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$\gamma\delta^+$ and CD4$^+$ $\alpha\beta^+$ human T cell subset responses upon stimulation with various *Mycobacterium tuberculosis* soluble extracts

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SUMMARY

By using a flow cytometric technique which allows direct identification of proliferating cells within mixed cell populations, we have previously described that soluble extracts obtained from *Mycobacterium tuberculosis* or *M. avium* represent strong stimuli for human $\gamma\delta^+$ T cells. In the present study, we demonstrate that the protocol used for the preparation of *M. tuberculosis* soluble extracts may have an impact on their $\gamma\delta^+$ T cell stimulatory capacity. In agreement with our previous data, soluble extracts prepared from bacteria killed at 85°C and directly disrupted by prolonged sonication (TBe), elicited a strong proliferation of $\gamma\delta^+$ T cells after 6–7 days of stimulation. In contrast, when soluble extracts were obtained from bacteria autoclaved (121°C, 25 min) and then washed by centrifugation, a predominant proportion of CD4$^+$ $\alpha\beta^+$ T cells was achieved in the responding population. The stimulatory activity for $\gamma\delta^+$ T cells was recovered in the supernatant of the autoclaved bacteria, indicating that autoclaving of *M. tuberculosis* bacilli releases an antigen(s) into the supernatant which stimulates human $\gamma\delta^+$ T cells. While protease digestion of TBe only partially reduced its stimulatory capacity on $\gamma\delta^+$ T cells, the stimulatory component(s) released into the supernatant after autoclavation of bacilli was found to be sensitive to protease digestion. Interestingly, in contrast to the preponderant proportion of $\gamma\delta^+$ T cells induced in the responding population by unfractionated TBe, when the extract was fractionated by fast performance liquid chromatography (FPLC), most of the fractions exhibited a strong stimulatory capacity on CD4$^+$ $\alpha\beta^+$ T cells only. The $\gamma\delta^+$ T cell stimulatory activity was confined to the low molecular weight range FPLC fractions. Such results may suggest a possible regulatory role of $\gamma\delta^+$ T cells on CD4$^+$ $\alpha\beta^+$ T cells.

Keywords *Mycobacterium tuberculosis* $\gamma\delta^+$ T cell CD4$^+$ T cell T cell subsets proliferation

INTRODUCTION

Different cell subsets contribute to the overall immune response to intracellular bacteria, such as *Mycobacterium tuberculosis* [1,2]. Besides a well established role for CD4$^+$ $\alpha\beta^+$ T cells and, at least in the mouse, for CD8$^+$ $\alpha\beta^+$ T cells, it has become evident over the last few years that T cells bearing $\gamma\delta$ T cell receptors (TCR) may represent a significant component of the cellular immune response to intracellular pathogens [3–6]. Although $\gamma\delta^+$ T cells have been reported to respond to a variety of bacterial, viral, parasitic and tumoural antigens, they seem to be particularly reactive towards mycobacterial components [3,7–14]. A number of protein and non-protein mycobacterial antigens for $\gamma\delta^+$ T cells have been described. In mice, peptides derived from the 60-kD mycobacterial heat shock protein (hsp) are recognized by certain $\gamma\delta^+$ T cells [15], while recognition of mycobacterial and/or homologous hsp by human $\gamma\delta^+$ T cells has been reported in some [16,17], but not in other studies [18,19]. Recently, a cell-associated, heat-stable 10–14-kD protein antigen of *M. tuberculosis* has been described as a major stimulus for human $\gamma\delta^+$ T cells [20]. Besides protein antigens, protease-resistant, phosphorylated low molecular weight molecules of mycobacterial origin have been repeatedly described as potent stimulators of a major subset of human $\gamma\delta^+$ T cells expressing Vγ9 and Vδ2 regions [21–24]. Sequencing of cDNA encoding these $\gamma\delta$ TCR chains demonstrated marked junctional diversities consistent with a T cell response to a superantigen [25]. To date, the role of these novel classes of non-protein TCR ligands in the protective immunity or immunopathology of mycobacterial infections is completely unknown. By comparison of the different species of the *M. tuberculosis* group, it was demonstrated that the load of the non-protein, phosphorylated antigens correlates positively with the degree of pathogenicity [26]. This observation

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strongly suggests that such antigens represent an immunological determinant of mycobacterial virulence for humans [26].

Little is known about the physiological role of $\gamma\delta^+$ T cells. A selective anatomical distribution in areas in strict contact with the external environment (skin, gut), together with a limited V-gene expression and the ability to interact with non-processed antigens, support the hypothesis that they are involved in early phases of infections [27]. Evidence for a protective role of $\gamma\delta^+$ T cells in early tuberculosis (TB) has been provided from studies of knock-out mice. Infection of TCR-$\delta^{-/-}$ mutant mice having normal levels of $\alpha\beta^+$ T cells with high doses of *M. tuberculosis* causes rapid death of the animals [6]. In humans, $\gamma\delta^+$ T cells are present in increased proportions in peripheral blood of a fraction of patients with TB [28] and in the granulomatous skin lesions of leprosy patients [29].

We have previously described that different kinds of mycobacterial antigen preparations differ in their ability to trigger *in vitro* proliferation of distinct human peripheral blood cell subsets [30]. In particular, while both $CD4^+$ and $CD8^+$ $\alpha\beta^+$ T cells mainly proliferate in response to intact (live and killed) mycobacteria, a preponderant proportion of $\gamma\delta^+$ T cells is achieved in the responding population using soluble extracts of *M. tuberculosis* or *M. avium*. In this study, we further characterize the $\gamma\delta^+$ T cell stimulatory activity of various *M. tuberculosis* soluble extracts. Using a flow cytometric technique which allows direct identification of distinct proliferating cell subsets within mixed cell populations, we demonstrate that the protocol used for preparation of the soluble extract appears to have an impact on the composition of the responding T cell subsets ($\gamma\delta^+$ *versus* $CD4^+$ $\alpha\beta^+$ T cells). In addition, indications of a possible regulatory activity of $\gamma\delta^+$ T cells on $\alpha\beta^+$ T cells will be presented.

MATERIALS AND METHODS

Mycobacterial cultures

Mycobacterium tuberculosis, strain NBL 27/94, was isolated from the blood of a Swedish child affected with disseminated TB. The strain was grown in standing cultures in Middlebrook 7H9 broth supplemented with oleic acid albumin dextrose complex (OADC; Difco Labs, Detroit, MI) for 3–4 weeks at 37°C. Bacteria were harvested, washed three times by centrifugation and resuspended in water at a concentration of ≈ 3 mg/ml (wet weight).

Preparation of mycobacterial soluble extracts

The main steps in the preparation of mycobacterial soluble extracts are depicted in Fig. 1. Bacterial suspension was divided into two identical aliquots. Bacteria in the two aliquots were killed by incubation at 85°C for 20 min or by autoclaving at 121°C for 25 min, respectively. The bacterial suspension heated at 121°C was further divided into two aliquots. Bacteria in the first aliquot were washed by centrifugation at 2600 ***g*** for 10 min. The bacterial pellet was resuspended in water and the supernatant was collected. Bacteria from all the suspensions were disrupted by 5 h sonication (45 W) in a waterbath sonicator at 4°C. Membranes and insoluble components were removed by centrifugation at 12 000 ***g*** for 10 min and the supernatants were collected. Extracts were designated, respectively, as: TBe (from bacteria killed at 85°C), SN1 (from autoclaved and then washed bacteria), SN2 (supernatant of autoclaved bacteria), and SN3 (from autoclaved bacteria). Protein contents of the extracts were estimated according to Lowry *et al.* [31]. Extracts were lyophilized and reconstituted in PBS pH 7·4 at a concentration of 500 μg/ml. They were then filtered through a 0·22-μm filter (Millipore, Eschborn, Germany) and stored in aliquots at −70°C until use.

Protease digestion

Mycobacterial extracts (500 μg/ml) or purified protein derivative (PPD; 500 μg/ml; Statens Seruminstitutet, Copenhagen, Denmark) were treated with pronase (Boehringer GmbH, Mannheim, Germany) at a 50:1 protein:pronase ratio at 37°C for 20 h. The samples were then heated at 65°C for 3 min and treated with a second aliquot of pronase. After an additional incubation of 8 h, samples were heated at 65°C for 3 min and used in the stimulation assay.

Gel filtration of M. tuberculosis *soluble extract by fast performance liquid chromatography*

TBe was lyophilized and resuspended in PBS. Extract (1·2 mg) was fractionated by gel filtration on a Superose 12 column (Pharmacia LKB, Uppsala, Sweden) equilibrated with PBS pH 7·4. PBS was used to elute 0·5-ml fractions at a flow rate of 0·3 ml/min. Elution from the column was monitored at A_{280} nm. Molecular mass standards from 2000 kD to 6 kD (Pharmacia) were used to calibrate the column with PBS as a buffer. Two hundred microlitres of each fraction were used to stimulate peripheral blood mononuclear cells (PBMC) from *M. bovis* bacille Calmette–Guérin (BCG)-vaccinated healthy blood donors in a 7-day stimulation assay.

Cell populations and stimulation assay

PBMC were isolated from heparinized blood of healthy BCG-vaccinated blood donors by centrifugation on standard Ficoll–Hypaque (Pharmacia). Cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine (GIBCO BRL, Paisley, UK) and seeded in 48-well plates (Nunc, Roskilde, Denmark) at a density of 1×10^6 cells/cm^2. After 1 h incubation at 37°C in humidified air containing 5% CO_2, non-adherent cells were removed by gentle repetitive washes with prewarmed RPMI, centrifuged and passed over a nylon wool column (Biotest AG, Dreieich, Germany) to enrich for T cells. Effluent cells were washed, resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated autologous serum, and added ($1·5–2\times10^6$ cells/well) to the autologous plastic adherent cells. TBe, SN1, SN2 and SN3 were added to the cultures at the optimal concentration of 9 μg/ml determined in dose–response experiments. BCG-short-term culture filtrate (STCF-BCG), prepared as described previously [32], and PPD were used at a concentration of 5 μg/ml. Phytohaemagglutinin (PHA; 5 μg/ml) was used as a positive control for cell reactivity. As negative control, antigen-free cultures were established. Cultures were maintained in humidified 5% CO_2 at 37°C for 6–7 days before the proliferation assay was performed.

Proliferation assay and identification of cell subsets responding to mycobacterial antigens

Proliferative responses of the nylon wool effluent population following stimulation with mycobacterial antigens were assayed by flow cytometric measurement of bromodeoxyuridine (BdU) uptake, as previously described [30]. Briefly, stimulated cultures were incubated for 16 h with BdU (Sigma, St Louis, MO) at a final concentration of 30 μg/ml. Non-adherent cells were collected, washed and resuspended in a known amount of PBS. Each cell suspension was divided in identical aliquots and each was stained

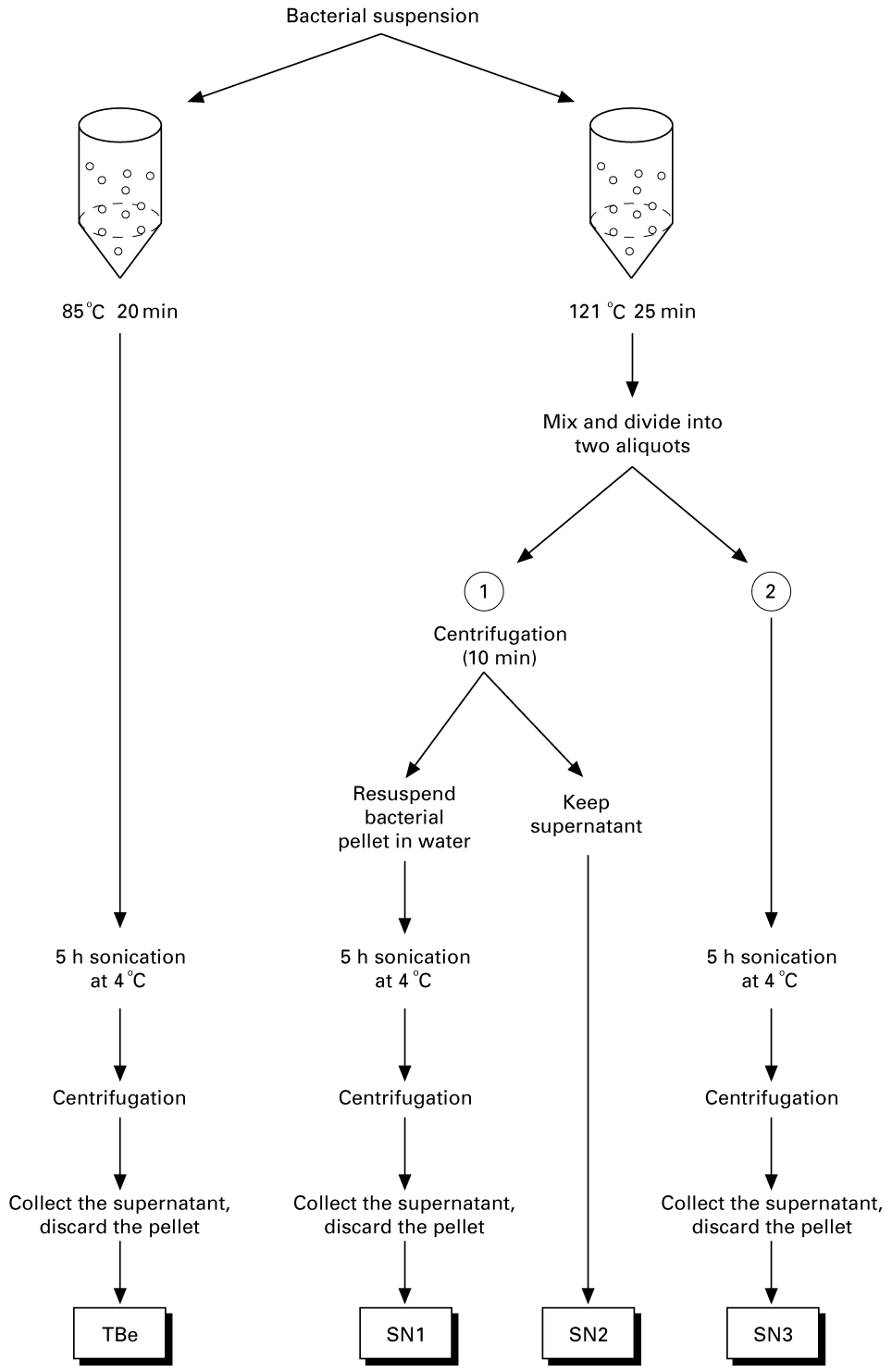

Fig. 1. Main steps in the preparation of mycobacterial soluble extracts.

with a PE-conjugated MoAb directed against different phenotypic surface markers (see below). One aliquot from each stimulated culture was transferred to a Falcon tube (Becton Dickinson, Mountain View, CA) and used to assess the absolute number of cells/well after 6–7 days of stimulation (see below). Cells were fixed overnight with 1% paraformaldehyde (PFA), 0·01% Tween 20, in PBS and then subjected to DNA digestion by resuspension in PBS with Ca^{2+} and Mg^{2+} containing 50 Kunitz units/ml bovine pancreatic DNase-I (Sigma). Digestion was carried on for 45 min at 37°C. After washing, cells were resuspended in 150 μl of 10% bovine serum albumin (BSA), 0·5% Tween 20 in PBS and stained with an FITC-labelled anti-BdU MoAb (Becton Dickinson). After incubation for 45 min at room temperature, cells were washed, resuspended in PBS and analysed by flow cytometry.

Immunofluorescence staining

Cells ($1–5 \times 10^5$) were resuspended in PBS and incubated with saturating amounts of antibodies for 30 min at 4°C. For indirect staining, cells were washed twice and additionally incubated for 30 min at 4°C with PE-labelled $F(ab')_2$ fragments of goat anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark). The following MoAbs were used for the staining: TCSδ1 (anti-Vδ1) (T Cell Diagnostics, Cambridge, MA), IMMU389 (anti-Vδ2) and IMMU360 (anti-Vγ9) (Immunotech, Marseille, France), anti-Vγ8 [33] (kindly provided by Dr K. Söderström, Karolinska Institute, Stockholm, Sweden), UCHT1 (anti-CD3), MT310 (anti-CD4), DK25 (anti-CD8), TÜK4 (anti-CD14), B-Ly1 (anti-CD20), isotype-matched PE- and FITC-conjugated mouse IgG as negative controls (Dakopatts), anti-TCR-γ/δ-1 (11F2, recognizing all $\gamma\delta^+$ T cells), Leu-11c (anti-CD16), and Leu-45RO (UCHL-1, anti-CD45RO) (Becton Dickinson).

FACS analysis and estimation of the absolute number of cells responding to mycobacterial antigens

Twenty thousand events were acquired ungated for each cell surface marker in a FACScan flow cytometer (Becton Dickinson). For analyses, viable cells were selected by a widely set gate on a two-parameter plot of side scatter *versus* forward angle scatter to include small as well as large cells and kept constant for each condition. LYSYS-II and CellQuest softwares (Becton Dickinson) were used for computer-assisted analyses. The percentage of FITC anti-BdU^+ cells gave the total proportion of proliferating cells, while the percentage of double-positive (FITC anti-BdU^+/PE-antisurface $marker^+$) cells represented the proportion of each distinct proliferating cell subset in relation to the gated population. Finally, the percentage of each cell subset in the proliferating population was calculated as follows: (FITC anti-BdU^+ PE-antisurface $marker^+$/FITC anti-BdU^+) $\times$ 100.

In order to assess the absolute number of proliferating cells after 6–7 days of stimulation, an aliquot (usually 50 μl) of each stimulated culture was fixed by adding 200 μl of 1% PFA, 0·01% Tween 20, in PBS. During the flow cytometric analysis of the corresponding stained culture, this aliquot was used to assess the absolute count by using a flow rate-calibrated (usually 0·75 μl/s) flow cytometer. In some experiments the absolute number of cells estimated by this method was compared with that obtained by direct counting of the cells by light microscopy, and a good correlation between the two methods was detected. The absolute number of proliferating cells for each surface marker was calculated by multiplying the absolute count of cells by the percentage of FITC anti-BdU and PE antisurface marker double-positive cells of the corresponding stimulated culture.

Statistical analysis

Statistical significance of the data was determined by two-tailed Student's *t*-test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Proliferative responses of distinct human T cell subsets to different M. tuberculosis *soluble extracts*

Soluble extracts of *M. tuberculosis*, prepared as schematically indicated in Fig. 1, as well as PPD and STCF-BCG, were used for *in vitro* stimulation of nylon wool-enriched T cell populations from healthy, BCG-vaccinated blood donors. Proliferation of different cell subsets was evaluated by flow cytometric determination of BdU incorporated into DNA of dividing cells and simultaneous identification of cell surface markers.

All the mycobacterial antigen preparations tested induced a strong proliferation of $CD3^+$ T cells with similar kinetics, the response reaching a peak by 6–7 days of *in vitro* stimulation (data not shown). Intracellular BdU staining of $CD4^+$ and $\gamma\delta^+$ T cell subsets in response to various soluble extracts is represented in Fig. 2. The proportion of different T cell subsets among the proliferating BdU^+ cells is depicted in Fig. 3 (as mean values of different blood donors tested). In agreement with our previous report [30], a prevalent proportion of $\gamma\delta^+$ T cells was obtained in the responding population when a soluble extract of *M. tuberculosis*, prepared from bacteria killed at 85°C and directly disrupted by prolonged sonication (TBe), was used as a stimulant. In contrast, soluble extracts obtained from bacteria autoclaved and then washed by centrifugation (SN1) elicited a strong and almost exclusive proliferation of $CD4^+ \alpha\beta^+$ T cells. The stimulatory activity for $\gamma\delta^+$ T cells was recovered in the supernatant of the autoclaved bacteria (SN2), indicating that autoclaving of *M. tuberculosis* bacilli leads to a release of antigen(s) into the supernatant which stimulates human $\gamma\delta^+$ T cells. An intermediate level of $\gamma\delta^+$ T cells in the proliferating population was observed when SN3 (extract prepared by direct sonication of autoclaved bacilli) was used as a stimulant (Fig. 3a). Interestingly, a statistically significant difference ($P = 0{\cdot}0014$) was detected in the proportion of $\gamma\delta^+ BdU^+$ T cells out of the total BdU^+ cells, when TBe and SN3 were compared. In order to assess whether the reduction of $\gamma\delta^+$ T cell stimulatory capacity of SN3 compared with TBe could be due to the effect of autoclaving, TBe was heated at 121°C for 25 min and the proportion of $CD4^+$ and $\gamma\delta^+$ T cells in the proliferating population was assessed after *in vitro* stimulation. As reported in Table 1, heating at 121°C of TBe markedly reduced its capacity to stimulate $\gamma\delta^+$ T cells, indicating the existence of a heat-sensitive $\gamma\delta$-stimulatory component in the TBe.

As depicted in Fig. 3b, PPD as well as STCF-BCG were particularly effective in stimulating $CD4^+ \alpha\beta^+$ T cells, with very low proliferation of $\gamma\delta^+$ T cells. In contrast to what was observed with the polyclonal stimulator PHA, which elicited proliferation in both $CD4^+$ and $CD8^+ \alpha\beta^+$ T cell subsets, all the mycobacterial antigen preparations tested exhibited a very poor stimulatory activity of $CD8^+ \alpha\beta^+$ T cells (Fig. 3). The composition pattern of the proliferating T cell subsets in response to the various mycobacterial antigen preparations was highly reproducible among different batches of extracts (data not shown).

In some experiments the absolute cell count of $CD4^+$ and $\gamma\delta^+$ T cell subsets was calculated after 6–7 days of *in vitro* stimulation. Absolute cell number ratio obtained by dividing the absolute number of cells in the stimulated cultures by the absolute number of cells in the antigen-free cultures (RPMI) is graphically illustrated in Fig. 4. In concordance with the experiments based on calculation of relative percentages, a strong proliferation of $\gamma\delta^+$ T cells was observed upon stimulation with TBe and SN2 (20–30-fold increment in comparison with unstimulated cultures), while SN1 or PPD preferentially elicited proliferation of the $CD4^+$ T cell subset.

$\gamma\delta^+$ T cells responding to both TBe and SN2 predominantly expressed a $\delta 2^+/\gamma 9^+$ TCR, as assessed by simultaneous staining for BdU and variable regions of γ- and δ-chains (data not shown). Moreover, the expression of the surface marker CD45RO on the

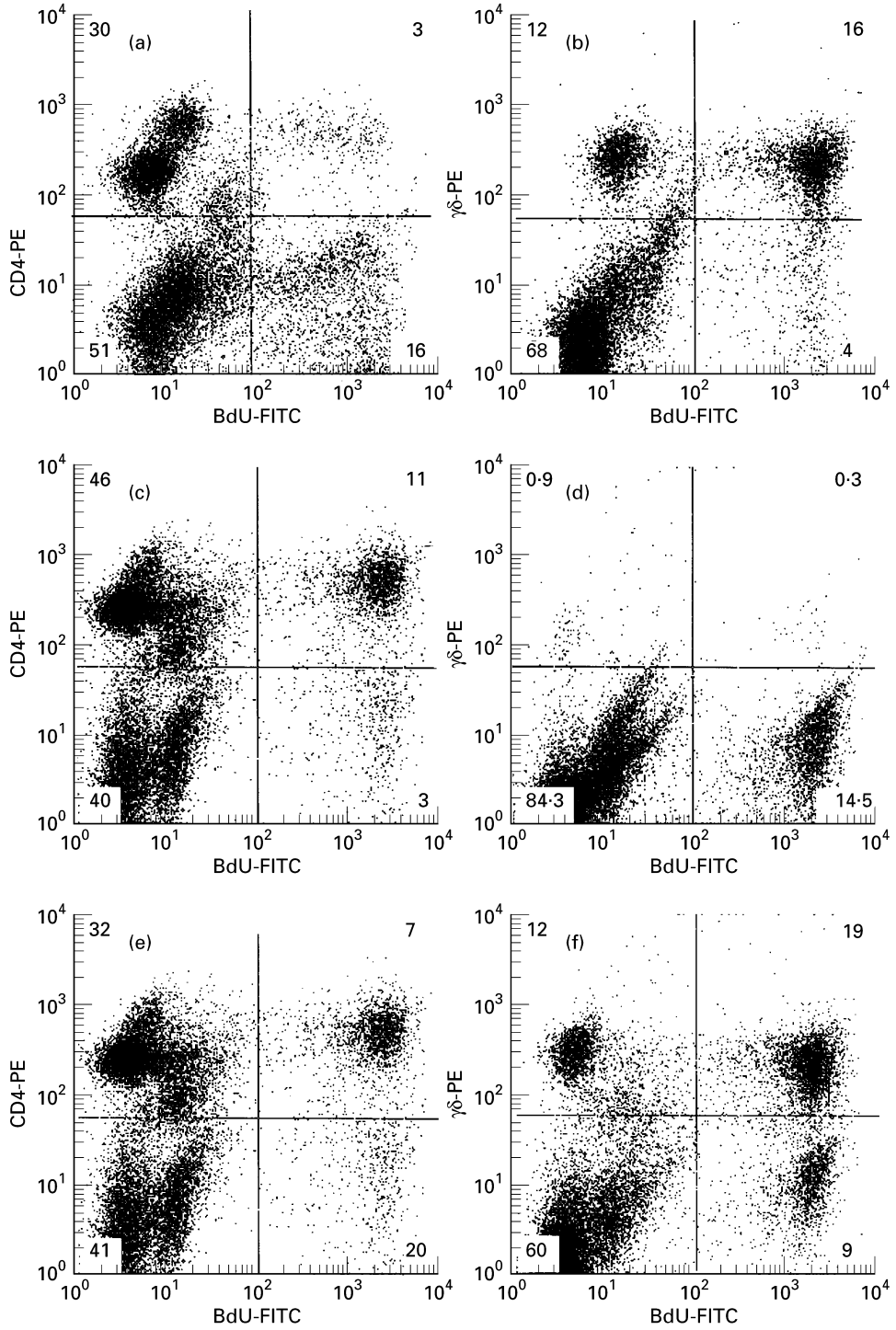

Fig. 2. Intracellular bromodeoxyuridine (BdU) staining of $CD4^+$ and $\gamma\delta^+$ T cell subsets responding to TBe (a,b); SN1 (c,d); and SN2 (e,f). Cells were stimulated with 9 μg/ml of each extract for 6 days and stained with MoAbs (PE-conjugated, ordinate) to CD4 (a,c,e) or $\gamma\delta$ (b,d,f) T cell surface markers, followed by a MoAb (FITC-conjugated, abscissa) to BdU. Total $CD4^+$ and $\gamma\delta^+$ T cells were 44·6% and 1·16%, respectively, before culture (day 0). The percentage of BdU^+ cells in the corresponding antigen-free culture (RPMI) was < 0·5% after 6 days of *in vitro* culture.

proliferating cells was analysed. While upon stimulation with PHA < 45% of the BdU^+ cells were $CD45RO^+$, TBe, SN2 and PPD elicited an almost exclusive expression of CD45RO within the proliferating cells ($BdU^+ CD45RO^+ = 85–90\%$) (data not shown).

Protease digestion of mycobacterial extracts

In order to investigate the nature of the antigen(s) involved in $\gamma\delta^+$/$CD4^+$ T cell stimulatory activity, TBe, SN2, SN1 and PPD were digested with pronase and the absolute number of total BdU^+ cells as well as of proliferating $CD4^+$ and $\gamma\delta^+$ T cell subsets was

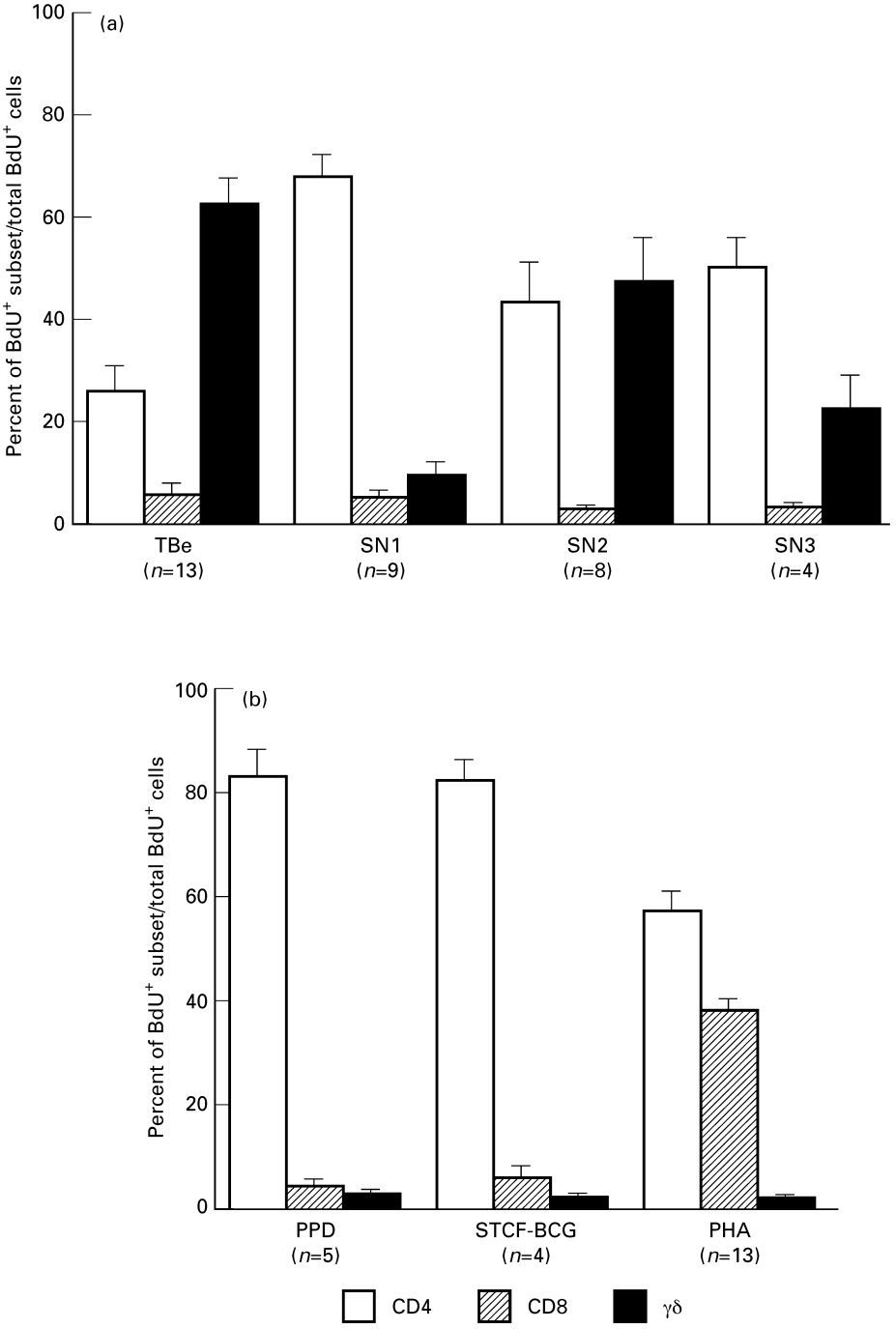

Fig. 3. Composition of proliferating cells after 6–7 days of *in vitro* stimulation with different kinds of mycobacterial antigen preparations or 3 days of stimulation with phytohaemagglutinin (PHA). TBe, SN1, SN2 and SN3 were prepared as depicted in Fig. 1 and used at a concentration of 9 μg/ml. Bacille Calmette–Guérin-short-term culture filtrate (STCF-BCG), purified protein derivative (PPD) and PHA were used at a concentration of 5 μg/ml. *n*, Number of donors tested for each antigen preparation; BdU, bromodeoxyuridine.

evaluated after 7 days of *in vitro* stimulation (Fig. 5). Controls included both wells with pronase alone to check for non-specific activation and mock treatment of the extract to control for the treatment conditions. In parallel cultures, pronase was added to the wells together with mycobacterial extract or PPD at the time of the assay to test for non-specific inhibition. While pronase treatment completely abolished the stimulatory activity of PPD to a level comparable to that of antigen-free cultures (RPMI), digestion of TBe only partially reduced its stimulatory activity (Fig. 5a). The reduction in the absolute number of BdU^+ cells after digestion was mainly due to a reduction in the absolute number of proliferating $CD4^+$ T cells and only to a minor, but reproducible extent, to inhibition of $\gamma\delta^+$ T cells (Fig. 5b,c). These results suggest that the $\gamma\delta^+$ T cell stimulatory activity of TBe was probably due to a

Table 1. Stimulatory capacity of TBe before and after autoclaving at 121°C for 25 min

Donor	Extract	Percent $\gamma\delta^+$ (day 0)	Percent $\gamma\delta^+$ (day 6)	Percent $\gamma\delta^+$ BdU^+/BdU^+ (day 6)	Percent $CD4^+BdU^+/BdU^+$ (day 6)
1	TBe	1·22	41·89	78·71	11·26
	TBe au*		16·45	45·3	37
2†	TBe	2·44	11·3	54·92	38·02
	TBe au		5·13	21·58	72·83
2	TBe	2·44	11·41	51·29	29·54
	TBe au		9·64	42·72	42·78

* TBe au, TBe autoclaved at 121°C for 25 min.
† Donor 2 was tested with two different batches of TBe.

mixture of protease-resistant and protease-sensitive components which differentially contributed to the overall response of $\gamma\delta^+$ T cells. In contrast, digestion of SN2 almost completely abolished its stimulatory activity of both $CD4^+$ and $\gamma\delta^+$ T cells (Fig. 5d,e,f), indicating that the bioactivity of SN2 on $\gamma\delta^+$ T cells was mainly derived from protease-sensitive component(s). Digestion of SN1 completely abolished its stimulatory capacity of $CD4^+$ T cells (data not shown).

Responses of $CD4^+$ and $\gamma\delta^+$ T cell subsets to fractionated TBe

To identify the active fractions for $\gamma\delta^+$ T cell stimulatory activity, TBe was fractionated on a Superose 12 column by fast performance liquid chromatographyy (FPLC) and each fraction was used in a 7-day *in vitro* stimulation assay. Proliferative responses were evaluated as absolute number of $CD4^+$ BdU^+ and $\gamma\delta^+$ BdU^+ T cells, respectively. As illustrated in Fig. 6, while stimulation with the unfractionated extract, again, induced a preponderant proportion of $\gamma\delta^+$ T cells in the responding population, most of the single fractions exhibited a strong stimulatory capacity of only $CD4^+$ T cells. $\gamma\delta^+$ T cell stimulatory capacity was mainly confined to the low molecular weight range, showing two peaks corresponding to molecular masses of $\approx$ 10–14 kD and $<$ 6 kD, respectively.

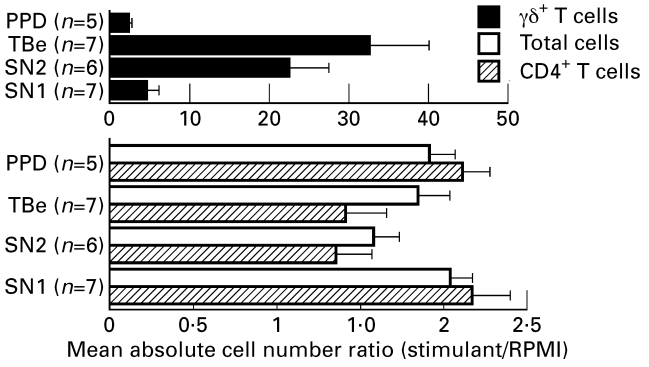

Fig. 4. Ratio between the absolute number of total, $CD4^+$ and $\gamma\delta^+$ T cells in stimulated and unstimulated cultures. Mean values of the different donors tested are illustrated. For the doses of stimulants, see legend to Fig. 3.

DISCUSSION

In the present study, we analysed the proliferative responses of $\gamma\delta^+$ and $CD4^+$ $\alpha\beta^+$ T cell subsets to different *M. tuberculosis* soluble extracts. We demonstrated that the protocol used for the preparation of the extract has an impact on the T cell subset preferentially expanded following *in vitro* stimulation. In agreement with our previous data [30], a soluble extract of *M. tuberculosis* (TBe), obtained from bacilli killed at 85°C, represented a strong stimulus for human $\gamma\delta^+$ T cells. In contrast, the $\gamma\delta^+$ T cell stimulatory capacity was almost completely abolished when the extract was prepared from autoclaved and washed bacilli (SN1), a treatment that did not seem to greatly affect the bioactivity on the $CD4^+$ T cell subset. Although the $\gamma\delta^+$ T cell stimulatory activity of the TBe appeared to be mainly dependent on protease-resistant component(s), a contribution of protease-sensitive component(s) was also observed. Interestingly, a selective release of protease-sensitive component(s), strongly active on $\gamma\delta^+$ T cells, was observed in the supernatant of autoclaved bacilli of *M. tuberculosis* (SN2). The latter observation confirms previous data from Boom *et al*. [20], who described a 10–14-kD protease-sensitive antigen from *M. tuberculosis* H37Ra efficiently released from the bacilli by heating at 121°C and highly active on human $\gamma\delta^+$ T cells.

The experiments with fractionated TBe revealed that the components active on $\gamma\delta^+$ T cells were localized in the low molecular range, with two major peaks of bioactivity. The first peak, localized at a molecular mass of $\approx$14 kD, may correspond to the heat-stable, protease-sensitive antigen described by Boom *et al.* [20], while the second peak, at a molecular mass $<$ 6 kD, may represent the component(s) of the extract resistant to protease treatment, recently identified by several authors as the major mycobacterial stimulus for human $\gamma\delta^+$ T cells [8,30,34,35]. A complete digestion of the component active on $\gamma\delta^+$ T cells in SN2, together with the partial loss of $\gamma\delta^+$ T cell stimulatory activity of SN3 (extract from autoclaved and unwashed bacteria) in comparison with TBe, suggest that the protease-resistant component of the extract may be relatively less heat-stable than the protease-sensitive one which, instead, efficiently maintains its $\gamma\delta$-stimulating capacity in the supernatant of autoclaved bacteria.

We have previously demonstrated that intact mycobacteria, both live and killed, do not represent a good stimulus for human $\gamma\delta^+$ T cells, and that only by disruption of the bacilli can a release of the components active on such cells be achieved [30]. Several

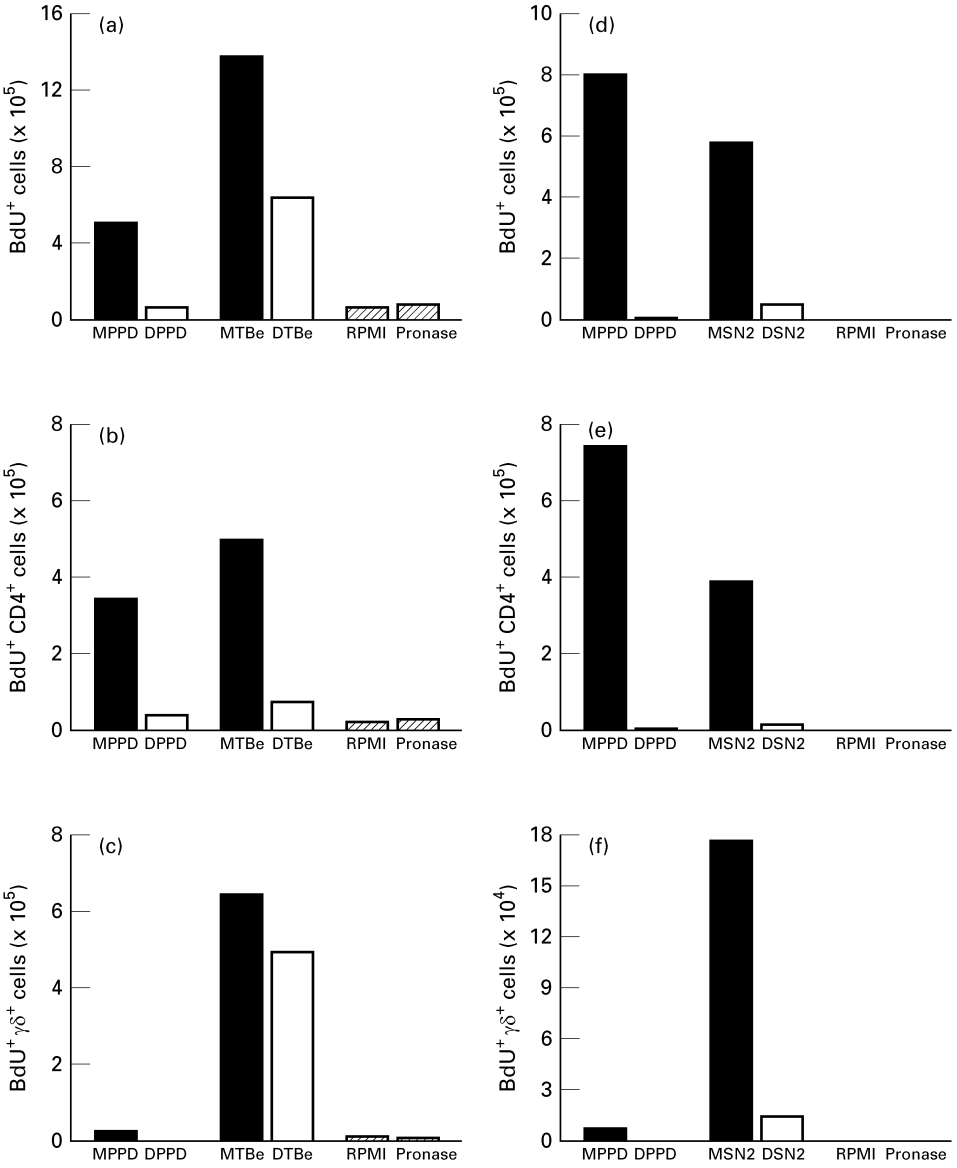

Fig. 5. Stimulatory capacity of pronase-digested purified protein derivative (PPD) (a–f), TBe (a,b,c) and SN2 (d,e,f). For each digested antigen preparation the absolute number of total bromodeoxyuridine $(BdU)^+$ cells (a,d), $CD4^+$ (b,e) and $\gamma\delta^+$ (c,f) T cells was calculated after 6 days of stimulation and compared with that of the corresponding mock-treated sample. M, Mock-treated sample; D, digested sample; RPMI, antigen-free cultures; pronase, cultures with pronase alone. Addition of pronase to the wells, at the time of the assay, together with PPD, TBe or SN2, did not significantly alter the stimulatory capacity of the stimulants (data not shown). Results illustrated in (a,b,c) and (d,e,f) were obtained from two different blood donors.

studies, both *in vitro* and in animal models, have supported the hypothesis that $\gamma\delta^+$ T cells play a role in the early phase of infections [3,6,24,34]. Such observations raise the question of how the antigens for $\gamma\delta^+$ T cells become available during the early stages of an infection, when most of the bacilli are still likely to be intact. Secretion of $\gamma\delta$-stimulating components by live mycobacteria could be one possibility. Although some authors [24] have observed a secretion of the non-peptide prenyl pyrophosphate antigen for $\gamma\delta^+$ T cells by live mycobacteria, others [26] have demonstrated a total absence of the non-peptidic $\gamma\delta$-stimulatory antigens in the culture fluids of several strains of *M. tuberculosis*, *M. bovis* and BCG, even after prolonged culture times during which cell wall antigen shedding could occur. Under the same culture conditions, only *M. fortuitum* was demonstrated to secrete efficiently the non-peptidic component active on $\gamma\delta^+$ T cells [26]. Additionally, the 10–14-kD protease-sensitive antigen was associated with the cytosolic fraction of *M. tuberculosis* rather than being secreted into the culture fluid [20]. In the present study, no $\gamma\delta^+$ T cell stimulatory activity was observed when short-term culture filtrate of BCG or late culture filtrate antigens in PPD were used as stimulants, supporting the data that $\gamma\delta$-stimulatory antigens are associated with the bacterial cells, are not secreted and only become available after disruption and/or partial alteration of the bacilli. If this is also the case *in vivo*, it can be argued that the $\gamma\delta^+$

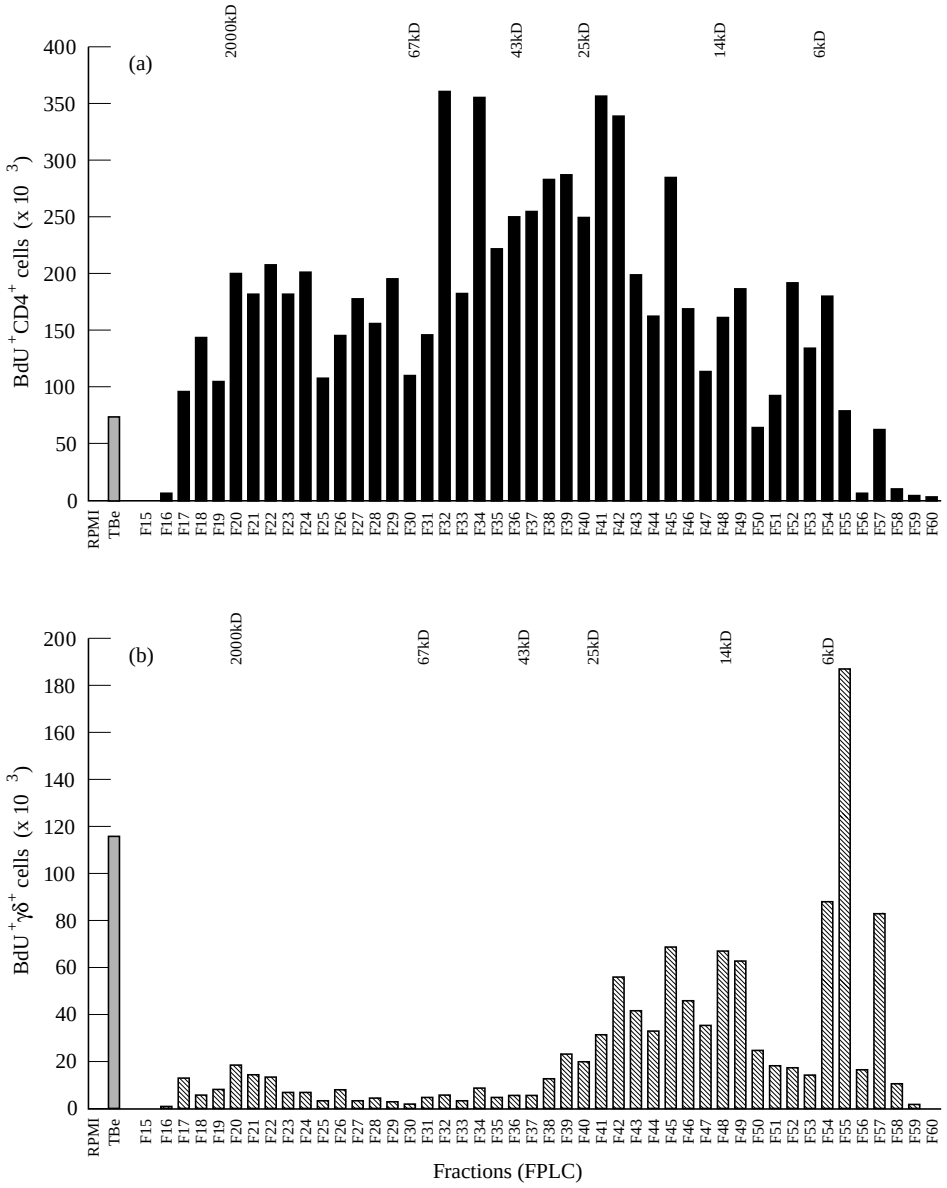

Fig. 6. Responses of $CD4^+$ and $\gamma\delta^+$ T cell subsets to fractionated *Mycobacterium tuberculosis* extract (TBe). Concentrated TBe was fractionated on a Superose 12 HR column by fast performance liquid chromatography (FPLC). Fractions, as well as unfractionated extract, were tested in a 7-day proliferation assay and the results were expressed as absolute number of proliferating $CD4^+$ (a) and $\gamma\delta^+$ T cells (b), respectively. The column was calibrated with proteins of known molecular masses indicated above the graphics.

T cell response occurs during later stages of the disease course, after the $\alpha\beta^+$ T cell response and after clearance of the bacteria has been achieved. Such a hypothesis is supported by several studies. In the mouse model, a contribution of $\gamma\delta^+$ T cells to the optimum control of late stages of the BCG infection has been demonstrated [36], while an influence of the dose of infection on an early or late $\gamma\delta^+$ T cell response has been proved in experimental infection with *Listeria monocytogenes* [37]. In humans, appearance of $\gamma\delta^+$ T cells subsequent to $\alpha\beta^+$ T cells has been demonstrated in skin lepromin reactions [38]. It is possible that $\gamma\delta^+$ T cells may act at different stages during the course of an infection, and that several factors (e.g. dose or site of infection, bacterial strain, immunocompetence of the host) modulate the release of $\gamma\delta^+$ T cell ligands and the response of such cells.

As assessed in fractionation experiments of TBe, most of the single fractions elicited an intense and exclusive proliferative response of the $CD4^+$ T cell subset, whereas stimulation with unfractionated extract triggered a preferential proliferation of $\gamma\delta^+$ T cells. The apparent low $CD4^+$ T cell response to unfractionated TBe did not seem to be due to a depletion or lack of native protein constituents as a consequence of the heat treatment, as most of the single fractions were strongly active on $CD4^+$ T cells. Rather, it may suggest an inhibition of potentially reactive $\alpha\beta^+$ $CD4^+$ T cells by activated $\gamma\delta^+$ T cells or, alternatively, inhibitory effects of $\gamma\delta^+$ T cell stimulatory molecules on $\alpha\beta^+$ $CD4^+$ T cells. Most of the data available regarding the biological properties of $\gamma\delta^+$ or $CD4^+$ T cell subsets are based on the use of clones or cell lines. Different subpopulations of cells produce, after stimulation, various cytokines

which may exhibit stimulatory as well as inhibitory effects on other cell subset(s); consequently, the proliferation of an isolated population might not exactly reflect what actually happens in unseparated cultures, even if exogenous cytokines are added. In the present study, the methodology employed to assess proliferation of different cell subsets allowed evaluation of the response of $\gamma\delta^+$ and $CD4^+$ T cells in heterogeneous cultures, thus providing appropriate conditions for the occurrence of cell–cell interactions.

The consequences of the reciprocal interaction(s) between $\alpha\beta^+$ and $\gamma\delta^+$ T cells are still a major question in the immune response to mycobacteria. While it seems that an efficient activation of $\gamma\delta^+$ T cells requires the interaction with $\alpha\beta^+$ T cells (via a cell–cell contact or soluble factors released by $CD4^+$ cells) [35,39], it is also possible that activated $\gamma\delta^+$ T cells may exhibit a regulatory function and affect the proliferative responses of $\alpha\beta^+$ T cells [5,40]. Increased proliferative responses of $\alpha\beta^+$ T cells in mice depleted of $\gamma\delta^+$ T cells have been described, suggesting a possible role for the $\gamma\delta^+$ T cells in the regulation of $\alpha\beta^+$ T cell activation *in vivo* [40]. If this is the case, as has also been proposed by others, $\gamma\delta^+$ T cells seem to be extremely powerful in their regulatory functions, despite being far less numerous than the much larger population of $\alpha\beta^+$ T cells [27]. We are currently investigating the possible mechanisms of the $\gamma\delta^+$–$\alpha\beta^+$ T cell interactions *in vitro* and the eventual role, in such interactions, of different (protein/non-protein) $\gamma\delta$-stimulatory ligands.

In conclusion, in this study we analysed the proliferative response of $\alpha\beta^+$ and $\gamma\delta^+$ T cells in mixed heterogeneous cultures in response to different kinds of *M. tuberculosis* soluble extracts. Our results provide further indications that protease-sensitive ligands may also account for stimulation of $\gamma\delta^+$ T cells, and that such cells may play important roles during the course of infections.

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