Role of IL-10 on costimulation and cytotoxicity in tuberculosis

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**SUMMARY**

Activation of T cells requires both TCR-specific ligation and costimulation through accessory molecules during T cell priming. IFNγ is a key cytokine responsible for macrophage activation during *Mycobacterium tuberculosis* (*Mtb*) infection while IL-10 is associated with suppression of cell mediated immunity in intracellular infection. In this paper we evaluated the role of IFNγ and IL-10 on the function of cytotoxic T cells (CTL) and on the modulation of costimulatory molecules in healthy controls and patients with active tuberculosis (TB). γ-irradiated-*Mtb* (*i-Mtb*) induced IL-10 production from CD14+ cells from TB patients. Moreover, CD3+ T cells of patients with advanced disease also produced IL-10 after *i-Mtb* stimulation. In healthy donors, IL-10 decreased the lytic activity of CD4+ and CD8+ T cells whereas it increased γδ-mediated cytotoxicity. Furthermore, we found that the presence of IL-10 induced a loss of the alternative processing pathways of antigen presentation along with a down-regulation of the expression of costimulatory molecule expression on monocytes and macrophages from healthy individuals. Conversely, neutralization of endogenous IL-10 or addition of IFNγ to either effector or target cells from TB patients induced a strong lytic activity mediated by CD8+ CTL together with an up-regulation of CD54 and CD86 expression on target cells. Moreover, we observed that macrophages from TB patients could use alternative pathways for *i-Mtb* presentation. Taken together, our results demonstrate that the presence of IL-10 during *Mtb* infection might contribute to mycobacteria persistence inside host macrophages through a mechanism that involved inhibition of MHC-restricted cytotoxicity against infected macrophages.

**Keywords** IL-10, macrophages, coreceptors, cytotoxicity, tuberculosis

**INTRODUCTION**

In general, humans infected with *M.tuberculosis* (*Mtb*) display a strong delayed-type hypersensitivity response to the bacteria, as measured by the PPD skin test. Moreover, the majority of *Mtb*-infected individuals show a chronic bacterial burden. Activation of T cells, a process mediated through two critical signals provided by antigen presenting cells (APC), plays an important role in the protective immune response against *Mtb*. The first signal, is Ag-specific and requires TCR binding to the MHC/Ag complex presented on the APC. The second signal, is Ag independent and involves the interaction of adhesion molecules and costimulatory molecules that bind to their respective ligands on T cells [1]. Thereafter, development of Th1 cytokine responses are enhanced by CD40/CD40L interactions through IL-12 induction by macrophages and dendritic cells [2,3] and by augmentation of CD80/CD86 expression [4,5].

During active tuberculosis (TB), CD4+ and CD8+ T cells participate in the host defense against *Mtb* but these cells are also involved in the immunopathology of the disease through the release of cytokines and/or the lysis of infected-target cells [6,7]. IFNγ is a key cytokine that participates in macrophage activation mediating host defenses against *Mtb* [8]. In contrast, production of immunosuppressive/macrophase deactivating molecules up-regulated during active TB contributes to the establishment of chronic mycobacterial infections [9,10]. In fact, overproduction of IL-10 by T cells has been associated with suppressive immu-
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nity and increased susceptibility to mycobacterial infection [11]. IL-10 has been reported to inhibit proliferation and IL-2 production by activated T lymphocytes by down-regulating the major histocompatibility complex (MHC) molecules [12]. In TB and leprosy, depressed IFNγ responses seem to be mediated by IL-10 since *Mtb* and *M. leprae* elicit IL-10 production by macrophages [13,14]. Besides, it has been suggested that IL-10 produced by APC could play a major role as an autocrine regulator of macrophage activation by controlling the clearance of *Mtb* [12]. Therefore, triggering of IL-10 during the early stages of mycobacterial infection could strongly influence the generation of effector T cells during the subsequent adaptive immune response [12–14].

Antigen-specific CD4+ T lymphocytes are thought to be the main effector cells in *Mtb* infection through their ability to produce cytokines that activate macrophages [6,15] and by contributing to maintain optimal CD8+ T cell responses [16]. On the other hand, γδT cells are readily activated by *Mtb* [17] and are potent sources of early IFNγ production and competent cytotoxic effector cells. Therefore, γδT cells might complement CD4+ and CD8+ T cells’ functions during TB [18]. It is well known that the way in which particulate or soluble mycobacterial antigens are taken up by monocytes might influence the antigen processing pathway for *Mtb*-specific CD4+ and CD8+ αβ T cells as well as for γδT cell lines from PPD+ healthy individuals. Actually, it has been demonstrated that particulate *Mtb* escapes the classical pathways for MHC class-I and class-II processing and, alternative pathways for antigen presentation in PPD+ healthy individuals have been described [19,20].

In a previous study, we have demonstrated an inverse correlation between the impairment in the specific lytic activity of cells from TB patients and the severity of the disease [21]. However, this finding was not related to differences in the expression of MHC class I or class II molecules on APC. Given that binding of accessory molecules expressed on APC to their coreceptors on T cells plays an important role in T cell activation [22], we evaluated the role of IL-10 and IFNγ on the function of CTL as regulators of the expression of costimulatory molecules on APC. Moreover, we investigated the function of IL-10 and IFNγ on cytotoxicity in human tuberculosis.

**MATERIALS AND METHODS**

**Patients**

Thirty patients with pulmonary tuberculosis were studied. Patients were diagnosed on the basis of the presence of recent clinical symptoms of tuberculosis, a positive sputum smear test for acid-fast bacilli confirmed by a positive culture of tuberculosis bacilli and characteristic chest radiograph. Informed consent for experimentation was obtained from patients according to the Ethics Committee of the Hospital Francisco J. Muñiz. All patients had active tuberculosis and 20 out of 30 were under multidrug treatment at the moment of the study (2–15 days). Pulmonary disease was classified according to the extent and type of X-ray findings into moderate (M) and advanced (A) tuberculosis according to the American Tuberculosis Society criteria. Routine blood tests were performed and patients who tested positive for human deficiency virus (HIV) or with concurrent infectious diseases were excluded. Patients were classified into two groups: M-TB; patients with moderate tuberculosis (*n* = 10, 28–53 years) and A-TB: patients with advanced tuberculosis (*n* = 20, 22–68 years). Twelve healthy individuals (25–60 years) were also studied as controls. Five of them were tuberculin skin positive (PPD+).

**Mononuclear cells**

Heparinized blood was drawn and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation [23]. Cells were collected from the interphase and suspended in RPMI 1640 tissue culture medium (Gibco Laboratory, NY, USA) containing gentamycin (85 μg/ml) and 15% heat inactivated fetal calf serum (FCS) (Gibco Laboratory, NY, USA) (complete medium).

**Antigen**

The γ-irradiated *Mycobacterium tuberculosis* H37-Rv strain (i-Mtb) employed in this study was kindly provided by DrBelisle (Colorado University, Denver, CO, USA). Mycobacteria were resuspended in pyrogen free phosphate buffered (PBS), sonicated and adjusted to a concentration of 1 ¥ 10^6 bacteria/ml.

**PBMC culture**

PBMC (2 ¥ 10^6 cells/ml) were cultured in Falcon 2036 tubes (Becton Dickinson, Lincoln, PK, NJ, USA) at 37°C in humidified 5% CO₂ atmosphere, in complete medium with or without i-Mtb (1 ¥ 10^6 bacteria/ml, equivalent to 5 μg/ml). IL-10 (10 ng/ml, Peprotech, Rocky Hill, NJ, USA), IFNγ (100 U/ml, Peprotech) or a monoclonal antibody specific for human IL-10 (10 ng/ml, Peprotech). On day 6, i-Mtb-stimulated and/or cytokine treated and control cells were washed three times with RPMI 1640, suspended in complete medium (2 ¥ 10^6 cells/ml) and tested for their cytotoxic activity.

**Coculture of monocytes and lymphocytes**

Adherent cells (85–95% monocytes) were obtained from PBMC by plastic adherence. PBMC (5 ¥ 10^6 cells/well) were plated at the bottom of 24 well Falcon plates (2 h at 37°C) and after removing the non adherent cells, monocytes were extensively washed with warm medium and cultured in complete medium in the presence of IL-10 (10 ng/ml) or IL-10 (10 ng/ml) plus IFNγ(100 U/ml) for 24 h. Meanwhile, autologous nonadherent mononuclear cells were cultured in complete medium alone. Then, non adherent cells (2 ¥ 10^5 cells/ml) were added to the 24 h cultured monocytes and the cell suspensions were incubated for further 5 days in the presence of i-Mtb.

**Purification of CD4+ or CD8+αβ TCR+ and CD4+CD8-γδ TCR+ T lymphocytes**

Cultured CD4+ and CD8+ T cells expressing the αβ TCR and γδ T lymphocytes were isolated by negative selection with magnetic beads (Dynal, Oslo, Norway) from bulk PBMC or cocultures of monocytes and nonadherent cells. For CD4+ and CD8+ T cell enrichment, cells were treated first with anti-γδ TCR (Panγδ, IgG1, clone Immum 510, Immunotech, Marseille, France) and anti-CD16 (IgG1, clone 3G8, Immunotech) monoclonal antibodies (MAb), followed by goat anti-mouse IgG-coated beads, and anti-CD8 or anti-CD4-coated beads (for CD4+ and CD8+, respectively). For γδ T cell enrichment cells were treated first with anti-αβ TCR (Panαβ, IgG2b, clone BMA 031, Immunotech) and anti-CD16 followed by goat-anti-mouse IgG-coated beads and anti-CD4- plus anti-CD8-coated beads. In both cases, cells were also depleted of B cells using anti-pan B-coated beads. Generally, one cycle of treatment was sufficient for an effective depletion as
assessed by flow cytometry. Purity of isolated cells was 85–95% in each case. Isolated CD4+, CD8+ and γδT cells were resuspended in complete medium ensuring that the number of cell/ml of each subset was the same as in total cultured PBMC in order to compare their lytic activity. Then CD4+, CD8+ and γδT lymphocytes were employed as effector cells in the cytotoxic assay.

**Target cells**

Monocytes were allowed to adhere to the bottom of 96 well flat bottom Falcon plates by incubation of 50 µl of a PBMC (1 x 10^6/ml) suspension for 2 h at 37°C. After removing nonadherent cells, cells remaining in the plates (10% of the original cell suspension) were extensively washed and incubated at 37°C in a humidified 5% CO2 atmosphere for 6 days. On day 5, macrophages were pulsed overnight with i-Mtb (1 x 10^6 bac/ml [5 mg/ml]) in the presence or absence of IL-10 (10 ng/ml) or IFNγ (100 U/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. On day 6 plates macrophages were washed with warm medium and labelled with 1 µCi of Na2131I (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. Then the cells were washed thee times and used as target cell.

**Cytotoxic assay**

CD4+, CD8+ or γδT cells effector cells were added in triplicate at a target cell ratio of 40:1 in 200 µl final volume to 11Cr labelled target cells (5 x 10^6) seeded into each well of 96 well microtitre plates (Corning, USA). Plates were centrifuged at 50x g for 5 min and incubated at 37°C in 5% CO2 for 4 h. After centrifugation at 200x g for 5 min, 100 µl of supernatants were removed from each well. The radioactivity of supernatants and pellets was measured in a gamma counter. Results were expressed as percentage of cytotoxicity (C x):

\[
\% Cx = \frac{cpm \ exp - cpm \ spont. \ release}{cpm \ total - cpm \ spont. \ release}
\]

The radioactivity released from target cells incubated with complete medium alone was considered as spontaneous release. It ranged from 8 to 15%. Total 11Cr release was obtained by treating target cells with Triton X-100 5% (Sigma, Chemical Co., St.Louis, MO, USA). In all cases, the cytotoxic assays performed with PBMC cultured in the absence of i-Mtb or with macrophages not pulsed with antigen rendered negligible cytotoxicity (0%–6%). Data presented in Tables 2 and 4 were obtained by subtracting the cytotoxicity against non antigen-pulsed macrophages from the experimental values determined using antigen-pulsed targets.

**Inhibition of antigen-presentation**

Inhibition of antigen processing was performed by incubating autologous macrophages with Chloroquine (400 µM) (Sigma) or Brefeldin A (5 µg/ml) (Sigma) 30 min before addition of i-Mtb, IL-10 (10 ng/ml) or IFNγ (100 U/ml). After that, cells were coinubated overnight in the presence of inhibitors and i-Mtb. Then, macrophages were washed and labelled with 11Cr and employed as target cells.

**Measurement of IFNγ by ELISA**

PBMC were cultured in the presence or absence of i-Mtb (5 µg/ml). After 48 h supernatants were frozen until their use. IFNγ (Endogen) ELISA was performed according to the manufacturer’s instructions. Briefly, flat bottom 96 well microtiter plates were coated with 100 µg/ml of mouse anti-human IFNγ mAb (Endogen #M-700 A) at 5 g/ml in sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C, followed by blocking with PBS containing 1% BSA for 1 h at room temperature. Samples and IFNγ standard (human recombinant IFNγ; Endogen #R-IFNγ-50) were serially diluted and incubated at room temperature for 3 h. Subsequently, biotinylated anti-IFNγ mAb (Endogen M-701-B) was added at 2 µg/ml for 1 h at room temperature. Avidin-peroxidase conjugate (Sigma, #A-3151) was then added at 2 µg/ml for 30 min at room temperature. Peroxi-
dase substrate solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was added and the plates were read in an ELISA reader (Cambridge Technology, Inc. Watertown, MA, USA) at a wavelength of 405 nm. Washing steps (PBS containing 0.1% BSA and 0.05% Tween 20) were included between each step of the ELISA. A standard curve was made by plotting and regression analysis was applied. The IFNγ concentration of each sample was calculated by regression analysis using the mean absorbance (average of triplicate readings) of the sample. The sensitivity of this assay was 10 pg/ml.

**Immunofluorescence analysis**

**Determination of IL-10+ cells**

In order to determine the expression of intracytoplasmatic IL-10 in control and i-Mtb induced CD3+ effector cells or CD14+ monocytes, PBMC were cultured for 48 h with or without i-Mtb. Brefeldin A (5 µg/ml) was added to the PBMC cultures for the final 4 h of culture to block IL-10 secretion. Then cells were washed and 5 x 10^6 cells sus-
pended in 100 µl of PBS-azide were incubated with anti-CD3 or anti-CD14 MoAb (Ancell, Bayport, MN, USA) for 15 min at room temperature. Thereafter, the cells were fixed according to the manufacturer’s instructions (IntraPrepTM permeabilization reagent, Immunotech). Then, cells were washed with PBS supplemented with 1% FCS and 0.01% of azide (PBS-FCS-azide) and suspended in 100 µl of PBS-FCS-azide. Phycocerythrin-conjugated antibody for IL-10 (Caltag, Burlinghame, CA, USA) was added together with 100 µl of permeabilizing solution (IntraPrep). Cells were incubated for 30 min at 4°C, washed once with PBS-FCS-azide and finally suspended in Isoflow. The samples were analysed by flow cytometry as mentioned above. 20000 events were acquired for each sample, gates were set with respect for the for-
ward and side-scatter to exclude cell debris and apoptotic cells. Results are expressed as percentage of positive cells.

**Expression of CD86, CD54, CD40 antigens on CD14+ monocytes and macrophages**

In order to evaluate the expression of CD86, CD54 and CD40 antigens on monocytes, PBMC were cultured for 18 h with or without i-Mtb in the presence of either IL-10 (N controls) or IFNγ (TB patients and N controls). On the other hand, monocytes isolated by plastic adherence from PBMC were cultured in 24 wells Falcon plates for 5 days with complete medium, then they were pulsed or not with i-Mtb and IL-10 or IFNγ for further 18 h. On day 6, macrophages were recovered from the plates. Plates were cooled for 3 h to facilitate the detachment of cells by vigorous pipetting with ice cold medium, cells were washed and then tested for their expression of CD86, CD54 and CD40. Either 18 h cultured-PBMC or 6 days cultured macrophages were incubated for 30 min at 4°C with anti-human CD14 (FITC conjugated anti-CD14, Ancell, MN, USA or PE-conjugated, Immunotech, France) and anti-human CD86 (B7-2/ PE, Ancell), anti-CD54 (ICAM-1/PE, Ancell) or anti-CD40 (FITC-anti-CD40, Ancell). FITC- or PE-labelled-isotype
matched antibodies were also tested to evaluate nonspecific staining. Stained cells were analysed by flow cytometry by acquiring 20 000 events. Results are expressed as relative fluorescence (RF):

$$RF = \frac{(MFI_{\text{specific antigen}} - MFI_{\text{isotype antibody}}) \times 100}{MFI_{\text{isotype antibody}}}$$

where MFI is mean fluorescence intensity.

**Statistics**

Comparisons of TB and N were performed using Student’s *t*-test. Cytotoxicity values obtained from the different subsets of effector cells of each individual were compared using the Wilcoxon signed rank test.

**RESULTS**

**IL-10 impairs the lytic activity of CD4\(^+\), CD8\(^+\) and \(\gamma\delta T\) cells in healthy individuals**

Since IL-10 could influence cytotoxic responses, we first investigated its role in the generation of CD4\(^+\) cells. PBMC from 10 healthy individuals (N) were stimulated with i-Mtb in the presence or absence of IL-10 for 6 days. Then, CD4\(^+\), CD8\(^+\) and \(\gamma\delta T\) cells were isolated by negative selection and their ability to lyse autologous i-Mtb-pulsed macrophages was analysed. As shown in Table 1, the lytic activity from isolated CD4\(^+\) and CD8\(^+\) T cells was inhibited by exogenous addition of IL-10 to the bulk culture during the induction stage in a dose dependent manner (data not shown). In contrast, the lytic activity of \(\gamma\delta T\) cell was significantly enhanced in N individuals (Table 1). These results suggest that IL-10 impairs the lytic activity of CD4 and CD8 T cells while it enhances the lytic activity of \(\gamma\delta T\) cell in normal controls.

**Pre-treatment of monocytes or macrophages with IL-10 modifies the CTL profile in PPD\(^+\) N controls**

Considering that IL-10 markedly inhibits a broad spectrum of monocyte-macrophage functions including antigen-presentation [12], we next evaluated whether IL-10 could modulate monocyte functions during CTL development. Therefore, adherent-monoocytes from 5 PPD\(^+\) healthy individuals (N-PPD\(^+\)) were preincubated with IL-10 for 24 h, nonadherent autologous mononuclear cells were then added and cells were stimulated with i-Mtb. After 5 days, CD4\(^+\), CD8\(^+\) and \(\gamma\delta T\) cells were isolated and antigen-specific cytotoxicity was determined employing i-Mtb-pulsed autologous macrophages. As shown in Fig. 1a, IL-10 pretreated monocytes inhibited CD4\(^+\) and CD8\(^+\) CTL activity while the lysis mediated by \(\gamma\delta T\) cells was not modified. To determine whether this negative effect of IL-10 on monocytes could be modified by IFN\(\gamma\), IL-10 and IFN\(\gamma\) were simultaneously added to adherent-monocytes in culture. While the activity of CD8\(^+\) CTL was significantly increased by the coaddition of INF\(\gamma\) neither CD4\(^+\) CTL nor \(\gamma\delta T\) CTL were significantly modified (Fig. 1a).

These results demonstrate an impairment in the generation of MHC-restricted CTL activity after IL-10 treatment of human monocytes, suggesting that IL-10 might interfere with IFN\(\gamma\) dependent pathways.

To evaluate whether IL-10 could modify the up-take or presentation of Mtb antigen to cytotoxic T cells, macrophages to be used as target cells, were pulsed overnight with i-Mtb in the presence of IL-10. As shown in Fig. 1b, this treatment induced a significant inhibition of CD4\(^+\) CTL activity and a complete abolishment of CD8\(^+\) CTL activity. However, a significant enhancement of \(\gamma\delta T\) lytic activity was detected when IL-10 was present during i-Mtb up-take by macrophages from N-PPD\(^+\). Therefore, these results indicate that IL-10 might modulate the generation of effector cells and the presentation of Mtb antigens to cytotoxic T cells.

**Table 1. Inhibitory effect of IL-10 on i-M.tuberculosis-induced CTL activity from healthy individuals**

| PBMC inc. with | Macrophages inc. with | % Cytotoxicity |
|---------------|----------------------|---------------|
| i-Mtb         | i-Mtb                | 45 ± 5        |
| i-Mtb + IL-10 | i-Mtb                | 50 ± 3*       |
| i-Mtb         | CD4                  | 19 ± 2        |
| i-Mtb + IL-10 | CD4                  | 17 ± 3*       |
| i-Mtb         | CD8                  | 22 ± 3        |
| i-Mtb + IL-10 | CD8                  | 26 ± 5*       |
| i-Mtb         | \(\gamma\delta T\)   | 11 ± 1*       |
| i-Mtb + IL-10 | \(\gamma\delta T\)   | 28 ± 4*       |

PBMC from 10 healthy controls (N) were incubated with i-Mtb with or without IL-10 (10 ng/ml) for 6 days. CD4, CD8 and \(\gamma\delta T\) cells were isolated by magnetic methods and used as effector cells. 5 day cultures of autologous macrophages were pulsed with i-Mtb (18 h) and then they were employed as target cells during the cytotoxic assay. Spontaneous release from i-Mtb-stimulated macrophages was %15%. Data were obtained subtracting spontaneous release to experimental values. Results are expressed as x – SEM. Statistical differences between percentage cytotoxicity from PBMC – i-Mtb + IL-10 and percentage cytotoxicity from PBMC – i-Mtb: *P < 0.05.

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Table 2. Percentage of IL-10+ CD14+ and IL-10+ CD3+ cells

| PBMC from        | % IL-10+ CD14+ cells | % IL-10+ CD3+ cells |
|------------------|-----------------------|---------------------|
| M-TB             |                       |                     |
|                  | C                     | i-Mtb               |
|                  | 4·6–3·2               | 4·8–0·4†            |
|                  | 0·2–0·2               | 0·5–0·3*            |
| A-TB             |                       |                     |
|                  | C                     | i-Mtb               |
|                  | 7·8–1·5†              | 12·0–1·4††          |
|                  | 3·9–1·7††             | 5·9–2·3††           |
| N                | 4·2–1·1               | 2·9–0·7             |
|                  | 0·05–0·05             | 0·1–0·1             |

Table 3. Modulatory effect of anti-IL-10 and IFNγ on the induction stage of i-Mtb specific-CTL activity in patients with tuberculosis

| PBMC from     | PBMC inc. with     | % Cytotoxicity |
|---------------|--------------------|---------------|
|                | CD4                | CD8           | γδT           |
| TB severity    |                    |               |               |
| Moderate       | i-Mtb              | 43±5          | 24±3          | 31±3          |
| (n = 8)        | i-Mtb + a-IL-10    | 53±4*         | 37±3*         | 38±3          |
|                | i-Mtb + IFNγ       | 50±5          | 34±3*         | 38±3          |
| Advanced       | i-Mtb              | 30±3          | 9±1           | 43±3          |
| (n = 18)       | i-Mtb + a-IL-10    | 44±4*         | 29±3*         | 43±3          |
|                | i-Mtb + IFNγ       | 42±3*         | 29±2*         | 42±3          |

PBMC from 8 moderate (M-TB) and 18 advanced (A-TB) tuberculosis patients were incubated with i-Mtb with or without anti-IL-10 (10 ng/ml) or IFNγ (100 U/ml) for 6 days. CD4, CD8 and γδ T cells were isolated by magnetic beads and used as effector cells. Macrophages were pulsed with i-Mtb (18 h) and then these cells were employed as target cells during the cytotoxic assay. Results are expressed as mean ± SEM. Statistical differences between percentage cytotoxicity from PBMC + i-Mtb + a-IL-10 or IFNγ and percentage cytotoxicity from PBMC + i-Mtb: *P < 0.05.

Effect of i-Mtb on the modulation of costimulatory molecules on monocytes and macrophages

Given that overproduction of IL-10 by T cells has been associated with suppressed immunity [11] and that IL-10 produced by APC could act as an autocrine regulator of macrophage activation, we investigated whether the observed effects of IFNγ and IL-10 on the lysis of i-Mtb-pulsed macrophages could be related to the modulation of T cell costimulatory molecules during CTL generation or i-Mtb-up-take. Therefore, either PBMC or 5 day adherent-monocytes from N-PPD+ and TB patients were anti-IL-10 nor IFNγ modified γδ T cell-dependent cytotoxicity (Table 3). Furthermore, we found an inverse correlation between the production of IFNγ against i-Mtb and the severity of the disease (M-TB, 1390±396 pg/ml; A-TB, 305±181 pg/ml). However, neutralization of endogenous IL-10 or addition of IFNγ during i-Mtb up-take increased CD8+ CTL activity in A-TB patients (Table 4).
stimulated with i-Mtb in the presence or absence of IFNγ or IL-10 for 18 h, and cell surface expression of CD86, CD54 and CD40 on CD14+ cells was examined by flow cytometry.

As shown in Fig. 3, during maturation of monocytes to macrophages we detected an spontaneous increased expression of CD86 (in N-PPD+ individuals) CD54 (in A-TB patients and N-PPD+ controls). i-Mtb down-regulated the expression of CD86 and CD54 on monocytes from M-TB patients and up-regulated the expression of CD86 on monocytes from N-PPD+. Moreover, i-Mtb stimulation also increased the levels of CD40 on monocytes from M-TB and N-PPD+. Furthermore, the addition of IFNγ to i-Mtb-stimulated monocytes, increased CD86 and CD40 levels on cells from TB patients. While IFNγ did not modify the expression of costimulatory molecules on i-Mtb stimulated N-PPD+ monocytes, the addition of IL-10 down-regulated the CD86 and CD40 levels (Fig. 3). Like IFNγ, the neutralization of endogenous IL-10 in monocytes from TB patients increased the expression of CD86, CD54 and CD40 (Fig. 4). Besides, in macrophages pulsed with i-Mtb, we found an increased expression of CD86 in M-TB and N-PPD+ compared with control macrophages, while no changes in the levels of these molecules were observed by effect of IFNγ. However, IFNγ did induce up-regulation of CD86 and CD54 on macrophages from A-TB. Furthermore, a down-regulation of CD86, CD40 and CD54 expression on N-PPD+ macrophages was induced by IL-10 during i-Mtb up-take. Therefore, our data indicate that the down-regulation of the expression of costimulatory molecules by i-Mtb may be abrogated by the addition of IFNγ or by neutralization of endogenous IL-10 (Fig. 4). Furthermore, the low expression of CD86 and CD54 on A-TB macrophages, suggests an incapacity of monocytes from TB patients to differentiate into macrophages.
IL-10 inhibits alternative antigen-presentation pathways

It has been previously demonstrated that monocyte-derived macrophages from healthy individuals can process peptides derived from Mtb bacilli by alternate pathways and present them to CD8\(^+\) and CD4\(^+\) T cells [19,20]. To explore whether the inability to generate MHC-restricted effector cells in TB patients could be related to differential use of antigen-presentation pathways, macrophages were treated with Brefeldin A or Chloroquine (inhibitors of classical MHC class-I and class-II antigen-presentation pathways, respectively), and after 2 h, cells were pulsed with i-Mtb. As shown in Table 5, in TB patients and N controls i\(^\beta\)T cell-mediated cytotoxicity was markedly inhibited by Brefeldin A. On the contrary, neither Brefeldin A nor Chloroquine inhibited CD4\(^+\) mediated cytotoxicity was markedly inhibited by Brefeldin A. On the contrary, neither Brefeldin A nor Chloroquine inhibited CD4\(^+\) and CD8\(^+\)-dependent cytotoxicity in N (Table 5), suggesting an effective use of alternate pathways to process i-Mtb. Conversely, blockade of the classical class-I and class-II antigen presentation pathways significantly inhibited CD8\(^+\) and CD4\(^+\)-mediated cytotoxicity in TB patients. Only macrophages from M-TB patients and from healthy donors employed class-II alternative presentation pathway.

To explore whether IL-10 could be involved in the inhibition of the alternate pathways, 5-day-cultured macrophages from N control were first treated with IL-10, then i-Mtb was added together with the inhibitors, and after 18 h the cells were used as target in cytotoxic assays. As shown in Table 5, pre-treatment of macrophages with IL-10 modulated the ability of N macrophages to employ the alternate pathways of i-Mtb processing/presentation to CD4\(^+\) or CD8\(^+\) CTL. On the other hand, in TB macrophages previously incubated with anti-IL-10 or IFN\(\gamma\), no inhibition of CD4\(^+\) CTL by Chloroquine and of CD8\(^+\) CTL by Brefeldin A was observed. Taken together, these results suggest that IL-10 can modify the capacity of macrophages from TB patients to employ alternate pathways of antigen presentation.

**DISCUSSION**

Infection with Mtb is accompanied by a local inflammatory response where cytokines play an important role. In contrast to IFN\(\gamma\), which is a key cytokine in the control of Mtb infection, IL-10 is associated with suppression of CMI in TB patients [11]. In this study we evaluated the role of IL-10 on the modulation of the expression of costimulatory molecules during the development of CTL. Moreover, we analysed whether IL-10 participated in the lysis of i-Mtb-pulsed macrophages.

Our results showed that, in healthy individuals, IL-10 modified the lytic activity of CD4\(^+\), CD8\(^+\) and i\(^\beta\)T cells. Furthermore, we found that IL-10 modulated the expression of costimulatory molecules on monocytes/macrophages and influenced the pathways for antigen presentation. Addition of IL-10 during CTL induction inhibited the ability to lyse i-Mtb-pulsed macrophages mediated by CD4\(^+\) and CD8\(^+\) cells but increased i\(^\beta\)T lytic activity.

### Table 4. Modulation of CD4 and CD8 CTL activity by addition of anti-IL-10 or IFN\(\gamma\) to i-Mtb-pulsed macrophages

| PBMC from | % Cytotoxicity | CD4 | CD8 | i\(^\beta\)T |
|-----------|----------------|-----|-----|------------|
| TB        |                |     |     |            |
| Moderate  | (n = 8)        |     |     |            |
| i-Mtb     | 43 ± 5         | 24 ± 3 | 31 ± 3 |          |
| i-Mtb + a-IL-10 | 44 ± 6  | 26 ± 3 | 40 ± 2 |          |
| i-Mtb + IFN\(\gamma\) | 46 ± 8 | 31 ± 3 | 40 ± 3 |          |
| Advanced  | (n = 20)       |     |     |            |
| i-Mtb     | 30 ± 3         | 10 ± 3 | 43 ± 3 |          |
| i-Mtb + a-IL-10 | 42 ± 5* | 28 ± 6* | 42 ± 3 |          |
| i-Mtb + IFN\(\gamma\) | 42 ± 4* | 29 ± 5* | 39 ± 4 |          |

CD4, CD8 and i\(^\beta\)T cells were isolated from 6-day i-Mtb-cultured PBMC from 8 M-TB and 20 A-TB and used as effector cells. Five day cultures of macrophages were pulsed 18 h with i-Mtb in the presence or absence of anti-IL-10 (10 ng/ml) or IFN\(\gamma\) (100 U/ml) and then they were employed as target cells during the cytotoxic assay. Results are expressed as mean ± SEM. Statistical differences between percentage cytotoxicity from PBMC + i-Mtb + a-IL-10 or IFN\(\gamma\) and percentage cytotoxicity from PBMC + i-Mtb: *P < 0.05.

### Table 5. Differential inhibition of CD4 and CD8 CTL activity by Brefeldin A and Chloroquine in patients with tuberculosis and healthy individuals

| PBMC from | % CD4-CTL against macrophages treated 1 h with | % CD8-CTL against macrophages treated 1 h with | % i\(^\beta\)T-CTL against macrophages treated 1 h with |
|-----------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|           | Mac. stimulated 18 h with                     | B | C | B | C | B | C |
| M-TB (n = 8) | i-Mtb                                              | 45 ± 4 | 39 ± 5 | 44 ± 5 | 26 ± 3 | 11 ± 5* | 27 ± 2 | 30 ± 7 | 11 ± 5* | 33 ± 6 |
|           | i-Mtb + a-IL-10                                   | 45 ± 4 | 46 ± 4 | 49 ± 5 | 28 ± 5 | 29 ± 5 | 26 ± 2 | 40 ± 2 | 13 ± 3 | 27 ± 5 |
|           | i-Mtb + IFN\(\gamma\)                              | 48 ± 4 | 52 ± 2 | 53 ± 4 | 30 ± 3 | 29 ± 5 | 34 ± 4 | 41 ± 3 | 13 ± 2 | 29 ± 4 |
| A-TB (n = 16) | i-Mtb                                              | 30 ± 4 | 32 ± 3 | 14 ± 3* | 10 ± 1 | 5 ± 1* | 13 ± 1 | 44 ± 4 | 10 ± 2* | 35 ± 6 |
|           | i-Mtb + a-IL-10                                   | 39 ± 3 | 38 ± 5 | 32 ± 5 | 26 ± 4 | 20 ± 3 | 26 ± 2 | 44 ± 3 | 11 ± 3 | 35 ± 5 |
|           | i-Mtb + IFN\(\gamma\)                              | 40 ± 3 | 41 ± 3 | 35 ± 3 | 25 ± 3 | 19 ± 1 | 27 ± 4 | 42 ± 4 | 10 ± 3 | 34 ± 4 |
| N (n = 10) | i-Mtb                                              | 40 ± 5 | 36 ± 3 | 37 ± 3 | 25 ± 4 | 25 ± 3 | 29 ± 2 | 20 ± 2 | 7 ± 2* | 22 ± 3 |
|           | i-Mtb + a-IL-10                                   | 24 ± 5 | 20 ± 1 | 13 ± 3* | 16 ± 1 | 5 ± 3* | 14 ± 2 | 32 ± 4 | 5 ± 3 | 27 ± 5 |

PBMC from patients with tuberculosis and N controls were incubated with i-Mtb for 6 days and then used as effector cells. Five day cultures autologous macrophages were pulsed (18 h) with i-Mtb in the presence or absence of either a-IL-10 or the cytokines (CKs) IFN\(\gamma\) for patients or IL-10 for N controls, and Brefeldin A (B) or Chloroquine (C) as described in Materials and Methods. Then, macrophages were used as target cells in the cytotoxic assay. Results are expressed as mean ± SEM. Statistical differences between percentage cytotoxicity against macrophages + i-Mtb + anti-IL-10/CKs + Brefeldin A or Chloroquine and percentage cytotoxicity against macrophages + i-Mtb + anti-IL-10/CKs without inhibitors: *P < 0.05.
Given that we did not find a high percentage of CD14^+ IL-10^+ or CD3^+ IL-10^+ cells in N-PPD^+ in response to i-Mtb, our results suggest that the IL-10 would be responsible for the observed effect on the lytic activity. Accordingly, in TB patients where CD3^+ IL-10^+ cells were detected, neutralization of IL-10 led to an increase in the lytic activity of CD4^+ and CD8^+ CTL. Moreover, neither IL-10 nor IL-10 plus IFNγ affected the γδT-mediated lytic activity on monocytes from N-PPD^+ suggesting that γδ cells may represent an in vivo polarized type-1 population [24] expanded through a pathway that does not require antigen uptake, processing or known presenting molecules [25]. Therefore, the early production of IL-10 detected in response to i-Mtb, could be related to the high γδT lytic activity in TB patients.

Costimulatory molecules are important for the initiation and maintenance of an immune response [1–5,22,26]. Our results showed a decrease in the expression of CD86 and CD40 on monocytes from PPD^+ healthy donors pretreated with IL-10 upon i-Mtb stimulation, which would be affecting the lytic activity of CD4^+ and CD8^+ CTL. CD86, a molecule constitutively expressed on APC, plays a key role in early interactions between APC and T cells [26,27] and has been shown to be the major CD28 costimulatory ligand in the clonal expansion of Ag-specific cells [28]. Furthermore, CD86 delivered as vaccine adjuvant was shown to play a prominent role in the Ag-specific induction of CD8^+ CTL [29]. In accordance, we found that the up-regulation of CD86 on i-Mtb-stimulated monocytes from N-PPD^+ correlated with a strong CD8^+ lytic activity. Enhanced expression of B7 molecules on APC (due to CD40/CD40L interactions) results in the induction of NF-κB, STAT-3 activation [30–32], and cytokine production, including IL-10 [33]. However, CD40 may also redirect the cytokine response towards a Th1 profile through stimulation of IL-12 which leads to IFNγ production [34].

Fig. 3. Mtb modulates T cell costimulatory molecules on monocytes and macrophages. Recently isolated PBMC (a–c) or 5 day adherent-macrophages (d–f) from 4 M-TB, 6 A-TB and 5 N-PPD^+ were cultured for 18 h in complete medium alone, with i-Mtb or with i-Mtb plus IFNγ or IL-10, and cell surface marker expression of CD86 (a,d), CD54 (b,e) and CD40 (c,f) on CD14^+ cells was examined by flow cytometry. Open histograms represent the monocytes stained with specific antibodies; grey dotted histograms represent the respective isotype control. Results are expressed as relative fluorescence (RF) (mean – SEM) as mentioned in Materials and Methods. Statistical differences: RF from i-Mtb-pulsed macrophages versus RF from control macrophages: *P < 0.05; RF from IFNγ or IL-10 treated-i-Mtb-pulsed macrophages versus RF from i-Mtb-pulsed macrophages: ^P < 0.05; RF A-TB versus N: b^P < 0.05, A-TB versus M-TB: c^P < 0.05.
up-regulation of CD40 on monocytes from N-PPD+ upon i-Mtb stimulation, suggesting that the signals triggered by the bacteria in monocytes (regarding the production of IL-10), might be overriden by the IFNγ produced by N-PPD+ cells. In agreement, the addition of IFNγ to IL-10 pretreated monocytes overcame the inhibition of CD8+ cells lytic activity increasing the CD4+ lytic activity. Similar to healthy donors, i-Mtb increased the expression of CD40 in CD14+ cells from M-TB patients. Conversely, neither i-Mtb nor IFNγ modified the low expression of CD40, CD86 and CD54 molecules on monocytes from A-TB patients. It has been demonstrated that mice deficient in CD40L achieved Th1 protection against Mtb [35]. However, depressed expression of CD40L in TB patients correlated with reduced IFNγ production and blockade of CD40 in N-PPD+ reduced IFNγproduction [36]. Our results showed that the low CD40 expression in control and i-Mtb-stimulated monocytes from A-TB patients, correlated with reduced IFNγ levels and low expression of CD86. Together, these results suggest that diminished CD40 expression on monocytes during i-Mtb stimulation might contribute to reduce IFNγ production, affecting the lytic activity. Therefore, IL-10 would regulate the lytic activity of CTL at least in part through the down-regulation of costimulatory molecules during CTL induction.

In macrophages from N-PPD+, the presence of IL-10 during the uptake of i-Mtb inhibited the CD8+ cytotoxicity but increased the γδT-mediated lytic activity. Moreover, IL-10 induced a down-regulation of CD86, CD54 and CD40 expression on macrophages. In agreement with these results, the neutralization of endogenous IL-10 in A-TB patients increased the lytic activity of CD8+ T cells. Thus, the lowest expression of CD86 and CD54 correlated with the highest amount of CD14+ IL-10+ cells and the lowest IFNγ production in A-TB patients, suggesting that endogenous IL-10 might participate in delaying the differentiation of monocytes to macrophages. Moreover, addition of IFNγ up-regulated the expression of CD86 and CD54 on macrophages from A-TB patients and improved the lytic activity from CD8+ cells. CD54 has a crucial role in granuloma formation in Mtb-infected mice [37,38]. In addition, CD54 is a strong driver of CTL induction and CD8+ effector functions and its expression induces IFNγproduction by costimulated T cells [39]. In the current study, we showed that IFNγincreased CD54 expression on i-Mtb stimulated monocytes from M-TB patients or on macrophages from A-TB patients.

Fig. 4. Recently isolated PBMC from 3 M-TB (a–c) and 3 A-TB (d–f) were cultured during 18 h in medium alone (- - - -), i-Mtb (———) or with i-Mtb and anti-IL-10 MAb (——). Then, the surface expression of CD86, CD54 and CD40 on CD14+ cells was examined by flow cytometry. □ the monocytes stained with specific antibodies; ■ the respective isotype control. A representative example for each group is shown.
leading to enhancement of MHC-restricted lytic activity. These results could explain in part the improvement in clinical symp- toms observed in TB patients exposed to r-IFNγ aerosols in vivo [40]. Therefore, our data indicate that if IFNγ is present at the site of infection, as in M-TB patients, the expression of CD54 molecules on APC could provide costimulatory signals to CD8+ CTL [41,42]. Hence, the coexpression of CD86 and CD54 would amplify the MHC-restricted cytotoxic activity contributing to the lysis of infected cells.

Finally, our results regarding the antigen presentation pathways for i-Mtb in N controls are in accordance with Balaji & Boom [19] and Canaday et al. [20] who demonstrated alternate pathways for Mtb presentation to MHC-restricted cytotoxic T cells. Neither Brefeldin A nor Chloroquine inhibited the presentation to CD8+ and CD4+ CTL. Nevertheless, if IL-10 was present during i-Mtb uptake, processing of Mtb-antigens followed the classical presentation pathways. As in N controls, macrophages from TB patients are able to use the class-I and class-II alternate presentation pathways when endogenous IL-10 is neutralized or IFNγ is added. Therefore, our data suggest that IL-10 may also participate in the modification of Mtb processing. Considering that IFNγ and IL-10 signalling are similar [43], our data suggest that mycobacteria could have developed strategies to inhibit IFN-γ signalling [44] or to enhance IL-10 triggering, by modifying the processing pathway of the bacteria through inhibition of alternate MHC-class I and class II processing pathways. In this way, this pathogen would successfully persist inside host macrophages. However, other mechanisms, such prolonged exposure to pathogen-associated molecular patterns that signal through Toll-like receptors may also be involved [45,46].

In conclusion, either inappropriate IL-10 expression or signalling in PPD+ individuals contribute to the progression of tuberculosis by inhibiting IFNγsignalling and by the down-regulation of costimulatory molecules. Moreover, the ineffective maturation from monocytes to macrophages may also be related to the presence of IL-10 at the site of infection, preventing the differentiation of these cells to dendritic cells [47,48], leading to ineffective specific cytotoxicity and favouring the perpetuation of the disease.

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