Clin Exp Immunol 2004; **138**:128–138 doi:10.1111/j.1365-2249.2004.02577.x

IL-10 down-regulates costimulatory molecules on *Mycobacterium tuberculosis*-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients

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(Accepted for publication 21 June 2004)

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 $\gamma\delta$-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 local 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/macrophage 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, $\gamma\delta$T cells are readily activated by *Mtb* [17] and are potent sources of early IFNγ production and competent cytotoxic effector cells. Therefore, $\gamma\delta$T 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 $CD8^+$ $\alpha\beta$T cells as well as for $\gamma\delta$T 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 and 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 1×10^8 bacteria/ml.

PBMC culture

PBMC (2×10^6 cells/ml) were cultured in Falcon 2063 tubes (Becton Dickinson, Lincoln, PK, NJ, USA) at 37°C in humidified 5% CO_2 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 thee 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 (5×10^5 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 $CD8^+$/$\alpha\beta$ TCR^+ and $CD4^-CD8^-$/$\gamma\delta$ TCR^+ T lymphocytes

Cultured $CD4^+$ and $CD8^+$ T cells expressing the $\alpha\beta$TCR and $\gamma\delta^+$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-$\gamma\delta$ TCR (Pan $\gamma\delta$, IgG1, clone Immun 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 $\gamma\delta$T cell enrichment cells were treated first with anti-$\alpha\beta$ TCR (Panα/β, 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 $\gamma\delta$T 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 $\gamma\delta$ T 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 (1×10^6/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% CO_2 atmosphere for 6 days. On day 5, macrophages were pulsed overnight with i-*Mtb* (1×10^6 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 $Na_2{}^{51}CrO_4$ (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 $\gamma\delta$T cells effector cells were added in triplicate at a target cell ratio of 40 : 1 in 200 μl final volume to ^{51}Cr labelled target cells (5×10^3) seeded into each well of 96 well microtitre plates (Corning, USA). Plates were centrifuged at 50× g for 5 min and incubated at 37°C in 5% CO_2 for 4 h. After centrifugation at 200× 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 (% Cx):

$$\% \text{ Cx} = \frac{\text{cpm exp} - \text{cpm spont. release}}{\text{cpm total} - \text{cpm spont. release}}$$

The radioactivity released from target cells incubated with complete medium alone was considered as spontaneous release. It ranged from 8 to 15%. Total ^{51}Cr 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) o 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 coincubated overnight in the presence of inhibitors and i-*Mtb*. Then, macrophages were washed and labelled with ^{51}Cr 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-γ-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. Peroxidase 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 5×10^5 cells suspended 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 (IntraPrep™ 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. Phycoerythrin-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. 20·000 events were acquired for each sample, gates were set with respect for the forward 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):

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

where MFI is mean fluorescence intensity.

Statistics

Comparisons of TB and N were performed using Student's *t*-test. Cytoxicity 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 γδ T cells in healthy individuals

Since IL-10 could influence cytotoxic responses, we first investigated its role in the generation of CD4$^+$, CD8$^+$ and $\gamma\delta^+$ cytotoxic T lymphocytes (CTL). To do this, peripheral blood mononuclear 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-monocytes 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 IFNγ, IL-10 and IFNγ were simultaneously added to adherent-monocytes in culture. While the activity of CD8$^+$ CTL was significantly increased by the coaddition of INFγ 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 IFNγ 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.

*i-*Mtb*-induced IL-10 production by CD14$^+$ monocytes and by CD3$^+$ T cells in tuberculosis patients*

To determine whether *Mtb* stimulation could induce the generation of IL-10$^+$ cells, PBMC from TB patients and N-PPD$^+$ were incubated with i-*Mtb* for 48 h, and IL-10 production by CD14$^+$ and CD3$^+$ cells was analized by flow cytometry. As shown in Tables 2, i-*Mtb*-stimulated CD14$^+$ cells from TB patients produced IL-10. On the other hand, no increase in IL-10$^+$ cells was detected after i-*Mtb* stimulation of PBMC from N-PPD$^+$. The highest percentage of IL-10$^+$ CD14$^+$ cells was detected among cells from A-TB patients which were significantly increased after i-*Mtb* stimulation. i-*Mtb* stimulation did not modify the levels of IL-10$^+$ CD14$^+$ in M-TB patients and N-PPD$^+$ healthy individuals. Interestingly, IL-10$^+$ CD3$^+$ cells were only observed in TB patients and the highest percentage was detected in A-TB patients (Fig. 2).

Effect of anti-IL-10 or IFNγ on the modulation of the lytic activity in tuberculosis patients

Previously we had demonstrated a loss of CD8$^+$ CTL activity, an abrogation of CD4$^+$ lytic activity and an enhancement of $\gamma\delta$T cytotoxic activity during severe pulmonary tuberculosis [21]. Since IL-10 inhibited the induction of MHC-restricted cytotoxic T cells in N-PPD$^+$ and considering the high percentage of IL-10$^+$ CD14$^+$ cells detected in TB patients (Table 2), we next evaluated the role of IL-10 in the cytotoxicity during active TB. As shown in Table 3, the pattern of MHC and non-MHC-restricted CTL was related to the severity of the disease in accordance with previous data from our laboratory [21]. Neutralization of endogenous IL-10 during the generation of CTL increased CD4$^+$ and CD8$^+$ lytic activity in tuberculosis patients (Table 3). Moreover, the addition of IFNγ induced a significant increase in CD4$^+$ CTL from A-TB and in CD8$^+$ CTL in both groups of patients. Conversely, neither

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

PBMC inc. with	Macrophages inc. with	% Cytotoxicity		
		CD4	CD8	γδ T
i-*Mtb*	i-*Mtb*	45 ± 5	28 ± 5	19 ± 2
i-*Mtb* + IL-10	i-*Mtb*	26 ± 5*	11 ± 1*	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 8–15%. Data were obtained subtracting spontaneous release to experimental values. Results are expressed as × – SEM. Statistical differences between percentage cytotoxicity from PBMC – i-*Mtb* + IL-10 and percentage cytotoxicity from PBMC – i-*Mtb*: *$P < 0.05$.

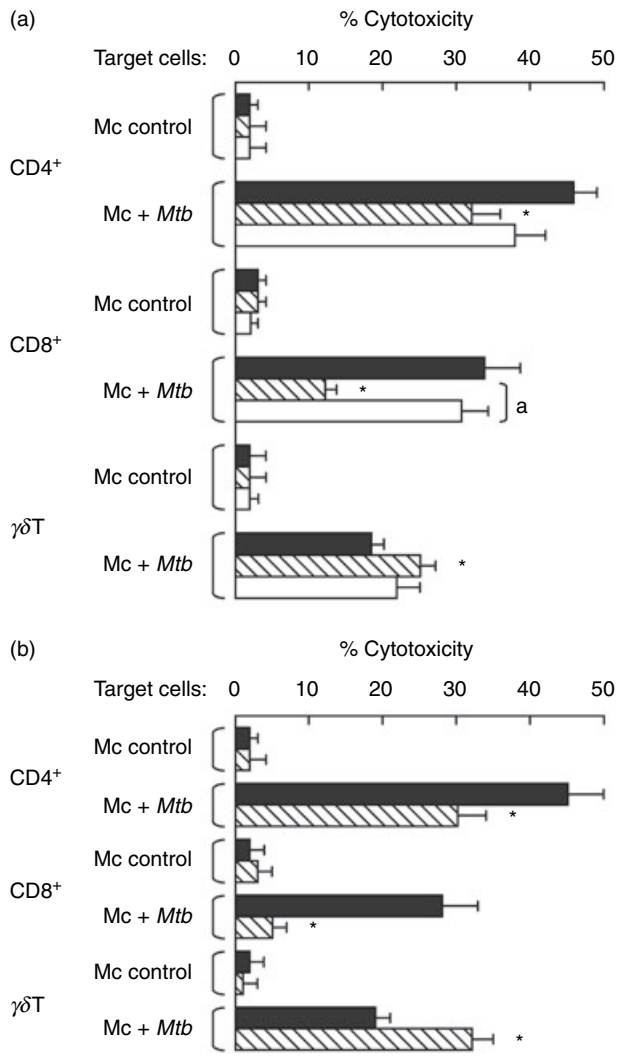

Fig. 1. Lytic activity of *Mycobacterium tuberculosis*-stimulated PBMC (a) Monocytes from 5 PPD$^+$ normal individuals were allowed to adhere to 24 wells Falcon plates and cultured for 24 h with either complete medium alone (■), IL-10 (▨) or IL-10 plus IFNγ (□). Then, autologous non adherent mononuclear cells (2×10^5 cells/ml) were added to the 24 h cultured monocytes and the cell suspensions were incubated with medium alone or i-*Mtb* for a further 5 days. On day 6, CD4$^+$, CD8$^+$ and $\gamma\delta$ T lymphocytes were isolated from these monocytes/nonadherent cocultures and tested for their lytic activity against non pulsed (Mc control) and i-*Mtb* pulsed macrophages (Mc + *Mtb*). Results are expressed as percentage of cytotoxicity. Statistical differences:% cytotoxicity from (nonadherent cells + IL-10 treated monocytes) *versus*% cytotoxicity from (nonadherent cells + non treated monocytes): $^*P < 0{\cdot}05$;% cytotoxicity from (nonadherent cells + IFNγ and IL-10) *versus* % cytotoxicity from (nonadherent cells + IL-10 treated monocytes): $^aP < 0{\cdot}05$. (b) Monocytes from 5 PPD$^+$ normal individuals were isolated from PBMC by plastic adherence and cultured for 5 days in complete medium. Then, macrophages were incubated for 18 h with or without i-*Mtb* (Mc + *Mtb* and Mc control, respectively) in the absence (■) or presence of IL-10 (▨). On day 6, macrophages were recovered from the plates and used as target cells in the cytotoxic assay. CD4$^+$, CD8$^+$ and $\gamma\delta$T lymphocytes were isolated from autologous PBMC cultured for 6 days with i-*Mtb* and employed as effector cells. Results are expressed as percentage of cytotoxicity. Statistical differences between percentage cytotoxicity employing IL-10 treated macrophages *versus* % cytotoxicity employing nontreated macrophages: $^*P < 0{\cdot}05$.

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

	% IL-10+ CD14+ cells		% IL-10+ CD3+ cells	
PBMC from	C	i-*Mtb*	C	i-*Mtb*
M-TB	4·6 – 3·2	4·8 – 0·4†	0·2 – 0·2	0·5 – 0·3*
A-TB	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

PBMC from 5 moderate (M-TB) and 6 advanced (A-TB) tuberculosis patients and 6 normal controls were cultured with medium (control, C) or with i-*Mtb* for 48 h. Then, the percentage of IL-10$^+$ CD14$^+$ and IL-10$^+$ CD3$^+$ cells was determined by flow cytometry. Results are expressed as percentage of double positive (x – SEM). Statistical differences: i-*Mtb*-stimulated *versus* control cells: $^*P < 0{\cdot}05$; A-TB *versus* N: † $P < 0{\cdot}05$, A-TB *versus* M-TB: ‡$P < 0{\cdot}05$.

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

		% Cytotoxicity		
PBMC from	PBMC inc. with	CD4	CD8	$\gamma\delta$ T
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 $\gamma\delta$ 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:* $^*P < 0{\cdot}05$.

anti-IL-10 nor IFNγ modified $\gamma\delta$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).

*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

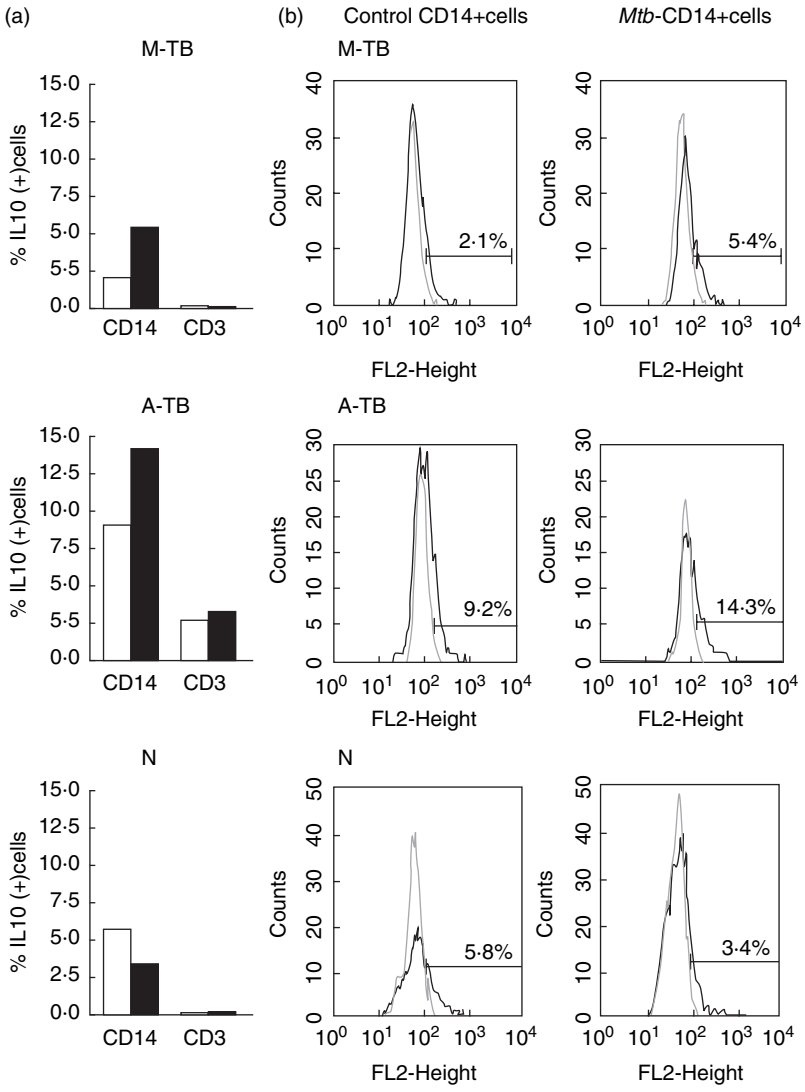

Fig. 2. *Mtb*-induced IL-10 production by CD14$^+$ monocytes and CD3$^+$ T cells in tuberculosis patients. (a) PBMC from patients with moderate (M-TB) or advanced (A-TB) tuberculosis and PPD$^+$ normal individuals (N) were incubated in complete medium alone (□) or with i-*Mtb* (■) for 48 h, thereafter IL-10 produced by CD14$^+$ and CD3$^+$ cells was determined by flow cytometry. Results are expressed as percentage of IL-10 (+) cells in CD14 or CD3 populations (individual data are shown). (b) PBMC from M-TB, A-TB or N were cultured without (control) or i-*Mtb* for 48 h and then IL-10 producing monocytes were determined by flow cytometry. Black open histograms show IL-10 producing CD14$^+$ monocytes while grey open histograms show the isotype-matched antibody. A representative example for each group is shown.

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$^+$) and CD40 antigens (in 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 $\gamma\delta$ T cell-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, pretreatment of macrophages with IL-10 modified 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 IFNγ, 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.

Table 4. Modulation of CD4 and CD8 CTL activity by addition of anti-IL-10 or IFNγ to i-*Mtb*-pulsed macrophages

PBMC from	Macrophages inc. with	% Cytotoxicity CD4	CD8	$\gamma\delta$ 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* + IFNγ	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* + IFNγ	42 ± 4*	29 ± 3*	39 ± 4

CD4, CD8 and $\gamma\delta$ 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 IFNγ (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 IFNγ and percentage cytotoxicity from PBMC + i-*Mtb*: $*P < 0{\cdot}05$.

DISCUSSION

Infection with *Mtb* is accompanied by a local inflammatory response where cytokines play an important role. In contrast to IFNγ, 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 $\gamma\delta$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 $\gamma\delta$T lytic activity.

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

PBMC from	Mac. stimulated 18 h with	% CD4-CTL against macrophages treated 1 h with –	B	C	% CD8-CTL against macrophages treated 1 h with –	B	C	% $\gamma\delta$-CTL against macrophages treated 1 h with –	B	C
M-TB	i-*Mtb*	45 ± 4	39 ± 5	44 ± 5	26 ± 3	11 ± 5*	27 ± 2	30 ± 7	11 ± 5*	33 ± 6
($n = 8$)	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* + IFNγ	48 ± 4	52 ± 2	53 ± 4	30 ± 3	29 ± 5	34 ± 4	41 ± 3	13 ± 2	29 ± 4
A-TB	i-*Mtb*	30 ± 4	32 ± 3	14 ± 3*	10 ± 1	5 ± 1*	13 ± 1	44 ± 4	10 ± 2*	33 ± 6
($n = 16$)	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* + IFNγ	40 ± 3	41 ± 3	35 ± 3	25 ± 3	19 ± 1	27 ± 4	42 ± 4	10 ± 3	34 ± 4
N	i-*Mtb*	40 ± 5	36 ± 3	37 ± 3	25 ± 4	25 ± 3	29 ± 2	20 ± 2	7 ± 2*	22 ± 3
($n = 10$)	i-*Mtb* + 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) IFNγ 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: $*P < 0{\cdot}05$.

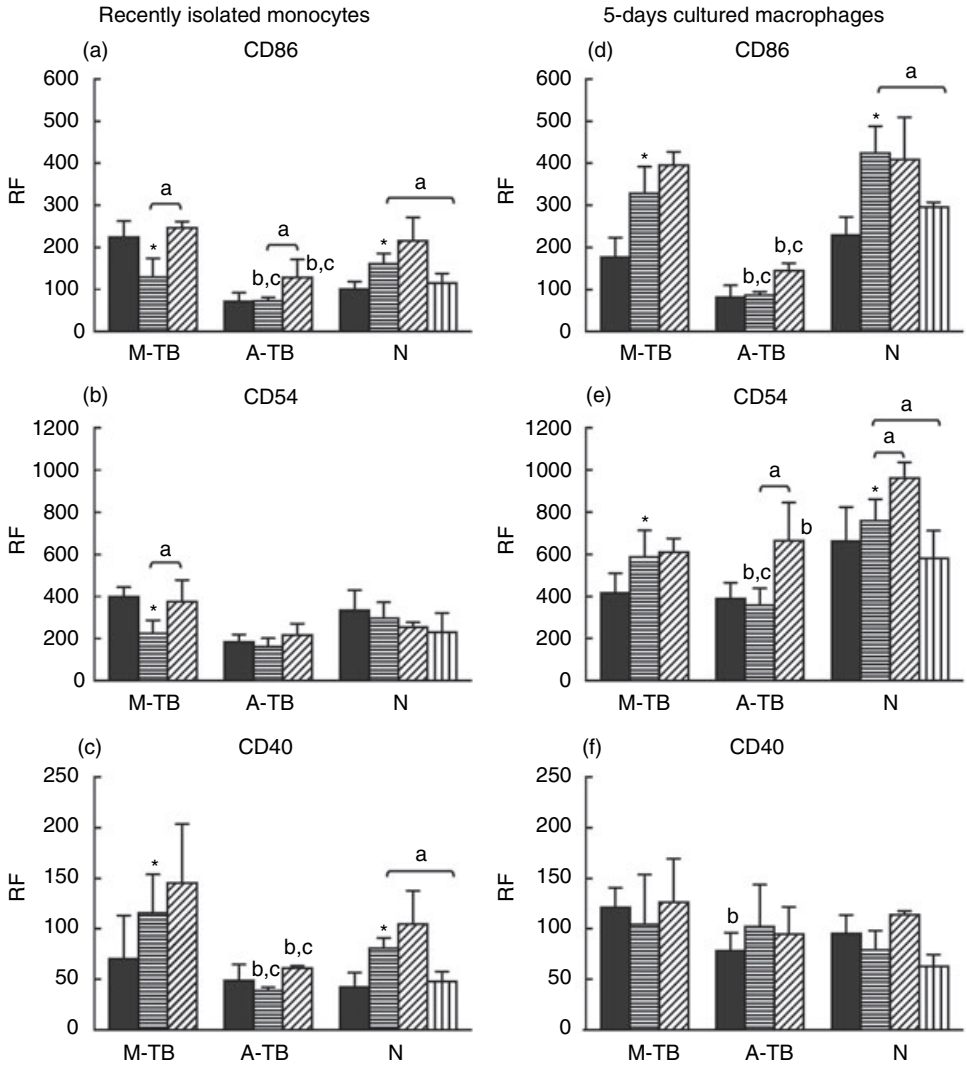

Fig. 3. *Mtb* modulates T cell costimulatory molecules on monocytes and macrophages. Recently isolated PBMC (a–c) or 5 day adherent-monocytes (d–f) from 4 M-TB, 6 A-TB and 5 N-PPD$^+$ were cultured for 18 h in complete medium alone, with i-*Mtb* or with i-*Mtb* plus IFNγ or IL-10, and cell surface marker 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: $^*P < 0{\cdot}05$; RF from IFNγ or IL-10 treated-i-*Mtb*-pulsed macrophages *versus* RF from i-*Mtb*-pulsed macrophages: $^aP < 0{\cdot}05$; RF A-TB *versus* N: $^bP < 0{\cdot}05$, A-TB *versus* M-TB: $^cP < 0{\cdot}05$.

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$^+$cells, but it did not modify $\gamma\delta$T activity. Moreover, neither IL-10 nor IL-10 plus IFNγ affected the $\gamma\delta$T-mediated lytic activity on monocytes from N-PPD$^+$ suggesting that $\gamma\delta$ 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 $\gamma\delta$T 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]. Our results showed

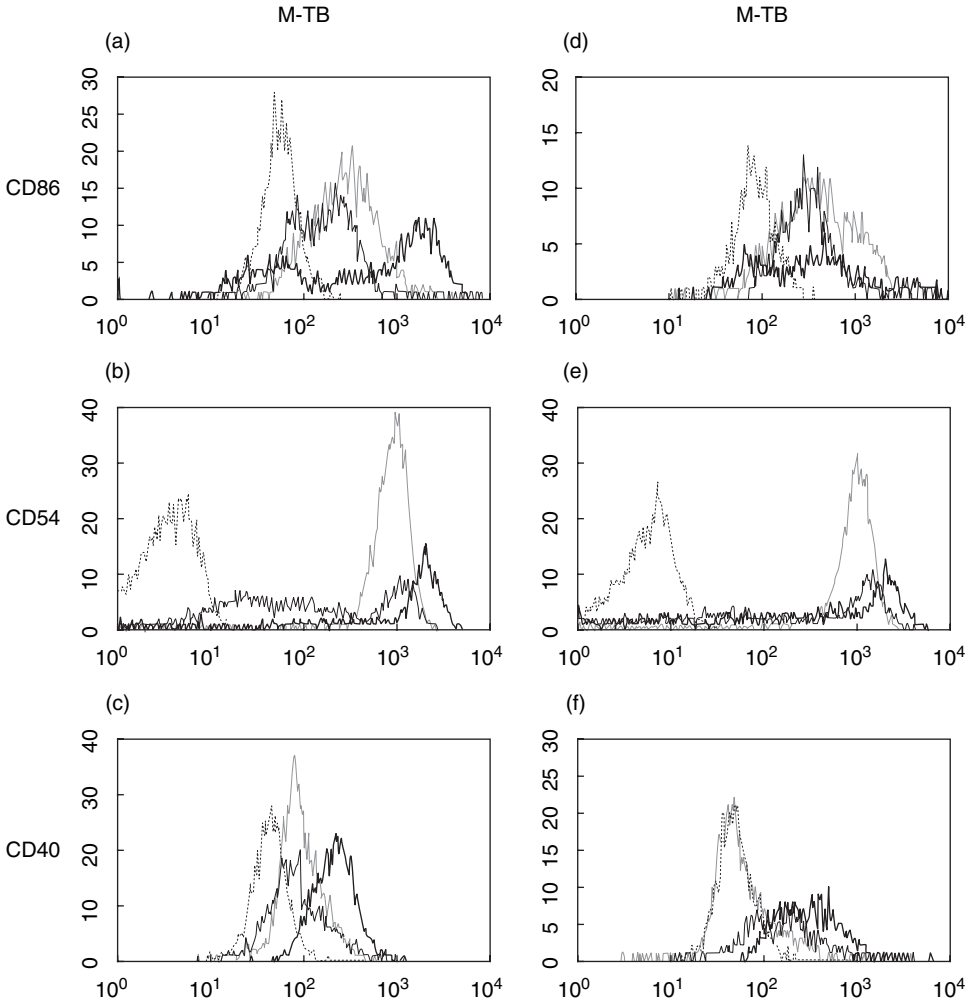

Fig. 4. Recently isolated PBMC from 3 M-TB (a–c) and 3 A-TB (d–f) were cultured during 18 h in medium alone (**- - - -**), i-*Mtb* (——) or with i-*Mtb* and anti-IL-10 MAb (——). Then, the surface expression of CD86, CD54 and CD40 on $CD14^+$ cells was examined by flow cytometry. □ the monocytes stained with specific antibodies; ■ the respective isotype control. A representative example for each group is shown.

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 overridden 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 IFNγ production [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 $\gamma\delta$T-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 IFNγ production by costimulated T cells [39]. In the current study, we showed that IFNγ increased CD54 expression on i-*Mtb* stimulated monocytes from M-TB patients or on macrophages from A-TB patients

leading to enhancement of MHC-restricted lytic activity. These results could explain in part the improvement in clinical symptoms 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 IFNγ signalling 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.

ACKNOWLEDGEMENTS

We thank the medical staff of División Tisioneumonología of Hospital F.J.Muñiz, for their great help providing clinical samples from patients with tuberculosis. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0711/98) and from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, 05–04816).

REFERENCES

1 Greenfield EA, Nguyen KA, Kuchoo VK. CD28/B7 costimulation: a review. Crit Rev Immunol 1998; **18**:389–418.
2 McDyer JF, Goletz TJ, Thomas E, June CH, Seder RA. CD40 ligand/CD40 stimulation regulates the production of IFNγ from human peripheral blood mononuclear cells in an IL-12 and/or CD28-dependent manner. J Immunol 1998; **160**:1701–7.
3 Stüber E, Strober W, Neurath M. Blocking the CD40L/CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. J Exp Med 1996; **183**:693–8.
4 Morse MA, Lyerly K, Gilboa E, Thomas E, Nair SK. Optimization of the sequence of antigen loading and CD40-ligand induced maturation of dendritic cells. Cancer Res 1998; **58**:2965–8.
5 Yang Y, Wilson JM. CD40 ligand-dependent T cell activation requirement of B7-CD28 signalling though CD40. Science 1996; **273**:1862–4.
6 Tan JS, Canady DH, Boom WH, Balaji KN, Schwander SK. Human alveolar T lymphocyte response to *Mycobacterium tuberculosis*. Role for CD4+ and CD8+ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. J Immunol 1997; **159**:290–7.
7 Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility class I restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA 1992; **89**:12013–7.
8 Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart T, Bloom BR. An essential role for Interferon-γ in resistance to *Mycobacteriun tuberculosis* infection. J Exp Med 1993; **178**:2249–54.
9 Toosi Z, Gogate P, Shiratsuchi H, Young T, Ellner JJ. Enhanced production of TGF-β by blood monocytes from patients with active tuberculosis and presence of TGF-β in tuberculosis granulomatous lung lesions. J Immunol 1995; **154**:465–73.
10 Dlugovizky DA, Torrez-Morales A, Rateni L, Farroni MA, Largacha O, Molteni O, Bottasso O. Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. FEMS Immunol Med Microbiol 1997; **18**:203–7.
11 Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC, Berezovskaya A, Rousset D, Reynes JM, Goldfeld AE. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest 2000; **105**:1317–25.
12 Moore KW, de Wall Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the Interleukin-10 receptor. Annu Rev Immunol 2001; **19**:683–765.
13 Shaw T, Thomas LH, Friedland JL. Regulation of IL-10 secretion after phagocytosis of *Mycobacterium tuberculosis* by human monocytic cells. Cytokine 2000; **12**:483–6.
14 Sieling PA, Abrams JS, Yamamura M, Salgame P, Bloom BR, Rea TH, Modlin RL. Immunosuppressive roles for IL-10 and IL-4 in human infection. In vitro modulation of T cell responses in leprosy. J Immunol 1993; **150**:5501–10.
15 Orme I, Roberts A, Grieffen J, Abrams JS. Cytokine secretion by CD4+ T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J Immunol 1993; **151**:518–25.
16 Serbina N, Lazarevic V, Flynn J. $CD4^+$ T cells are required for the development of cytotoxic CD8+ T cells during *Mycobacterium tuberculosis* infection. J Immunol 2001; **167**:6991–00.
17 Kabelitz D, Bender A, Schondelmaier S, Schoe B, Kaufmann SHE. A large fraction of human peripheral blood γ/δ T cells is activated by *Mycobacterium tuberculosis* but not by its 65-kD heat shock protein. J Exp Med 1990; **171**:667–79.
18 Tsukaguchi K, Balaji KN, Boom WH. CD4+ $\alpha\beta$ T cell and $\gamma\delta$ T cell responses to *Mycobacterium tuberculosis*. similarities and differences in antigen recognition, cytotoxic effector function, and cytokine production. J Immunol 1995; **154**:1786–96.
19 Balaji K, Boom WH. Processing of *Mycobacterium tuberculosis* by human monocytes for CD4+ $\alpha\beta$ and $\gamma\delta$ T cells role of particulate antigen. Infect Immun 1998; **66**:98–106.
20 Canaday DH, Ziebold Ch, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation of human CD8+ $\alpha\beta$ TCR+ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. J Immunol 1999; **162**:372–9.
21 de la Barrera SS, Finiasz M, Frias A *et al.* Specific lytic activity against mycobacterial antigens is inversely correlated with the severity of tuberculosis. Clin Exp Immunol 2003; **132**:450–61.
22 Damle NK, Klussmann K, Linsley PS, Aruffo A. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3 and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. J Immunol 1992; **148**:1989–92.
23 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Invest 1968; **97**:77–89.
24 Dagna L, Iellem A, Biswas P *et al.* Skewing of cytotoxic activity and chemokine production, but not of chemokine receptor expression, in

human type-1/-2 gamma delta T lymphocytes. Eur J Immunol 2002; **32**:2934–43.
25 Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. Immunity 1995; **3**:495–507.
26 Van Gool SW, Vandenberghe P, de Boer M, Ceuppens JL. CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. Immunol Rev 1996; **153**:47–83.
27 Freeman GJ, Borriello F, Hodes RJ *et al.* Murine B7–2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. J Exp Med 1993; **178**:2185–92.
28 Bluestone JA. New perspectives of CD28-B7-mediated T cell costimulation. Immunity 1995; **2**:555–9.
29 Kim JJ, Bagarazzi ML, Trivedi N *et al.* Engineering of in vivo immune responses to DNA immunization via co-delivery of costimulatory molecule genes. Nat Biotechnol 1997; **15**:641–6.
30 Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J Exp Med 1993; **177**:925–35.
31 Arinobu Y, Sugimoto R, Akaiwa M, Arima K, Otsuka T, Hamasaki N, Izuhara K. Augmentation of signal transducer and activation of transcription (STAT) 6 and STAT3 expression in stimulated B and T cells. Biochem Biophys Res Commun 2000; **277**:317–24.
32 Francis DA, Karras JG, Ke XY, Sen R, Rothstein TL. Induction of the transcription factors NF-kappa B, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. Int Immunol 1995; **7**:151–61.
33 Foey AD, Feldmann M, Brennan FM. Route of monocyte differentiation determines their cytokine production profile: CD40 ligation induces interleukin 10 expression. Cytokine 2000; **12**:1496–505.
34 McDyer JF, Goletz TJ, Thomas E, June CH, Seder RA. CD40 ligand/CD40 stimulation regulates the production of IFN-gamma from human peripheral blood mononuclear cells in an IL-12- and/or CD28-dependent manner. J Immunol 1998; **160**:1701–7.
35 Campos-Neto A, Ovendale P, Bement T, Koppi TA, Fanslow WC, Rossi MA, Alderson MR. CD40 ligand is not essential for the development of cell-mediated immunity and resistance to *Mycobacterium tuberculosis*. J Immunol 1998; **160**:2037–41.
36 Samten B, Thomas EK, Gong J, Barnes PF. Depressed CD40 ligand expression contributes to reduced gamma interferon production in human tuberculosis. Infect Immun 2000; **68**:3002–6.
37 Saunders BM, Frank AA, Orme IM. Granuloma formation is required to contain bacillus growth and delay mortality in mice chonically infected with *Mycobacterium tuberculosis*. Immunology 1999; **98**:324–8.
38 Johnson CM, Cooper AM, Frank AA, Orme IM. Adequate expression of protective immunity in the absence of granuloma formation in *Mycobacterium tuberculosis*-infected mice with a disruption in the intracellular adhesion molecule 1 gene. Infect Immun 1998; **66**:1666–70.
39 Kim JJ, Tsai A, Nottingham LK *et al.* Intracellular adhesion molecule-1 modulates beta-chemokines and directly costimulates T cells in vivo. J Clin Invest 1999; **103**:869–77.
40 Condos R, Raju B, Canova A, Zhao BY, Weiden M, Rom WN, Pine R. Recombinant gamma interferon stimulates signal transduction and gene expression in alveolar macrophages in vitro and in tuberculosis patients. Infect Immun 2003; **71**:2058–64.
41 Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. Am J Respir Crit Care Med 1998; **157**:729–35.
42 Law KF, Jagirdar J, Weiden MD, Bodkin M, Rom WN. Tuberculosis in HIV-positive patients: cellular response and immune activation in the lung. Am J Respir Crit Care Med 1996; **153**:1377–84.
43 Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, Larner AC, Finbloom DS. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 1999; **93**:1456–63.
44 Ting LM, Kim AC, Cattamanchi A, Ernst JD. *Mycobacterium tuberculosis* inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 1999; **163**:3898–906.
45 Tobian AA, Potter NS, Ramachandra L, Pai RK, Convery M, Boom WH, Harding CV. Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen-associated molecular patterns. *Mycobacterium tuberculosis* 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. J Immunol 2003; **171**:1413–22.
46 Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT, Boom WH, Harding CV. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. J Immunol 2001; **167**:910–8.
47 Fortsch D, Rollinghoff M, Stenger S. IL-10 converts human dendritic cells into macrophage-like cells with increased antibacterial activity against virulent Mycobacterium tuberculosis. J Immunol 2000; **165**:978–87.
48 De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. Eur J Immunol 1997; **27**:1229–35.