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Mycobacterium tuberculosis induces IL-17A responses through TLR4 and dectin-1 and is critically dependent on endogenous IL-1

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ABSTRACT

In the present study, we dissected the pathways that trigger the IL-17A responses by MTB. Dectin-1 and TLR4 were shown to be involved in MTB-induced IL-17A production, and blockade of the NOD2, TLR2, or MR had no effect on IL-17A. The MAPK Erk, known to mediate transcription of IL-1 β mRNA, was strongly involved in the IL-17A production induced by MTB. The intracellular enzymes caspase-1 and serine proteases, which process pro-IL-1 β into the active IL-1 β , were also crucial for the induction of IL-17A. Lastly, the MTB-induced IL-17A response was strongly dependent on signaling through the IL-1R but not the IL-6R pathway. In conclusion, the MTB-induced IL-17A response relies strongly on the endogenous IL-1 pathway and IL-1R signaling. TLR4 and dectin-1 are the main receptors responsible for mediating the signals responsible for IL-17A production by MTB. These findings contribute to a better understanding of the host response to mycobacteria and provide the opportunity to explore potential, novel, therapeutic strategies against TB. *J. Leukoc. Biol.* **88**: 227–232; 2010.

Introduction

TB remains an important public health problem with 2 million deaths per year [1]. Although several mechanisms of MTB immune control have been elucidated, there remains a critical need to better understand the host defense mechanisms leading to elimination of the pathogen, latent disease, or active TB [2, 3]. Recently, a distinct IL-17A-producing subset of memory CD4⁺ T cells (Th17 cells) with specificity for MTB has been described, and it has been suggested that they have an important contribution to the human antimycobacterial immune response [4].

In addition, novel vaccination strategies have focused on inducing TB-specific Th17 responses, as the IL-17A pathway induced after immunization has been shown to contribute to the protection at the site of infection [5]. In this respect, IL-17A does not appear to play a major role in primary TB infection but seems mainly to be involved in the maintenance of the inflammatory response. In line with this, granuloma formation in the lungs of IL-17A-deficient mice infected with BCG was reported to be impaired [6], and IL-17A was shown to play a role in the trafficking of Th1 cells to the site of infection [7]. These data strongly underline the importance of the IL-17A response in host defense against TB and provide the rationale for developing vaccines that induce a strong TB-specific Th17 response.

Although many studies have focused on the importance of IL-17A in antimycobacterial host defense, little is known about the mechanisms through which MTB triggers the Th17-specific host response. The induction of Th17 responses by microorganisms has been reported to be induced by several TLRs, NLRs, and C-type lectin receptors. The main receptors reported for inducing a pathogen-specific Th17 response are TLR2, TLR4, NOD2, dectin-1, and MR [8–11]. Interestingly, recognition of MTB has been associated with recognition by these receptors, but which of them are responsible for the MTB-induced IL-17A responses is not yet known [12–18]. Regarding the cytokines that play a role in the induction of Th17 responses, IL-1 β and IL-6 have been implicated as the main Th17-polarizing cytokines for memory CD4⁺ T cells in humans [19]. Recently, it has been shown that IL-1 signaling is crucial for the early development of Th17 cells [20]. In the present study, we have focused on the induction of IL-17A by MTB and tried to identify the mechanisms involved in triggering the IL-17A response by MTB.

Abbreviations: BCG=bacillus Calmette-Guerin, DPP1=dipeptidyl peptidase I, -i=inhibitor, IL-1RA=IL-1R antagonist, Man-LAM=mannose capping of lipopolysaccharide, MR=mannose receptor, MTB=*Mycobacterium tuberculosis*, NLR=Nod-like receptor, PRR=pattern recognition receptor, TB=tuberculosis, TDM=trehalose 6,6'-dimycolate

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MATERIALS AND METHODS

Volunteers

Blood was collected after informed consent by venipuncture into 10 ml EDTA syringes (Monoject) from seven healthy volunteers. None of the volunteers had a history of a positive tuberculin skin test or was vaccinated with BCG. Five patients with Crohn's disease with *3020msC* mutation, without immunosuppressive medication, were investigated further in the cytokine studies. The Ethics Committee of Radboud University Nijmegen (The Netherlands) approved the study.

Animals

Balb/c control mice (Charles River Wiga, Sulzfeld, Germany) and DPPI-deficient mice (Balb/c background; Christine T. Pham), 10–14 weeks, were bred and housed (Radboud University Nijmegen Medical Centre) in filter-top cages, and water and food were supplied ad libitum. The Radboud University Animal Ethical Committee approved all animal experiments.

MTB

MTB H37Rv was grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton Dickinson, Palo Alto, CA, USA), heat-killed at 100°C for 1 h, washed three times in sterile saline, and resuspended in RPMI 1640.

In vitro cytokine production

Separation and stimulation of PBMCs were performed as described previously [21]. PBMCs (5×10^5) in a volume of 200 μ l/well were incubated

at 37°C in round-bottom 96-wells plates (Greiner, Nuremberg, Germany) with 10% human pooled serum. In blocking experiments, PBMCs were stimulated with heat-killed MTB (1×10^5 microorganisms/ml) in the absence or presence of inhibitors [preincubation 1 h: anti-TLR4 inhibitor *Bartonella quintana* LPS 100 ng/ml; mannose 100 nM (Sigma Chemical Co., St. Louis, MO, USA); anti-TLR2 10 μ g/ml and control anti-IgA 10 μ g/ml (InvivoGen, San Diego, CA, USA); laminarin 10 μ g/ml (Dr. David Williams, University of Tennessee, Knoxville, TN, USA); IL-1RA 10 μ g/ml (Prof. Charles Dinarello, University of Colorado, Boulder, CO, USA); 10 μ g/ml humanized IL-6R antibody tocilizumab (Roche Nederland BV, The Netherlands); and 20 nM SB202190 (p38-i), SP600125 (JNK-i), and 10 nM U0126 (Erk-i; Superarray Bioscience, Bethesda, MD, USA); no preincubation: caspase-1 inhibitor Tyr-Val-Ala-Asp (Alexis Biochemicals, San Diego, CA, USA); protease inhibitor α 2 macroglobulin (Sigma Chemical Co.); and equivalent concentration of vehicle (0.01–0.1% DMSO) where appropriate]. Viability of the cells was not affected by the MAPK inhibitors, as shown by unaffected lactate dehydrogenase measurements in the supernatants of cells incubated with the various inhibitors (not shown). Mouse spleen cells (1 mL), resuspended in RPMI 1640, adjusted to 5×10^6 mL⁻¹, were stimulated for 48 h with heat-killed MTB.

Cytokine assays

Cytokines were measured by commercial ELISA kits according to the instructions of the manufacturer. Human IL-1 β , IL-6, IL-17A, and IL-22 and mouse IL-17A concentrations (R&D Systems, Minneapolis, MN, USA) and IFN- γ (Bio-

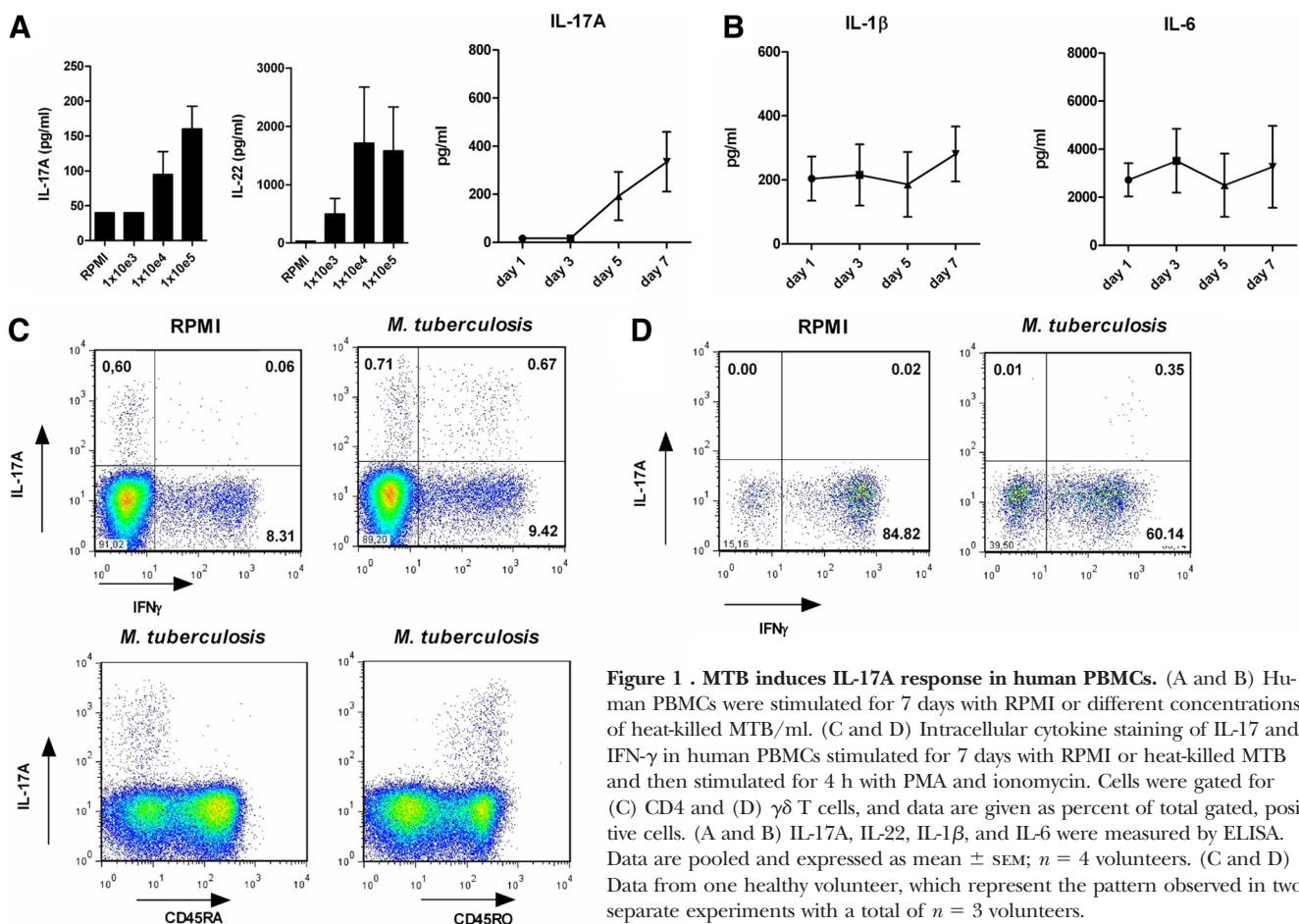


Figure 1 . MTB induces IL-17A response in human PBMCs. (A and B) Human PBMCs were stimulated for 7 days with RPMI or different concentrations of heat-killed MTB/ml. (C and D) Intracellular cytokine staining of IL-17 and IFN- γ in human PBMCs stimulated for 7 days with RPMI or heat-killed MTB and then stimulated for 4 h with PMA and ionomycin. Cells were gated for (C) CD4 and (D) $\gamma\delta$ T cells, and data are given as percent of total gated, positive cells. (A and B) IL-17A, IL-22, IL-1 β , and IL-6 were measured by ELISA. Data are pooled and expressed as mean \pm SEM; $n = 4$ volunteers. (C and D) Data from one healthy volunteer, which represent the pattern observed in two separate experiments with a total of $n = 3$ volunteers.

source, Camarillo, CA, USA) were measured by commercial ELISA kits according to the instructions of the manufacturer.

Intracellular cytokine staining

Four hours before the staining, the supernatant was aspirated, and culture medium with brefeldin A (10 $\mu\text{g/ml}$), PMA (0.05 $\mu\text{g/ml}$), and ionomycin (1 $\mu\text{g/ml}$) was added. Cells were stained with live/dead fixable dead cell stain kits Aqua (Invitrogen, Carlsbad, CA, USA) for 30 min on 4°C, washed with PBS containing 0.5% albumin for bovine serum (Sigma Chemical Co.), and stained with IOTest anti-TCR Pan γ/δ -PE (Beckman-Coulter, Fullerton, CA, USA), IOTest CD45RO-phycoerythrin Texas Red (Beckman-Coulter), PerCP anti-human CD3 (BioLegend, San Diego, CA, USA), PE-Cy7 anti-human CD45RA (eBioscience, San Diego, CA, USA), and Pacific Blue anti-human CD4 (eBioscience) for 20 min on 4°C. After washing, cells were incubated with fixation Medium A (Caltag, S. San Francisco, CA, USA) and subsequently, incubated with FITC anti-human IFN- γ (eBioscience) and Alexa Fluor 647 anti-human IL-17A (BD PharMingen, San Diego, CA, USA) in permeabilization Medium B (Caltag) for 20 min on 4°C. Cells (100,000) gated on a lymphocyte gate by forward- and side-scatter were acquired on a CyAn ADP 9 color flow cytometer (Beckman-Coulter). Data were analyzed using the FlowJo software program.

Statistical analysis

Differences between groups were analyzed by a two-tailed paired *t*-test or the Mann-Whitney U test. Differences were considered statistically significant when $P \leq 0.05$. All experiments were performed at least twice, and the data are presented as the cumulative result of all experiments performed.

RESULTS AND DISCUSSION

Th17 cells, characterized by the production of IL-17A, IL-17AF, IL-21, and IL-22, contribute to the human antimycobacterial immune response [4]. In line with this, we were able to demonstrate that MTB induces IL-17A and IL-22 production in a dose-dependent manner (Fig. 1A). In addition, production of the proinflammatory cytokines IL-1 β and IL-6, which are involved in driving CD4-positive memory T cells toward Th17 cells, was also induced by MTB (Fig. 1B). It has been reported that the main cellular source of MTB-induced IL-17A production in human PBMCs was a subset of CD4⁺ T cells (Fig. 1C) [4, 22]. In the present study, we show that there is an increase in double IL-17A/IFN- γ -positive CD4⁺ memory T cells after stimulation with MTB *in vitro*; CD4⁺ naïve T cells did not express IL-17A (Fig. 1C). These data support the concept that in humans, the CD4⁺ memory T cells are the main source responsible for IL-17A production during infection with MTB. However, the presence of CD4⁺ memory T cells responding with IL-17A release upon stimulation with MTB in individuals who have a negative purpose of tuberculin skin test and have not been vaccinated with BCG is likely to mirror the presence of a nonspecific, primed subset of memory Th cells, which is able to express IL-17 after MTB exposure. We are currently investigating this important observation. In addition, animal models have provided evidence that $\gamma\delta$ T cells rather than CD4⁺ memory T cells are the main source for IL-17A production induced by MTB [23], and $\gamma\delta$ T cells have been recognized as an important innate source of IL-17A production [24, 25]. Interestingly, IL-17A was also expressed in a small subpopulation of $\gamma\delta$ T cells after stimulation with MTB (Fig. 1D), indicating that $\gamma\delta$ T cells contribute to IL-17A production during MTB infection in humans. It remains to be

elucidated which subset is predominantly responsible for the observed IL-17A production after a MTB infection.

The recognition of MTB is mediated by a complex network of TLRs (among which include TLR2 and TLR4), lectin receptors (dectin-1 and MR), and the NLR receptor NOD2 [12–18]. We assessed which of the various PRRs are involved in the induction of IL-17A by MTB. Dectin-1 blockade led to inhibition of mycobacteria-induced IL-17A (Fig. 2A), which is in line with the fact that dectin-1 has been recognized as one of the main receptors that skews Th responses toward a more Th17 profile [26]. Notably, the Syk-caspase recruitment domain-containing protein 9 pathway through which dectin-1 signals has been suggested recently as a rational target for vaccine development against

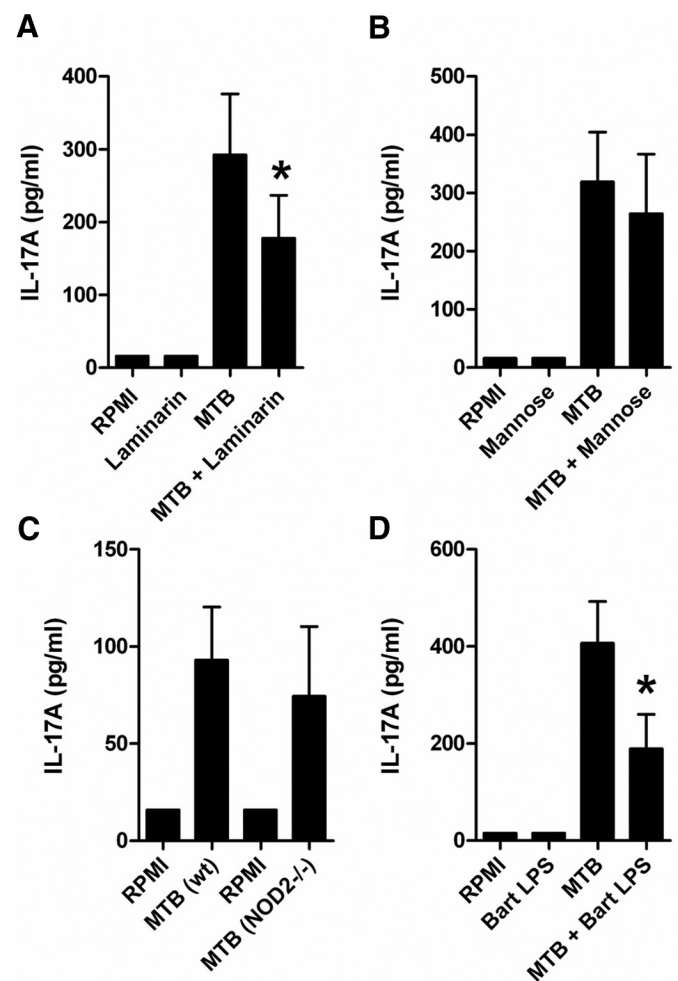


Figure 2. IL-17A induced by MTB is dependent on TLR4 and dectin-1 but independent of NOD2 and MR. Human PBMCs were stimulated for 7 days with 10^5 heat-killed MTB/ml in the presence or absence of (A) dectin-1 inhibitor 50 $\mu\text{g/ml}$, (B) mannose 100 nM (C; $n=6$ volunteers), (D) TLR4 inhibitor 20 ng/ml [*B. quintana* LPS (Bart LPS)]. (C) Human PBMCs from five patients with NOD2 deficiency were stimulated for 7 days with 10^5 heat-killed MTB/ml and compared with healthy volunteers ($n=9$); wt, wild-type. IL-17A was measured by ELISA. Data are pooled and expressed as mean \pm SEM; *, $P < 0.05$.

TB, as it is critical for inducing a robust Th17 response to a MTB subunit vaccine [5].

The MR is involved in the recognition of Man-LAM of mycobacteria [27] and can mediate phagocytosis of mycobacteria [28]. Furthermore, the MR has been reported to be the main pathway involved in the induction of IL-17A by *Candida albicans* [11]. However, there was no significant difference in IL-17A production when MR was blocked by high concentrations of mannose. This observation is supported by the recent evidence that the Man-LAM does not dominate the mycobacteria-host interaction [29].

NOD2 has been suggested to be able to induce Th17 responses [10], and it can mediate recognition of mycobacteria [17]. Interestingly, PBMC from individuals defective in NOD2 function (Crohn's disease patients homozygous for the 3020insC NOD2 mutation) [30], stimulated with MTB, released the same amounts of IL-17A as healthy volunteers (Fig. 2C), making it unlikely that NOD2 plays an important role in the induction of the Th17 response induced by MTB.

It is generally accepted that TLR2 is one of the main receptors that recognizes MTB [31]. A commercial antibody described to antagonize TLR2 had no effect on MTB-induced IL-17A production (not shown). However, the inhibitory profile on the cytokine production induced by the specific TLR2 ligand tripalmitoyl-S-glycerol cysteine revealed only 30–40% inhibition using this antibody, which precludes us to draw definitive conclusions about the role of TLR2 for the induction of IL-17A by MTB. On the other hand, the same approach has clearly demonstrated TLR2 involvement in the IL-17A stimulation by *C. albicans* [11], which suggests that the lack of effect of TLR2 in the case of MTB is genuine.

Less anticipated was the observed role for TLR4 in the MTB-induced IL-17A response. When TLR4 was blocked, a

significant reduction in IL-17A production was observed (Fig. 2D). TLR4 recognizes a heat-labile component from MTB [32], and one study has reported the potency of TLR4-mediated signals to induce Th17 responses [8]. As IL-17A has been suggested to be involved in the maintenance of the inflammatory response that provides long-lasting protection against TB, these data suggest that TLR4 is not involved in primary TB host defense but rather, plays a role in secondary immune responses providing protection.

TLR4 and dectin-1 most likely contribute to the IL-17A response by inducing a specific cytokine profile that is able to skew the Th response toward a Th17 profile. Further investigation is needed to determine whether PRRs also play a specific role in antigen presentation during the induction of the Th17 response by MTB. Recently, Mincle has been identified as the receptor for the mycobacterial cell wall glycolipid cord factor (TDM) [33]. As this receptor is also expressed on T cells, it has been suggested that T cells might recognize TDM in a Mincle-dependent manner, which could trigger T cell activation and cytokine production directly. Whether MTB could induce IL-17A production directly in a TCR-independent mechanism remains to be elucidated.

The IL-1R has been shown recently to be critical for the early development of the Th17 response [20]. Furthermore, IL-1 and also IL-6 are suggested to be involved in the production of IL-17A by memory T cells [19]. When PBMC were stimulated with MTB, blocking the IL-6R did not affect IL-17A production, and blocking IL-1 signaling with IL-1RA led to an almost complete inhibition of IL-17A (Fig. 3A). This demonstrates that endogenous IL-1 plays a central role for the Th17 responses induced by mycobacteria and that the processes affecting IL-1 production would be likely to have a strong influence on IL-17A production induced by MTB. We have undertaken a sys-

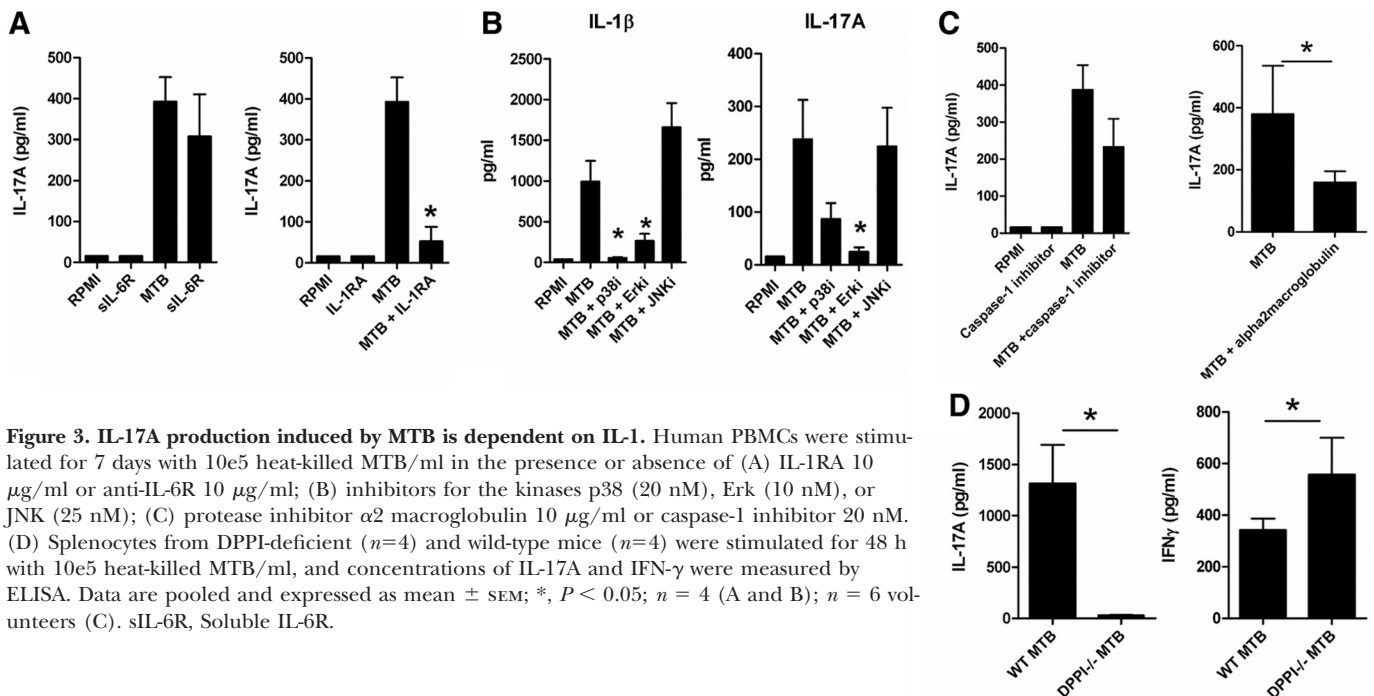


Figure 3. IL-17A production induced by MTB is dependent on IL-1. Human PBMCs were stimulated for 7 days with 10e5 heat-killed MTB/ml in the presence or absence of (A) IL-1RA 10 μ g/ml or anti-IL-6R 10 μ g/ml; (B) inhibitors for the kinases p38 (20 nM), Erk (10 nM), or JNK (25 nM); (C) protease inhibitor α 2 macroglobulin 10 μ g/ml or caspase-1 inhibitor 20 nM. (D) Splenocytes from DPP1-deficient ($n=4$) and wild-type mice ($n=4$) were stimulated for 48 h with 10e5 heat-killed MTB/ml, and concentrations of IL-17A and IFN- γ were measured by ELISA. Data are pooled and expressed as mean \pm SEM; *, $P < 0.05$; $n = 4$ (A and B); $n = 6$ volunteers (C). sIL-6R, Soluble IL-6R.

tematic assessment of the transcriptional and post-transcriptional mechanisms known to mediate the release of bioactive IL-1 β from monocytes. Recently, it was demonstrated that the MAPK Erk and p38 but not JNK are important for the induction of transcription of IL-1 mRNA [34]. In line with this, Erk inhibition lowered the production of IL-1 β and IL-17A induced by MTB, and JNK inhibition had no effect (Fig. 3B). Results from experiments that blocked p38 MAPK were less clear but showed a trend toward lower IL-17A production (Fig. 3B). In addition, when blocking the enzymes responsible for the processing of the inactive pro-IL-1 β into the active IL-1 β , such as caspase-1 [35] and the inflammatory serine proteases (a family comprising protease 3, elastase, cathepsin G) [36], IL-17A production was strongly down-regulated (Fig. 3C). In line with this, cells from DPP1 knockout mice, which are also deficient in serine proteases, showed a strong defect in inducing IL-17A (Fig. 3D). This effect was specific for the IL-17A responses, as IFN- γ production was not affected (Fig. 3D). Altogether, these data underline the central role of endogenous IL-1 in the induction of the IL-17A response by MTB.

In the present study, we elucidate the pathways through which MTB activates the IL-17A response. The PRRs involved in triggering the IL-17A response are TLR4 and dectin-1 but not NOD2, TLR2, or MR. Release of endogenous IL-1 is a central event for the induction of the IL-17A response by MTB, as blocking of the various steps responsible for the production and release of IL-1 β also inhibited IL-17A. These findings contribute to a better understanding of the host response to mycobacteria and provide the opportunity to explore novel treatment and vaccination strategies that could be beneficial in fighting the global burden of TB.

AUTHORSHIP

F.L.V. designed, performed, and analyzed experiments and prepared the manuscript. A.C.T. designed, performed, and analyzed experiments. J.K., B.J.K., R.C., J.W.M.M., L.A.B.J., and M.G.N. designed experiments, interpreted results, and prepared the manuscript.

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KEY WORDS:
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