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Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette–Guérin

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SUMMARY

Causes of individual variation in susceptibility to mycobacterial diseases are only partly understood. An efficient cell-mediated immune response is crucial for resistance. Macrophages and T cells interact to eliminate the mycobacteria, partially through the effects of secreted cytokines. A vigorous anti-bacterial inflammatory response is sometimes accompanied by severe tissue damage, while immunosuppression leads to progressive infection. Here, live, attenuated *Mycobacterium bovis*, bacillus Calmette–Guérin (BCG), was used as a model antigen to study cytokine production at the single-cell level in response to mycobacteria. Peripheral blood mononuclear cells from healthy individuals were challenged *in vitro* and the kinetics and frequencies of cytokine-producing cells were studied by immunofluorescent visualization of intracellular cytokines. Fourteen cytokines were assayed; interleukin-1α (IL-1α), IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), TNF-β and granulocyte–macrophage colony-stimulating factor (GM-CSF). A sequential production of T helper-1 (Th1) and T helper-2 (Th2) cytokines was induced by BCG. Early, at days 1–2 after stimulation, the response was dominated by monokines and a low IFN-γ and TNF-β production. At days 4–5 there was a marked production of Th1 lymphokines, with approximately 6% IFN-γ^+ cells, 4% TNF-β^+ cells and 2% IL-2^+ cells. Late in the reaction, at days 10–12, a Th2 response with IL-4, IL-5 and IL-10 was detected, while the synthesis of Th1 lymphokines and monokines declined. Overall, our results provide further evidence of IFN-γ as the major cytokine induced by mycobacteria in healthy individuals, but also suggest that Th2 cytokines participate in the response.

INTRODUCTION

Individual susceptibility to *Mycobacterium tuberculosis* varies greatly, in part due to genetic factors that determine the clinical outcome of the disease (reviewed in ref. 1). Experimental data show that cell-mediated immunity is essential for resistance.[2,3] Some of the immunoregulatory effects of macrophages and T cells might be mediated through cytokine secretion. In mice, two types of $CD4^+$ T-cell clones have been described on the basis of cytokine gene expression.[4,5] The T helper-1 (Th1) cells secrete interleukin-2 (IL-2) interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), and the Th2 cells secrete IL-4, IL-5 and IL-10. The Th1 and Th2 cytokines often have opposite effects on macrophage activation, immune reactions to parasitic diseases and in allergic responses.[6–8] Human T-cell clones responding to purified protein derivative (PPD) from *M. tuberculosis* preferentially produced the Th1 cytokines IL-2 and IFN-γ.[9,10] In murine tuberculosis it is known that IFN-γ and TNF are required for activation of macrophages to produce reactive nitrogen intermediates and to kill intracellular bacteria.[11–14] Challenge with PPD *in vivo* induced mainly mRNA for IFN-γ and IL-2 in human skin, but also some IL-3, IL-4, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF).[15] However, no attempt to verify the cytokine production at the protein level was made.

The attenuated strain of *M. bovis*, bacillus Calmette–Guérin (BCG), is used as a vaccine against tuberculosis (reviewed in ref. 16) and also as a therapeutic agent against neoplastic disease, such as superficial cancer of the urinary bladder.[17] However, like infectious tuberculosis, there is marked interindividual variation in the response to BCG in vaccination[16] and in cancer therapy.[18] The variables that induce resistance or susceptibility to mycobacteria are only partly understood, although it seems to be beyond doubt that an efficient interaction between mononuclear phagocytes and T cells is crucial (reviewed in ref. 19). There is increasing evidence that these two cell types are bidirectionally regulating each

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other during the mycobacterial response via cytokines. As many cytokines are able to potentiate or antagonize each other it is of importance to determine a complete cytokine profile in order to understand the net result of the response. Our study was undertaken to investigate the production of a large repertoire of lymphokines and monokines in response to mycobacteria, many of which might have agonistic or antagonistic effects *in vivo*. To address this question we determined the BCG-induced intracellular production of 14 cytokines in peripheral blood mononuclear cells (PBMC) from healthy donors. The evaluation of the anti-mycobacterial reaction from these individuals provides insights into important components of the response to tuberculosis.

MATERIALS AND METHODS

Cell culture and cytokine induction

PBMC were prepared from buffy coats of healthy adult blood donors ($n = 7$) by lymphoprep centrifugation (Nycomed AS, Oslo, Norway). The cells were cultured at $1{\cdot}5 \times 10^6$/ml at 37° in humified air atmosphere containing 5% CO_2, for the indicated periods of time in RPMI-1640 medium (Flow Laboratories, Irvine, UK), supplemented with 5% polymyxin B-treated fetal calf serum (FCS; Flow Laboratories) and 2 mM L-glutamine, in 25-cm^2 cell culture flasks (Costar, Cambridge, MA).

The cells were stimulated to produce cytokines with live BCG, strain TICE (OncoTICE; Organon Teknika, Boxtel, the Netherlands), diluted in NaCl to pharmacological concentrations [25 mg/ml, approximately 10^7 colony-forming units (CFU)/ml] according to the recommendations of the manufacturer. As a negative control unstimulated cells were cultured in media alone for the indicated time periods. To minimize the risk of endotoxin contamination we used polymyxin B-treated FCS and filtered all reagents.

Cytokine-specific antibodies

The following cytokine-detecting monoclonal antibodies (mAb) were used for indirect immunofluorescence staining: IL-α (1277-89-7, 1277-82-29, 1279-143-4, all mouse IgG1), IL-1β (2-D-8, mouse IgG1), IL-1 receptor antagonist (IL-1rα) (mouse IgG1) (all from H. Towbin, Ciba-Geigy, Basel, Switzerland),[20] IL-2 (17.H.12), IL-3 (3G11), IL-4 (25.D.2), IL-5 (39.D.10), IL-6 (6.A.3) (all rat IgG mAb from J. Abrams, DNAX, Palo Alto, CA),[21] IL-8 (NAP-1, mouse IgG1 mAb from M. Ceska, Sandoz, Vienna, Austria),[22] IL-10 (19.F.1. + 12.G.8.) (rat IgG mAb from J. Abrams),[21] TNF-α (20.A.4), GM-CSF (21.C.11) (all rat IgG mAb from J. Abrams),[21] TNF-β (LTX-1, mouse IgG2b mAb from G. Adolf, Boehringer-Ingelheim, Vienna, Austria,[23] and IFN-γ (DIK-1, mouse IgG1 mAb from G. Andersson, KABI, Stockholm, Sweden).[24] The cytokine-specific mAb were used at a final concentration of 1–5 μg/ml.

Immunofluorescent staining of cytokines

Our staining technique has been described previously[25] and is summarized here. Cultured PBMC were harvested after the indicated time periods and washed in Hank's balanced salt solution (HBSS; Gibco, Paisley, UK) supplemented with 0·01 M HEPES buffer. Cells were washed protein-free by repeated centrifugation and were transferred to adhesion slides (Bio-Rad, Munich, Germany) and allowed to adhere electrostatically to the slides for 10 min at room temperature. Excess cells were washed away, and the unbound surface area on the adhesive fields was blocked by incubation with 2% FCS in HBSS for 10 min at 37°.

The cells were fixed on each field with phosphate-buffered 4% paraformaldehyde at pH7·4 for 5 min. After subsequent washes with HBSS, the cells were permeabilized with HBSS supplemented with 0·1% saponin (Riedel-de Haen Ag, Seelze, Germany) (HBSS–saponin) to allow the intracellular entrance of the cytokine-specific mAb. Ten microlitres of the mAb diluted to a final concentration of 1–5 μg/ml was added and left to incubate for 20–30 min at 37°. The cells were then washed in HBSS–saponin. Ten microlitres of fluorescein isothiocyanate (FITC)–conjugated second-step antibody was added for 30 min of exposure in darkness at room temperature. Dilution of all antibodies was performed in HBSS–saponin with the addition of 10% human AB serum. The FITC-labelled anti-mouse IgG1 or anti-mouse IgG2b (Caltag Lab., South San Fransisco, CA) were used at a final concentration of 1 : 300 and FITC-labelled anti-rat immunoglobulin (Vector Laboratories, Burlingame, CA) at 1 : 100. After several washes with HBSS–saponin, the final washes were performed with HBSS exclusively, which prevented leakage of stained cytokine from the cells. The cells were left to dry on the slides. Buffered glycerol containing 2% diazobicyclo-octane (Sigma, St Louis, MO) was used as the mounting medium to reduce ultraviolet (UV) quenching.

Fluorescence microscopy and evaluation of results

The results are presented as the percentage of total cells counted that were positively stained. The frequency of cytokine-producing cells was determined by UV microscopic evaluation of 2000 cells. Slides were examined by a Polyvar fluorescent microscopy (Reichert-Jung, Vienna, Austria) equipped with a 200 W mercury lamp.

RESULTS

Cytokine-staining morphology in individual cells

The cytokine production induced by BCG was determined at

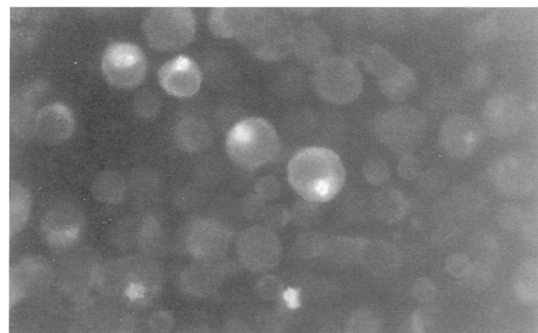

Figure 1. Intracellular IFN-γ in BCG-stimulated cells. Cells were cultured with live BCG as described in the Materials and Methods. Four days later the cells were harvested, washed, fixed with 4% paraformaldehyde and stained with the mAb DIK-1 anti-human IFN-γ in the presence of saponin, followed by indirect immunoflourescence. Note that the IFN-γ^+ cells exhibit maximal fluorescence in the cytoplasm close to the nucleus, reflecting the accumulation in the Golgi apparatus.

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the single-cell level with PBMC obtained from seven healthy blood donors. Detection of intracellular cytokines in producing cells was facilitated by a specific juxtanuclear staining pattern, which has been shown to represent cytokine accumulation in the Golgi stacks (Fig. 1).[26] However, cells producing IL-1α and IL-1β showed an intense, generalized or granular cytoplasmic staining,[20,27] which is explained by the fact that these cytokines do not enter the endoplasmic reticulum–Golgi route.[28,29] Cells that do not produce, but have been exposed to exogenously derived cytokines, do not show any of these specific staining patterns.[26]

BCG induced prolonged cytokine production

Previous results have shown that cytokine production induced by mitogens or bacterial toxins *in vitro* has a rapid onset and declines before 96 hr of culture (reviewed in ref. 20). The BCG response of four donors was therefore assayed every 24 hr until day 4. Unexpectedly, high frequencies of cytokine-producing cells were still detected at 96 hr, indicating that BCG induced a prolonged cytokine response. Cytokine synthesis in PBMC from three additional donors was therefore examined daily from day 1 until the response declined at days 10–12 (figs 2 and 3 and Table 1).

Spontaneous IL-1ra production was down-regulated by BCG

The PBMC were cultured in RPMI-1640 supplemented with 5% heat-inactivated, polymyxin B-treated FCS with or without 10^7 CFU/ml of live BCG, strain TICE. No cytokine-producing cells were detected in freshly isolated PBMC. In cells cultured with medium alone, a substantial production of the monokines IL-1α, IL-1β, IL-8 and, in particular, IL-1ra occurred (Fig. 2). Spontaneous production of IL-1, IL-8 and IL-1ra has previously

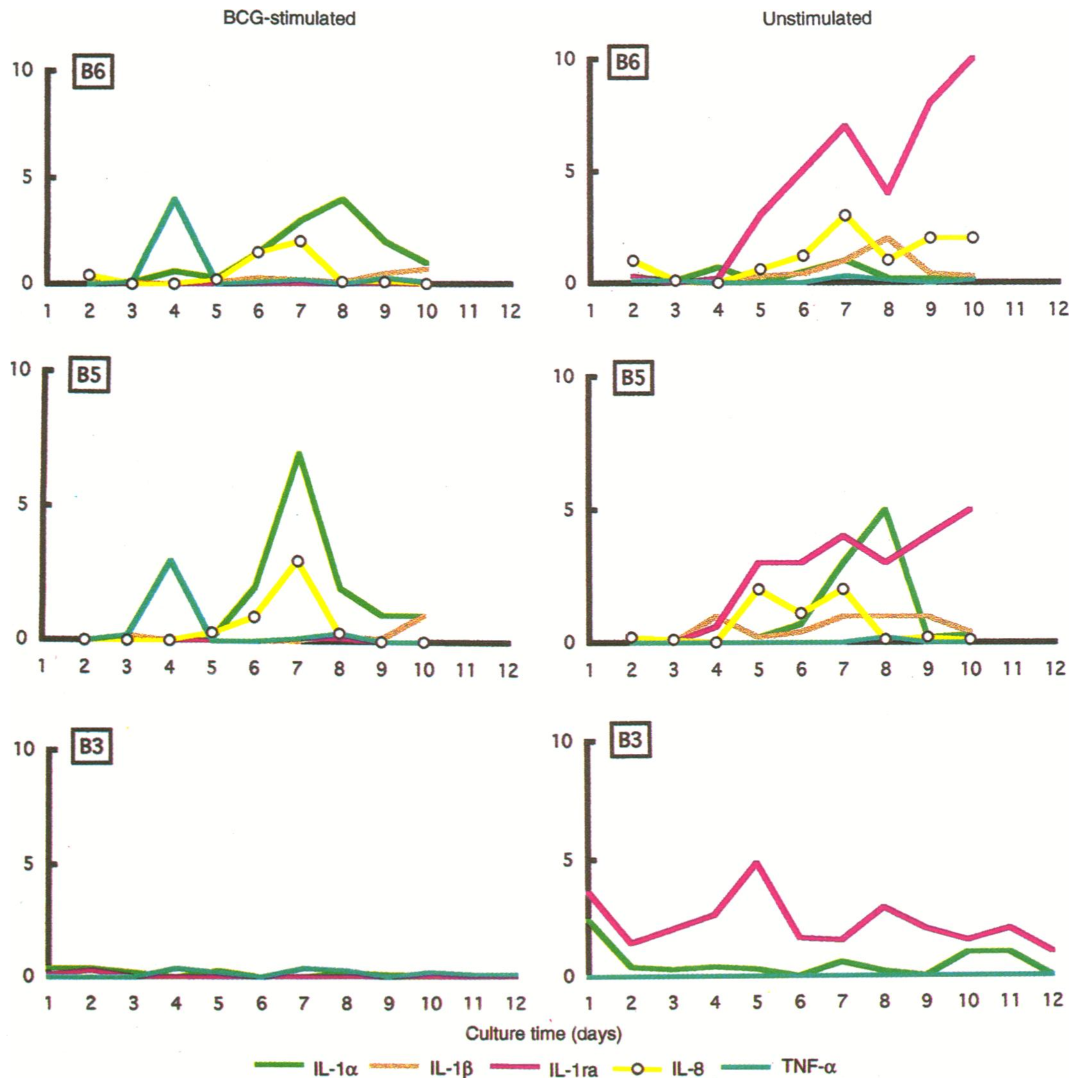

Figure 2. Kinetic study of monokine production in BCG-stimulated and unstimulated cells from three different donors. Cells were cultured in flasks in the presence of live BCG or medium alone for 10–12 days. Each day the cells in one flask were harvested, washed and stained for intracellular cytokines as described in the Materials and Methods. Frequencies of positive cells (%) is given on the *y*-axis, culture time (days) on the *x*-axis. Note that the production of IL-1ra is higher in cells cultured in medium alone.

been reported by us and others.[20,30,31] The spontaneous IL-1ra production could then be prevented by eliminating contaminating lipopolysaccharide (LPS) and plastic adherence of the cells. It is therefore possible that some of the monokine production in the cells cultured in medium alone was induced by plastic adherence. However, as blood monocytes are extremely sensitive to LPS and start producing monokines in the presence of very low endotoxin levels (10–100 pg/ml) we cannot exclude contaminating low levels of LPS as the cause of the observed monokine production in our control cultures.

BCG-stimulated cells produced both monokines and lymphokines of the Th1 and Th2 type (figs 2 and 3). In two of the three donors that were followed beyond 96 hr, IL-1α reached a peak at days 7–8 and then gradually declined (Fig. 2), while the third donor (B3) had a smaller response. Less cells produced IL-1β and even fewer IL-1ra. Not only was IL-1ra synthesized by few cells, the production was also transient and occurred mainly during the first 2–3 days.

Sequential production of Th1 and Th2 lymphokines

The dominating lymphokine was IFN-γ, which was detected from day 2 until day 10 of BCG stimulation. The response was biphasic, with one peak at days 4–5 and one even higher peak at day 8 of culture (Fig. 3). TNF-β and IL-2 production was also biphasic (Fig. 3).

Th2 lymphokine production was small but after the first week of BCG stimulation two or more Th2 lymphokines were detected in PBMC from all three donors.

IL-3 and GM-CSF could be produced by both Th1 and Th2 clones and GM-CSF also by activated macrophages. In the BCG response low frequencies of IL-3-producing cells were detected in two of the seven donors. In contrast, GM-CSF production was evident throughout days 1–10 of BCG stimulation (Fig. 3).

The number of cytokine-producing cells varied from different donors (Table 1). Interestingly, however, the kinetics of the response and the sequential order by which the cytokines appeared in the cultures were highly reproducible. In Table 1 the frequencies of cytokine-producing cells at different time-points in the BCG-stimulated cell cultures are presented. Early, at days 1–2 after stimulation, the response was dominated by monokines but some cells produced IFN-γ and TNF-β. At days 4–5 there was a marked Th1 response. TNF-α production was also high at this time-point. Late in the response, at days 7–8, there were even higher frequencies of Th1-type cytokines, dominated by IFN-γ in two out of three donors while in one donor TNF-β- and IL-2-producing cells were more numerous. However, also in this donor, cells producing Th1 cytokines were more frequent than monokine or Th2 producers.

At the end of the response, days 10–12, the Th1 production had declined and the cells producing the Th2 cytokines IL-4, IL-5 and IL-10 were in the same order of magnitude as the Th1 and monokine producers.

DISCUSSION

Live BCG induced rapid and prolonged production of lymphokines and monokines in PBMC from healthy subjects.

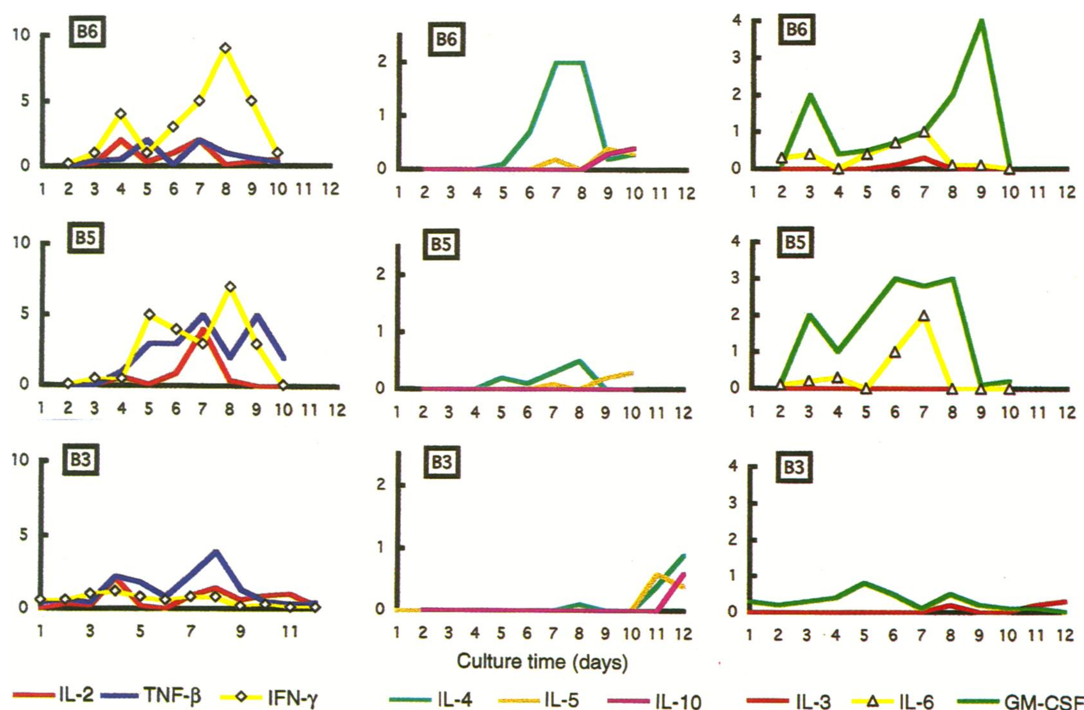

Figure 3. Frequencies of cells producing IL-2, TNF-β or IFN-γ (Th1 cytokines), IL-4, IL-5 or IL-10 (Th2 cytokines) or IL-3, IL-6 or GM-CSF at various time-points after BCG stimulation. Cells from three different donors were cultured in flasks with live BCG and harvested at different time-points after stimulation. Cytokine-staining was performed as described in the Materials and Methods. Frequencies of positive cells (%) is given on the y-axis, culture time (days) on the x-axis.

Table 1. Frequencies of cytokine-producing cells in response to BCG

Cytokine	% at culture time (days)* 1–2	4–5	7–8	10–12
IL-1α	0·4 (0·3)	0·4 (0·4)	**3·7** (3·4)†	0·7 (0·5)
IL-1β	0·7 (0·5)	< 0·1	0·2 (0)	**0·8** (0·2)
Il-1ra	0·5 (0·5)	< 0·1	0	0
IL-6	0·6 (0·6)	0·2 (0·1)	**1·5** (0·7)‡	0‡
IL-8	1·6 (1·5)	0·7 (0·7)	**2·5** (0·7)‡	0
TNF-α	0·4 (0·3)	**2·5** (1·8)	0·3 (0·1)	0·1 (0·1)
IL-2	0·1 (0·1)	**1·9** (1·0)	**2·5** (1·4)	0·5 (0·5)
IFN-γ	0·8 (0·7)	**2·4** (1·8)	**5·6** (3·4)	0·5 (0·4)
TNF-β	0·9 (1·1)	**3·1** (2·9)	**3·6** (1·5)	0·9 (0·9)
IL-4	0	0·1 (0·1)	**0·8** (0·8)	0·4 (0·4)
IL-5	0	0	0·2 (0·1)	**0·4** (0·1)
IL-10	0	0	0	**0·3** (0·2)
IL-3	0	0	0·2 (0·1)	0·1 (0·1)
GM-CSF	1·4 (1·0)§	1·1 (0·8)	1·8 (1·2)	0·1 (0·1)

*Determination of numbers of cytokine-producing cells was performed by indirect immunofluorescence technique. At least 2000 cells were counted for each cytokine. values are mean and SD of three to seven different donors.

†Bold figures represent peak frequencies.

‡Data represent mean and SD of two donors.

§Days 2–3.

As BCG vaccination was until recently incorporated into the routine childhood immunization programme in Sweden, the *in vitro*-detected immune reactions most probably represented recall responses to BCG. However, the buffy coats were obtained from the local blood bank and the immune status of the individual donors was therefore unknown. For many cytokines the frequencies of producer cells in BCG-stimulated cultures were approximately 10-fold higher than has been reported for recall responses to a conventional antigen, such as tetanus toxoid,[32] but lower than the cytokine response to streptococcal and staphylococcal superantigens.[20,33] As live BCG has the capacity to produce a vast array of microbial proteins that potentially could be presented by monocytic major histocompatibility complex (MHC) class II or class I molecules, polyclonal activation of T cells might be expected.

While the magnitude of cytokine response differed between cells isolated from different individuals, the balance between different cytokines seemed to remain intact; that is, low lymphokine responders also had a low monokine response and vice versa. Similar results have been observed in other studies of microbial cytokine responses[32] and could indicate that a tight regulation of cytokine production occurs not only *in vivo* but also in an *in vitro* immune reaction.

The response was dominated by the Th1 cytokines IFN-γ, TNF-β and IL-2. IL-2 is a growth factor for T cells[34] and could be responsible for expansion of antigen-specific clones. IL-2 may also enhance production of other cytokines such as IFN-γ and IL-4. In several donors the frequencies of IFN-γ-producing cells was as high or even higher than cells synthesizing TNF-β. In contrast, during most superantigen responses and in recall reactions to tetanus toxoid, TNF-β was the most abundant cytokine detected.[20,32,33] It is likely that this early and stable BCG-induced IFN-γ production strongly favoured the activation and development of Th1 cells and suppressed the Th2 response early in the immune reaction. Indeed, in mice, IFN-γ has been shown to play a central role in the resistance to *M. tuberculosis*/*M. bovis*.[12–14,35] TNF-β production appears to be restricted to lymphocytes, while TNF-α production is a property of both T lymphocytes and monocytes.[36,37] TNF has cytolytic capacity and induces cachexia (reviewed in ref. 38). This suggests that TNF could be important in the killing of infected monocytes which fail to eliminate intracellular bacteria, and that high levels of TNF in chronic TBC may be responsible for the tuberculosis cachexia. *In vivo* TNF has been detected in *M. bovis*-induced granulomas in mice, and treatment with anti-TNF antibodies interfered not only with granuloma development but also with persistence of already formed granulomas.[39]

In all donors the IL-1ra response was markedly diminished by BCG and instead high frequencies of IL-1α$^+$ and IL-1β$^+$ cells were induced, most probably resulting in high levels of bioavailable IL-1. IL-1 and other monokines produced in response to BCG are likely to play a role in the inflammatory response to tuberculosis. IL-1 and IL-6 mediate the acute-phase response and may contribute to the fever, anaemia and elevation of hepatic acute-phase proteins characteristic of tuberculosis.[40,41] IL-8 stimulates chemotaxis in neutrophils and lymphocytes and may thus enhance the local inflammatory response.[42] GM-CSF and IL-6 have been reported to induce anti-mycobacterial activities in murine monocytes.[19]

The production of Th2 cytokines late in the response to BCG might reflect a dynamic *in vivo* balance, preventing an overexpression of inflammatory, potentially tissue-damaging Th1 cytokines. In mice aquired immunity tuberculosis infection has been suggested to stimulate a CD4$^+$ IFN-γ-secreting T-cell population early in the response, and CD4$^+$ IL-4-producing cells 20–40 days later.[43] However, recent results suggest that induction of a mixed response with IFN-γ, IL-4 and IL-10 in response to mycobacteria makes the site of immune reaction highly sensitive to TNF-α-mediated necrosis.[3] Also, in pneumonia caused by virus a mixed Th1 and Th2 response has been reported to cause severe tissue damage.[44] Induction of Th2 cytokines may therefore not only stimulate a humoral immune reaction but also potentiate the cellular, inflammatory response to mycobacteria. Even though Th2 cytokines were produced by few cells in comparison to Th1 cytokines in the present study, the fact that they were reproducibly induced requires interest. In skin reactions to PPD, a mixed pattern of cytokines in response to mycobacterial proteins has also been reported to occur.[15] It is likely that an efficient immunological response to mycobacteria requires a complex balance between inflammatory and inhibitory cytokines in order to avoid harmful tissue destruction while still eliminating the infection. This might be difficult to accomplish in susceptible individuals with a dysregulated cytokine production. Analyses of mycobacterial-induced cytokine profiles and effects of immunomodulators in such individuals would help to clarify some of the pathogenic mechanisms in mycobacterial disease.

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