Mycobacterial antigens induce apoptosis in human purified protein derivative-specific αβ T lymphocytes in a concentration-dependent manner

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SUMMARY

The morbidity and lethality of tuberculosis is partially the result of an ineffective delayed-type hypersensitivity reaction which causes caseating granulomas in the lung and other organs. Recently we showed that during caseation besides macrophages numerous Fas[+] FasL[+] lymphocytes undergo apoptosis and postulated that this phenomenon may be due to activation-induced cell death (AICD) as a consequence of T-lymphocyte reactivation via bacillary antigens. As purified protein derivative of *Mycobacterium tuberculosis* (Mtb-PPD) provokes caseation in tuberculosis patients, the question arose as to whether bacillary antigens are responsible for AICD within caseous areas. In the present study Mtb-PPD-specific T helper 1 (Th1)-differentiated T lymphocytes were generated *in vitro*. Reactivation of these cells with Mtb-PPD resulted in a concentration-dependent hyporesponsiveness, which was due to an increase in apoptosis of γδ^{+}, αβ^{+} CD4^{+} as well as αβ^{+} CD8^{+} T lymphocytes as assessed by the demonstration of the apoptosis-associated mitochondrial membrane protein 7A6 and DNA fragmentation. Blocking experiments demonstrated that Mtb-PPD antigens exploited the Fas/FasL system to induce apoptosis in Mtb-PPD-specific T lymphocytes. These results may support the hypothesis that in tubercle granulomas with caseation T lymphocytes undergo AICD following reactivation by bacillary antigens, thus contributing to the persistence of tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis, the main aetiological agent of tuberculosis, is responsible for eight million new cases and more than two million deaths each year.[1] After entering into the host organism, mycobacteria may be immediately eliminated by macrophages, but if not, the immune system reacts with a cell mediated immunity.[2–4] Subsequently, epithelioid cell granulomas are generated under the influence of lymphocytes expressing T helper 1 (Th1) type cytokines such as interferon-γ (IFN-γ). In parallel the

immune system develops a delayed-type hypersensitivity (DTH) reaction causing necrosis. The morphological hallmark of DTH-associated necrosis is the presence of a soft to moderately firm cheese-like material termed caseous necrosis.[5] On the one hand, caseous necrosis may lead to extensive tissue damage, resulting in organ failure. On the other hand, caseous lesions may be liquefied, thereby forming cavities, the content of which is known to serve as an excellent growth medium for the bacilli. At this stage mycobacteria proliferate extracellularly within cavities, which rupture into blood or lymph vessels and/or break into airways, thus spreading the infectious pathogens throughout the body and/or releasing them in aerosols.[5]

In a recent communication we showed that during the course of caseation not only CD68^{+} macrophages but also numerous CD3^{+} CD45RO^{+} T lymphocytes expressing both death receptor Fas and death ligand FasL undergo apoptosis. Based on this evidence we hypothesized that apoptosis of Fas[+] FasL[+] T lymphocytes may be caused by activation-induced cell death (AICD) as a consequence of T-lymphocyte reactivation via bacillary antigens.[6]

Because the purified protein derivative of *Mycobacterium tuberculosis* (Mtb-PPD) is known to be capable to provoke caseous necrosis in infected patients, but not in healthy individuals,[3,5,7] the question arises as to whether Mtb-PPD proteins are responsible for T-lymphocyte apoptosis during caseation.[5] To address this question Mtb-PPD-specific T lymphocytes were generated using autologous dendritic cells (DC) loaded with bacillary antigens. As the most potent antigen-presenting cells,[8,9] DC are capable of taking up and processing not only whole mycobacteria but also Mtb-PPD, and are known to deliver Mtb-PPD and other bacillary antigens to naïve T lymphocytes resulting in a Th1 immune response *in vivo* and *in vitro*.[10–14] Herein we report that on re-exposure to bacillary antigens Mtb-PPD-specific $\gamma\delta^+$, $\alpha\beta^+$ CD4$^+$, and $\alpha\beta^+$ CD8$^+$ T lymphocytes become reactive and apoptotic via Fas-dependent cytolytic mechanisms in an antigen-concentration-dependent manner.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody against human leucocyte antigen (HLA)-DR (clone L243) was purchased from Leinco Technologies (St Louis, MO), the monoclonal antibody against CD40 (clone EA-5) from Hoelzel Diagnostika (Cologne, Germany), and the monoclonal antibody against CD56 (clone T199) from Bio Trend (Cologne, Germany). The monoclonal antibodies against CD3 (clone B-B11), CD4 (clone 13B8.2), CD8 (clone B9.11), CD14 (clone RMO52), CD45RA (clone 4KB5), CD45RO (clone UCHL-1), CD54 (clone 84H10), CD86 (clone Bu63), CD95/Fas (clone UB2 and clone ZB4) and the mitochondrial membrane protein 7A6 (clone APO2.7) were obtained from Coulter-Immunotech (Marseilles, France) and the monoclonal antibodies recognizing $\alpha\beta$ T-cell receptor (TCR; clone T10B9.1A-31), $\gamma\delta$ TCR (clone B1) or CD95L/FasL (clone NOK-1) from Pharmingen (Hamburg, Germany).

Staining for phenotypic markers

Cells were transferred to 96-well round-bottom microtitre plates (Nunc, Wiesbaden, Germany) which had been precoated by blocking buffer (10% heat-inactivated rabbit serum and 0·1% NaN$_3$ in phosphate-buffered saline (PBS)). They were then incubated for 30 min with primary antibodies which were either conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). After washing with PBS, the cells were fixed with 2% paraformaldehyde, and subjected to flow cytometric analysis using FACStar^{plus} (Becton Dickinson, San Jose, CA).

Preparation of lymphocytes and monocytes and generation of DC

Lymphocytes and monocytes were isolated from venous blood of 11 tuberculin-negative, healthy volunteers (22–35 years old). Peripheral blood mononuclear cells (PBMC) were separated by centrifugation on a Ficoll-Hypaque discontinuous gradient. PBMC (5×10^7) were cultured in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 2·5% heat-inactivated autologous serum in flat-bottom plates (Hereaus, Hanau, Germany).

T lymphocytes were separated by 'rosetting' using sheep erythrocytes as described previously.[15] Subsequent flow cytometry revealed that 19–26% of the isolated cells were CD3$^+$ CD45RA$^+$ and 45–50% CD3$^+$ CD45RO$^+$ T cells (not shown). After characterization T cells were portioned into cryotubes (Nunc) and preserved in liquid nitrogen for further usage. To generate DC, the adherent PBMC fractions were cultured in RPMI-1640 medium (Biochrom) supplemented with granulocyte–macrophage colony-stimulating factor (GM-CSF; 300 U/ml), interleukin-4 (IL-4; 300 U/ml) (Genzyme, Cambridge, UK), and 2·5% autologous serum as described.[16]

After 7 days, more than 90% of cultured cells could be characterized as DC because they expressed high levels of major histocompatibility complex (MHC) class I and class II (HLA-DR), CD40, and CD86, but were negative for CD3, CD14, CD16, CD20, or CD56. Furthermore they were functionally active in stimulating allogeneic T-cell proliferation as described elsewhere.[15] Flow cytometric analysis indicated that on exposure to Mtb-PPD DC were activated, resulting in up-regulation of MHC I and II, CD40, and CD86 during up to 2 days in the follow-up (data not shown).

Sensitization of T lymphocytes against mycobacterial antigens

After characterization DC from each donor were cultured in 100 μl RPMI-1640 medium supplemented with 2·5% autologous serum in 96-well flat-bottom plates. After adding Mtb-PPD (Behring, Marburg, Germany) DC were cocultured with autologous lymphocytes at a ratio of 1 : 10 (mixed leucocyte culture, MLC). To determine the optimum concentration of Mtb-PPD leading to the highest lymphocyte proliferation, Mtb-PPD was added to the cultures at a final concentration of 6·25, 12, 25, 50, 100, 200 or 400 μg/ml. After 6 days cocultured lymphocytes were analysed by [^{3}H]thymidine assay for their proliferation activity. Results indicated that too low concentrations of Mtb-PPD lead to low proliferation for the same numbers cells seeded whereas 25–400 μg/ml Mtb-PPD was the range in which lymphocytes showed the strongest proliferation activity (not shown). In the next step, the highest Mtb-PPD concentration (400 μg/ml) was added to MLCs to sensitize lymphocytes against mycobacterial antigens. After 6 days lymphocytes were isolated from MLCs by rosetting (see above), and analysed by [^{3}H]thymidine assay for their proliferation activity, by *in vitro* cytokine release assay for their Th1/Th2 differentiation and by flow cytometry for their phenotype.

Proliferation assay

To determine the proliferation activity of lymphocytes, 0·2 μCi [^{3}H]thymidine was added to the autologous MLC on day 5. After 24 hr, cells were harvested by an automated Inotech cell harvester (Dunn, Ansbach, Germany), and [^{3}H]thymidine incorporation was measured in triplicate

cultures by a β Counter (Hewlett-Packard, Meriden, CT). Mixed cultures composed of T cells and autologous DC without Mtb-PPD served as controls. Further controls included cultures consisting solely of either DC, or T lymphocytes, which were pulsed with Mtb-PPD in some experiments. Results were given in counts per minute (c.p.m.) ± SEM.

Th1/Th2 differentiation of Mtb-PPD-specific T lymphocytes

To determine the Th1/Th2 differentiation of lymphocytes,[17] the supernatants (500 μl) of day 6 autologous MLCs were harvested and assessed in commercial enzyme-linked immunosorbent assay (ELISA) for production of IFN-γ, IL-5 and IL-10 as described by the manufacturer (R & D, Wiesbaden, Germany). The lower limit of sensitivity for each assay amounted to: IFN-γ, <3 pg/ml; IL-5, <3 pg/ml; IL-10, <0·5 pg/ml. Supernatants removed from autologous DC–T-cell mixed cultures without Mtb-PPD served as controls. These assays were also carried out on supernatants from cultures consisting solely of either DC, or T lymphocytes, which were pulsed with Mtb-PPD in some experiments.

Reactivation of Mtb-PPD-specific T lymphocytes

T lymphocytes from each autologous MLC were reactivated with different concentrations of Mtb-PPD (0, 25, 50, 100, 200 and 400 μg/ml). In some control experiments reactivation was carried out in the presence of DC. When indicated, neutralizing mouse anti-Fas antibody (50 ng/ml) or irrelevant mouse immunoglobulin G (IgG) was also added to the cultures. 24 and 48 hr after reactivation, Mtb-PPD-specific lymphocytes were tested by flow cytometry for the expression of the apoptosis-associated mitochondrial membrane protein 7A6 recognized by the monoclonal antibody APO2.7 and for DNA fragmentation by TdT-mediated FITC-dUTP nick-end labelling (TUNEL). Forty-eight hr after reactivation Mtb-PPD-specific lymphocytes were pulsed with [3H]thymidine for 24 hr and incorporated radioactivity was assessed by a Matrix 96 Direct β counter (see above).

TUNEL

Reactivated T lymphocytes (1×10^6) were suspended in 0·5 ml PBS. Then they were incubated for 15 min with 5 ml of 1% paraformaldehyde. After centrifugation (3×5 min, 300 *g*), cells were re-suspended in 0·5 ml PBS and added to 5 ml ice-cold 70% ethanol. After 30 min, the cell suspension was centrifuged (3×5 min, 300 *g*). The supernatant was then removed by aspiration and cells were re-suspended in 50·75 μl of the staining solution of the APO-DIRECT™ Kit (Pharmingen). The staining solution contained 10 μl reaction buffer, 0·75 μl TdT enzyme, 8 μl FITC–dUTP and 32 μl H_2O. After incubation for 60 min at 37°, cells were washed with rinse buffer (2×5 min, 300 *g*) of the APO-DIRECT™ Kit. To characterize cell populations underwent DNA-fragmentation, the TUNEL technique was combined with the staining for several phenotypic markers (see above). Then the cells were subjected to flow cytometric analysis.

Statistical analysis

Differences in proliferative and apoptotic rates of lymphocytes reactivated by Mtb-PPD in the presence of neutralizing anti-Fas antibody or irrelevant IgG were examined by two-tailed *t*-test. Results were considered significant for *P*-values < 0·05.

RESULTS

Generation and characterization of Mtb-PPD-specific T lymphocytes

In vivo naïve T lymphocytes can be sensitized against bacillary antigens by antigen-presenting cells delivering Mtb-PPD antigens.[10–14] Peripheral blood lymphocytes from 11 tuberculin-negative, healthy individuals were cocultured with autologous DC and 400 μg/ml Mtb-PPD (MLC). After 6 days, lymphocytes from each MLC experiment were analysed for their proliferation activity, Th1/Th2 differentiation, and phenotype. The [3H]thymidine assay demonstrated that in MLC lymphocytes proliferate strongly in the presence, but not in the absence of Mtb-PPD. Only weak [3H]thymidine incorporation was measured in cultures consisting of either lymphocytes or dendritic cells, solely, regardless of the presence or absence of Mtb-PPD (Fig. 1a). To determine the Th1/Th2 differentiation of Mtb-PPD-primed lymphocytes,[17] *in vitro* cytokine release assays were performed on culture supernatants. Significantly higher concentrations of the Th1-associated cytokine IFN-γ could be noted in supernatants of autologous mixed cultures consisting lymphocytes, DC, and Mtb-PPD, when compared to controls (Fig. 1b). In contrast, no significant difference could be noted between concentrations of the Th2-associated cytokines IL-5 or IL-10 in autologous MLC and controls (Fig. 1c, d). Flow cytometric analysis indicated that Mtb-PPD-primed cells consisted of $38 \pm 5\%$ $\alpha\beta^+$ $CD4^+$, $56 \pm 5\%$ $\alpha\beta^+$ $CD8^+$, $8 \pm 5\%$ $\gamma\delta^+$ $CD4^-$, and $7 \pm 5\%$ $\gamma\delta^+$ $CD8^-$ lymphocytes (Fig. 2). In comparison with peripheral blood lymphocytes[18,19] only the percentage of $\alpha\beta^+$ $CD8^+$ cells increased during MLC, thus indicating that the proliferation of $\alpha\beta^+$ $CD8^+$ lymphocytes was higher than the other lymphocyte populations in the course of sensitization (Fig. 2). Analysis of the expression of the death receptor Fas and its ligand FasL illustrated that during MLC the percentage of Fas^+ $CD3^+$ and $FasL^+$ $CD3^+$ Mtb-PPD-primed lymphocytes increased from approximately $19 \pm 6\%$ and $9 \pm 5\%$ at the beginning to $65 \pm 4\%$ and $59 \pm 6\%$ on day 6, respectively (Fig. 2).

Concentration-dependent recall response of Mtb-PPD-primed T lymphocytes

Following separation from MLC sensitized T lymphocytes were reactivated by different concentrations of Mtb-PPD. In the presence of DC Mtb-PPD did not affect the proliferation rate of lymphocytes (Fig. 3). In the absence of
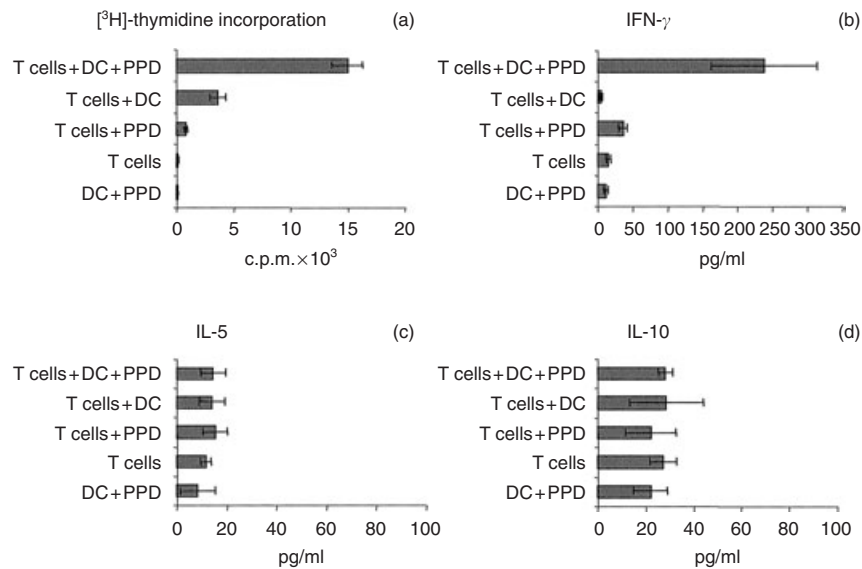
Figure 1. Proliferation and differentiation of Mtb-PPD-specific T lymphocytes. Human lymphocytes were cultured with autologous DC and Mtb-PPD (autologous MLC). After 6 days, uptake of [³H]thymidine and release of Th1/Th2-associated cytokines were measured. The results of a representative autologous MLC are expressed as mean ± SD of three replicates. The [³H]thymidine assay shows high proliferative activity in MLC when compared to controls (a). Cytokine release assays demonstrate that in MLC the production and secretion of IFN-γ (b), but not of IL-5 (c) or of IL-10 (d) are increased.

DC, however, increasing Mtb-PPD concentrations correlated with less proliferative activity of reactivated lymphocytes (Fig. 3). Based on these findings we asked whether Mtb-PPD-dependent defective T-cell proliferation seen in experiments without DC is caused by an increased apoptotic rate of reactivated lymphocytes. Consequently, in the next step lymphocytes reactivated by different Mtb-PPD concentrations were analysed for apoptosis.

Results indicated that the apoptosis-associated mitochondrial 7A6 epitope, recognized by the monoclonal antibody APO2.7,[20] became accessible, and DNA fragmentation, demonstrated by TdT-mediated FITC–TUNEL technique,[21] was detectable in significantly higher numbers of reactivated Mtb-PPD-specific T lymphocytes, when compared to resting T lymphocytes. Interestingly, quantitative analyses of 7A6 expression and DNA fragmentation demonstrated that the death rate of reactivated T lymphocytes was positively correlated with higher Mtb-PPD concentrations (Fig. 4).

Characterization of apoptotic Mtb-PPD-specific T lymphocytes

To characterize T-cell populations undergoing DNA-fragmentation, the TUNEL technique was combined with cell surface staining for different markers. Two-colour dot plot analysis of MLC-derived lymphocytes restimulated by 400 μg/ml Mtb-PPD revealed that among DNA-fragmented cells 64 ± 6% were CD3$^+$, 25 ± 6% CD4$^+$, 40 ± 4% CD8$^+$, 56 ± 6% αβ$^+$, and 5 ± 3% γδ$^+$ (Fig. 5). Taking these data into account, the TUNEL-positive fraction of each lymphocyte population was determined. Results indicated that

53 ± 5% of CD4$^+$, 72 ± 6% of CD8$^+$, 69 ± 4% of αβ$^+$, and 73 ± 6% of γδ$^+$ cells undergo apoptosis.

Role of Fas and FasL in apoptosis of Mtb-PPD-specific lymphocytes

To prove whether Fas–FasL interaction is involved in apoptosis of reactivated lymphocytes the death receptor Fas was blocked on Mtb-PPD-specific lymphocytes. Results demonstrated that following Fas-blockade the proliferative rate of lymphocytes reactivated by Mtb-PPD at high concentrations (100 μg/ml and 400 μg/ml Mtb-PPD) increased significantly, when compared to the control ($P < 0.05$). As expected this increase correlated with a decline in T-lymphocyte apoptosis, because Fas-blockade reduced the apoptotic rate of primed lymphocytes reactivated by Mtb-PPD at the same high concentrations, when compared to controls ($P < 0.05$) (Fig. 6).

DISCUSSION

T lymphocytes represent the most important cell population in immunity to tuberculosis. An extensive T cell death may therefore crucially affect the labile balance between the immunity and mycobacteria. Based on our recent report that in tuberculosis lesions with caseous necrosis numerous Fas$^+$, FasL$^+$ T lymphocytes undergo apoptosis,[6] and the evidence that Mtb-PPD provokes caseation in infected patients, but not in healthy individuals,[3,5,7] we asked whether mycobacterial antigens are capable to cause apoptosis in Mtb-PPD specific T lymphocytes. At first lymphocytes from healthy donors were sensitized against
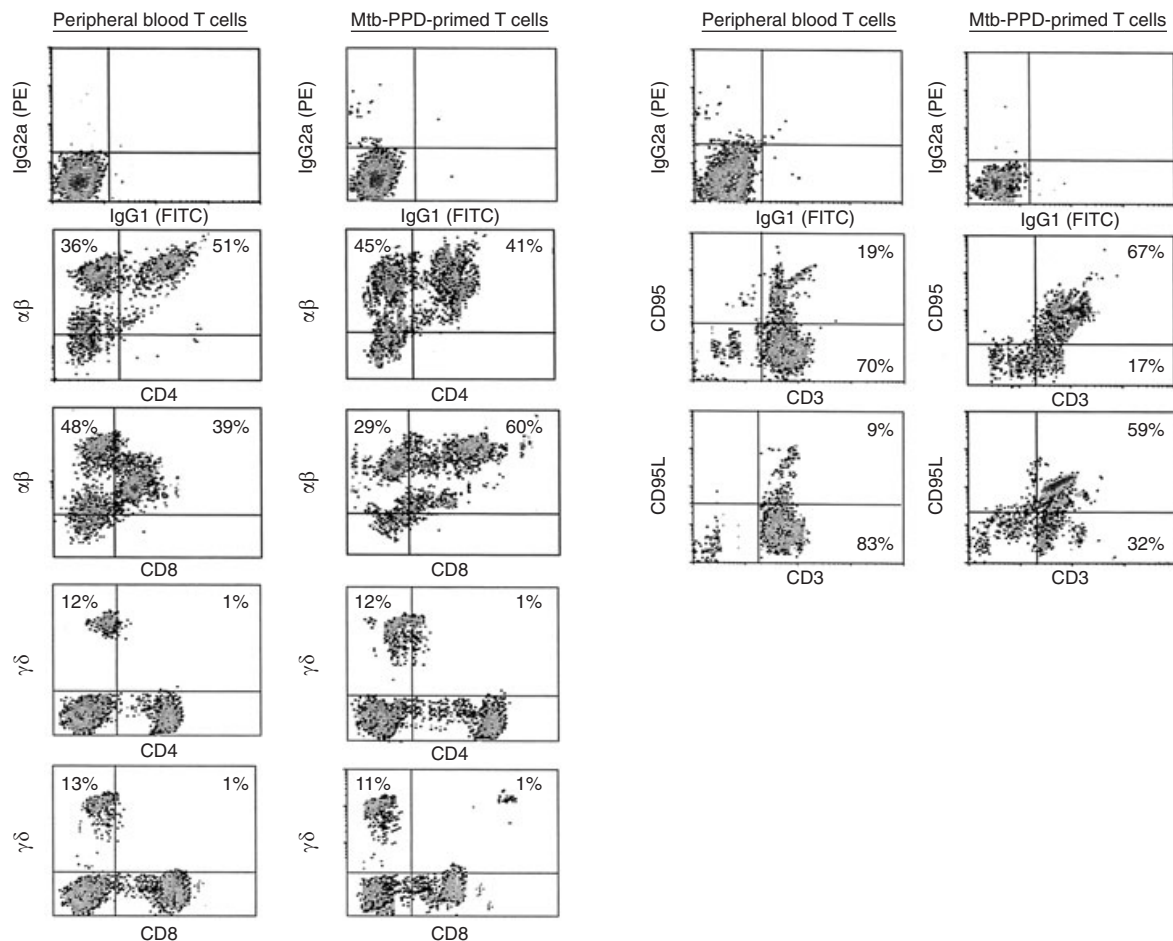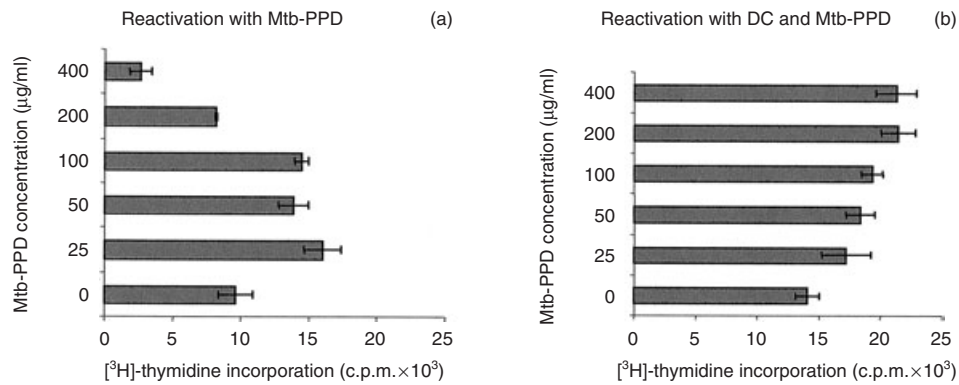
Figure 2. Characterization of Mtb-PPD-specific T lymphocytes. Human lymphocytes were cultured with autologous DC and Mtb-PPD (autologous MLC) for 6 days. Two-colour dot plot analysis of a representative autologous MLC shows that in the course of culturing the percentage of $\alpha\beta^+$ CD4$^+$, $\alpha\beta^+$ CD8$^+$, and $\gamma\delta^+$ CD4$^-$ CD8$^-$ cells shifts in favour of $\alpha\beta^+$ CD8$^+$ cells (left panel), and the expression of the Fas-receptor and its ligand FasL increases in MLC-derived CD3$^+$ T lymphocytes when compared to peripheral blood lymphocytes (right panel).

bacillary antigens in an autologous MLC containing DC, peripheral blood lymphocytes and Mtb-PPD. In this system Mtb-PPD-loaded DC induced an effective T-cell proliferation, as assessed by [^{3}H]thymidine assay. As a slight increase in proliferation activity was also observed in mixed cultures without antigens and in lymphocytes stimulated only by Mtb-PPD, it cannot be excluded that the lymphocyte proliferation seen in autologous MLCs is partially caused by antigen-independent costimulatory signals delivered by dendritic cells and/or by some mitogenic effects of Mtb-PPD, as suggested previously.[22,23] Nevertheless, comparison between MLCs and controls demonstrated that in the presence of Mtb-PPD-loaded DC T lymphocytes not only proliferate more strongly but also produce higher concentrations of the Th1-associated cytokine IFN-γ,[17] suggesting that they mainly represent Mtb-PPD-specific lymphocytes. In accordance, phenotypic characterization of these lymphocytes showed that in the course of sensitization they up-regulated the expression of both Fas and FasL, two cell surface molecules which are highly expressed on activated

Th1- but not Th2-differentiated lymphocytes.[24,25] Importantly the MLC-derived lymphocytes consisted of $\alpha\beta^+$ and $\gamma\delta^+$ T cells, thus representing both major T-cell populations involved in immunity to tuberculosis.[4,26–28] Of importance was also the finding that the proliferation rate of $\alpha\beta^+$ CD8$^+$ lymphocytes was higher in our *in vitro* system than those of other T-cell populations. This phenomenon was surprising, because two previous studies demonstrated that Mtb-PPD preferentially activates CD4$^+$ and not CD8$^+$ lymphocytes.[29,30] This difference, however, may be caused by the fact that in our study DC and not macrophages served as antigen-presenting cells. Although both DC and macrophages are derived from monocytes, DC however, possess the highest efficacy in cross-priming soluble exogenous antigens for presentation on MHC class I molecules, and therefore in activating CD8$^+$ lymphocytes.[31]

After generation and characterization primed T lymphocytes were reactivated by different Mtb-PPD concentrations. Results indicated that the proliferative response of reactivated T cells was significantly diminished by Mtb-PPD

Figure 3. Proliferation of Mtb-PPD-specific T lymphocytes following reactivation. Human lymphocytes were cultured with autologous DC and Mtb-PPD (autologous MLC) for 6 days. Following separation from MLC sensitized T lymphocytes were reactivated by different concentrations of Mtb-PPD in the absence of DC. The proliferative response of reactivated lymphocytes were determined by applying [³H]-thymidine assay (a). As control Mtb-PPD-specific T lymphocytes were reactivated by DC pulsed with the same concentrations of Mtb-PPD (b). The results of a representative experiment with lymphocytes from a donor are expressed as c.p.m. $\pm$ SD of three replicates. Evidence shows that in the absence of DC the more the Mtb-PPD concentration the less the proliferative rate of reactivated lymphocytes. Conversely in the presence of DC Mtb-PPD does not affect the proliferation activity of lymphocytes.
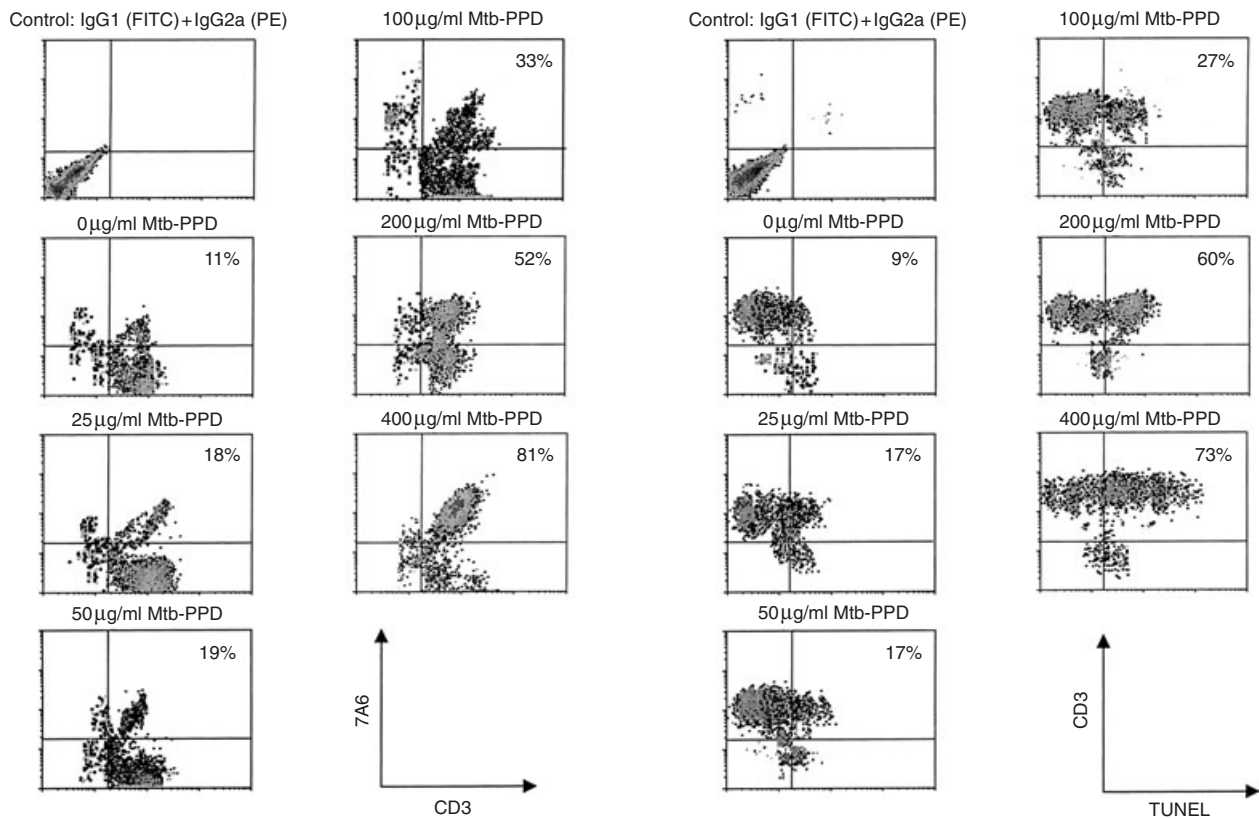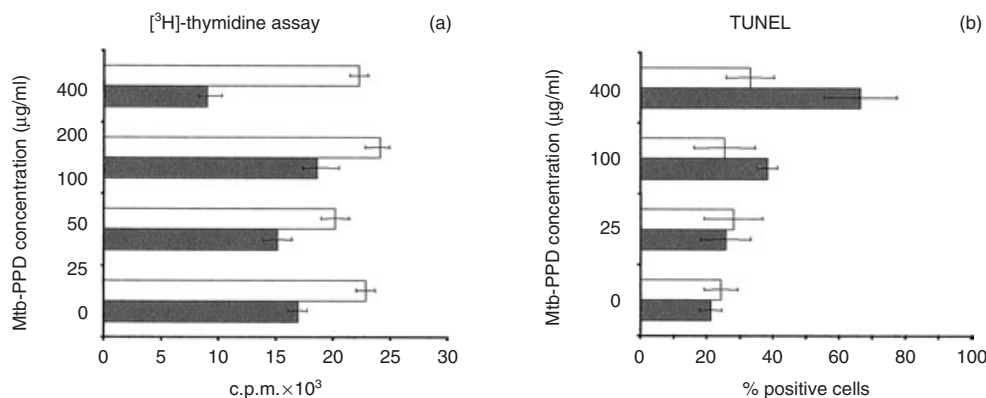

Figure 4. Apoptosis of Mtb-PPD-specific T lymphocytes following reactivation. Human lymphocytes were cultured with autologous DC and Mtb-PPD (autologous MLC) for 6 days. Following separation from MLC sensitized T lymphocytes were reactivated by different concentrations of Mtb-PPD. The apoptotic rate of reactivated lymphocytes were determined by flow cytometric analysis of the apoptosis-associated mitochondrial membrane protein 7A6 (left panel) and TUNEL (right panel). Two-colour dot plots of a representative experiment with lymphocytes from a donor indicate that the apoptotic rate of reactivated CD3$^+$ lymphocytes is positively correlated with increasing Mtb-PPD concentrations.
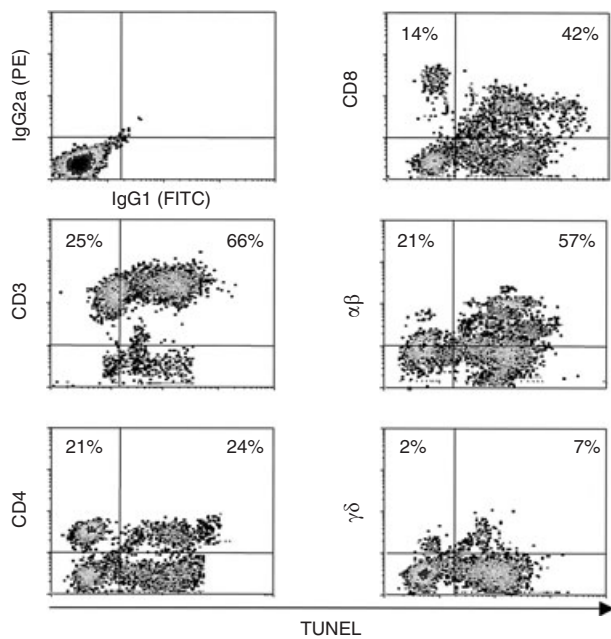
Figure 5. Characterization of reactivated T lymphocytes undergoing apoptosis. Mtb-PPD-specific human T lymphocytes were reactivated by 400 μg/ml Mtb-PPD. To characterise apoptotic T-cell populations, the TUNEL technique was combined with cell surface staining for different T-cell markers. Two-colour dot plots show a representative experiment with lymphocytes from a donor following reactivation by 400 μg/ml Mtb-PPD. Results indicate that CD3^{+}, CD4^{+}, CD8^{+}, αβ^{+} as well as γδ^{+} cells undergo apoptosis.

at high concentrations. This evidence posed the question as to whether the hyporesponsiveness of lymphocytes lies in the increased apoptotic rate of them following TCR ligation by Mtb-PPD-associated antigens, a phenomenon which is called AICD.[32–35] According to the current knowledge, when TCR ligation together with appropriate costimulation takes place, reactivation results in proliferation rather than AICD. In the absence of costimulatory signals, however, TCR ligation leads to cell death rather than to cell proliferation.[36–40] In accordance, application of bacillary antigens in the absence of DC led to apoptosis of Mtb-PPD-specific T lymphocytes, as proved by demonstration of the apoptosis-associated mitochondrial membrane protein 7A6 and DNA fragmentation.[20,21] Interestingly, *in vitro* the higher the applied Mtb-PPD concentration, the more the rate of apoptosis, which was noted in γδ[+] as well as in αβ[+] T lymphocytes. Thus, on the one hand, our findings confirm reports that tuberculosis-associated γδ[+] T lymphocytes undergo AICD following reactivation.[41–43] On the other hand, the findings extend previous data because they show that in consequence of reactivation αβ^{+} CD4^{+} and αβ^{+} CD8^{+} T lymphocytes also die via apoptosis. Notably this AICD was mainly Fas-dependent, because Fas-blockade significantly reduces the apoptotic rate of lymphocytes reactivated by Mtb-PPD at high concentrations. The fact, however, that the apoptotic rate of reactivated lymphocytes could not be completely diminished via Fas-blockade poses the question as to whether other members of the tumour necrosis factor receptor family also contribute to the Mtb-PPD-induced AICD, as previously shown.[44,45] The other question which remains unclear is how the TCR is reactivated by bacillary antigens in the absence of antigen-presenting cells. In this regard one may speculate that Mtb-PPD-associated antigens are presented by lymphocytes

Figure 6. Effect of Fas on re-call response of reactivated Mtb-PPD-specific T lymphocytes. Mtb-PPD-specific human T lymphocytes were reactivated by different concentrations of Mtb-PPD. The death receptor Fas was blocked on Mtb-PPD-specific lymphocytes by a neutralizing anti-Fas antibody (a and b, white bars). As control irrelevant mouse IgG was added to the cultures (a, b, black bars). The proliferative and apoptotic rates of reactivated lymphocytes were determined by applying [³H]thymidine assay (a) and TUNEL (b), respectively. The results of a representative experiment with lymphocytes from a donor are expressed as ± SD of three replicates. Evidence demonstrates that following Fas-blockade the proliferative rate of Mtb-PPD-specific lymphocytes reactivated by 100 or 400 μg/ml Mtb-PPD increases (a, white bars), when compared to the controls (a, black bars) ($P < 0·05$). Conversely Fas-blockade leads to a decrease in apoptotic rate of Mtb-PPD-specific reactivated by 100 or 400 μg/ml Mtb-PPD (b, white bars), when compared to the controls (b, black bars) ($P < 0·05$).

throughout their own surface molecules, as previously shown for different soluble antigens such as human immunodeficiency virus-derived gp120 and hepatitis B envelope protein.[46,47]

Together, the data suggest that high concentrations of soluble bacillary antigens may *in vivo* mediate Fas-dependent and Fas-independent apoptosis (AICD) in tuberculosis-associated T lymphocytes. In this regard, Mtb-PPD-associated proteins secreted by *M. tuberculosis* might be of particular interest,[48] because increased secretion of such proteins can be responsible for the extensive T-lymphocyte apoptosis seen in disintegrated granulomas with caseous necrosis, where following apoptosis of macrophages costimulatory signals are reduced while bacilli and their antigens are released into the extracellular space.[6] In such granulomas apoptosis of $\alpha\beta^+$ T cells is probably more relevant than apoptosis of $\gamma\delta^+$ T cells, because lymphocytes involved in granulomatous reactions in tuberculosis are predominantly $\alpha\beta^+$ cells.[49] Although this model is supported by our recent report showing that in tuberculosis associated necrosis numerous CD3$^+$ CD45RO$^+$ Fas$^+$ FasL$^+$ T lymphocytes undergo apoptosis,[6] by previous data suggesting that continued exposure of immunoreactive lymphocytes to *M. tuberculosis* leads to apoptosis of CD4 and non-CD4 cells,[50,51] and by clinical studies demonstrating that injection of Mtb-PPD causes necrosis in infected but not healthy individuals,[3,5,7] we cannot rule out divergences between *in vitro* findings presented here and *in vivo* 'realities' in the human organism. Thus, studies are needed to assess the concentration of bacillary proteins within epithelioid cell granulomas and its relation to apoptosis of tuberculosis-associated T lymphocytes.

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