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Original article

Non-tuberculous mycobacteria and their surface lipids efficiently induced IL-17 production in human T cells

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Abstract

Interleukin-17 (IL-17) is produced by a subset of $CD4^+$ T helper (Th) lymphocytes known as Th17 cells. In humans, IL-1β, enhanced by IL-6 and IL-23 is crucial for differentiation of these cells. IL-17 evokes inflammation and is involved in host defence against microorganisms, although little is known about its role in diseases caused by non-tuberculous mycobacteria. The genus *Mycobacterium* contains both obligate and opportunistic pathogens as well as saprophytes, and the mycobacterial cell envelope is unique in its abundance of lipids. Here we investigated IL-17 and IL-23 production in human PBMC in response to intact UV-inactivated mycobacteria and mycobacterial surface lipids from two opportunistic (*Mycobacterium avium* and *Mycobacterium abscessus*) and one generally non-pathogenic (*Mycobacterium gordonae*) species. Representative Gram-positive (*Enterococcus faecalis*, *Streptococcus mitis*) and Gram-negative (*Escherichia coli*) bacteria were included as controls. Intact mycobacteria induced production of large amounts of IL-17, while IL-17 responses to control bacteria were negligible. Purified $CD4^+$ T cells, but not CD4-depleted cell fractions, produced this IL-17. Isolated mycobacterial surface lipids induced IL-17, but not IL-23 production. The ability of the non-tuberculous mycobacteria to induce IL-17 production in $CD4^+$ T cells was the same regardless of the pathogenic potential of the particular mycobacterial species.

Keywords: Mycobacteria; Lipids; Cytokine; IL-17; $CD4^+$ T cells

1. Introduction

Interleukin-17 (IL-17) is produced by a subset of $CD4^+$ T helper (Th) lymphocytes known as Th17 cells [1–3]. Activation of Th17 cells induces production of antimicrobial β-defensins in keratinocytes and recruitment of neutrophils [4,5]. In addition to its pro-inflammatory role in autoimmune diseases, IL-17 producing T cells can function with Th1 cells to mediate protective immunity to pathogenic organisms and IL-17 is found to be of crucial importance combating fungal infections, whereas its role in defence against other pathogens is less clear [5,6]. Although focus has been on IL-17-secreting $CD4^+$ T cells, other cells such as $CD8^+$ T cells, γδ T cells, and NKT cells are all capable of secreting IL-17 [4,5,7]. Furthermore, IL-1α or IL-1β in synergy with IL-23 can promote IL-17 secretion from memory T cells [5].

Both in human and mouse T cells the combination of IL-6 with IL-1β and IL-23 is capable of inducing Th17 differentiation [8]. The importance of growth factor β (TGF-β), in human Th17 differentiation remains elusive, and recent studies have shown that TGF-β might not be an obligate specification factor for Th17 differentiation but a modulator in Th17 development (reviewed in [9]). IL-6 and IL-1β are mainly produced by monocytes/macrophages in response to microbial danger signals and tissue damage [10,11].

IL-23 is produced by antigen-presenting cells in response to microorganisms (reviewed in Ref. [12]), but the microbial structures that induce production of this cytokine have not

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been defined [12]. Although IL-23 seems to play an important role in maintaining effector function and survival of Th17 cells, it is not required for the differentiation of naïve $CD4^+$ Th cells into mature Th17 cells [13].

The genus *Mycobacterium* contains the human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*, as well as the so-called "non-tuberculous" mycobacteria (NTM), which are ubiquitous in nature [14,15]. Some of these NTMs are considered non-pathogenic, for example *Mycobacterium gordonae*, whose presence in clinical specimens is generally considered as contamination from environmental sources [14]. Others are opportunistic pathogens in selected patient groups (reviewed in Ref. [16]), such as *Mycobacterium avium*, which can cause cervical lymphadenitis in children, traumatic soft tissue infections, bacteraemia in AIDS patients as well as chronic pulmonary disease in certain slender middle-aged women without known risk factors. *M. avium* can also cause chronic pulmonary disease in patients with cystic fibrosis; a genetic disorder affecting airway bacterial clearance (due to mutations in the cystic fibrosis transmembrane conductance regulator protein i.e. CFTR) as well as CFTR heterozygotes [16–18] *Mycobacterium abscessus* is another opportunistic pathogen which generally does not cause disease in immuno-competent individuals, and its isolation from clinical samples may reflect transient colonization after natural environmental exposure [15]. In selected patient groups, however, *M. abscessus* is able to cause wound infections, mastoiditis, and pulmonary infections, and has more recently emerged as a major pathogen in patients with cystic fibrosis [14,16,19]. At present, 6% of the cystic fibrosis patients in South West Sweden are colonized with this species, which in many cases have resulted in deteriorating lung-function with future risk of fatal outcome [20,21]. As *M. abscessus* is inherently resistant to most antibiotics, these infections are nearly impossible to eradicate [16,21,22].

Mycobacteria have a unique cell envelope containing an abundance of waxes, with branched long-chained lipids (mycolic acids), lipoarabinomannans and a great variety of other complex lipids more or less loosely associated with the mycobacterial cell envelope [23,24].

Little is known on the capacity of NTMs to induce IL-17 production, especially concerning *M. abscessus*, which is our main focus. We have however recently shown that *M. avium* strains isolated from lymphadenitis in children induced significantly less IL-17 but significantly more IL-10 production by human PBMCs than did isolates from birds and other animals, suggesting that genetic differences in these bacteria could play an important role in human pathogenicity [25]. Other researchers have shown that patients with non-tuberculous mycobacterial lung disease had higher levels of IL-10 and IFN-γ, but reduced levels of IL-17 compared to adult offspring, indicating a reduced Th17 immunity and a predisposition towards IL-10 production [26]. IL-17 production has been observed in healthy tuberculin positive individuals and in patients with active pulmonary tuberculosis [27]. A recent study has shown that regulatory T cells inhibit protective Th1 responses but not pro-inflammatory Th17 responses in humans during active tuberculosis, a trait which was thought to facilitate mycobacterial replication and cause tissue damage [28–30]. In another study, >20% of specific cytokine-producing CD4+ T cells of healthy, mycobacteria-exposed adults expressed IL-17 or IL-22, whereas these cells were reduced in patients with active tuberculosis [31].

We have recently investigated cytokine production by human peripheral blood mononuclear cells (PBMC) in response to the opportunistic mycobacteria *M. avium* and *M. abscessus,* the non-pathogenic *M. gordonae* including extracted surface lipids from the same species. Our results showed that non-tuberculous mycobacteria regardless of pathogenic potential to some extent evade triggering production of phagocyte activating cytokines (IL-12, TNF and IFN-γ) and that the mycobacterial lipids extracted from the cell envelope significantly inhibited the expected Th1 responses to another microorganism (in this case the Gram-positive bacterium *Enterococcus faecalis*) [32].

The aim of the present study was to investigate if intact non-tuberculous mycobacteria *(M. avium*, *M. abscessus*, *M. gordonae*) could induce production of IL-17 by freshly isolated human peripheral blood mononuclear cells (PBMC), and if the mycobacterial species used differed concerning this capacity. Furthermore we analyzed whether the same mycobacteria could induce IL-1β, IL-6 and/or IL-23 production. The responses were compared to those induced by representative species of Gram-positive and Gram-negative bacteria, as it has previously been found that Gram-positive bacteria induces a Th1 (IL-12/IFN-γ − pathway) response, while Gram-negatives induces a Th2 ditto [33]. Furthermore, we have recently shown that non-tuberculous mycobacteria induced a cytokine response in human PBMCs, *in vitro*, differing from both "normal" Th1 and Th2 responses. Mycobacterial surface lipids extracted from the cell envelope of the same species were also investigated concerning their capacity to evoke production of IL-17 and IL-23, as we have previously shown that extract of these lipids could inhibit Th1 responses by PBMCs to the Gram-positive bacterium *E. faecalis*, and thereby might have antigenic properties [32].

It is likely that IL-17 is of importance in the defence against mycobacteria. Thus we hypothesized that all non-tuberculous mycobacteria investigated are able to induce IL-17, IL-1β, IL-6 and IL-23 production by PBMCs from healthy humans, and that surface lipids in the mycobacterial cell envelope may in part be responsible for this response.

2. Materials and methods

2.1. Bacteria, bacterial culture and preparation

Three strains each of *M. avium* (smooth opaque strains), *M. abscessus* (smooth strains) and *M. gordonae*, representing both human and veterinary isolates were studied (Table 1). Mycobacteria were cultured aerobically at 37 °C for 5 d (*M. abscessus*) or 4 weeks (*M. avium* and *M. gordonae*) on Middlebrook 7H10 agar. All culture media were prepared in-house at the Department of Bacteriology, Sahlgrenska University Hospital, Sweden. Bacteria were harvested in Dulbecco's

Table 1
Bacteria used to stimulate human peripheral blood mononuclear cells.

Bacterial species	CCUG[a] strain no.	Isolation site
Mycobacterium avium 1 (subsp. avium)	20 992	Poultry liver Type strain (ATCC[b] 25 291, NCTC[c] 13 034)
M. avium 2	47 945	Human lymph node; cervical lymphadenitis
M. avium 3	47 948	Human lung tissue; lung infection
Mycobacterium abscessus 1	20 993	Human knee abscess/ Type strain (ATCC 19 977)
M. abscessus 2	50 644	Human blood; septicaemia
M. abscessus 3	50 643	Human sputum; lung colonization
Mycobacterium gordonae 1	21 811	Human gastric lavage Type strain (ATCC 14 470)
M. gordonae 2	47 949	Human bronchial ravage; contaminant
M. gordonae 3	47 950	Human bronchial lavage; contaminant
Enterococcus faecalis	19 916	Unknown
Streptococcus mitis	31 811	Human oral cavity; commensal
Escherichia coli	24	Human urine; cystitis

[a] CCUG: the Culture Collection of the University of Gothenburg, Department of Infectious Medicine, University of Gothenburg, Göteborg, Sweden.
[b] ATCC: American Type Culture Collection.
[c] NCTC: Health Protection Agency Culture Collection.

endotoxin-free PBS (PAA laboratories, GmbH, Pasching, Austria). Mycobacterial suspensions were vortexed to disrupt bacterial aggregates and clumps were allowed to sediment for 15 min at room temperature. Bacterial suspensions were UV-irradiated for 1 h (in preliminary experiments found to be required to obtain a negative viable count after 8 weeks of incubation on Middlebrook agar) in 6-well tissue culture plates (Nunc, Roskilde, Denmark). *E. faecalis* and *Streptococcus mitis* were used as representatives of the Gram-positive bacteria and *Escherichia coli* as a representative of the Gram-negative bacteria (Table 1). They were cultured aerobically overnight at 37 °C on horse blood agar, harvested, and inactivated by exposure to UV-light for 15–18 min. The inactivation was confirmed by a negative viable count after 48 h of incubation on horse blood agar. Bacteria were counted in a haemocytometer, adjusted to 1×10^9/ml, and stored frozen at −70 °C, until used. Previous experiments have shown that freezing of inactivated Gram-positive and Gram-negative bacteria do not affect their cytokine inducing capacity, compared to freshly prepared bacteria [34]. Live and UV-killed mycobacteria were compared and no difference in cytokine responses could be revealed.

2.2. Mycobacterial surface lipids

Mycobacterial surface lipids were extracted by a slight modification of a method, which yields a mixture of non-polar and polar lipids devoid of mycolic acids, proteins and pyogenes [35]. In short: suspensions of *M. avium* subsp. avium (CCUG 20 992), *M. abscessus* (CCUG 20 993) and *M. gordonae* (CCUG 21 811) were inactivated 1.5 h at 60 °C. After washing, lipids were extracted by treating 2–3 g of mycobacteria (wet weight) with 6 ml of chloroform–methanol (2:1) for 15 min at 55 °C. The extraction was repeated and the organic phases from both extractions were pooled and washed twice with 1.25 ml of water. The solvent was evaporated and the dry lipid was weighed, dissolved in chloroform, and aliquoted into glass vials. After evaporation of the chloroform, vials were stored at 4 °C until used. For incubation with PBMC, 1 ml of Dulbecco's endotoxin-free PBS (PAA laboratories) was added to the lipid extracts, and an emulsion was obtained by sonication for 60 min.

2.3. Isolation of PBMC and $CD4^+$ T cells

Peripheral blood mononuclear cells (PBMC) were prepared from blood donor buffy coats (Blood banks at Sahlgrenska University Hospital and Kungälv Hospital) by density gradient centrifugation on Lymphoprep (Fresenius Kabi, Oslo, Norway). According to Swedish law no information concerning healthy blood donors is allowed to be recorded for research purposes. Tuberculin Skin Test (TST)-status, BCG-vaccination-status and Quantiferon-screening results were therefore not possible to obtain. More than 2/3 of healthy young adults in Sweden are, however, TST negative [36].

$CD4^+$ cells were purified from PBMCs using magnetic beads (MACS CD4 MicroBeads – human, Miltenyi Biotec, Bergisch Gladbach, Germany), which yielded approximately 95% pure $CD4^+$ cells as assayed by immunofluorescence microscopy.

2.4. Stimulation of PBMC/$CD4^+$ T cells

Human PBMCs, or $CD4^+$ T cells, were washed × 3 (460 × *g*, 10 min) in ice-cold endotoxin-free RPMI 1640 medium with 2 mM glutamine (Invitrogen, Carlsbad, CA) and suspended at 2×10^6/ml in RPMI -medium with 2 mM glutamine, supplemented with 0.1% gentamicin (Sigma–Aldrich, St. Louis, MO), and 5% inactivated endotoxin-free foetal calf serum (Invitrogen; endotoxin level of <0.1 EU/ml). Cell suspensions were aliquoted (200 μl/well, 2×10^6 cells/ml) in flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Bacteria were added to achieve final concentrations of 5×10^6 or 5×10^7/ml, corresponding to 2.5 and 25 bacteria per mononuclear cell, respectively. Crude lipid extracts in PBS were added to achieve final concentrations of 4 and 40 μg/ml. We calculated (considering losses during the extraction process) that 40 μg lipid roughly corresponded to 5×10^7 mycobacteria.

PBMC/$CD4^+$ cultures were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2. Supernatants were harvested after 1, 2, 3, 4, and 5 days to assess the kinetics of IL-23 and IL-17 production by PBMCs. IL-1β and IL-6 were measured after 24 h, a time previously found to be optimal for a range of Gram-positive and Gram-negative bacteria [33]. All supernatants were stored frozen at −20 °C until analyzed.

2.5. Blocking of IL-23

For inhibition of human IL-23, goat anti-human IL-23p19 antibody was used (both from R&D systems, Minneapolis, MN). Antibodies were added to the PBMC preparations at a final concentration of 10 μg/ml at 4 °C, 45 min prior to addition of bacteria.

2.6. Cytokine measurement

Concentrations of human IL-1β and IL-6 in cell culture supernatants were determined by an in-house sandwich ELISA [37]. IL-1β antibodies and standards were purchased from R&D systems (Minneapolis, MN) while IL-6 antibodies came from BD Pharmingen (San Diego, CA). The limits of detection were 6 pg/ml, for both IL-1β and IL-6.

Human IL-23 (p19/p40) and IL-17A were analyzed using commercially available ELISA kits (Human IL-23, p19/p40 and Human IL-17A ELISA Ready-SET-Go!, both from eBioscience, San Diego, CA), according to the manufacturer's instructions. The limits of detection were 62 and 16 pg/ml for IL-23, and IL-17 respectively.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (Graphpad Software, Inc., San Diego, CA). Statistical significance was determined by using oneway ANOVA followed by the Tukey's post-test. $p < 0.05$ was considered to be statistically significant.

To visualize the overall cytokine response pattern induced by mycobacteria compared to Gram-positive and Gram-negative control bacteria, principal component analysis (PCA) was performed [38] using the SIMCA-P 12.0 software (Umetrics AB, Umeå, Sweden). This method permits assessment of a large number of response variables simultaneously. The variables are condensed into composite variables, termed "principal components", that contain as much variation as possible in the data set. The first principal component is depicted on the *x*-axis and the second on the *y*-axis [38].

2.8. Ethics

This study was approved by The Ethical Committee, University of Gothenburg, Göteborg, Sweden (S 557-02).

3. Results

3.1. Induction of IL-17, IL-23, IL-1β, and IL-6 by human PBMC in response to non-tuberculous mycobacteria

PBMCs from eight blood donors were stimulated, *in vitro*, with nine strains of UV-inactivated mycobacteria representing the opportunistic species *M. avium*, *M. abscessus* and the non-pathogenic species *M. gordonae* (Table 1). Two typical Gram-positive bacteria (*E. faecalis* and *S. mitis*) and one Gram-negative bacterium (*E. coli*) served as controls (Table 1). All bacterial strains were UV-inactivated to leave bacterial cells intact and to avoid bacterial growth during the experiments. In general, 5×10^6 bacteria/ml (2.5 per mononuclear cell) and 5×10^7 bacteria/ml (25 per mononuclear cell) induced similar responses, while 5×10^5 bacteria/ml gave low responses. If not otherwise stated, responses to 5×10^6 bacteria/ml, corresponding to approximately 2.5 bacteria/PBMC, are reported. IL-23 (p19 component), IL-1β, and IL-6 were analyzed in 24 h supernatants, while IL-17 was measured after 5 d.

PBMC responses of eight (IL-17, seven) blood donors to 5×10^6 bacteria/ml of each individual bacterial strain are shown in Fig. 1. IL-17 production after stimulation of PBMC with mycobacteria was similar among the three mycobacterial species as well as among the mycobacterial strains of the same species, except for *M. abscessus* strain 2 (blood isolate, septicaemia), which induced less of this cytokine than the others (Fig. 1A).

A significant difference in IL-23 response was seen between the mycobacterial species; the non-pathogenic *M. gordonae* induced less IL-23 than did the other two (Fig. 1B). A small variation within species was seen in response to *M. abscessus* (Fig. 1B).

Concerning IL-1β (Fig. 1C) and IL-6 (Fig. 1D) no significant strain-to-strain variation in PBMC responses were seen to strains of the same mycobacterial species. A significant species-to-species variation was, however, noted for both IL-1β and IL-6 *M. avium*, induced more of these two cytokines than did the other mycobacterial species (Fig. 1C and D). Considerable variations in cytokine responses were seen between blood donors (Fig. 1A–D).

3.2. Non-tuberculous mycobacteria induce IL-17 more strongly than do conventional Gram-positive and Gram-negative bacteria

PBMCs from eight blood donors were stimulated, *in vitro*, with the nine strains of UV-inactivated mycobacteria which all were capable of inducing IL-17 responses with no apparent difference between the species (Fig. 1A). Two typical Gram-positive bacteria (*E. faecalis* and *S. mitis*) and one Gram-negative bacterium (*E. coli*) served as controls (Table 1). These bacteria were included as controls because stimulation of human PBMCs with these species of Gram-positive bacteria was previously shown to induce a typical Th1 response while stimulation with the Gram-negative *E. coli* induced a typical Th2 response [33]. IL-17 production was measured in the supernatants each day during 5 days. As seen in Fig. 2A, the Gram-positive and Gram-negative control bacteria elicited negligible IL-17 responses in contrast to the mycobacteria. The mycobacterial induced IL-17 was detectable in the supernatant from day 2, and increased up to day 5 (Fig. 2A). The average IL-17 response to the mycobacteria was significantly stronger than those elicited by the Gram-positive (*E. faecalis*, *S. mitis*) and Gram-negative control (*E. coli*) bacteria (Fig. 2B).

3.3. $CD4^+$ T cells produce IL-17

The kinetics of IL-17 production showed slow onset (4–5 days), indicating a so-called recall response produced by

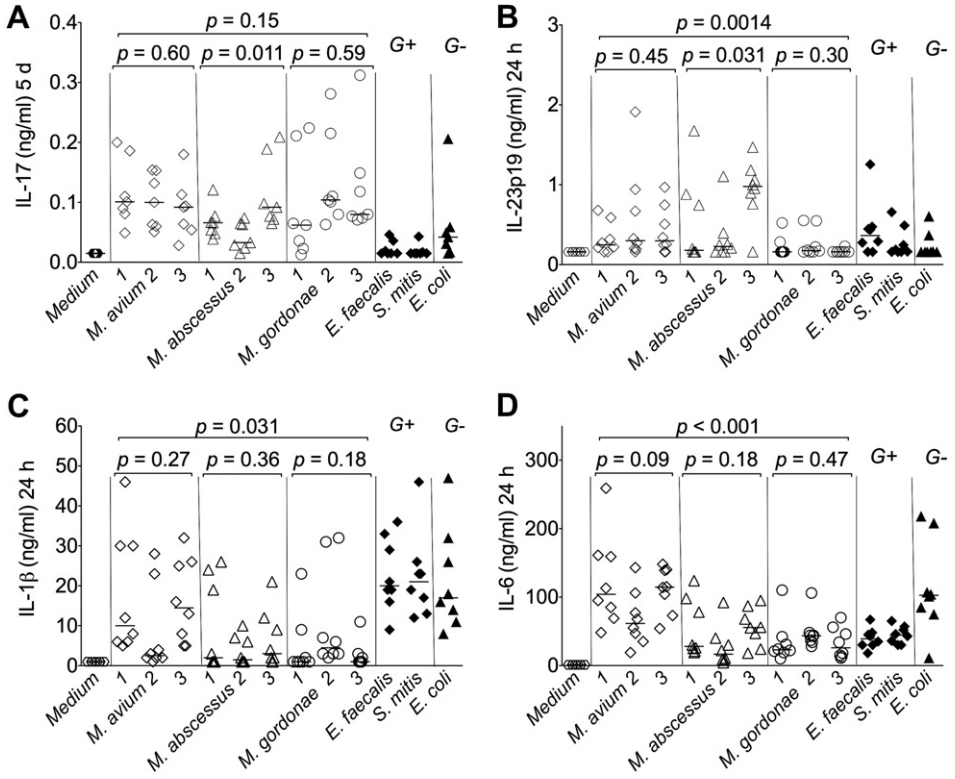

Fig. 1. Production of (A) IL-17, (B) IL-23p19, (C) IL-1β and (D) IL-6 by human PBMCs, in response to all bacterial strains in Table 1 analyzed by ELISA. Oneway ANOVA with Tukey's post-test. Each point represents one blood donor ($n = 8$; IL-17, $n = 7$). Horizontal bar represents the median.

memory T cells in response to microbial products (Fig. 2A) [39]. Further, we noted pronounced variability between blood donors in IL-17 production, indicating an acquired rather than innate immune response (Fig. 1A). To determine whether $CD4^+$ T cells were responsible for the IL-17 secretion, $CD4^+$ cells were isolated from PBMC by magnetic bead separation. The $CD4^+$ cell fraction, the CD4-depleted fraction and unseparated PBMC were all stimulated with non-tuberculous mycobacteria. The CD4-depleted fraction failed to produce any IL-17 in response to mycobacteria, while the $CD4^+$ fraction produced large quantities of IL-17 (>0.5 ng/ml) (Fig. 2C).

3.4. Production of IL-23, IL-1β and IL-6 in relation to IL-17 production

Whether or not the strong IL-17 responses to mycobacteria could be triggered by IL-23 was investigated. The IL-23 (e.g. IL-23p19) responses to UV-inactivated Gram-positive/Gram-negative control bacteria and one strain each of the three mycobacterial species by blood donor PBMCs were first measured at different time-points (Fig. 3A). The highest levels of IL-23 were seen on day one. In this experiment the most efficient inducer of IL-23, was the Gram-positive bacterium *E. faecalis*, while *S. mitis*, *E. coli* and the mycobacteria induced relatively low levels (Fig. 3A). However, as seen in Fig. 1B, the overall IL-23 responses on day one, did not significantly differ among any of the control bacteria and the nine mycobacterial strains.

The capacity of IL-23 to stimulate IL-17 production was tested by using a neutralizing antibody against IL-23 (α-IL-23p19). It was added to the PBMC cultures before stimulation with bacteria (5×10^6 cells/ml). IL-17 was measured in the supernatants after 5 days. As seen in Fig. 3B, IL-17 did not decrease in the presence of blocking antibodies compared to antibody free controls. The strong IL-17 inducing capacity of mycobacteria could not be explained by the capacity of these bacteria to induce IL-23 production.

Besides IL-23, maturation of human Th17 cell and IL-17 production are considered to be dependent on IL-1β and IL-6 [40]. We therefore stimulated PBMC with the mycobacteria and Gram-positive and Gram-negative control bacteria and measured IL-1β and IL-6 in the cell supernatants. The pooled data of the mycobacteria, was compared to the Gram-positive and Gram-negative control data. We found that the mycobacteria induced significantly lower amounts of IL-1β than did both Gram-positive and Gram-negative control bacteria (Fig. 3C), but approximately equal amounts of IL-6 compared to Gram-positive bacteria and lower amounts compared to the Gram-negative *E. coli* (Fig. 3D).

The overall cytokine response induced was compared between bacterial strains using PCA (Fig. 3E). The analysis shows that the mycobacteria formed a cluster clearly distinct from Gram-positive bacteria (*E. faecalis*, *S. mitis*; right in the diagram) and the Gram-negative *E. coli* (left in the diagram). The variables causing the separation are superimposed in the same diagram. The positions of the variables (cytokines) and observations (strains) denote their association, such that, high

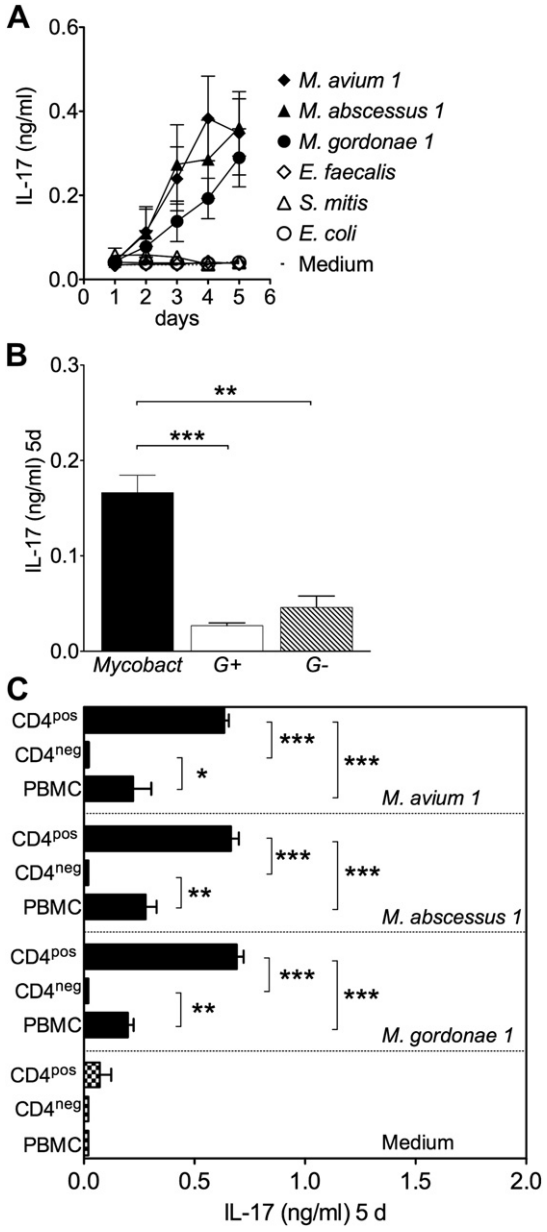

Fig. 2. Production of IL-17 and the role of $CD4^+$ T cells. IL-17 responses by human PBMCs to mycobacteria, *E. faecalis*, *S. mitis* and *E. coli* (A) during 5 d (B) summarized at day 5. Cytokines analyzed by ELISA. Oneway ANOVA with Tukey's post-test (A, $n = 4$; B, $n = 7$). (C) IL-17 responses by the $CD4^+$ fraction ($CD4^{pos}$), the CD4-depleted fraction ($CD4^{neg}$) and un-fractioned PBMC at day 5; oneway ANOVA with Tukey's post-test ($n = 3$). Error bars represent SEM ($n = 3$); $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

levels of IL-23 characterize the response of the blood donors to bacteria positioned to the lower right, i.e. the classical Gram-positives *E. faecalis* and *S. mitis.* In contrast, low levels of IL-23 and high levels of IL-6 characterize the response to bacteria positioned to the left, i.e. the Gram-negative *E. coli*. IL-17 responses were unrelated to IL-1β, IL-6, and IL-23 responses. The same results were obtained using Spearman's rank correlation; IL-17 responses were neither related to IL-23, nor to IL-1β or IL-6 responses (data not shown).

3.5. Mycobacterial surface lipids induce production of IL-17 but not IL-23

The mycobacterial cell envelope is unique in containing an abundance of complex lipids. The outermost layer contains a poorly defined mixture of lipids; here termed surface lipids. Crude surface lipid extracts from one strain each of *M. avium*, *M. abscessus* and *M. gordonae* were used in two concentrations (4 and 40 μg/ml) to stimulate PBMC. The higher concentration corresponded roughly to the amount present in 5×10^7 per ml of intact UV-inactivated mycobacteria, and we used this bacterial concentration as reference stimulation. As seen in Fig. 4A, surface lipid extracts from mycobacteria induced pronounced production of IL-17. In fact, the amounts of IL-17 induced by mycobacterial surface lipids were comparable to those seen in response to intact mycobacteria (Fig. 4A). No significant difference in IL-17 production was seen in response to the two concentrations of surface lipids.

The crude lipid extracts were also tested for IL-23 inducing capacity. They were found to be poor inducers of IL-23 compared to intact mycobacterial cells (Fig. 4B).

4. Discussion

In this study, the production of IL-17 in freshly isolated human PBMC in response to non-tuberculous mycobacteria of varying pathogenic potential was analyzed. Two opportunistic pathogenic species, *M. abscessus* and *M. avium* and one species generally regarded as non-pathogenic, *M. gordonae*, were investigated. We found a pronounced production of IL-17 by freshly isolated human blood mononuclear cells in response to all mycobacteria, regardless of pathogenic potential. The IL-17 production in response to conventional Gram-positive and Gram-negative control bacteria was in contrast negligible.

IL-17 is produced by $CD4^+$ T cells, designated Th17 cells, but also by $CD8^+$ T cells, γδ T cells and NKT cells [5,41,42]. Concentrations of IL-17 in the supernatants of stimulated PBMCs increased successively up to 5 days, indicating that a recall response by memory T cells was the principal sources of this IL-17. Indeed, $CD4^+$ cells seemed to be the main producers of IL-17 in response to mycobacteria in human PBMCs, *in vitro*, as CD4-depleted PBMCs produced no IL-17 while $CD4^+$ cells purified from PBMC produced large amounts (0.6 ng/ml). The purity of the $CD4^+$ cell clone was, however, approximately 95%, which indicates that other cells were present in this cell fraction, although in low numbers. A few isolated cells of suspected monocyte origin were seen in the $CD4^+$ fraction. These antigen-presenting cells do not produce IL-17, but it cannot be excluded that they to some extent have contributed to the IL-17 secretion by the $CD4^+$ cells.

The surface lipids induced IL-17 in amounts comparable to the mycobacterial cells. Our results, thus, show that mycobacterial lipids might act as antigens that induce IL-17 production in $CD4^+$ T cells. Crude surface lipid extract fractions from *M. avium*, *M. abscessus* and *M. gordonae*, were all

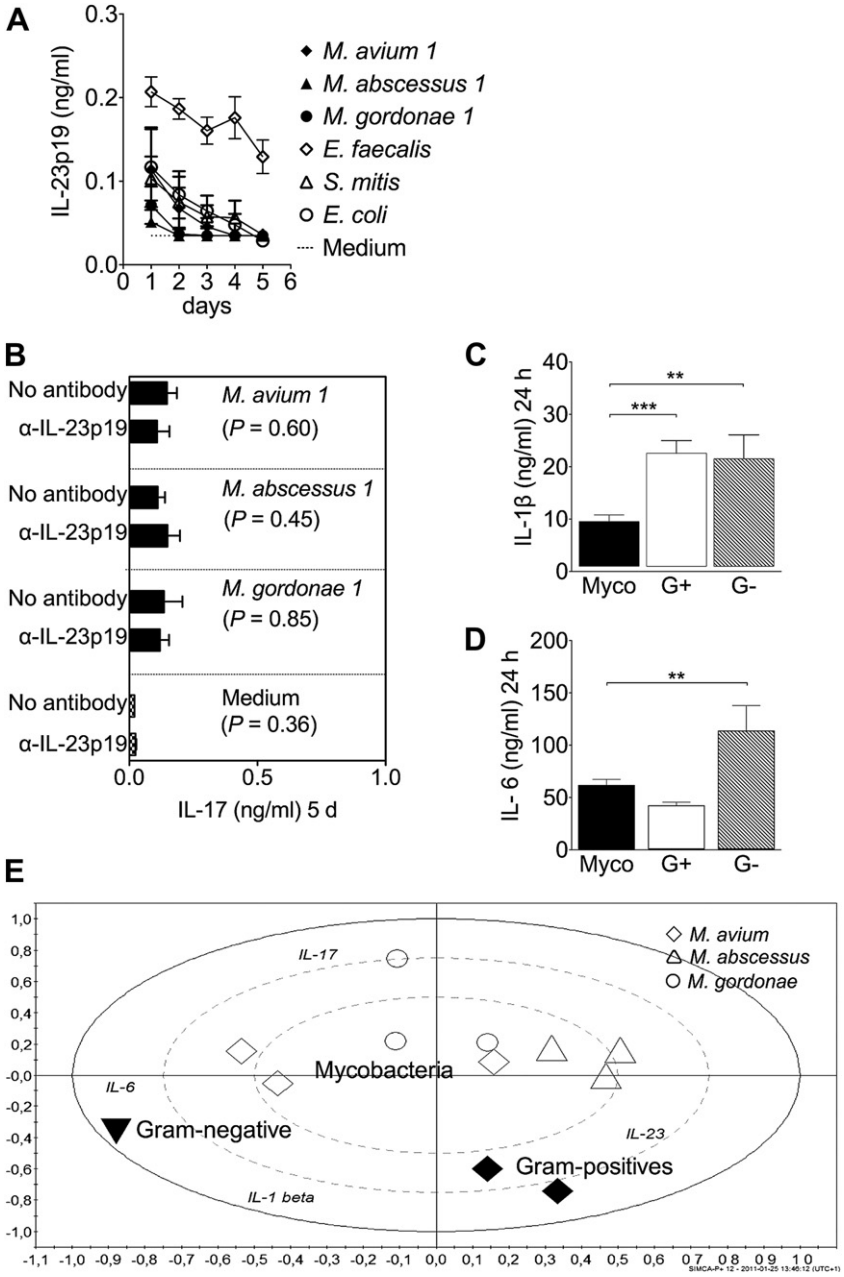

Fig. 3. Production of IL-23, IL-1β and IL-6 in relation to IL-17 production. (A) IL-23p19 during 5 d. (B) Effect on IL-17 production (5 d) by human PBMCs after pre-neutralization of IL-23p19. (C) Induction of IL-1β and (D) IL-6 at 24 h. Fig. 3A, C, D: oneway ANOVA with Tukey's post-test; Fig. 3A ($n = 7$), 3C and 3D ($n = 8$). Fig. 3B: oneway ANOVA with Tukey's post-test ($n = 6$). Error bars represent SEM. $^{**}p < 0.01$; $^{***}p < 0.001$. (E) Overall cytokine profile according to principal component analysis, PCA. The variables (cytokines) that determine the position of the bacteria are superimposed in the plot.

capable of inducing IL-17 in quantities similar to those seen in response to intact mycobacteria. The generally non-pathogenic *M. gordonae* and its surface lipids were equally efficient in inducing IL-17 as the two opportunistic species *M. avium* and *M. abscessus* (whole bacterial cells and surface lipids).

All three *M. abscessus* strains used to stimulate human PBMCs were isolated from patients infected with these strains (Table 1). This study is the first one showing the IL-17 inducing capacity of *M. abscessus* and *M. gordonae*, according to our knowledge. Other studies indicate that pulmonary disease caused by *Mycobacterium avium-intracellulare* could be due to reduced Th17 immunity [26]. We have recently shown that *M. avium* strains isolated from lymph nodes extirpated from children with mycobacterial infection induced less IL-17 in human PBMCs (healthy blood donors) than *M. avium* strains isolated from birds and other animals, indicating genetic differences between *M. avium* isolates [25]. The fact that the generally non-pathogenic *M. gordonae* is a potent IL-17 inducer in human PBMC complicates the picture. To elucidate the role of IL-17 in the pathogenesis and host defence against non-tuberculous mycobacteria is therefore a future challenge. Studies on human PBMCs stimulated with

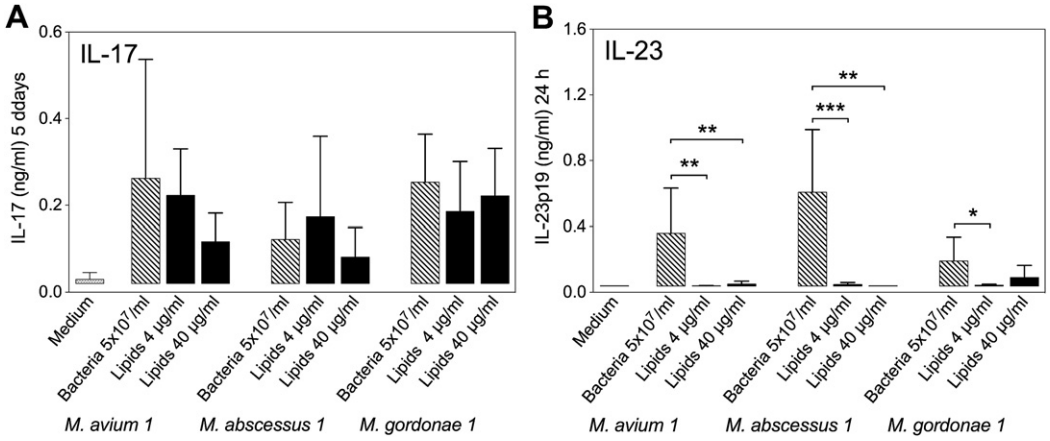

Fig. 4. Cytokine responses to mycobacterial cells and mycobacterial surface lipids. (A) IL-17 at 5 d and (B) IL-23 at 24 h. Cytokine analyses by ELISA. Oneway ANOVA with Tukey's post-test ($n = 7$); error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

a lysate from *M. tuberculosis* have shown that the IL-17 production was augmented in patients with active tuberculosis compared to BCG-vaccinated healthy blood donors [43]. Furthermore, another study indicated that IL-17 producing T cells could play an immunopathological role in multi resistant tuberculosis in promoting severe tissue damage [44]. The role of IL-17 in mycobacterial disease is complex and the variations might be due to differences between mycobacterial species, differences in host immunity, as well as the fact that these diseases are chronic and change over time.

Lineage commitment of T cells depends on co-stimulatory signals delivered by the antigen-presenting cells in the form of accessory molecules and T cell modulating cytokines. Thus, maturation of human Th17 cells and IL-17 production might depend on IL-23, IL-1β, and IL-6 [3]. The strong IL-17 production in response to mycobacteria and their lipids could, however, not be explained by particularly strong IL-23 responses to these bacteria. All the mycobacterial strains were able to induce IL-23, but the highest responses were induced by the Gram-positive *E. faecalis*, which did not induce IL-17. IL-23 has been reported to induce both proliferation and IFN-γ production in human PHA stimulated blast T cells indicating that IL-23 might have Th1 polarizing properties apart from its ability to stimulate Th17 cells [45]. Furthermore, mycobacterial surface lipids were strong IL-17 inducers. It is of interest to note that the mycobacterial lipids did not induce any IL-23 despite being strong IL-17 inducers. It is known that different bacterial structures induce different cytokine responses, but the background of the present result needs to be investigated and elucidated in further studies.

Blocking of IL-23 did not reduce IL-17 production triggered by mycobacteria or any of the other bacteria tested, results which are in discordance with a previous report in which IL-17 production by $CD4^+$ cells was inhibited by anti-IL-23 in *M. tuberculosis*-stimulated monocyte cultures [29]. Whether IL-17 plays a protective or pathogenic role in mycobacterial infection remains to be elucidated, but there is probably a balance between protection and destruction. Mycobacteria induced relatively moderate amounts of both IL-1β and IL-6, why it is unlikely that the production of IL-17 solely is dependent on any of these two cytokines. Furthermore, no positive association between IL-17 responses on one hand and IL-23, IL-1β or IL-6 on the other was found. Thus, the propensity of mycobacteria and their lipids to stimulate IL-17 production is probably complex and remains to be explained, but it is likely that IL-17 is of importance in the defence against mycobacterial infection.

In summary, we here show that three different species of non-tuberculous mycobacteria induce a unique cytokine profile in human PBMC characterized by large amount of IL-17 compared with "conventional" Gram-positive and Gram-negative bacteria. Lipids in the mycobacterial cell envelope may act as antigens and play, in all probability, a crucial role in eliciting IL-17. We also confirmed that $CD4^+$ T cells are responsible for the IL-17 production, as has been previously shown to be the case in disease caused by *M. tuberculosis*. Neutralization of IL-23 did not effect the IL-17 induction indicating that other mediators were responsible for triggering of the $CD4^+$ T cells. The exact mechanisms in which mycobacterial lipids induce IL-17 by human PBMCs and their importance in pathogenesis and host defence need to be elucidated in further studies.

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