Clin Exp Immunol 2000; **119**:270–279

Identification of distinct lymphocyte subsets responding to subcellular fractions of *Mycobacterium bovis* bacille Calmette–Guérin (BCG)

G. BATONI*, S. ESIN*†, M. PARDINI*, D. BOTTAI*, S. SENESI*, H. WIGZELL† & M. CAMPA*
*Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, University of Pisa, Pisa, Italy, and †Microbiology and Tumourbiology Centre, Karolinska Institute, Stockholm, Sweden

(Accepted for publication 28 October 1999)

SUMMARY

In order to investigate the ability of *Mycobacterium bovis* BCG vaccination to induce immune responses toward different classes of mycobacterial antigens and the cell populations involved in such responses, proliferation of distinct human lymphocyte subsets from BCG-vaccinated donors in response to different subcellular fractions of BCG was analysed and compared with that of not sensitized subjects. Proliferation of different cell subsets was evaluated by flow cytometric determination of bromodeoxyuridine incorporated into DNA of dividing cells and simultaneous identification of cell surface markers. Although a certain degree of variability was observed among different donors, after 6 days of *in vitro* stimulation BCG-vaccinated subjects displayed, as a mean, a stronger blastogenic response to all the classes of antigens compared with non-sensitized ones. PPD, culture filtrates and membrane antigens induced a predominant proliferation of $CD4^+$ T cells. In contrast, preparations enriched in cytosolic antigens elicited strong proliferation of $\gamma\delta^+$ T cells which, as a mean, represented 55% of the proliferating cells. Although to a lesser extent, proliferation of $\gamma\delta^+$ T cells was also elicited by preparations enriched in membrane and cell wall antigens. In response to the latter preparation proliferation of $CD4^+$ T cells and $CD16^+/CD3^-$ (natural killer (NK)) cells was observed, as well. In particular, cell wall antigens were found to induce significantly higher levels of proliferation of NK cells compared with all the other classes of antigens.

Keywords *Mycobacterium bovis* (BCG) T cell subsets subcellular fractions proliferation flow cytometry

INTRODUCTION

BCG, an attenuated strain of *Mycobacterium bovis*, is the vaccine used against tuberculosis (TB) world wide [1]. Several vaccination trials have however revealed that the efficacy of the vaccine is extremely variable and various attempts are in progress to develop a new subunit vaccine composed of defined mycobacterial antigens [1,2]. In general, mycobacterial antigens have been classified in somatic proteins, which are part of the bacterial body, and secreted proteins which can be obtained from culture filtrates at early times during growth *in vitro* when contamination with autolysis products is low [3]. The observation that immunization of mice with live *M. tuberculosis* induces protection and DTH, whereas heat-killed organisms induce DTH only [4], has focused the interest on the study and identification of proteins released in culture filtrate by live microorganisms. Although it has been demonstrated that secreted proteins exert strong immunogenic capacity both in animal models and in humans [5–7], the emerging evidence suggests that other classes of proteins (i.e. cytosolic, cell wall or membrane proteins) can also be highly immunogenic [8,9]. In addition to protein antigens, novel classes of non-proteinaceous T cell ligands of mycobacterial origin have also been described [10–13], but their role in protective immunity or immunopathology of TB is still largely unknown. Although a large number of mycobacterial components are able to induce immune responses, it is necessary to identify the key antigens able to elicit protective immunity and a long-lived state of immunological memory.

Protection against mycobacteria strongly depends on T cells, and although it is generally accepted that different T cell populations are needed for an efficient response to mycobacteria, the precise role of these T cell subsets in protection requires further investigation. The creation of knockout mice has enormously contributed to clarification of the role of distinct immune

Correspondence: Giovanna Batoni, Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, University of Pisa, Via S. Zeno 35–39, 56127 Pisa, Italy.
E-mail: batoni@biomed.unipi.it

components in the development of protective immunity to mycobacteria. For example, it has become clear that beside $\alpha\beta^+$ $CD4^+$ T lymphocytes, other T cell subsets such as $\alpha\beta^+$ $CD8^+$ or $\gamma\delta^+$ T cells contribute to the cell-mediated immunity against TB [14–16]. Such findings have suggested that among a number of reasons responsible for the variable protection observed with BCG (differences in BCG strains used and/or in the proportion of live bacilli in the vaccine, lack of important antigens in BCG which are expressed in *M. tuberculosis*, degree of sensitization to environmental non-tuberculous mycobacteria prior to vaccination) [1], a low capacity of the vaccine to stimulate a broad spectrum of cellular types essential for inducing an efficient response might be a determinant factor [16,17]. Results obtained in animal models cannot be simply extrapolated to humans and consequently many aspects of the nature of immunity to BCG in man remain to be elucidated.

By using an *in vitro* stimulation assay which allows direct identification of proliferating cell subsets by flow cytometric determination of bromodeoxyuridine (BdU) incorporation into DNA, we have previously described a preferential proliferation of both $CD4^+$ and $CD8^+$ T cells in response to intact (live and killed) mycobacteria, while disrupted bacilli, in the form of a sonic extract, were found to elicit preferential proliferation of $\gamma\delta^+$ T cells [18]. In order to investigate the nature of the immunity elicited by BCG vaccine in man, in the present study *M. bovis* BCG was fractionated into different subcellular fractions (cell wall, cytosol, membrane) and proliferation of distinct cell subsets in response to the different classes of somatic antigens, as well as to secreted antigens, was evaluated in donors sensitized to mycobacterial antigens and compared with that of non-sensitized donors. Although all the classes of antigens were found to elicit strong proliferative responses and interferon-gamma (IFN-γ) production in most of the sensitized donors, a differential proliferation of distinct cell subsets in response to the various antigen preparations was observed.

SUBJECTS AND METHODS

Mycobacterial cultures

Mycobacterium bovis BCG (Pasteur Merieux, Lyon, France) was grown in standing cultures in modified Sauton's medium enriched with 0·5% sodium pyruvate and 0·5% glucose [19]. Tween 80 was added to a final concentration of 0·05% v/v only for preparation of standard inocula. To this end bacteria were harvested in logarithmic growth phase, washed by centrifugation and resuspended in fresh medium at 2×10^9 colony-forming units (CFU)/ml. Aliquots were kept frozen at −70°C until use. The number of CFU was determined by plating 10-fold dilutions, in duplicate, in Middlebrook 7H11 agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) enriched with oleic acid, dextrose, albumin and catalase (Acumedia; Dalynn Laboratory Products Ltd, Calgary, Canada).

Preparation of mycobacterial antigens

Subcellular fractions and culture filtrates of *M. bovis* BCG were essentially prepared as previously described [19]. Briefly, 2×10^9 CFU of BCG were inoculated in 200 ml of modified Sauton's medium and grown in standing cultures for 10 days. The culture was filtered through a 0·22 μm pore size filter to separate bacteria from culture supernatant. Bacteria were washed several times in PBS, vortexed for 30 min with glass beads to dissolve clumps and killed at 80°C for 1 h. After centrifugation, the bacterial pellet was resuspended in cold sterile PBS at a concentration of approximately 250 mg/ml (wet weight). Bacilli were disrupted by sonication with a probe sonicator (Ultrasonic Processor XL; Hertz Systems, Farmingdale, NY) at 1 min pulser on, 10 s pulser off intervals for 60 cycles in an ice bath. Unbroken cells were removed by low speed centrifugation (1000 ***g***). The sonicate was centrifuged at 20 000 ***g*** for 20 min at 4°C. The resulting pellet was resuspended in PBS and washed again by centrifugation as above to obtain the bacterial cell wall (CW). The supernatant, containing the cytosol and the membrane fractions, was further centrifuged at 20 000 ***g*** for 20 min at 4°C to remove residual CW and ultracentrifuged at 100 000 ***g*** for 2 h at 4°C to obtain the cytoplasmic (Cyt) and membrane (ME) components. Preparations enriched in cytosolic antigens of *M. tuberculosis* (TBe) were prepared as previously described [18]. Supernatants from BCG cultures used for the preparation of the different subcellular fractions were collected as source of secreted proteins (CF). They were concentrated 100-fold by ultrafiltration through an Amicon YM3 membrane (Amicon, Grace Italiana SpA, Milan, Italy), extensively dialysed with sterile filtered PBS and further concentrated five times by low speed centrifugation under vacuum. A commercial preparation of PPD (Statens Seruminstitut, Copenhagen, Denmark) was used as reference reagent to discriminate between sensitized and non-sensitized donors. The protein concentration of the different preparations was determined by the method of Lowry *et al*. [20] and in the case of ME and CW a modification of the procedure was used to allow protein quantification of lipid-containing samples [21].

Subjects

The study group consisted of 15 healthy volunteers ranging from 23 to 37 years old. Eight subjects had been vaccinated with *M. bovis* BCG at least 3 years before the donation. Seven subjects had no history of previous BCG vaccination and exhibited a negative skin test reaction to PPD. Informed consent was obtained and the protocol was approved by the local ethical committee.

Cell culture and proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors by centrifugation on standard density gradient (Lymphoprep, Cedarlane, Ontario, Canada). Cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine (HyClone Europe Ltd, Cramlington, UK) and seeded in 48-well plates (Costar, Cambridge, MA) 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. Mycobacterial antigen preparations were used as stimulants at the following optimal concentrations determined in dose–response experiments: PPD 10 μg/ml; CF 10 μg/ml; Cyt 9 μg/ml; CW 76 μg/ml; ME 8·5 μg/ml. Phytohaemagglutinin (PHA; 5 μg/ml) was used as a positive control for cell reactivity. As negative control, antigen-free cultures were set up. 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 BdU uptake, as previously described [18]. 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 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 30 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. The following PE-conjugated MoAbs were used for the staining: MT310 (anti-CD4), DK25 (anti-CD8), TÜK4 (anti-CD14), B-Ly1 (anti-CD20), UCHT1 (anti-CD3, FITC-conjugated), isotype-matched PE- and FITC-conjugated mouse IgGs as negative controls (Dakopatts, Glostrup, Denmark); anti-TCR-γ/δ-1 (11F2, recognizing all $\gamma\delta^+$ T cells), and Leu-11c (anti-CD16) (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 FACSort 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, kept constant for each condition. LYSYS-II and CellQuest software (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-anti-surface $marker^+$) 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-anti-surface $marker^+$/FITC-anti-BdU^+) $\times 100$.

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 150 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. 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-anti-surface marker double-positive cells of the corresponding stimulated culture.

IFN-γ assay

The amount of IFN-γ present in the culture supernatants was quantified by a commercial ELISA (ELISA Cytokine kit; Euroclone Ltd, UK) according to the instructions of the manufacturer. Supernatants were collected at day 5 from triplicates of parallel cultures stimulated with the different antigen preparations, pooled and stored in aliquots at −70°C until use. Recombinant IFN-γ was used as a standard in each assay. The detection level for the assay was 50 pg/ml. The results are given as means of duplicate wells and the difference between wells did not exceed 10% of the means.

Statistical analysis

Statistical significance of the data was determined by non-parametric two-tailed Mann–Whitney *U*-test and paired Wilcoxon matched pairs test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Identification of healthy donors sensitized and non-sensitized to mycobacterial antigens

Fifteen healthy Italian donors were included in the study. Eight of them had been vaccinated with *M. bovis* (BCG) when they were adult, at least 3 years before being included in the study. Seven subjects had no history of BCG vaccination and resulted negative when skin-tested with PPD. T cell-enriched populations from all of the subjects were stimulated *in vitro* with PPD and proliferation was evaluated by two-colour flow cytometric determination of $CD4^+/BdU^+$ cells after 6 days of culture. A percentage of 5% $CD4^+/BdU^+$ cells was arbitrarily chosen as a cut-off value to discriminate between sensitized (SD) and non-sensitized donors (NSD). As reported in Table 1, seven of eight BCG-vaccinated donors responded strongly to PPD *in vitro*, exhibiting a proliferation of $CD4^+$ T cells > 5% (BCG^+ SD group). Only one of eight BCG-vaccinated subjects responded at a very low level ($CD4^+/BdU^+ < 5\%$) to PPD and was included in the group of NSD together with the majority of non-vaccinated subjects. Finally, two of seven non-vaccinated donors, although PPD skin test-negative, responded powerfully to PPD *in vitro* and were therefore considered sensitized to mycobacterial antigens (BCG^- SD).

Comparison of the blastogenic response and IFN-γ production induced by different classes of BCG antigens in sensitized and non-sensitized donors

To evaluate the specificity of the response to the different antigen preparations, total proliferation, expressed as percentage of BdU^+ cells, and IFN-γ production were compared in SD and NSD. Although a certain degree of variability was observed among different donors, after 6 days of *in vitro* stimulation SD displayed, as a mean, a stronger blastogenic response to all classes of antigens compared with NSD (Fig. 1) ($P < 0{\cdot}01$). In contrast, similar levels of proliferation were observed for the two groups of donors in cultures without antigens (RPMI) or stimulated with a T cell polyclonal activator like PHA. However, while in response to PPD and CF all but one NSD displayed very low levels of proliferation (BdU^+ cells < 5%), some of the NSD displayed a modest proliferative response (BdU^+ cells > 5%) to Cyt (three of six subjects), ME (six of six subjects) and CW (four of six subjects).

IFN-γ production in the supernatant of cultures stimulated with the different antigen preparations was determined by ELISA after

Table 1. Identification of healthy donors sensitized and non-sensitized to mycobacterial antigens

	BCG-vaccinated, sensitized donors (BCG^+ SD)				BCG-non-vaccinated, sensitized donors (BCG^- SD)				Non-sensitized donors (NSD)		
	BCG vaccination	PPD test	% $CD4^+$/ BdU^+		BCG vaccination	PPD test	% $CD4^+$/ BdU^+		BCG vaccination	PPD test	% $CD4^+$/ BdU^+
D 1	Yes	ND	7·3	D 1	No	–	27·2	D 1	Yes	ND	2·69
D 2	Yes	ND	18·81	D 2	No	–	11·7	D 2	No	–	4·67
D 3	Yes	ND	24·2					D 3	No	–	1·19
D 4	Yes	ND	11·1					D 4	No	–	3·9
D 5	Yes	ND	11					D 5	No	–	1·56
D 6	Yes	+	13·1					D 6	No	–	1·93
D 7	Yes	+	18·4								

5 days of *in vitro* culture in the presence of the different antigen preparations. As depicted in Fig. 2, there was a significant difference in the production of IFN-γ by cells from SD and NSD stimulated with PPD or CF, but not with Cyt, ME, CW or unstimulated.

Identification of distinct lymphocyte subsets responding to subcellular fractions of M. bovis (BCG)

To evaluate whether mycobacterial antigen preparations enriched in cytoplasmic, membrane or cell wall antigens, respectively,

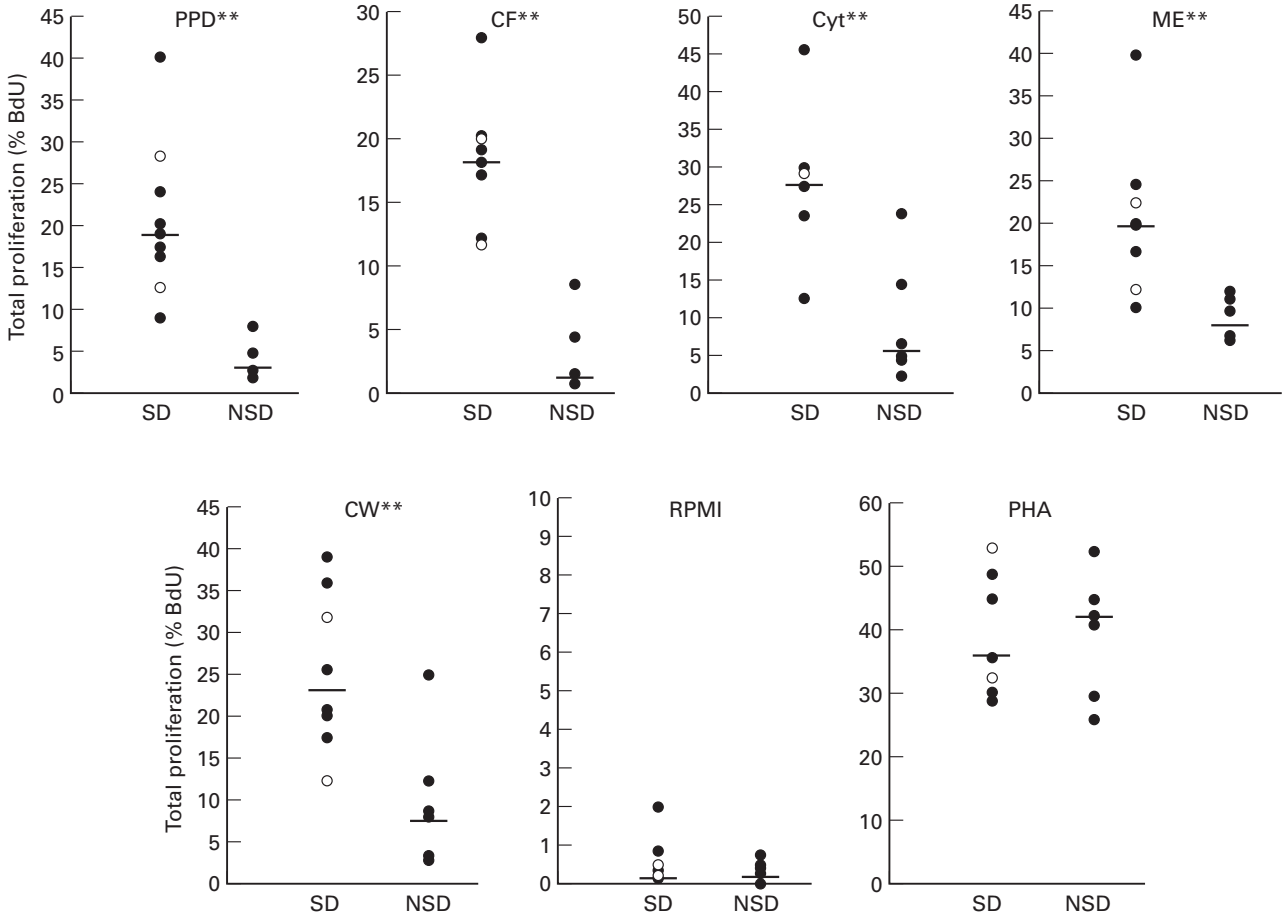

Fig. 1. Total percentage of proliferating cells after stimulation *in vitro* with various antigen preparations in sensitized (SD) and non-sensitized donors (NSD). T cell-enriched populations from healthy donors were stimulated for 6 days with antigen preparations enriched in cytosolic (Cyt), membrane (ME), and cell wall (CW) antigens as well as with PPD and culture filtrates (CF) from *Mycobacterium bovis* BCG. Phytohaemagglutinin (PHA) was used as positive control for cell reactivity. RPMI, Antigen-free cultures. ○, Two donors non-vaccinated with BCG, but sensitized to mycobacterial antigens according to the criteria specified in Results. $^{**}P < 0{\cdot}01$, non-parametric two-tailed Mann–Whitney *U*-test. Median values are indicated by horizontal lines.

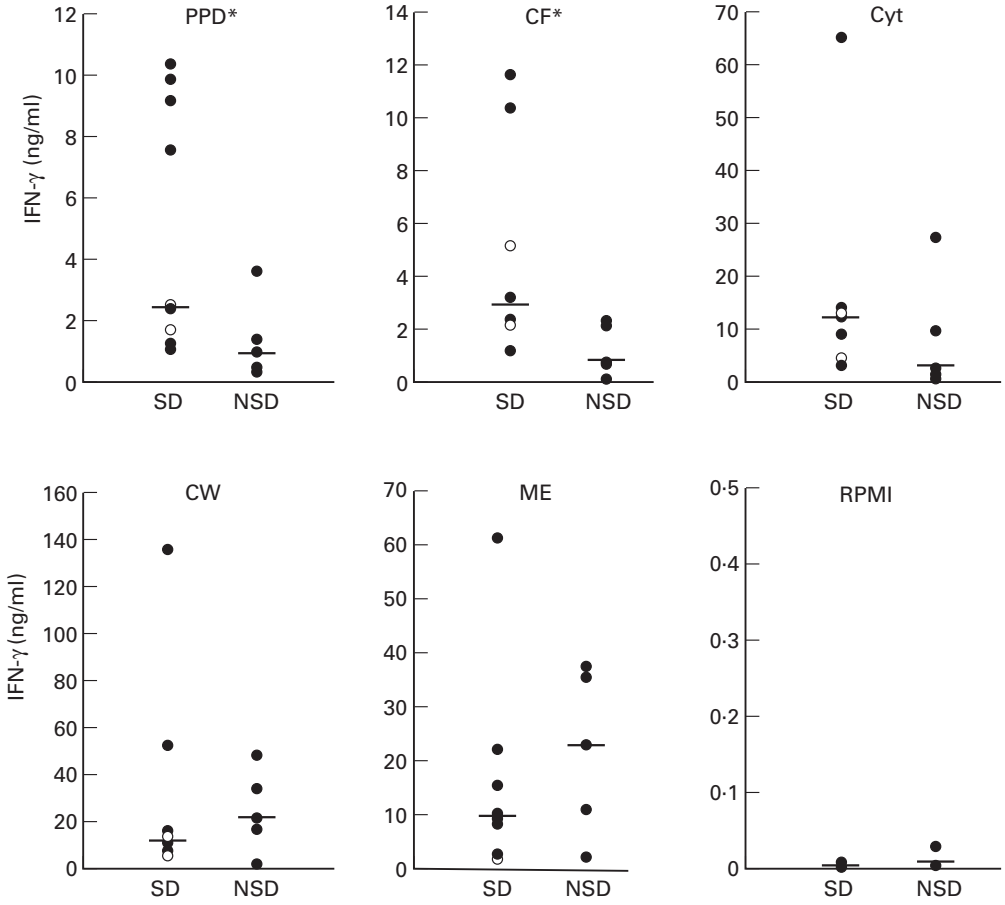

Fig. 2. Interferon-gamma (IFN-γ) production after stimulation *in vitro* with various antigen preparations in sensitized and non-sensitized donors. T cell-enriched populations from healthy donors were stimulated for 5 days with antigen preparations enriched in cytosolic (Cyt), membrane (ME), and cell wall (CW) antigens as well as with PPD and culture filtrates (CF) from *Mycobacterium bovis* BCG. ○, The two donors non-vaccinated with BCG, but sensitized to mycobacterial antigens according to the criteria specified in Results. $*P < 0{\cdot}05$, non-parametric two-tailed Mann–Whitney *U*-test. Median values are indicated by horizontal lines.

induced preferential proliferation of distinct cell subsets, the different subcellular fractions from *M. bovis* BCG were used to stimulate enriched T cell populations from the three groups of subjects (BCG^+ SD, BCG^- SD, NSD). Culture supernatants from the same bacterial cultures used to prepare the different fractions were collected and used in the stimulation assay as a source of secreted proteins. Proliferation of different cell subsets was evaluated by two-colour flow cytometric determination of BdU incorporated into DNA of dividing cells and simultaneous identification of cell surface markers. The proportion of different cell subsets among the proliferating BdU^+ cells is depicted in Fig. 3 as mean value of the different subjects tested. After 6 days of *in vitro* stimulation, T cell-enriched populations from BCG^+ SD responded to both PPD and culture filtrates (CF) with a strong and almost exclusive proliferation of $CD4^+$ T cells. Also membrane antigens (ME) induced high levels of proliferation of $CD4^+$ T cells. In contrast, preparations enriched in cytosolic antigens of *M. bovis* BCG (Cyt) elicited a predominant proliferation of $\gamma\delta^+$ T cells which, as a mean, represented > 55% of the proliferating cells. For some donors it was possible to test, in the same experiment, proliferation induced by cytosolic antigens from both BCG (Cyt) and *M. tuberculosis* (TBe). As shown in Table 2, no significant difference was observed between the two preparations in either the total extent of proliferation (percentage of total BdU^+ cells) or the percentage of proliferating $\gamma\delta^+$ T cells ($\gamma\delta^+/BdU^+$ T cells). Although to a lesser extent, proliferation of $\gamma\delta^+$ T cells was also elicited by preparations enriched in membrane (ME) and cell wall (CW) antigens (10·6% and 36·3% of the BdU^+ cells, respectively). In addition, CW induced proliferation of $CD4^+$ T cells (> 34% of the dividing cells) and, interestingly, of natural killer (NK) cells ($CD16^+/CD3^-$) which represented > 25% of the proliferating cells. In some subjects proliferation of NK cells in response to CW reached up to 37% of the BdU^+ cells. Figure 4 compares the absolute numbers of proliferating NK cells after 6 days stimulation with the different classes of antigens. In accordance with the experiments based on calculation of relative percentages in most of the subjects tested, CW was revealed to be the best inducer of NK cells compared with the other antigen preparations ($P < 0{\cdot}01$).

Analysis of the proliferating subsets was also made for the groups of BCG^- SD and NSD. In the latter group the composition of the responding cells was analysed only for those donors who displayed a total proliferation (percentage of BdU^+ cells) > 5%. While the two donors non-vaccinated but sensitized to mycobacterial antigens (BCG^- SD) displayed a pattern similar to that of the group of BCG^+ SD, analysis of the proliferating cell subsets in the group of NSD revealed a relative increase in the proliferation of

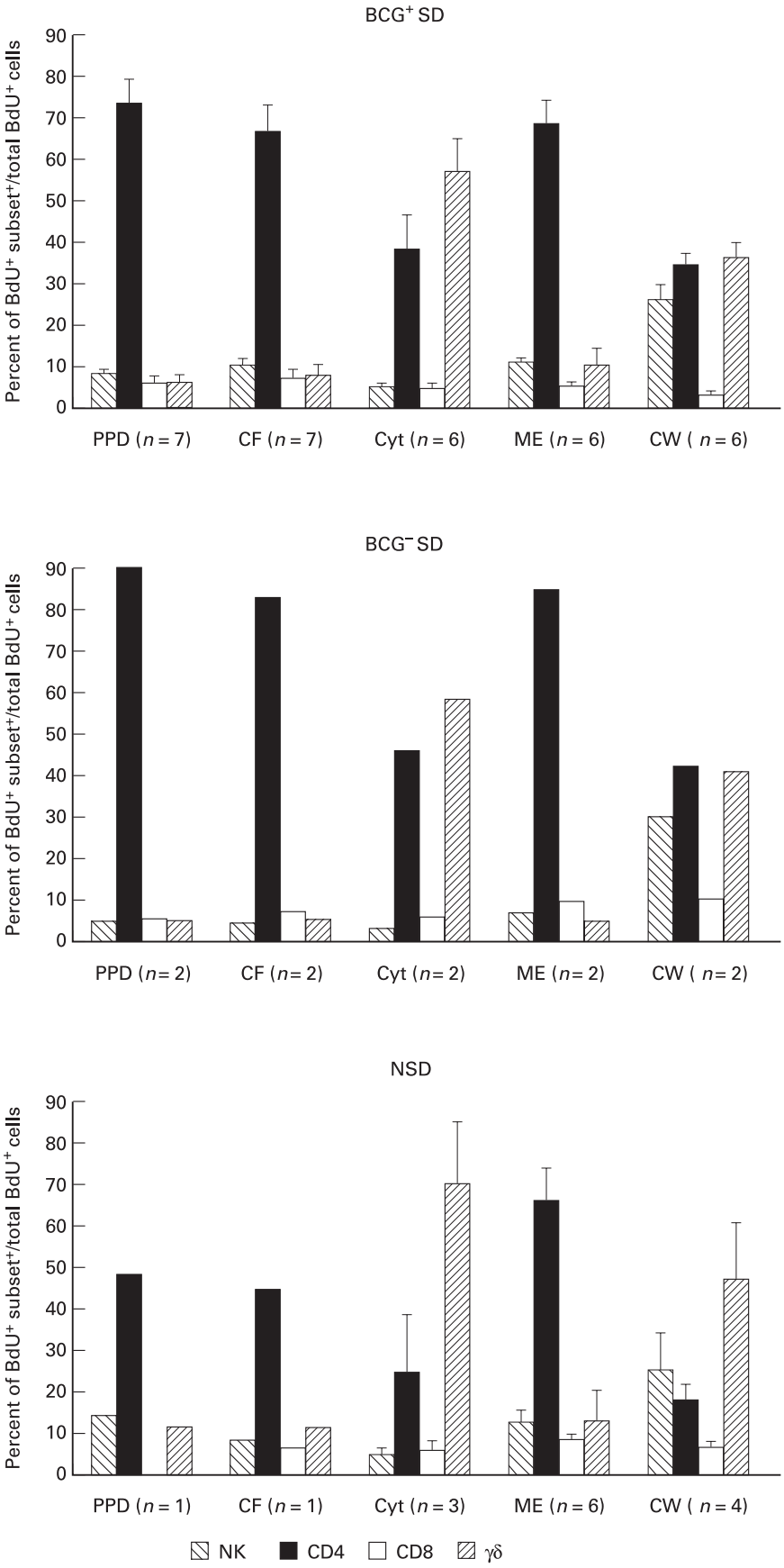

Fig. 3. (See next page for caption)

Table 2. Proliferation of $\gamma\delta^+$ T cells induced by *Mycobacterium bovis* BCG (Cyt) and *M. tuberculosis* (TBe) cytosolic antigens

	Cyt		TBe	
Donors	Total BdU^+ (%)	$\gamma\delta^+$ BdU^+ (%)	Total BdU^+ (%)	$\gamma\delta^+$ BdU^+ (%)
1	12·6	5·9	12·5	6·3
2	45·7	23	43·1	21
3	23·7	6·6	25·2	6
4	23·9	16	23·3	24

$\gamma\delta^+$ and NK cells and a parallel decrease in the proliferation of $CD4^+$ T cells (Fig. 3).

DISCUSSION

The study of the mechanisms by which individual bacterial components interact with the host immune system is becoming of increasing importance to identify candidate molecules to be included in a new subunit vaccine or to be used as reagents for diagnostic tests. Several studies in the mouse model indicate that the immune system recognizes different classes of mycobacterial antigens and that such recognition occurs in a sequential manner [3]. Secreted antigens are important target molecules at early phases during infection, while at later times, due to the host immune response, bacilli are killed and somatic antigens become available for recognition. Thus, vaccines based on antigens present in culture filtrates could be particularly effective in inducing memory cells for an 'immunological surveillance' of early phases of infection, while somatic antigens could be important for a control at later stages of the disease. For such reasons it is becoming evident that an efficient future subcellular vaccine against TB should include both early and late antigens. Only few studies deal with the analysis of the human immune responses induced by the vaccine strain *M. bovis* BCG [22]. Such studies may help to expand our understanding of the type of immunity induced by BCG vaccination and, eventually, of the reasons for its variable efficacy.

To investigate the ability of BCG vaccination to induce immune responses toward different classes of mycobacterial antigens, in the present study proliferation of human T cell-enriched populations to different subcellular fractions of *M. bovis* BCG was analysed. Most of the vaccinated subjects responded vigorously to PPD as well as to other classes of secreted and somatic antigens from *M. bovis* BCG *in vitro*. Although the difference in the extent of proliferation between SD and NSD donors was highly significant for all the classes of antigens, somatic antigens (Cyt, ME, CW) induced a certain degree of proliferation and IFN-γ production also in some of the NSD. Thus, the response to the latter antigen preparations seems to involve both specific and non-specific components.

IFN-γ levels in the supernatants of the cultures stimulated with the different antigen preparations only partially correlated with the proliferation values. In fact, while PPD and CF induced higher release of IFN-γ in SD compared with NSD, preparations of somatic antigens induced, as a mean, similar production of IFN-γ in both groups of subjects. Several reasons may explain such a discrepancy. First, while IFN-γ assay measures the cytokine accumulated in the supernatant during 5 days of culture, proliferation values reflect the percentage of cells which have incorporated BdU during the last 16 h of culture. Second, cell subsets other than $CD4^+$ T lymphocytes (i.e. $\gamma\delta^+$ T cells or NK cells) which proliferate in response to somatic antigens may also contribute, both in SD and NSD, to the total IFN-γ levels detected in the culture supernatants. Finally, possible induction by somatic antigens of Th2-type cytokines in addition to Th1-type ones in SD may offer another explanation.

In addition to cytokine production, human $CD4^+$ T lymphocytes have also been reported to be able to directly lyse macrophages infected with *M. tuberculosis* and are the preferential subset responsible for this function in man [3]. No analysis of cytotoxic effector functions induced by different classes of antigens in BCG-vaccinated donors was performed in the present study. Nevertheless, a PPD-specific cytotoxic activity induced by BCG vaccination has been described by others [22]. Thus, it is possible that beside priming $CD4^+$ T cells with a Th1-like cytokine profile, BCG vaccination may also induce cytotoxic effector functions.

Unlike the conventional ^{3}H-thymidine incorporation assay, the flow cytometric determination of BdU incorporation and the simultaneous identification of the surface phenotype of the proliferating cells has proved to be an extremely powerful means of assessing the relative contribution of particular cell subset(s) to the overall response to a given stimulus within a heterogeneous responder cell population [18,23,24]. Using this approach, in the present study analysis of the cell subsets involved in the response to different classes of BCG antigens demonstrated that, although $CD4^+$ T lymphocytes mainly proliferated to preparations enriched in secreted antigens (PPD, CF), they also contributed to the total proliferation induced by antigens associated to the bacterial body (Cyt, ME, CW). Among somatic antigens, ME turned out to be

Fig. 3. (See previous page) Composition of proliferating subsets in response to different classes of antigens from *Mycobacterium bovis* BCG. After 6 days of *in vitro* stimulation with the different antigen preparations, the percentage of each proliferating subset was calculated and divided by the total percentage of bromodeoxyuridine $(BdU)^+$ cells. BCG^+ SD, BCG-vaccinated sensitized donors; BCG^- SD, BCG non-vaccinated sensitized donors; NSD, non-sensitized donors. Mean values of the different donors tested are illustrated. *n*, Number of subjects tested for each antigen preparation. For the group of NSD *n* represents the number of subjects which displayed a total blastogenic response to the various antigen preparations of more than 5%. CF, Culture filtrates; Cyt, cytosolic; ME, membrane; CW, cell wall antigens from *M. bovis* BCG.

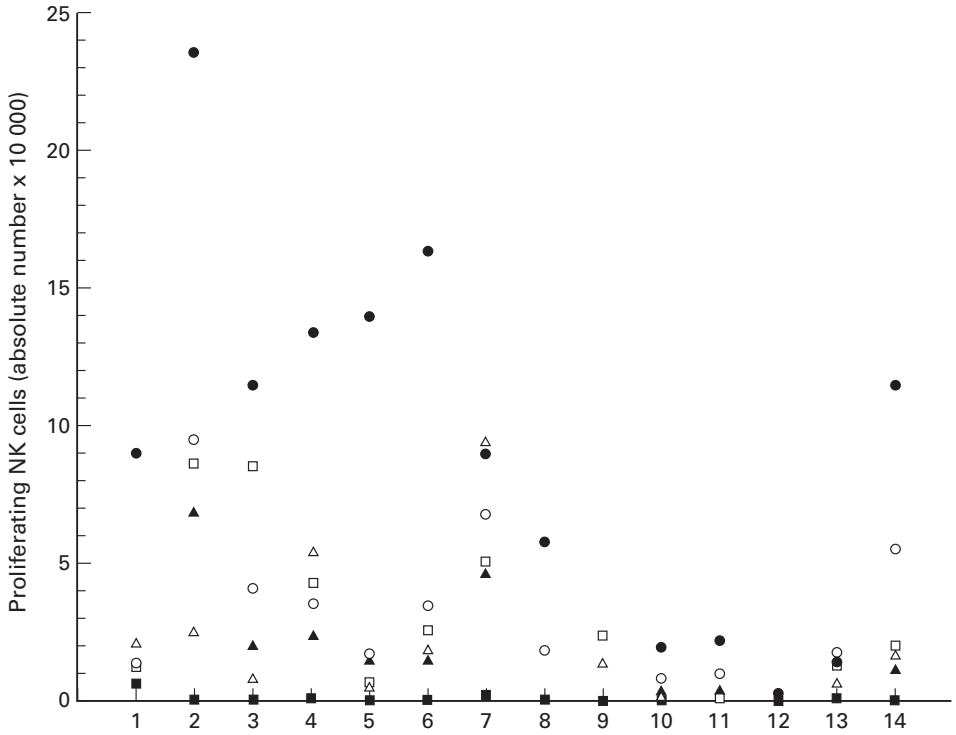

Fig. 4. Absolute number of proliferating natural killer (NK) cells (bromodeoxyuridine $(BdU)^+/CD16^+$ cells) in response to the different antigen preparations after 6 days of *in vitro* culture. Each number represents a different donor. For one donor the absolute number of proliferating cells was not calculated. RPMI, Antigen-free cultures (■); CF, culture filtrates (Δ); Cyt, cytosolic (▲); ME, membrane (○); CW, cell wall (●) antigens from *Mycobacterium bovis* BCG. □, PPD.

particularly effective in triggering proliferation of $CD4^+$ T lymphocytes. The observed high immunogenicity of membrane components confirms the emerging evidence that membrane proteins from a variety of pathogens can be highly immunogenic, indeed of vaccine value [25,26]. Membrane proteins, especially integral ones, isolated from *M. fortuitum* have been previously reported as potent stimulators of human T cell proliferative responses [8,27]. In a recent study analysing the kinetics of recognition of different classes of mycobacterial antigens following vaccination with *M. bovis* BCG, high levels of proliferation of human PBMC were induced by membrane preparations obtained from *M. tuberculosis*, with a peak at day 28 after BCG vaccination [22]. In that report, donors were followed up to 1 year after vaccination. In the present study all the donors had been vaccinated with *M. bovis* BCG at least 3 years before the donation (range 3–6 years), indicating that BCG vaccination is able to induce a quite long $CD4^+$ T cell response to membrane antigens as well as secreted ones. Such results stress the importance of mycobacterial membrane antigens other than secreted proteins as potent stimulators of $CD4^+$ T lymphocytes.

$\gamma\delta^+$ T lymphocytes preferentially proliferated in response to cytosolic antigens of both *M. bovis* and *M. tuberculosis* and, although to a lesser extent, in response to cell wall and membrane preparations. We previously demonstrated that very low levels of proliferation of $\gamma\delta^+$ T lymphocytes were induced by intact bacteria (both live and killed) [18] and by preparations enriched in secreted antigens (PPD, CF). Such findings indicate that antigens responsible for $\gamma\delta^+$ T cell proliferation are mainly associated with the bacterial body, are not secreted and seem to be available to the immune system only when bacteria are destroyed, such as in a sonic extract. If this is true also *in vivo* it can be argued that recognition of cytosolic antigens by $\gamma\delta^+$ T lymphocytes occurs in late phases of mycobacterial infection when disruption of the bacteria has been achieved. In this case $\gamma\delta^+$ T lymphocytes would represent an important immunoregulatory element in the activation and/or proliferation of $CD4^+$ T lymphocytes [24,28,29]. In agreement with the hypothesis of a late recognition of $\gamma\delta^+$ T cell-stimulating antigens, the study of the kinetics of recognition of different classes of mycobacterial antigens following vaccination with *M. bovis* BCG [22] demonstrated that, in contrast to other classes of antigens, the cytosolic preparation gave rise to delayed responses detectable only after 1 year following vaccination.

Not only $CD4^+$ T lymphocyte-stimulating preparations (PPD, CF, ME) but also cytosolic antigens induced low responses in NSD in comparison with vaccinated ones. Such reduction was due to a lower proliferating rate of both $CD4^+$ and $\gamma\delta^+$ T lymphocytes (data not shown), indicating that proliferation of $\gamma\delta^+$ T cells in individuals previously sensitized to mycobacterial antigens might be, at least in part, consistent with a recall response. It is well established that non-peptidic phosphorylated components of mycobacterial origin are the main activators of $\gamma\delta^+$ T cells. In particular, four related phosphorylated molecules, termed TUBag 1–4, have been identified in *M. tuberculosis* as well as in a variety of different mycobacteria, including environmental species [10]. A cell-associated, heat-stable 10–14 kD protein antigen of *M. tuberculosis* has also been suggested as a major stimulus for human $\gamma\delta^+$ T cells [24,30]. Thus, the observed $\gamma\delta^+$ T cell response to Cyt in sensitized donors could be the result of both specific and non-specific response to protein as well as to non-protein superantigen-like acting components present in the cytosolic preparation

from *M. bovis* BCG. Testing of sensitized and non-sensitized donors with purified protein as well as with non-protein γδ-stimulating components will help in the understanding of the physiologic role of $\gamma\delta^+$ T cells and in the decision if mycobacterial components stimulating such cell subsets should be included in a future subunit vaccine against TB.

Although it has been reported that the anti-tuberculous *M. bovis* BCG vaccine is an attenuated mycobacterial producer of the $\gamma\delta^+$ T cell-stimulating non-peptidic antigens [31], in our hands cytosolic preparations from both BCG and *M. tuberculosis* repetitively gave rise to comparable blastogenic responses of $\gamma\delta^+$ T cells when tested in parallel in the same blood donors.

In the present study, a marked proliferation of NK cells in response to preparation enriched in cell wall antigens was also observed. Using the same *in vitro* proliferation assay employed in this study, we have previously reported that human PBMC, depleted of monocytes/macrophages and cultured for 6–7 days with live mycobacteria, respond with a preferential proliferation of NK ($CD16^+$) cells [18]. Altogether these findings may indicate a direct interaction between NK cells and mycobacteria via component(s) of the bacterial cell wall. Such interaction would promote proliferation of NK cells and would occur when bacteria are extracellular, representing one of the first lines of defence against mycobacterial infection.

In conclusion, in the present study a broad spectrum of reactivity toward different classes of mycobacterial antigens in BCG-vaccinated donors was demonstrated. Such reactivity seems to be highly specific toward secreted antigens and to also involve non-specific components in response to somatic antigens. Moreover, such a reactivity appears to be long lasting after vaccination and to trigger a preferential proliferation of distinct cell types to different classes of antigens. It is possible that the outcome of a natural infection as well as the efficacy of a vaccination procedure depend on a balanced activation and regulation of distinct cell types responding to different classes of antigens.

ACKNOWLEDGMENTS

This work was supported by grants from EU BIOMED II Programme, contract no. BMH4-CT97-2671; Karolinska Institute; the Swedish Medical Research Council; the National Tuberculosis Project grant no. 96/D/T18 (Istituto Superiore di Sanità, Ministero della Sanità), Rome; Progetti M.U.R.S.T. (ex 40%) prot. 9706247700-002 and prot. 9806297296-003, Rome.

REFERENCES

1 Roche PW, Triccas JA, Winter N. BCG vaccination against tuberculosis: past disappointments and future hopes. Trends Microbiol 1995; **3**:397–401.

2 Kaufmann SHE, Andersen P. Immunity to mycobacteria with emphasis on tuberculosis: implications for rational design of an effective tuberculosis vaccine. Chem Immunol 1998; **70**:21–59.

3 Andersen P. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. Scand J Immunol 1997; **45**:115–31.

4 Orme IM. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccine. Infect Immun 1988; **56**:3310–2.

5 Horwitz MA, Lee B-WE, Dillon BJ, Harth G. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 1995; **92**:1530–4.

6 Boesen H, Jensen BN, Wilcke T, Andersen P. Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. Infect Immun 1995; **63**:1491–7.

7 Daugelat S, Gulle H, Schoel B, Kaufmann SHE. Secreted antigens of *Mycobacterium tuberculosis:* characterization with T lymphocytes from patients and contacts after two-dimensional separation. J Infect Dis 1992; **166**:186–90.

8 Mehrotra J, Bisht D, Tiwari VD, Sinha S. Serological distinction of integral plasma membrane proteins as a class of mycobacterial antigens and their relevance for human T cell activation. Clin Exp Immunol 1995; **102**:626–34.

9 Silva CL, Lowrie DB. A single mycobacterial protein (hsp 65) expressed by a transgenic antigen-presenting cell vaccinated mice against tuberculosis. Immunology 1994; **82**:244–8.

10 Constant P, Davodeau F, Peyrat M-A, Poquet Y, Puzo G, Bonneville M, Fournié J-J. Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands. Science 1994; **264**:267–70.

11 Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human γδ T cells. Nature 1995; **375**:155–8.

12 Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MD. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. Nature 1994; **372**:691–4.

13 Sieling PA, Chatterjee D, Porcelli SA *et al.* CD1-restricted T cell recognition of microbial lipoglycan antigens. Science 1995; **269**:227–30.

14 Ladel CH, Blum C, Dreher A, Reifenberg K, Kaufmann SHE. Protective role of γδ T cells and αβ T cells in tuberculosis. Eur J Immunol 1995; **25**:2877–81.

15 Ladel CH, Hess J, Daugelat S, Mombaerts P, Tonegawa S, Kaufmann SHE. Contribution of αβ and γδ T lymphocytes to immunity against *Mycobacterium bovis* bacillus Calmette–Guérin studies with T cell receptor-deficient mutant mice. Eur J Immunol 1995; **25**:838–46.

16 Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA 1992; **89**:12013–7.

17 Ladel CH, Daugelat S, Kaufmann SHE. Immune response to *Mycobacterium bovis* bacille Calmette Guérin infection in major histocompatibility complex class I- and II-deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance. Eur J Immunol 1995; **25**:377–84.

18 Esin S, Batoni G, Källenius G, Gaines H, Campa M, Svenson SB, Andersson R, Wigzell H. Proliferation of distinct human T cell subsets in response to live, killed or soluble extracts of *Mycobacterium tuberculosis* and *Mycobacterium avium*. Clin Exp Immunol 1996; **104**:419–25.

19 Florio W, Freer G, Dalla Casa B, Batoni G, Maisetta G, Senesi S, Campa M. Comparative analysis of subcellular distribution of protein antigens in *Mycobacterium bovis* bacillus Calmette–Guérin. Can J Microbiol 1997; **43**:744–50.

20 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; **193**:265–75.

21 Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; **87**:206–10.

22 Ravn P, Boesen H, Pedersen BK, Andersen P. Human T cell responses induced by vaccination with *Mycobacterium bovis* bacillus Calmette–Guérin. J Immunol 1997; **158**:1949–55.

23 Ottenhoff THM. Putting numbers on mycobacterium activated T cell subsets. Clin Exp Immunol 1996; **104**:381–3.

24 Batoni G, Esin S, Harris RA, Källenius G, Svenson SB, Andersson R, Campa M, Wigzell H. $\gamma\delta^+$ and $CD4^+$ human T cell subset responses upon stimulation with various *Mycobacterium tuberculosis* soluble extracts. Clin Exp Immunol 1998; **112**:52–62.

25 Smythe JA, Coppel RL, Brown GV, Ramasamy R, Kemp DJ, Anders RF. Identification of novel integral membrane proteins of *P. falciparum*. Proc Natl Acad Sci USA 1998; **85**:5195–9.

26 Murray PG, Spithill TW, Handman E. Characterization of integral membrane proteins of *Leishmania major* by Triton X-114 fractionation and analysis of vaccination effects in mice. Infect Immun 1989; **57**:2203–9.

27 Mehrotra J, Mittal A, Dhindsa MS, Sinha S. Fractionation of mycobacterial integral membrane proteins by continuous elution SDS-PAGE reveals the immunodominance of low molecular weight subunits for human T cells. Clin Exp Immunol 1997; **109**:446–50.

28 Kaufmann SH, Blum C, Yamamoto S. Crosstalk between $\alpha\beta$ T cells and $\gamma\delta$ T cells *in vivo*: activation of $\alpha\beta$ T-cell responses after $\gamma\delta$ T-cell modulation with the monoclonal antibody GL3. Proc Natl Acad Sci USA 1993; **90**:9620–4.

29 Mombaerts P, Arnoldi J, Russ F, Tonegawa S, Kaufmann SH. Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen. Nature 1993; **365**:536.

30 Boom WH, Balaji KN, Nayak R, Tsukaguchi K, Chervenak KA. Characterization of a 10- to 14-kilodalton protease-sensitive *Mycobacterium tuberculosis* H37Ra antigen that stimulates human $\gamma\delta$ T cells. Infect Immun 1994; **62**:5511–8.

31 Constant P, Poquet Y, Peyrat MA, Davodeau F, Bonneville M, Fournié JJ. The antituberculous *Mycobacterium bovis* BCG vaccine is an attenuated mycobacterial producer of phosphorylated nonpeptidic antigens for human $\gamma\delta^+$ T cells. Infect Immun 1995; **63**:4628–33.