Specific lytic activity against mycobacterial antigens is inversely correlated with the severity of tuberculosis

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SUMMARY
The ability of peripheral blood mononuclear cells (PBMC) from patients with active tuberculosis to display cytotoxic responses against autologous Mycobacterium tuberculosis (Mtb)-pulsed macrophages was evaluated. Non-MHC restricted cell-dependent lytic activity was observed in ex vivo effector cells from tuberculosis patients and was mediated mainly by CD3\^+\ g\delta TCR\^+ T (g\delta T) cells bearing CD56 and/or CD16 molecules. MHC-resticted and non-MHC restricted cytotoxic T cells (CTL) were differentially expanded upon stimulation with Mtb in tuberculosis patients and normal controls (N). Class-I restricted CD8\^+ CTL and class-II restricted CD4\^+ CTL were generated in PPD\^N and to a lesser extent in PPD\^N. Mtb-stimulated effector cells from tuberculosis patients became progressively non-MHC restricted CD4\^+CD8\^− g\delta T cells, while lytic activity of CD4\^+ and CD8\^+CTL decreased gradually as the disease became more severe. On the other hand, target cells were lysed by ex vivo cells from tuberculosis patients through the Fas-FasL and perforin pathways. Mtb-induced CD4\^+ CTL from tuberculosis patients and N controls preferentially employed the Fas-FasL mechanism. Mtb-induced CD8\^+ CTL effector cells from patients used the perforin-based mechanism while cells from N controls also used the Fas-FasL pathway. While Mtb-induced g\delta CTL from patients and PPD\^N employed the latter mechanism cells from PPD\^N individuals also used the perforin pathway. It can be concluded that shifts in the CTL response and the cytolytic mechanisms take place as the pulmonary involvement becomes more severe.

Keywords cytotoxicity lymphocytes tuberculosis

INTRODUCTION
Mycobacterium tuberculosis (Mtb), the aetiologic agent of tuberculosis, is an intracellular pathogen that resides and replicates within macrophages. Most healthy individuals infected with Mtb have the ability to control the infection by mounting an immune response capable of arresting the growth of bacilli within the granuloma. It was thought initially that antigen-specific CD4\^+ T lymphocytes were the main effector cells of protective responses against Mtb. However, there is increasing evidence that lysis of infected cells and killing of the invading pathogen also contribute to immune protection against Mtb [1]. CD4\^+ T cells participate in the control of Mtb infection not only through the production of type 1 cytokines, but also by their ability to lyse mycobacteria-infected and mycobacterial antigen-pulsed macrophages [2,3]. Although the role of CD8\^+ T cells in human tuberculosis remains controversial, their participation as cytotoxic T lymphocytes (CTL) is supported by studies in both murine and human tuberculosis, where Mtb-specific CD8\^+ CTL have been isolated [4–7]. A role for g\delta T cells in the protection of normal healthy individuals against tuberculosis has been established by their in vitro proliferation in response to antigenic preparations of Mtb [8,9]. Despite differences in the antigens recognized and cytokine production, it has been demonstrated in healthy purified protein derivative positive (PPD\^+) individuals, that CD4\^+ and g\delta T cells have similar effector functions such as cytotoxicity and interferon-gamma (IFN-\gamma) production [10]. CD4\^+ and CD8\^+ T cells recognize mycobacterial peptides in the context of major histocompatibility complex (MHC) class-II and class-I proteins, respectively [10,11] while CD3\^+ g\delta T cells recognize small peptides as well as phosphate containing antigens [12,13] in a non-MHC
restricted manner. The family of CD1 surface molecules (CD1a, CD1b, CD1c) react with double-negative, CD8+ or γδ T cells and have a high structural and conformational homology to MHC class-I molecules [14–16]. Moreover, CD1 has been shown to mediate specific T-cell recognition of non-peptide forms of mycobacterial cell wall constituents by which mycobacteria-infected macrophages can be lysed [14–16].

Two major mechanisms of lysis are recognized, exocytosis of cytotoxic granules, containing pore-forming perforin and serine esterase granzyme molecules and induction of apoptosis by ligation of CD95 (Fas) with CD95 ligand (CD95L, FasL) [17]. Expression of both perforin and CD95L has been demonstrated in PPD-specific CD4+ T cell clones [18]. In addition, Mtb-reactive CD4+ and CD8+ T cell lines from PPD+ healthy individuals, lysed Mtb-infected monocytes through perforin and Fas/FasL-dependent mechanisms [19]. It has also been reported that CD8+ T cells lyse and kill the mycobacterium inside Mtb-infected cells by the release of granulysin and perforin [20,21]. As γδ T cells possess cytolytic granules and express FasL [22], the lysis could be mediated by both pathways [23,24].

In this study we evaluated the ability of peripheral blood mononuclear cells (PBMC) freshly isolated from tuberculosis patients and PBMC cultured in the presence of Mtb (CTL), with or without previous pulmonary tuberculosis, to develop cytotoxic responses against autologous macrophages presenting Mtb antigens. In addition, we analysed the nature of the effector cells involved in the cytotoxic response as well as the lytic mechanisms employed by recently isolated and Mtb-induced CTL to lyse Mtb-pulsed macrophages from tuberculosis patients and healthy individuals. We demonstrate that in tuberculosis patients the in vivo activation of circulating CD3+ γδ T cells that bear the CD56 and/ or CD16 antigen are the main cytotoxic cells involved in the non-MHC restricted lysis of Mtb-pulsed macrophages. Upon in vitro stimulation of PBMC with Mtb a loss of CD4+ and CD8+ CTL with an increase in CD4+ CD8+ γδ CTL are observed and can be correlated with the severity of pulmonary involvement. Moreover, the lytic mechanisms used by the different subsets of cytotoxic T cell to lyse target cells differ between patients and healthy PPD+ individuals.

**METHODS**

**Patients**

A total of 51 patients with pulmonary tuberculosis were studied. Patients were diagnosed by 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 abnormal chest radiography. Informed consent was obtained from patients according to the Ethics Commission of the Hospital Francisco J. Muñiz and of IHema, Academia Nacional de Medicina. All patients had active tuberculosis and were under multidrug treatment at the time of study (2–45 days). Pulmonary disease was classified according to the extent and type of X-ray findings into mild (M) and advanced (A) tuberculosis according to the American Tuberculosis Society criteria. Of the patients, 18 had M and 33 had A tuberculosis. Patients positive for HIV (human immunodeficiency virus) or other concurrent infectious diseases were excluded. They were classified into two groups: (a) patients without previous pulmonary tuberculosis = TB – these include patients with M-TB (n = 9, eight men and one woman, 19–55 years) and with A-TB (n = 15, 13 men and two women, 22–68 years); and (b) patients with pulmonary tuberculosis overcome at least 3 years earlier with posterior reactivation of the disease = pre-TB – these were also separated in M-pre-TB (n = 9, seven men and two women, 20–45 years) and A-pre-TB (n = 18, 14 men and four women, 25–65 years). The controls included 16 healthy individuals (N) (10 men, six women, 25–60 years, eight tuberculin reactive (PPD+)) and 8 PPD–.

**Mononuclear cells**

PBMC were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation [25] and then resuspended in 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, CM).

**Antigen**

Mtb H37-Rv strain was kindly provided by the Mycobacteria Section of Instituto Nacional de Enfermedades Infecciosas, ANLIS, Dr. C. G. Malbrán (Buenos Aires, Argentina). Mycobacterial strain H37Rv was grown on 7H11 agar (Difco Laboratories, Detroit, USA) at 37°C in 5% CO2 air at mid-log phase. Mycobacteria were harvested, sonicated to disrupt the clumps, washed three times by centrifugation and resuspended in phosphate buffered saline (PBS) free of pyrogen at a concentration of 1 × 10^8 bacteria/ml. Bacteria were killed by heating at 80°C for 20 min and then aliquots of bacterial suspensions were stored at −20°C until their use. This mycobacterial suspension contains soluble as well as particulate antigens.

**Effector cells for cytototoxicity assays**

Either recently purified PBMC, resuspended in CM with 10% DMSO (4 × 10^6 cells/ml) and stored at −80°C until their use (subsequently referred to as ex vivo effector cells), or PBMC (2 × 10^6 cells/ml) cultured in Falcon 2063 tubes (Becton Dickinson, Lincoln, PK, NJ, USA) at 37°C in humidified 5% CO2 atmosphere, in CM with or without Mtb (1 × 10^6 bacteria/ml) (cultured cells) were employed as effector cells in the cytotoxic assay. On day 6, thawed ex vivo effector cells and cultured cells were washed three times with RPMI 1640, resuspended in CM (2 × 10^6 cells/ml) and tested for cytotoxic activity. Studies were performed at a 40 : 1 E/T ratio, unless otherwise stated.

**Depletion of CD3+/γδ T lymphocytes from ex vivo effector cells**

Ex vivo effector cells from tuberculosis patients were thawed and depleted of CD3+ T cells by a magnetic method. Of these, 2 × 10^6 cells were incubated with anti-CD3 antibody (clone 145–2C 11) during 30 min at 4°C, after which they were incubated for 30 min with goat antimouse IgG-coated magnetic beads (Dynal Oslo, Norway) and non-rosetted cells were separated using a magnet.

**Isolation of CD4+, CD8+ and γδ T lymphocytes from cultured PBMC**

CD4+ and CD8+ T cells were obtained by negative selection with magnetic beads (Dynal) from 6 day-cultured PBMC. For CD4+ and CD8+ T cell enrichment, cells were treated with anti-γδTCR (Pan γδ, IgG1, clone Immun 510, Immunotech, Marseille, France) anti-CD56 (Leu-19, IgG1, clone MY31, Becton Dickenson, CA, USA) and anti-CD16 (Leu-11b, IgM, clone G022, Becton Dickinson) monoclonal antibodies (MoAb), followed by goat
Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. After removing non-adherent cells, cells remaining in the plates (10% of the original cell suspension) were incubated at 37°C in a humidified 5% CO₂ atmosphere for 6 days. For the cytotoxic assays, on day 5 of incubation, macrophages were pulsed with Mtb (1 × 10⁸ bacteria/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. On day 6, plates were cooled for 2 h at 4°C to facilitate the detachment of adherent cells by vigorous pipetting using ice-cold medium. These cells were washed and pellets of 5–7 × 10⁵ cells were labelled with 100 μCi of Na²⁵³ CrO₄ (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. The cells were then washed three times and resuspended in CM at 1 × 10⁶ cells/ml.

**Cytotoxic assay**

**Target cells**

Monoctyes were allowed to adhere to the bottom of 24-well flat-bottom Falcon plates by incubation of PBMC (5 × 10⁵/ml) for 2 h at 37°C. After removing non-adherent cells, cells remaining in the plates (10% of the original cell suspension) were incubated at 37°C in a humidified 5% CO₂ atmosphere for 6 days. For the cytotoxic assays, on day 5 of incubation, macrophages were pulsed with Mtb (1 × 10⁸ bacteria/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. On day 6, plates were cooled for 2 h at 4°C to facilitate the detachment of adherent cells by vigorous pipetting using ice-cold medium. These cells were washed and pellets of 5–7 × 10⁵ cells were labelled with 100 μCi of Na²⁵³ CrO₄ (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. The cells were then washed three times and resuspended in CM at 1 × 10⁶ cells/ml.

**Spontaneous release**

Spontaneous release is the radioactivity released from target cells incubated with CM alone, ranging from 8 to 15%. In all cases, the cytotoxic assays performed with PBMC cultured in the absence of Mtb or with macrophages not pulsed with antigen rendered negligible cytotoxicity (0–6%). Data presented in Tables 1–4 and Figs 1–4 were obtained by subtracting the activity of 100 μCi of Na²⁵³ CrO₄ (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. The cells were then washed three times and resuspended in CM at 1 × 10⁶ cells/ml.

**Expression of CD4 and CD8 antigens on ex vivo effector cells**

Expression of CD4 and CD8 antigens on ex vivo effector cells, control and Mtb-stimulated PBMC were incubated with FITC-conjugated MoAb specific for the human CD4 (anti-CD4-FITC, Ancell) and CD8 (anti-CD8-FITC, Ancell), Mtb (1 × 10⁸ bacteria/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. On day 6, plates were cooled for 2 h at 4°C to facilitate the detachment of adherent cells by vigorous pipetting using ice-cold medium. These cells were washed and pellets of 5–7 × 10⁵ cells were labelled with 100 μCi of Na²⁵³ CrO₄ (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. The cells were then washed three times and resuspended in CM at 1 × 10⁶ cells/ml.

**Expression of intracellular perforin.**

Either ex vivo effector cells or control and Mtb induced effector cells were incubated during 4 h with monensin (3 μg/ml, Sigma, St Louis, MO, USA) at 37°C and were then incubated with anti-CD4, anti-CD8 or anti-γδ TCR MoAb for 15 min at room temperature. Then, the cells were fixed (Fix and Perm, Caltag) according to the manufacturer’s instructions. Cells were then washed, suspended in PBS-FCs-azide and PE-antiperforin MoAb (Ancell) was added together with permeabilizing solution (Fix and Perm). Cells were incubated for 30 min at 4°C and washed once with PBS-FCs-azide.

**Expression of class-I and class-II MHC antigens on ex vivo effector cells**

Recently isolated as well as 6 days-cultured PBMC (with or without Mtb) were incubated with anti-class-I (anti-HLA-ABC-FITC, Immunotech) or anti-class-II (anti-HLA-DR-FITC, Becton Dickinson) and anti-CD14-PE (Immunotech) (30 min, 4°C). Then, cells were washed and stained cells were analysed as mentioned above. FITC- or PE-labelled isotype matched labelled non-relevant antibodies were also tested to evaluate non-specific staining. Results are expressed as percentage of positive cells or as mean of fluorescence intensity (MFI) or as the percentage of the mean of fluorescence intensity (MFI) observed in normal individuals (% MFI).

**Statistics**

Comparisons of pre-TB, 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. Individual CD4+, CD8+ and γδ T cells. Ex vivo effector cells, control and Mtb-stimulated PBMC were incubated with FITC-conjugated MoAb specific for the human CD4 (anti-CD4-FITC, Ancell) and CD8 (anti-CD8-FITC, Ancell), Mtb (1 × 10⁸ bacteria/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. On day 6, plates were cooled for 2 h at 4°C to facilitate the detachment of adherent cells by vigorous pipetting using ice-cold medium. These cells were washed and pellets of 5–7 × 10⁵ cells were labelled with 100 μCi of Na²⁵³ CrO₄ (New England Nuclear, Boston, MA, USA) by incubation for 1 h at 37°C. The cells were then washed three times and resuspended in CM at 1 × 10⁶ cells/ml.

**Expression of surface membrane FasL antigen on CD4, CD8+ and γδ T cells.**

**Expression of CD16 on CD3+ and CD3− T cells.**

**Expression of CD16 on CD3− T cells.**

**Expression of CD16 on CD3+ T cells.**

**Expression of intracellular perforin.**

Either ex vivo effector cells or control and Mtb induced effector cells were incubated during 4 h with monensin (3 μg/ml, Sigma, St Louis, MO, USA) at 37°C and were then incubated with anti-CD4, anti-CD8 or anti-γδ TCR MoAb for 15 min at room temperature. Then, the cells were fixed (Fix and Perm, Caltag) according to the manufacturer’s instructions. Cells were then washed, suspended in PBS-FCs-azide and PE-antiperforin MoAb (Ancell) was added together with permeabilizing solution (Fix and Perm). Cells were incubated for 30 min at 4°C and washed once with PBS-FCs-azide.

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**Statistics**

Comparisons of pre-TB, 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. Individual CD4+, CD8+ and γδ T cell enrichment, cells were treated with anti-CD16 followed by goat-antimouse IgG-coated beads and anti-CD8+ plus anti-CD8 coated beads. In all cases, cells were also depleted of B cells using anti-pan B-coated beads (Dynal). One cycle of treatment was sufficient for an effective depletion as assessed by flow cytometry. Purity was 90–95% in each isolated. CD4+, CD8+ and γδ T cells were resuspended in CM, ensuring that the proportion of each subset was the same as in total cultured PBMC in order to compare the lytic activity.
Table 1. Depletion of CD3+ cells inhibited the lysis of Mtb-pulsed macrophages by ex vivo effector cells from patients with tuberculosis

| Ex vivo effector cells from | % cytotoxicity, whole cells | % cytotoxicity, CD3-depleted |
|-----------------------------|-----------------------------|-----------------------------|
|                            | Unpulsed macrophages | Mtb-pulsed macrophages | Unpulsed macrophages | Mtb-pulsed macrophages |
| 1 (M-TB)                   | 4                          | 70                        | 4                          | 10                        |
| 2 (M-TB)                   | 3                          | 72                        | 5                          | 8                         |
| 3 (M-TB)                   | 3                          | 78                        | 4                          | 8                         |
| 4 (M-pre-TB)               | 5                          | 83                        | 7                          | 10                        |
| 5 (M-pre-TB)               | 5                          | 89                        | 7                          | 17                        |
| 6 (A-TB)                   | 6                          | 69                        | 1                          | 15                        |
| 7 (A-TB)                   | 6                          | 71                        | 3                          | 12                        |
| 8 (A-TB)                   | 5                          | 70                        | 6                          | 11                        |
| 9 (A-TB)                   | 0                          | 73                        | 3                          | 18                        |
| 10 (A-pre-TB)              | 0                          | 55                        | 4                          | 15                        |
| 10 (A-pre-TB)              | 3                          | 65                        | 5                          | 13                        |
| 11 (A-pre-TB)              | 6                          | 81                        | 8                          | 12                        |

Ex vivo effector cells from patients with tuberculosis were depleted (CD3-depleted) or not (whole cells) of CD3+ cells by magnetic methods as described in Materials and methods. Then, both cell suspensions were tested for their cytotoxic ability to lyse unpulsed or Mtb-pulsed macrophages. Results are expressed as percentage of cytotoxicity and individual data are shown.

Fig. 1. Cytotoxic effector cells present in ex vivo cells from tuberculosis patients. Ex vivo cells from TB and pre-TB patients with M or A form of the disease were incubated with anti-CD56 and anti-CD16 or anti-γδ TCR MoAb and target cells were treated with anti-MHC class-I, anti-MHC class II, anti-CD1b MoAb or isotype-matched non-relevant control antibodies before the cytotoxic assay, as described in Materials and methods. Results are expressed as percentage of cytotoxicity (mean ± s.e.m.). Statistical differences between percentage cytotoxicity from untreated effector and target cells and from MoAb-treated effector or target cells: *P < 0.05.

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Table 2. *Ex vivo* effector cells from patients with tuberculosis lysed autologous and allogeneic *Mtb*-pulsed macrophages

| Effector cells from Mtb-pulsed macrophages from (% lysis) | 1 | 2 | 3 | 4 | 5 | 6 | N1 | N2 | N3 |
|----------------------------------------------------------|---|---|---|---|---|---|----|----|----|
| 1. M-TB                                                  | 77| – | 76| – | – | – | 75 | 70 | – |
| 2. M-TB                                                  | 84| – | 80| – | – | – | 80 | 83 | 85 |
| 3. M-pre-TB                                              | 69| – | 71| – | – | – | 72 | 68 | 70 |
| 4. A-TB                                                  | – | 80| 79| – | – | – | 80 | 73 | 75 |
| 5. A-TB                                                  | 71| 68| 71| – | – | – | 70 | 66 | 67 |
| 6. A-TB                                                  | – | – | – | 69| 65| 70| – | – | 66 |
| 7. A-pre-TB                                              | – | – | – | 55| 59| – | – | 53 | 60 |
| 8. A-pre-TB                                              | – | – | – | 53| – | – | – | – | 50 |

*Ex vivo* effector cells from patients with tuberculosis (1–8) were tested for their lytic activity against autologous or allogeneic macrophages from patients with tuberculosis or normal individuals (N1, N2 or N3). Results are expressed as percentage of cytotoxicity and individual data are shown.

Fig. 2. Expression of CD16⁺, CD56⁺ on CD3⁻ and γδ CD3⁺ T cells present on *ex vivo* cells. *Ex vivo* cells from 15 patients with tuberculosis and eight normal controls were tested for the presence of γδ T, γδ T/CD56⁺, γδ T/CD16⁺ and CD3⁻CD16⁺CD56⁺ cells, as mentioned in Materials and methods. Results are expressed as mean ± s.e.m. Statistical differences: *P* < 0·05, **P** < 0·01.
shown in Fig. 5a, analogous macrophages pulsed with normal individuals (N) were tested for their ability to lyse autologous or allogeneic macrophages from patients with mild (M) and advanced (A) tuberculosis and PPD–N individuals lysed from pre-TB patients than in cells from TB at all E/T ratios employed (Fig. 5a).

The ability of effector cells and 6-days cultured PBMC from patients with tuberculosis (1–6) or normal individuals (N1 to N5) were tested for their lytic activity against autologous or allogeneic macrophages. Results are expressed as percentage of lysis and individual data are shown.

Table 3. 56T cells from patients with tuberculosis and normal controls lysed autologous and allogeneic macrophages

| 56T effector cells from | Mtb-pulsed macrophages from (% lysis) |
|-------------------------|---------------------------------------|
|                         | 1 | 2 | 3 | 4 | 5 | 6 | N1 | N2 | N3 | N4 | N5 |
| 1. M-TB                 | 24| 20| 28| - | - | - | 18| 23| 27| - | - |
| 2. M-TB                 | 26| 26| - | - | - | - | 17| 20| - | - | - |
| 3. A-TB                 | 40| - | 46| - | - | - | 40| 48| - | - | - |
| 4. A-TB                 | - | - | 48| - | - | - | - | - | - | - | - |
| 5. A-pre-TB             | 16| 23| 20| - | 39| - | - | - | 31| 28| 32 |
| 6. A-pre-TB             | 20| 24| 21| - | - | 35| - | - | 32| 29| 26 |
| N1                      | 13| - | - | - | - | - | 22| 20| 15| - | - |
| N2                      | - | - | - | - | - | - | 25| 24| - | - | - |
| N3                      | - | - | - | 13| 29| 32| 16| - | 16| 15| 16 |
| N4                      | - | - | - | 10| 13| 10| - | - | 12| 13| 14 |
| N5                      | - | - | - | 18| 17| 15| - | - | 18| 16| 17 |

56 T cells from Mtb-stimulated PBMC from patients with tuberculosis (1–6) or normal individuals (N1 to N5) were tested for their lytic activity against autologous or allogeneic macrophages. Results are expressed as percentage of lysis and individual data are shown.

Table 4. Percentage of CD4, CD8 and 56T cells expressing Fas-Ligand or perforin

|                  | Mild          | Advanced      | Normal       |
|------------------|---------------|---------------|--------------|
|                  | Control | Mtb | Control | Mtb | Control | Mtb |
| Fas-L CD4 T cells|        |     |        |     |        |     |
| % (+) cells      | 20 ± 5 | 28 ± 5 | 26 ± 6 | 25 ± 7 | 31 ± 3 | 33 ± 4 |
| MFI              | 372 ± 17 | 328 ± 56 | 125 ± 20 | 148 ± 56 | 281 ± 63 | 394 ± 37 |
| CD8 T cells      |        |     |        |     |        |     |
| % (+) cells      | 19 ± 2 | 21 ± 2 | 16 ± 4 | 15 ± 2 | 22 ± 2 | 15 ± 2 |
| MFI              | 231 ± 65 | 202 ± 13 | 132 ± 7 | 128 ± 24 | 161 ± 76 | 131 ± 26 |
| 56 T cells       |        |     |        |     |        |     |
| % (+) cells      | 15 ± 2 | 31 ± 2 | 28 ± 2 | 43 ± 9 | 17 ± 7 | 28 ± 1 |
| MFI              | 165 ± 35 | 155 ± 18 | 178 ± 46 | 164 ± 5 | 143 ± 38 | 243 ± 53 |
| Perforin CD8 T cells|        |     |        |     |        |     |
| % (+) cells      | 24 ± 4 | 23 ± 4 | 12 ± 5 | 18 ± 6 | 22 ± 2 | 24 ± 1 |
| MFI              | 200 ± 67 | 247 ± 52 | 234 ± 63 | 216 ± 75 | 250 ± 38 | 283 ± 81 |
| 56 T cells       |        |     |        |     |        |     |
| % (+) cells      | 29 ± 2 | 27 ± 6 | 27 ± 4 | 32 ± 8 | 33 ± 9 | 33 ± 7 |
| MFI              | 183 ± 23 | 202 ± 15 | 223 ± 23 | 203 ± 58 | 239 ± 17 | 217 ± 33 |

CD4, CD8 and 56 T cells expressing FasL or perforin present in culture and Mtb-stimulated PBMC cultures from mild (n = 6) and advanced (n = 5) tuberculosis patients and N controls (n = 6) were evaluated as described in Methods. Results are expressed as the percentage of FasL and perforin positive cells present in the CD4, CD8 and 56T cell populations; the mean of fluorescence intensity is also shown (MFI). No statistical differences were observed.

RESULTS

Lytic activity of ex vivo and Mtb-stimulated PBMC

The ability of ex vivo effector cells and 6-days cultured PBMC from patients with mild (M) and advanced (A) tuberculosis and normal individuals (N) were tested for their ability to lyse autologous macrophages pulsed with Mtb at different E/T ratios. As shown in Fig. 5a, ex vivo effector cells from neither PPD+ nor PPD–N individuals lysed Mtb-pulsed macrophages. In contrast, those cells from TB and pre-TB patients with M and A disease could lyse antigen-pulsed macrophages in a dose-dependent manner. A lower lytic activity was observed in ex vivo effector cells from pre-TB patients than in cells from TB at all E/T ratios employed (Fig. 5a).

Development of antigen-specific effector cells (CTL) was induced by culture of PBMC with Mtb during 6 days. As shown in Fig. 5b, cytotoxic responses in N (PPD+ and PPD–) as well as in the four groups of patients were observed at different E/T cell ratios. Both cultured and ex vivo effector cells from pre-TB patients had a lytic activity lower than that observed in cells from TB patients.

Characterization of ex vivo cytotoxic effector cells

Further experiments were performed in order to analyse the nature of the cytotoxic cells present in ex vivo effector cells. For this purpose, ex vivo effector or target cells were treated with MoAb prior to the cytotoxic assay. As shown in Fig. 1, lysis of...
Mtb-pulsed macrophages was inhibited markedly when the TCR \( \gamma \delta \) was masked by treatment of \textit{ex vivo} effector cells in the four groups of patients studied. An inhibition of lysis was also observed when effector cells were treated with a combination of anti-CD16 and anti-CD56 (anti-16/56) MoAb. Therefore, an involvement of TCR \( \gamma \delta \), CD16 and CD56 molecules on the lysis of Mtb-pulsed macrophages by \textit{ex vivo} effector cells seems to be required. Conversely, lysis was not inhibited by blocking class I or class II antigens on target cells in all the groups studied. Isotype matched non-relevant control antibodies for each MoAb

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\text{Fig. 3. Mtb-induced CD4}^+ \text{ or CD8}^+ \text{ TCR}^+ \text{, CD8}^+ \text{CD9}^+ \text{, or } \gamma \delta \text{ TCR}^+ \text{cytotoxic effector T cells: CD4}^+ \text{ and CD8}^+ \text{ expressing the } \alpha \beta \text{ TCR and CD4}^+ \text{CD8}^+ \text{ expressing the } \gamma \delta \text{ TCR receptor were obtained from 6-day cultured PBMC by negative selection with magnetic beads as described in Methods. Nineteen TB patients [nine with mild (M-TB, □–□) and 10 advanced disease (A-TB, ▼–▼)] 15 pre-TB [five with mild (M-pre-TB, □–□) and 10 with advanced disease (A-pre-TB, ▼–▼)] patients, five PPD' N (●–●) and eight PPD N (○–○) were studied. The enriched populations were tested for their lytic activity against Mtb-pulsed macrophages in a 4-h cytotoxic assay at a 40 : 1 E/T ratio. Results are expressed as percentage of cytotoxicity (individual data). Statistical differences: CD4^+ CTL activity – patients versus PPD' N: M-TB, \( P < 0.05 \), A-TB, M-pre-TB and A-pre-TB, \( P < 0.002 \); patients versus PPD N: M-TB, \( P < 0.005 \); patients versus M-TB: A-TB, \( P < 0.05 \), M-pre-TB and A-pre-TB, \( P < 0.01 \); PPD' N versus PPD N, \( P < 0.002 \). CD8^+ CTL activity – patients versus PPD' N: M-TB, \( P < 0.05 \); A-TB, M-P and A-pre-TB, \( P < 0.002 \); patients versus PPD' N: A-TB, \( P < 0.05 \), M-pre-TB and A-pre-TB, \( P < 0.01 \); patients versus M-TB: A-TB, \( P < 0.05 \); M-pre-TB and A-pre-TB, \( P < 0.01 \); PPD' N versus PPD N, \( P < 0.05 \). γδ CTL activity – patients versus PPD' N: M-pre-TB, \( P < 0.05 \); patients versus PPD N: A-TB, M-TB, M-pre-TB and A-pre-TB, \( P < 0.05 \); M-TB and A-pre-TB, \( P < 0.01 \); PPD' N versus PPD N, \( P < 0.05 \).
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\text{Fig. 4. Correlation between activities of CTL subpopulations in patients with tuberculosis. (a) CD8}^+ \text{ and CD4}^+ \text{CTL; (b) } \gamma \delta \text{CTL and CD4}^+ \text{CTL. Individual CD4}^+ \text{CTL, CD8}^+ \text{CTL and } \gamma \delta \text{ CTL values (\% of cytotoxicity) from M-TB (□–□), A-TB (△–△), M-pre-TB (▼–▼) and A-pre-TB (▼–▼) patients were correlated employing the linear regression test.}
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activity was observed in CD3\(^+\) and/or CD16\(^+\) compared to N. Since activity mediated by NK cells, was dependent on LAK (lymphocyte activated killer cells) activity against autologous Mtb-pulsed macrophages (---) and non-pulsed macrophages (——) in a 4-h cytotoxic assay, employing different effector to target cell ratios (E/T) as described in Materials and methods. Results are expressed as percentage of cytotoxicity.

In order to delineate whether the lysis by ex vivo effector cells was dependent on LAk (lymphocyte activated killer cells) activity mediated by NK cells, ex vivo effector cells were depleted of CD3\(^+\) cells. As shown in Table 1, an important loss of the lytic activity was observed in CD3\(^+\) depleted cells, demonstrating that cytotoxic cells belonged to the CD3\(^+\) cells. Moreover, ex vivo effector cells from patients (n = 8) were not only able to lyse autologous but also allogeneic Mtb-pulsed macrophages confirming their non-MHC restricted nature (Table 2). Furthermore, in patients with pre-TB, lysis of Mtb-pulsed macrophages was inhibited when the CD1b antigen was blocked.

Taking into account that an in vitro expansion of \(\gamma\delta\) T cells by Mtb has been observed in PBMC from tuberculosis patients and N PPD\(^+\) [8,9] and, due to the differences observed in the lytic activity of ex vivo effector cells from patients and N, we undertook to evaluate the presence of lymphocyte subpopulations with lytic capacity such as \(\gamma\delta\) T and NK (CD16\(^+\)/CD56\(^+\)) cells. As can be observed in Fig. 2, the percentage of both \(\gamma\delta\) T (P < 0.05) and NK cells (P < 0.05) was increased in PBMC from tuberculosis patients compared to N. Since \(\gamma\delta\) T cells bearing the CD56 antigen have been described as potent cytotoxic cells, we determined the percentage of \(\gamma\delta\) T/CD56\(^+\) cells in ex vivo cells from tuberculosis patients which resulted higher than in N (P < 0.01). In addition, the percentage of \(\gamma\delta\) T cells bearing CD16 was higher than that observed in N controls (P < 0.05). Taking together all these results, we can assume that the lysis of Mtb-pulsed macrophages observed in ex vivo cells from tuberculosis patients can be due to a high percentage of CD3\(^+\) \(\gamma\delta\) T cells bearing the CD56 and/or CD16 antigens.

Characterization of Mtb-induced cytotoxic cells
To determine the nature of effector cells involved in the Mtb-induced cytotoxic response, CD4\(^+\), CD8\(^+\) and CD4 CD8\(^+\) \(\gamma\delta\) T cells were isolated from control and Mtb-stimulated 6-day cultures by negative selection. The involvement of the T cell subpopulations was quite different in patients and PPD\(^+\) N or PPD\(^-\) N controls. As shown in Fig. 3, significant differences in the development of antigen-specific CD4\(^+\)- and CD8\(^+\)-dependent cytotoxicity were detected between PPD\(^+\) N and PPD\(^-\) N, with the highest CD4\(^+\) CTL activity and CD8\(^+\) CTL activity in PPD\(^+\) N. In tuberculosis patients, Mtb-induced CD4\(^+\) and CD8\(^+\) CTL activity diminished with the severity of the disease and with a previous overcome tuberculosis. CD4\(^+\) and CD8\(^+\) cytotoxic activities in tuberculosis patients were lower compared to PPD\(^+\) N. While activity of CD4\(^+\) CTL from M and A-TB was higher or similar to that of cells from PPD N it was diminished in pre-TB. Furthermore, only a negligible CD8\(^+\) CTL activity was observed in cells from A-TB and all pre-TB patients even when compared to PPD N lytic activity, \(\gamma\delta\)-CTL activity was similar in PPD\(^+\) and PPD\(^-\) N controls while it was increased in cells from A-TB and M-pre-TB (Fig. 3). Besides, Mtb-induced \(\gamma\delta\) T cells from patients with tuberculosis and N individuals were able to lyse either autologous or allogeneic Mtb-pulsed macrophages demonstrating that lysis of target cells by \(\gamma\delta\)-CTL was non-MHC restricted (Table 3).

As can be seen in Fig. 4, CD8\(^+\)- and CD4\(^+\)-mediated cytotoxic activities correlated directly (P < 0.05), while an indirect correlation was observed between \(\gamma\delta\)-CTL and CD4\(^+\) CTL activities (P < 0.05) of PBMC from tuberculosis patients, suggesting that a defective MHC-restricted cytotoxic response could be compensated by a higher non-MHC restricted response.

Expression of class-I and class-II MHC molecules on CD14\(^+\) monocytes
Considering that the decrease of CTL activity might be due to a reduced expression of MHC molecules on the macrophages of tuberculosis patients, we analysed the expression of class-I and class-II molecules on CD14\(^+\) monocytes, which resulted similar on cells from nine TB and six pre-TB patients. Data are given as percentage of MFI observed in normal individuals, class-I, % MFI: TB = 95 ± 15, pre-
Lytic mechanisms used by ex vivo and Mtb-stimulated CD4\(^+\), CD8\(^+\) and γδ T cytotoxic effector cells

To investigate the lytic mechanisms used by ex vivo effector cells and Mtb-induced cytotoxic T cells, assays were performed in the presence of neutralizing concentrations of anti-Fas or antiperforin MoAb. In ex vivo effector cells the lytic activity observed from patients (\(n = 8\), % basal cytotoxicity = 83 ± 4) was inhibited in the presence of anti-Fas (43 ± 5, p < 0.05) or antiperforin (58 ± 3, P < 0.05). As shown in Fig. 6, treatment of target cells with anti-Fas inhibited Mtb-induced CD4\(^+\) and γδ CTL activity in all groups. In PPD\(^+\) as well as PPD\(^-\) N controls, Mtb-induced CD8\(^+\) T cell cytotoxicity was only partially inhibited by both anti-Fas and antiperforin; however, this activity was abrogated in the presence of antiperforin in all patients. Lysis of CD4\(^+\) T cells from M-TB, M-pre-TB patients and all N controls, as well as γδ CTL activity from PPD\(^+\) N, was slightly inhibited by antiperforin.

We then evaluated whether the impairment of CTL activity observed in patients could be attributed to a different percentage of effector cells expressing surface membrane FasL or intracellular perforin in patients and N controls. As shown in Table 4, a similar percentage of CD4\(^+\), CD8\(^+\) and γδ T cells expressing FasL or perforin was observed in all groups. Although no significant differences could be found, in patients...
with the advanced form of the disease a decrease in the percentage of perforin positive CD8\(^+\) T cells and an increase in the percentage of Fas-L \(\gamma\delta T\) cells were observed. However, FasL expression on *Mtb*-stimulated CD8\(^+\) and \(\gamma\delta T\) cells from patients and N controls was lower than that on CD4\(^+\) T cells. Besides, no differences in these parameters were observed in \textit{ex vivo} effector cells (data not shown).

**DISCUSSION**

It has been demonstrated that lymphocytes from the lung of healthy PPD\(^+\) individuals contain mycobacterial antigen-specific CTL precursors, which after being expanded can lyse alveolar or monocyte-derived macrophages, suggesting that CTL may have a role in protective immunity against *Mtb*, both in the lung and in the periphery [6]. In the present report we have demonstrated that both \textit{ex vivo} effector cells and *Mtb*-stimulated PBMC from tuberculosis patients were able to lyse *Mtb*-pulsed autologous macrophages, with differences according to the severity of the disease.

Our data show that monocyte-derived macrophages that present *Mtb* antigens can be lysed in a non-MHC restricted fashion by \textit{ex vivo} effector cells from tuberculosis patients, demonstrating an \textit{in vivo} activation of CD3\(^+\) \(\gamma\delta T\) cytotoxic effector cells. We also observed a high percentage of \(\gamma\delta T\) cells in PBMC from tuberculosis patients, which is consistent with reports by some authors [28,29] but differs from that of Li \textit{et al.} [30], who described a similar frequency of \(\gamma\delta T\) cells in patients and normal controls. In addition, we observed that more than half of the circulating \(\gamma\delta T\) cells from tuberculosis patients expressed the CD56 antigen. Although this antigen is usually detected in a low proportion of \(\gamma\delta T\) cells, an expansion of CD4\(^+\)CD8\(^-\) as well as CD8\(^-\) \(\gamma\delta T\) cells bearing CD56 can be induced by the combination of IL-12 and IL-2 [31,32]. The CD56 marker, which either alone or in combination with CD16 identifies the majority of mature NK cells, is able to modulate various \(\gamma\delta T\) cell functions including cytotoxic activity and cytokine release [32]. Although the role of the CD56 molecule on lysis is not well understood, the fact that it was inhibited by the addition of anti-CD16 during the cytotoxic assay suggests that this NK receptor is also involved in the recognition or lysis of *Mtb*-pulsed macrophages by \(\gamma\delta T\) lymphocytes. In this context, Mandelboin \textit{et al.} [26] have demonstrated that the blockade of CD16 receptor inhibited the lysis of virus-infected and tumour cells by NK cells suggesting that CD16 is involved not only in the antibody-dependent cell cytotoxic activity (ADCC) but also in the direct NK lysis. Therefore, as demonstrated with other activation markers [33] cytokines produced during the inflammatory response to *Mtb* might enhance the CD56 expression on \(\gamma\delta T\) cells, transforming them into more potent cytotoxic effector cells. Although down-regulation of CD1 molecule on macrophages by infection with live *Mtb* has been described [34], we observed that \textit{ex vivo} cytotoxic effector cells from pre-TB recognized the CD1b molecule on target cells. Therefore, \(\gamma\delta T\) bearing CD56\(^+\) and/or CD16\(^+\) cells activated by lipids or glycolipid antigens might have arisen \textit{in vivo} as a consequence of the mycobacterial disease. Although CD1 molecules have already been demonstrated for dendritic cells [14–16] perhaps, and as a late consequence of the previous disease, monocyte-derived macrophages that have not yet acquired the full characteristics of mature cells, might express CD1 on their surface. Hence, one may assume that under inflammatory conditions, monocytes from patients with tuberculosis and previous knowledge of the mycobacterial disease may have been preactivated by inflammatory cytokines [35] and/or by components of the bacilli [36] generating antigen presenting cells in a different stage of maturation that can be lysed by CD1-restricted \(\gamma\delta T\) cells. Besides, these \textit{ex vivo} cytotoxic effector cells, which might include NK effectors, kill the target cells through the Fas and perforin mechanisms.

It has already been demonstrated that antigen-specific T cells capable of lysing *Mtb*-pulsed macrophages can be generated in PBMC from healthy PPD\(^+\) donors upon *Mtb* stimulation [6,8,19,37]. However, the profile of cytotoxic T cells in PBMC from tuberculosis patients has not been studied in great detail. Our study confirms that both CD4\(^+\) and CD8\(^+\) cytotoxic cells were generated upon stimulation of PBMC from PPD-N with *Mtb*. As we also included PPD-N donors, the higher capacity of CD4\(^+\) and CD8\(^+\)CTL to lyse macrophages presenting *Mtb* antigens observed in PPD\(^+\) compared to PPD-N could be explained by the previous contact with the antigen. Conversely, in tuberculosis patients the decrease in CD4\(^+\) and CD8\(^+\)CTL activity can be associated to the severity of the disease and to an impairment to control the infection. In accordance with our results, a reduced cytotoxic CD8\(^+\) T lymphocyte activity was also obtained in tuberculosis patients compared to healthy PPD\(^+\) controls in response to *Mtb* H37Ra [7]. The decrease in the lytic activity of CD4\(^+\) and CD8\(^+\) cytotoxic T cells could not be ascribed to differences in the expression of MHC class I and II molecules because only a low class-II expression was observed in *Mtb*-stimulated patients’ macrophages [38], so that the weakened CD4\(^+\) and CD8\(^+\)CTL activities might be attributed to an inappropriate cytokine production by macrophages and CD4\(^+\) T cells, unable to provide help for CD8\(^+\)CTL development [39]. As a result, the faulty CD4\(^+\) cytotoxicity correlated with the progressive loss of CD8\(^+\)CTL activity. We have also demonstrated that \(\gamma\delta T\) CTL were generated in *Mtb*-stimulated PBMC from PPD\(^+\) and PPD-N controls suggesting that these cells, involved in early defense mechanisms against *Mtb* until the adaptive response is mounted, have the capacity to recognize and respond to molecules on antigen-presenting cells even without a previous exposure to the mycobacteria. As described by Kumararatne \textit{et al.} [37], in cell lines from tuberculosis patients, with the severity of pulmonary involvement the main CTL induced by *Mtb* are non-MHC restricted, but we identified this type of CTL as belonging to the CD4 CD8\(^-\) \(\gamma\delta T\) phenotype. The higher the non-MHC restricted lytic activity, the bigger the loss of CD4\(^+\) and CD8\(^+\)CTL activity, suggesting that \(\gamma\delta T\) cytotoxicity could be a compensatory lytic mechanism in tuberculosis patients.

Published data, mainly from studies with clones or cell lines from healthy PPD\(^+\) donors, are contradictory on whether the Fas and perforin pathways are involved in the restriction of *Mtb* growth [19,20,40–42]. As a similar expression of FasL and perforin in CD4\(^+\), CD8\(^+\) and \(\gamma\delta T\) cells was observed in patients and normal controls, we assumed that CTL from all individuals might be able to use both lytic pathways. Our results concerning the death of target cells induced by CD4\(^+\)CTL from PPD\(^+\) and PPD-N are in accordance with recent publications [19] where lysis by the two mechanisms was observed, even though in tuberculosis patients it was mediated mainly by the induction of apoptosis. Although the perforin pathway was employed by CD8\(^+\) CTL from tuberculosis patients and N controls, we have demonstrated a loss in the activity of these effector cells in most of the patients studied with a reduction in the expression of perforin in CD8\(^+\) cells observed in

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the advanced form of the disease. Therefore, we can speculate that other granule proteins such as granzyme and granulysin, responsible for the reduction in Mtb viability [20,21], are absent in CD8^+ CTL from tuberculosis patients. According to previous studies [24,40,41], we also found that CD4^+CD8^-^y^6^ T cells from healthy PPD^+ controls use both lytic pathways to lyse Mtb-pulsed macrophages. However, our results, obtained with T cells derived from tuberculosis patients, suggest that lysis of the infected macrophages by CD4^+CD8^-^y^6^ CTL, mediated by the Fas--FasL pathway, may only prevent the spread of Mtb infection without killing Mtb [20].

In conclusion, our results show that during a tuberculosis infection the in vivo activation of CD3^+^y^6^ T cell-bearing CD56^+^ and/or CD16 molecules is responsible for the lysis of Mtb-pulsed macrophages in a non-MHC restricted manner. Taking into account that human tuberculosis increases the homing capacities of peripheral blood T cells [33] we can infer that these cells, once migrated to the lung, could mediate the lysis of macrophages through mechanisms of apoptosis, suggesting that this type of lysis might control the inflammation avoiding tissue damage in an environment with a high number of mycobacteria. Furthermore, once the disease is installed, the first evidence observed in vitro is the loss of CD56^+CTL activity which together with an abrogation of CD4^+ lytic activity and a high contribution of non-MHC restricted cytotoxic activity suggest that shifts in the CTL response and the cytolytic mechanisms take place as the pulmonary involvement becomes more severe. Hence, we can infer that the ability to generate an antigen specific cytotoxic response in tuberculosis patients is determined by an interplay of several factors such as cytokines released during the inflammatory response and/or different mycobacterial antigens available as infection progresses. Our results, obtained in patients who had previously overcome tuberculosis, suggests strongly that non-MHC restricted lysis mediated by CD4^+CD8^-^y^6^ CTL induced by Mtb antigens is developed in an attempt to destroy the infected macrophages with a low spreading of the bacilli, avoiding the inflammatory process and extensive tissue damage.

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