ml labeled ESAT-6. The cells were washed and analyzed by flow cytometry. One representative result from three different experiments is shown for all panels.

that of IL-12. To link these findings, we added media from LPS/CD40LT-treated iDCs, with or without ESAT-6, to $CD3^+$ cells from six donors, stimulated with anti-CD3 plus anti-CD28. T cells treated with conditioned media from unstimulated DCs induced low IL-17 levels (132 ± 22 pg/ml), which were increased slightly by medium from DCs stimulated with LPS/CD40LT or ESAT-6 alone (Fig. 7A). However, medium from DCs treated with LPS/CD40LT in the presence of ESAT-6 increased IL-17 levels 6-fold (739 ± 80 pg/ml). In contrast to the effects on IL-17, medium from LPS/CD40LT-treated DCs almost doubled IFN-γ levels compared with that with DC medium without stimulation, but medium from DCs treated with LPS/CD40LT in the presence of ESAT-6 significantly reduced IFN-γ concentrations close to the level of T cells with conditioned medium from unstimulated DCs (Fig. 7B). Addition of neutralizing Abs to IL-23 or IL-1β to conditioned media from DCs treated with ESAT-6 and LPS/CD40LT modestly reduced IL-17 production by activated $CD3^+$ cells, and addition of both Abs together decreased IL-17 concentrations by 70–80% (Fig. 7C), suggesting that IL-23 and IL-1β contributed significantly to the capacity of ESAT-6–treated DCs to elicit IL-17 secretion by T cells. Taken together, these results indicated that DCs matured in the presence of ESAT-6 generate a condition that favors expansion of Th17 over Th1 cells.

ESAT-6 inhibits IL-12 production by DCs stimulated through CD40 and multiple TLRs

To determine whether ESAT-6 affects DC cytokine production in response to TLR ligands other than TLR4, we stimulated iDCs from three donors with nine different TLR ligands, together with CD40LT. Agonists for TLR1/2 (Pam3CSK4), TLR2 (heat-killed *Listeria monocytogenes*), TLR5 (flagellin), TLR6/2 (FSL-1 or Pam2CGDPKHPKSF), and TLR8 (single-stranded RNA) induced significant levels of both IL-12p70 (Supplemental Fig. 3A) and IL-23 (data not shown). ESAT-6 significantly reduced IL-12 production by DCs from six different donors in response to all five TLR ligands (Supplemental Fig. 3B), but it did not clearly affect IL-23 production (data not shown). Therefore, ESAT-6 may inhibit IL-12 production by DCs in response to multiple TLR agonists by targeting a common signaling pathway.

ESAT-6 inhibits transcription of p35 and enhances that of p19

IL-12 and IL-23 are heterodimeric cytokines composed of the p35 and p19 polypeptides, respectively, and a shared p40 chain (51, 52). To determine whether ESAT-6 regulated DC production of IL-12 and IL-23 through transcriptional regulation, we measured mRNA expression by real-time PCR. Consistent with previous reports (52, 53), LPS/CD40LT stimulation of iDCs induced robust transcription of all three polypeptide genes. ESAT-6 markedly inhibited p35 transcripts (Fig. 8A) in a dose-dependent manner, greatly enhanced p19 mRNA expression (Fig. 8C), and did not affect that of p40 (Fig. 8B). These results support previous findings that expression of p35 and p19 are the rate limiting factors for APC production of functional IL-12 and IL-23, respectively (54, 55), and that ESAT-6 reduced IL-12 and increased IL-23 production by DCs through differentially regulating expression of these peptides.

ESAT-6 affects transcription factors that control p35 and p19 mRNA expression

To understand the mechanisms by which ESAT-6 affects mRNA expression of p35 and p19, we evaluated the effect of ESAT-6 on

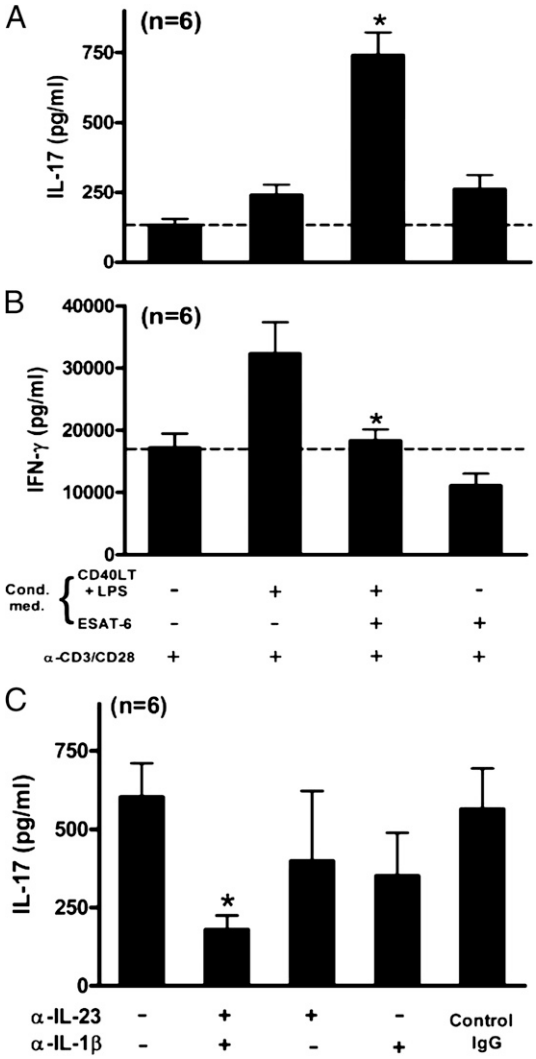

FIGURE 7. ESAT-6–treated DCs stimulate T cell IL-17 production through IL-23 and IL-1β. Purified $CD3^+$ cells from healthy donors were stimulated with plate-bound anti-CD3 plus anti-CD28 in a 96-well plate, with each well containing 100 μl RPMI 1640 and 10% human serum and 100 μl conditioned medium from DCs, matured under the conditions as indicated. Forty-eight hours later, supernatants were harvested and IL-17 (**A**) and IFN-γ (**B**) levels were measured by ELISA. (**C**) Purified $CD3^+$ cells were cultured in 180 μl RPMI 1640 with 10% human serum and 20 μl conditioned medium from DCs, matured by LPS/CD40LT in the presence of ESAT-6. Some cells were treated with neutralizing Abs or isotype control IgG for 1 h, prior to stimulation with anti-CD3 plus anti-CD28 in a 96-well plate. Forty-eight hours later, supernatants were harvested and IL-17 levels were measured by ELISA. Means and SEMs are shown for all three panels. $*p < 0.05$ compared with the cells stimulated in the presence of conditioned medium from DCs matured with LPS/CD40LT (A, B) or compared with the cells stimulated in the presence of control IgG (C).

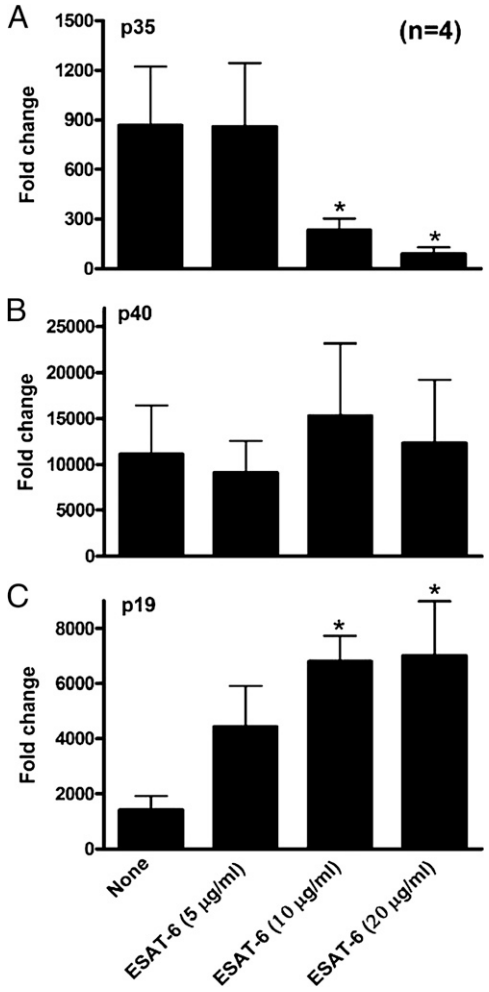

FIGURE 8. ESAT-6 inhibits DC transcription of p35 and enhances that of p19. iDCs were matured with LPS/CD40LT with or without ESAT-6 at 20 μg/ml. Sixteen hours later, the cells were collected and mRNA was quantified by real-time PCR for p35 (**A**), p40 (**B**), and p19 (**C**) after normalization for 18S rRNA content. Results are shown as the fold change relative to that of unstimulated DCs. Means and SEMs for four donors are shown. $*p < 0.05$ compared with cells stimulated with LPS/CD40LT only.

transcription factors that are downstream from the TLR signaling pathways and control transcription of these polypeptides. IRF-1 induces transcription of p35 by binding to the p35 promoter (56), but also inhibits p19 transcription by blocking RelA binding to the p19 κ-site (57). In contrast, STAT-3 enhances transcription of p19 through its promoter but inhibits that of p35 by preventing binding of c-Rel to the p35 promoter (58). The AP-1 transcription factors ATF-2 and c-Jun positively regulate transcription of p19 (59).

IRF-1 expression in DCs was increased by stimulation with LPS/CD40LT, but it was reduced by addition of ESAT-6 during DC maturation (Fig. 9A). Furthermore, binding of IRF-1 to the p35 promoter in live DCs was reduced, based on chromatin immunoprecipitation (Fig. 9D). LPS/CD40LT increased expression of phospho-STAT3 in DCs, but ESAT-6 inhibited STAT3 phosphorylation (data not shown), indicating that STAT3 does not mediate the effects of ESAT-6 on transcription of p35 and p19. Phosphorylation of ATF-2 and c-Jun was increased by stimulation with LPS/CD40LT, and it was further enhanced by ESAT-6 (Fig. 9A). To determine whether phosphorylation of these transcription factors correlates with promoter binding activity, we performed an EMSA using the AP-1 binding site of p19 as a probe. Stimulation of iDCs with LPS/CD40LT increased AP-1 binding activity, and this was further enhanced by ESAT-6 (Fig. 9B). DNA binding was specific, as excess unlabeled oligonucleotides with the consensus AP-1 sequence or the p19 AP-1 site blocked the formation of DNA/protein complexes, whereas the NF-κB consensus oligonucleotide did not (Fig. 9C). Anti–c-Jun supershifted and anti–ATF-2 blocked the formation of this complex (Fig. 9C), suggesting that

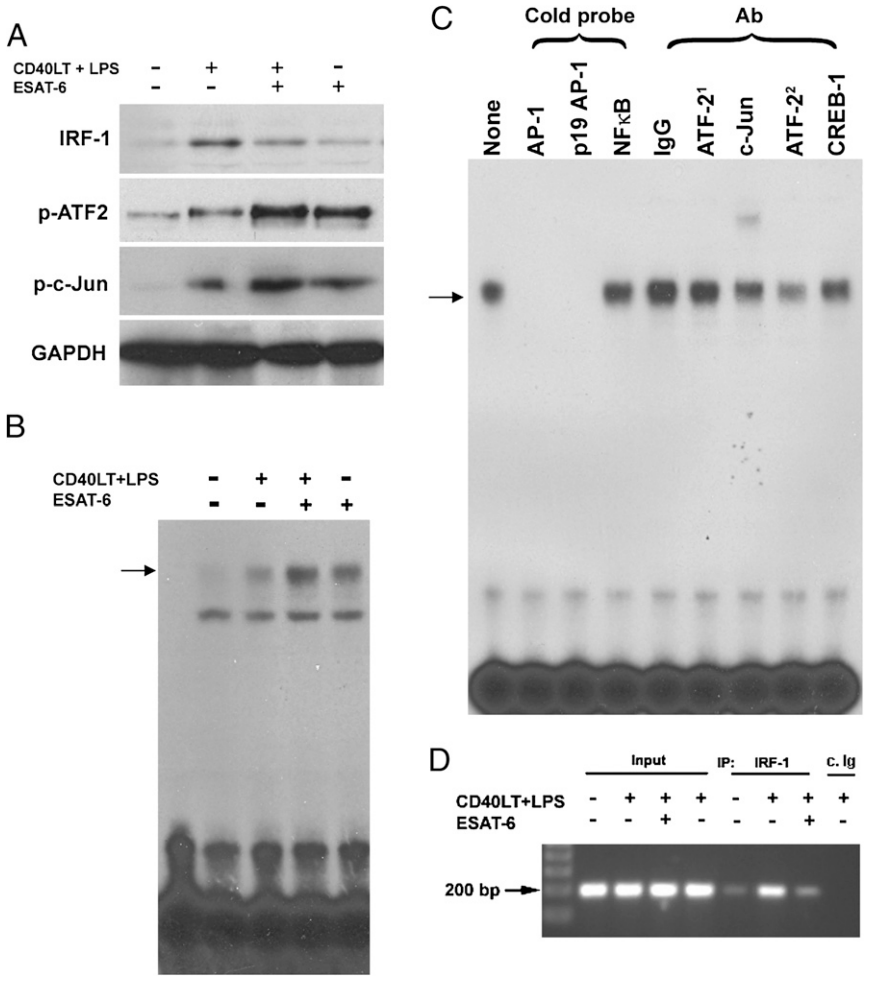

FIGURE 9. ESAT-6 differentially regulates expression and DNA binding activities of IRF-1 and AP-1 transcription factors. (**A**) iDCs from four donors were stimulated with LPS/CD40LT with or without ESAT-6. Four hours later, total cell protein extracts were prepared and Western blotting was performed to determine expression of IRF-1 and phosphorylation of the AP-1 transcription factors ATF-2 and c-Jun. The blots were stripped and reblotted for expression of GAPDH as a protein loading control. A representative result is shown. (**B**) iDCs from four donors were matured as in (A), and DNA binding activity of the nuclear protein extracts was evaluated by EMSAs using the radiolabeled AP-1 binding site of the p19 promoter as a probe. The DNA/protein complexes were resolved by 5% nondenaturing PAGE and visualized by autoradiography. A representative result is shown. (**C**) Nuclear extracts of DCs from four donors, matured with LPS/CD40LT and ESAT-6, were incubated with unlabeled dsDNA or different IgGs, as indicated, for 30 min, followed by incubation with the radiolabeled AP-1 site of p19 and visualized as in (B). A representative result is shown. ATF-2^1, polyclonal Ab; ATF-2^2, mAb. (**D**) Chromatin immunoprecipitation. iDCs from four donors were treated with LPS/CD40LT, with or without ESAT-6, as described in (A). Six hours later, the cells were incubated with 1% formaldehyde, followed by preparation of nuclear chromatin supernatants and immunoprecipitation with anti–IRF-1 or isotype-matched control IgG (c.Ig). The precipitated DNA samples were amplified with primers for a 187-bp DNA fragment that contains the IRF-1 binding site of the p35 promoter. The PCR product was visualized by 1% agarose gel analysis. A representative result is shown.

ATF-2 and c-Jun contribute to the increased DNA binding activity that accompanies iDC maturation. In summary, these results suggest that ESAT-6 induces differential transcription of p35 and p19 through inhibition of IRF-1 expression and upregulation of ATF-2 and c-Jun activation.

M. tuberculosis *lacking ESAT-6 elicits differential cytokine production by iDCs*

The studies above were performed with rESAT-6. To determine whether ESAT-6 produced by live *M. tuberculosis* had similar effects on DCs, we infected iDCs with *M. tuberculosis* H37Rv and its *esat-6* deletion mutant, H37RvΔ3875. Both H37Rv and H37RvΔ3875 inhibited LPS/CD40LT-induced IL-12 production, but inhibition was significantly greater with H37Rv (Fig. 10A), confirming our findings with rESAT-6. Similarly, H37Rv induced 3- to 4-fold more IL-1β than did H37RvΔ3875 (Fig. 10C), mimicking the effect of ESAT-6. In contrast, IL-23 levels produced by uninfected DCs and DCs infected with either H37Rv or H37RvΔ3875 were similar (Fig. 10B), suggesting that the effects of other *M. tuberculosis* components mask those of ESAT-6, or compensate for its deletion, in eliciting IL-23 production.

Discussion

DCs are pivotal to initiate, shape, and maintain protective T cell responses against microbial pathogens. During *M. tuberculosis* infection, DCs present mycobacterial peptides to T cells, activating them in lymph nodes that drain the lung (37). However, *M. tuberculosis* also evades immunity by interfering with these DC/T cell interactions (38). Previously we showed that ESAT-6 directly inhibits T cell IFN-γ production in a p38 MAPK-dependent manner (18, 19). In this study, we demonstrate that ESAT-6 programs human DCs to favor stimulation of Th17 cells at the expense of Th1 cells by increasing DC production of IL-23 and IL-1β while reducing production of IL-12. These effects of ESAT-6 on DCs are probably mediated through specific interaction of ESAT-6 with DCs (Fig. 6), followed by altered expression of the transcription factors IRF-1, ATF-2, and c-Jun. Our findings uncover a novel role for ESAT-6 in altering DC function to suppress protective immunity and elicit potentially immunopathologic responses.

Treatment with ESAT-6 during iDC maturation enhanced T cell production of IL-17 in response to allostimulation (Fig. 2E) or *M. tuberculosis* (Fig. 3), and conditioned medium from ESAT-6–treated DCs markedly increased IL-17 production by activated T cells, compared with medium from untreated DCs (Fig. 7A). This was not due to direct effects of free ESAT-6 in the DC conditioned medium on T cells, as medium from DCs treated with ESAT-6 but not LPS/CD40LT did not increase IL-17 production (Fig. 7A), and addition of ESAT-6 to T cells inhibits IL-17 production (18).

ESAT-6 enhanced DC secretion of IL-23 and IL-1β (Fig. 4B, 4C), and neutralization of these cytokines strongly inhibited T cell IL-17 production (Fig. 7A, 7C). These results are consistent with the important roles of IL-23 and IL-1β in expanding human Th17

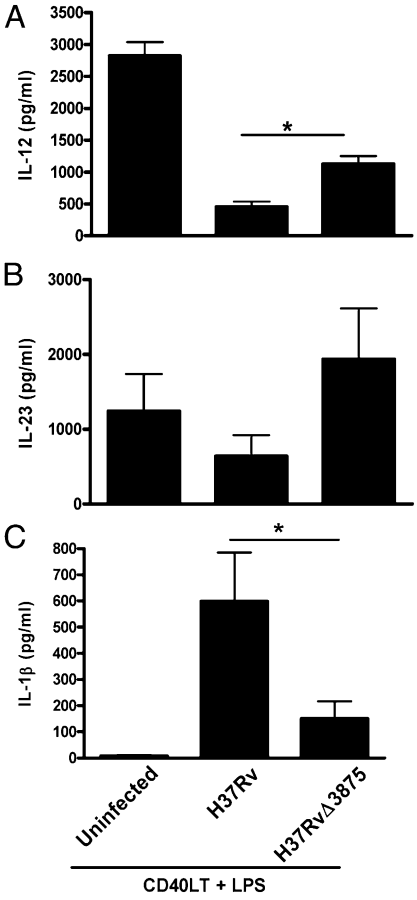

FIGURE 10. Differential effects of *M. tuberculosis* H37Rv and its *esat-6* deletion mutant on DC cytokine production. iDCs from four donors were infected with live H37Rv and its *esat-6* deletion mutant, H37RvΔ3875, at a multiplicity of infection of 10. Four hours later, the cells were stimulated with LPS plus CD40LT for 24 h, culture supernatants were collected, and levels of IL-12p70 (**A**), IL-23 (**B**), and IL-1β (**C**) were measured by ELISA. Means and SEMs of results from four donors are shown. $*p < 0.05$ compared with H37Rv.

cells (60, 61). IL-6 and TGF-β favor development of murine Th17 cells from naive T cells (62, 63), but their roles in human Th17 cells are more controversial (60, 61), perhaps because it is difficult to identify truly naive T cells in adult humans, and because serum used in cell culture contains variable levels of TGF-β (64). In our experimental system, ESAT-6 did not increase DC production of IL-6 (Fig. 4E), and anti–TGF-β did not reduce the capacity of ESAT-6–treated DCs to elicit IL-17 production (X. Wang, unpublished data), suggesting that these cytokines did not stimulate IL-17 production. The intracellular signaling molecules through which ESAT-6 increases IL-17 production remain uncertain. Inhibition of p38 MAPK did not reduce ESAT-6–induced IL-23 and IL-1β production by DCs (Supplemental Fig. 2), unlike the case in T cells, where ESAT-6 inhibits IFN-γ production through activation of p38 MAPK (19), suggesting that ESAT-6 may target different signaling molecules in different cells.

IL-12 is central to defense against many pathogens, which have developed several strategies to reduce its production, such as increased cAMP levels elicited by the adenylate cyclase toxin of *Bordetella pertussis* (47). However, blocking cAMP did not alter the effects of ESAT-6 on DC cytokine production (Supplemental Fig. 1). ESAT-6 also did not reduce DC IL-12 transcription by increasing IL-10 production (65, 66), as IL-10 levels of ESAT-6–treated DCs were not elevated (X. Wang, unpublished data). ESAT-6 inhibits LPS-stimulated production of IL-12p40 by the murine RAW macrophage cell line by binding to macrophage TLR2 through its C terminus 20-aa domain (67). However, deletion of the C-terminal 20 aas of ESAT-6 or neutralizing Abs to TLR2 and TLR1 did not abrogate the effects of ESAT-6 on LPS/CD40LT-stimulated DC IL-12 production (data not shown), suggesting that the mechanism for ESAT-6 inhibition of IL-12 production by human DCs differs from that described in murine macrophages.

Transcription factors of the NF-κB (68) and IRF families (69) are critical for development, differentiation, and activation of DCs. Production of IL-12 and IL-23 are regulated through transcription of p35 and p19, which are the rate-limiting factors in generating the mature IL-12 and IL-23 polypeptides, respectively. The NF-κB components, c-Rel and RelA, enhance transcription of p35 and p19, respectively. ESAT-6 did not affect the DNA-binding activity of NF-κB in DCs (X. Wang, unpublished data), but it reduced the expression and DNA binding activity of IRF-1 in live DCs (Fig. 9A, 9D). IRF-1 and IRF-8 regulate expression of IL-12 in DCs in response to TLR signaling through pathogen-associated molecular patterns, such as LPS (69). IRF-1 also enhances p35 transcription, but it inhibits that of p19 by interfering with binding of RelA (56, 57) to the promoter. Mice with a deleted IRF-1 gene are highly susceptible to tuberculosis (70–73), indicating that it regulates genes that are central for resistance to mycobacterial infection. Our results indicate that ESAT-6 can differentially regulate both IL-12 and IL-23 by targeting a single transcription factor, illustrating a novel and efficient means of manipulating the immune response to reduce protective immunity while enhancing immunopathology and inflammation. ESAT-6 also increased activation and DNA binding capacities of ATF-2 and c-Jun (Fig. 9A–C), which are known to facilitate p19 transcription by binding the p19 promoter (59).

Although most of our studies were performed with human DCs in vitro, experiments with DCs infected with live *M. tuberculosis* with a deleted *esat-6* gene showed that ESAT-6 expression inhibited DC production of IL-12 and enhanced that of IL-1β, mimicking the effects of rESAT-6. H37Rv and the *esat-6* deletion mutant did not increase IL-23 production by DCs (Fig. 10), perhaps because other mycobacterial components also affect IL-23 production and mask the effects of ESAT-6 deletion. Nevertheless, ESAT-6 is likely to affect IL-17 production in vivo, as this secreted protein is present in the lungs during mycobacterial infection (74), and aerosol infection of mice with *M. tuberculosis* H37Rv generated higher IL-17 levels in the lungs than did infection with an region of difference 1 deletion mutant or with BCG, both of which lack ESAT-6 (75).

In summary, we found that ESAT-6, a virulence determinant of *M. tuberculosis* that is also a candidate vaccine Ag, favors Th17 responses at the expense of protective Th1 responses by differentially regulating production of DC cytokines and expression of transcription factors that bind to the promoters of p35 and p19. These findings provide a novel mechanism through which *M. tuberculosis* modulates host immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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