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Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis

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As a hallmark of tuberculosis (TB), *Mycobacterium tuberculosis* (MTB) induces granulomatous lung lesions and systemic inflammatory responses during active disease. Molecular regulation of inflammation is associated with inflammasome assembly. We determined the extent to which MTB triggers inflammasome activation and how this impacts on the severity of TB in a mouse model. MTB stimulated release of mature IL-1β in macrophages while attenuated *M. bovis* BCG failed to do so. Tubercle bacilli specifically activated the NLRP3 inflammasome and this propensity was strictly controlled by the virulence-associated RD1 locus of MTB. However, *Nlrp3*-deficient mice controlled pulmonary TB, a feature correlated with NLRP3-independent production of IL-1β in infected lungs. Our studies demonstrate that MTB activates the NLRP3 inflammasome in macrophages in an ESX-1-dependent manner. However, during TB, MTB promotes NLRP3- and caspase-1-independent IL-1β release in myeloid cells recruited to lung parenchyma and thus overcomes NLRP3 deficiency in vivo in experimental models.

Key words: ESX-1 secretion system · Inflammasome · IL-1β · *Mycobacterium tuberculosis* · NLRP3

Supporting Information available online

Introduction

Host and pathogenic microbes have evolved a complex and intensive cross-talk. Ultimately, these interactions result in the induction of inflammation. Depending on the pathogen, this process is either rapid and involves host pattern recognition receptors (PRRs), which immediately launch an emergency response, or is protracted and characterized by silent pathogen entry resulting in dampened inflammation and delayed onset of host defense. *Mycobacterium tuberculosis* (MTB), one of the

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world's most successful pathogens, combines both strategies and thereby establishes chronic infection and active disease.

Despite major efforts to improve control measures, tuberculosis (TB) continues to cause high morbidity and mortality worldwide [1]. Animal models and genetic screens in humans have revealed that control of TB strongly depends on a robust Th1-cell response [2]. Active TB is primarily a disease of the lung, which can escalate to a generalized inflammatory disease at later stages. Yet, inflammation is necessary for pathogen control. Hence, inflammation needs to be tightly regulated to avoid collateral damage especially in the context of chronic infection [3, 4]. Despite detailed information about the kinetics and different types of inflammation, until recently the precise molecular events leading to IL-1β release, the central molecular mediator of inflammation, remained obscure. Identification of the inflammasome as a cytosolic platform essential for caspase-1 activation and cleavage of pro-IL-1β [5] considerably increased our knowledge about the inflammatory process. At the same time, it raised questions as to how pathogens can manipulate this cellular machinery. In line with this and regardless of the wealth of information about how inflammation shapes immune responses and pathology during TB, the mode of how MTB modulates inflammation at a molecular level remains ill-defined. Moreover, how this molecular regulation impacts on host responses remains virtually unknown.

Although the necessity of IL-1β and IL-1R in TB control has been described [6–9], there are few and contradictory insights into regulation of mature IL-1β during TB. One report suggests that both MTB and the vaccine *Mycobacterium bovis* BCG actively inhibit inflammasome activation via a zinc metalloprotease [10]. Others described that MTB actively induces caspase-1 activation [11, 12] for IL-1β release, similar to induction of mature IL-1β by *Mycobacterium marinum*, which depends on the region of difference 1 (RD1) locus (encoding part of the ESX-1 secretion system) [11, 13]. Furthermore, screening of a macrophage cell line with shRNAs revealed a role for CARD and NLR proteins, including NLRP3, in MTB-induced inflammasome activation [14]. Notably, in vivo experiments suggest that during murine TB mature IL-1β can be produced in a caspase-1-independent way [15]. These observations stress the complexity of the inflammatory process particularly in chronic infections such as TB and emphasize the need for a better understanding of how IL-1β release is regulated in molecular terms, both ex vivo and in vivo.

Here, we aim at a deeper molecular understanding of how MTB interferes with inflammasome activation and how this impacts on active TB disease. On the pathogen side, we identified a unique role of the ESX-1 secretion system in inflammasome activation. ESX-1 is of critical importance in TB virulence [16]. On the host side, we identified NLRP3 as critical factor for macrophage-dependent IL-1β secretion, which, however was compensated for during active TB in mice. NLRP3-independent release of mature IL-1β in lung parenchyma is probably a function of myeloid cells, which engage proteases other than caspase-1. We conclude that the importance of regulated inflammation for control of TB has led to the emergence of multiple factors, which facilitate alternative processing of mature IL-1β.

Results

M. tuberculosis activates the NLRP3 inflammasome in macrophages

We interrogated whether MTB interferes with cleavage of pro-IL-1β in macrophages. To this end, we used BM-derived macrophages (BMDMs) primed with ultrapure LPS for subsequent infection experiments. Short-term priming with ultrapure LPS induces pro-IL-1β and thus offers a model to study activation of the inflammasome. MTB infection of both primed and unprimed BMDMs led to the release of IL-1β into supernatants and IL-1β concentrations directly correlated with the multiplicity of infection (MOI) (Fig. 1A). Next, we determined whether infection with the attenuated vaccine strain *M. bovis* BCG caused release of mature IL-1β. LPS-primed BMDMs infected with *M. bovis* BCG induced scant IL-1β secretion, while the propensity of macrophages to release IL-1β following ATP stimulation remained unchanged. In a further step, we generated BMDMs from WT mice and *Nlrp3*-deficient mice (knockout, KO) and infected them with virulent MTB. Abundance of IL-1β was markedly reduced by NLRP3 deficiency (Fig. 1B). Analysis of cell-free supernatants by western blots confirmed that MTB infection of macrophages induced the secretion of both cleaved IL-1β and cleaved caspase-1, in an NLRP3-dependent manner (Fig. 1C). Using unprimed BMDMs, in order to circumvent multiple stimuli, which could interfere with each other and using different MOIs of virulent MTB, similar results were recorded (Fig. 1D). Thus, MTB activated the NLRP3 inflammasome to release mature IL-1β in macrophages and this propensity was apparently lost during attenuation of *M. bovis* BCG.

Phagocytosis, K^+ efflux and the mycobacterial RD1 locus modulate activation of the inflammasome

To investigate whether MTB specifically and exclusively activates the NLRP3 inflammasome, we generated BMDMs from mice deficient in ASC and caspase-1, as well as NLRC4 (*Ipaf*), P2X7 and NOD2. IL-1β was not detected in the absence of ASC and caspase-1, which are both involved in NLRP3-dependent release of mature IL-1β (Fig. 2A and C). In contrast, NLRC4, P2X7 or NOD2 had no impact on IL-1β secretion. ATP stimulation of macrophages from mice deficient in the aforementioned factors served as a control (Supporting Information Fig. 1). Similarly, we interrogated whether CARD9, a cytosolic adaptor that controls IL-1β secretion during MTB infection [17], or other PRRs such as TLR2, contribute to inflammasome activation by MTB. Neither CARD9 nor TLR2 deficiency impacted on inflammasome activation (Fig. 2B and C). Based on our findings that activation of the NLRP3 inflammasome was a unique feature of MTB but not BCG

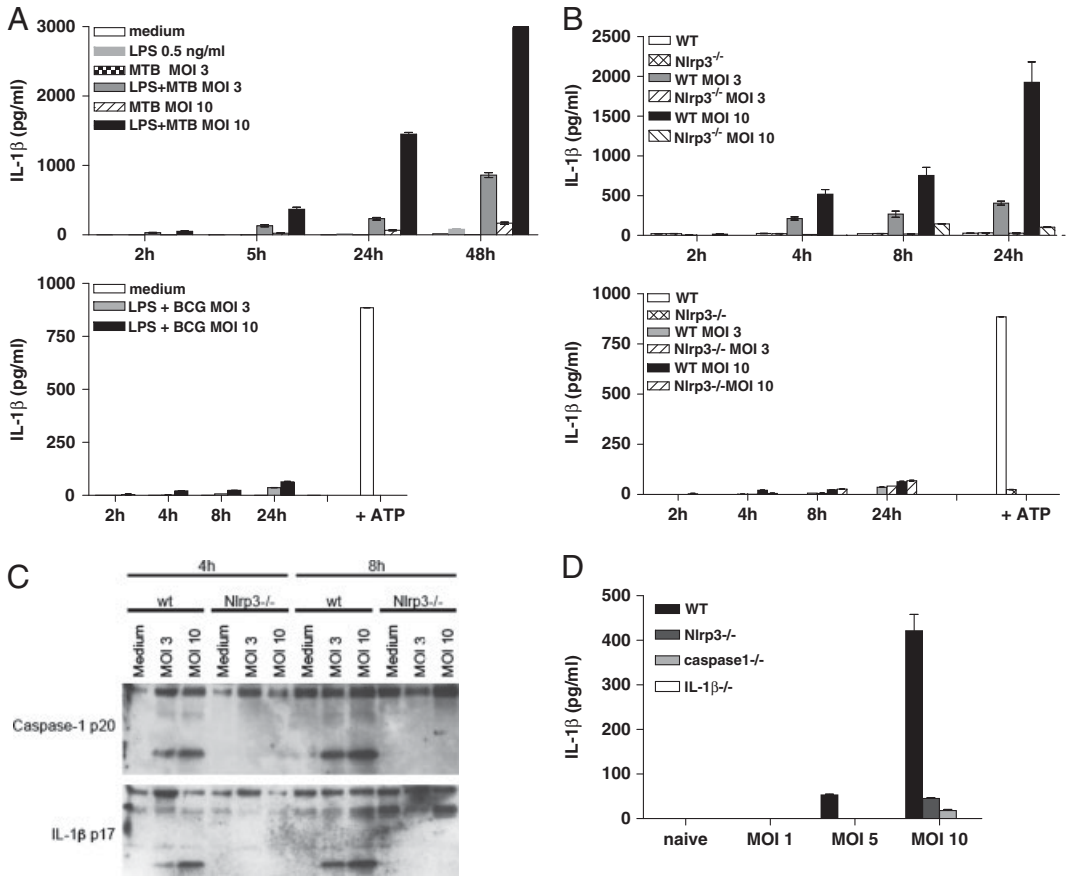

Figure 1. MTB activates the NLRP3 inflammasome in macrophages. (A, B) IL-1β levels were measured by ELISA at the indicated time points in cell-free supernatants of (A) LPS-primed or unprimed BMDMs isolated from WT C57BL/6 mice cultured in DMEM 10% FCS and infected with the virulent MTB strain H37Rv or *M. bovis* BCG, and (B) BMDMs isolated from WT C57BL/6 or Nlrp3$^{-/-}$ mice cultured in OptiMEM and infected with virulent MTB (C). Detection of cleaved caspase-1 and cleaved IL-1β by western blot in cell-free supernatants from LPS-primed WT or Nlrp3$^{-/-}$ BMDMs infected with MTB, (D) IL-1β levels were measured by ELISA in cell-free supernatants of BMDMs isolated from the indicated mice, cultured in DMEM 10% FCS and infected with the virulent MTB strain H37Rv at the indicated MOI. Data are (C) representative of or (A, B, D) the mean+SEM of one experiment representative of three with two to three biological replicates per experiment.

we went on to identify the bacterial gene products controlling this process. BMDMs were infected with MTB deleted in the RD1 locus (H37RvΔRD1). Both activation of caspase-1 and release of mature IL-1β were profoundly reduced when macrophages were infected with H37RvΔRD1 (Fig. 2A–C). Reciprocally, transfer of the RD1 locus into BCG (BCG::RD1) reestablished activation of the inflammasome. Importantly, BCG::RD1 did not modify the abundance of TNF-α (Fig. 2D). Stimulation of LPS-primed BMDMs with recombinant ESAT-6 and CFP-10, major proteins encoded by the RD1 locus, did not result in release of IL-1β (data not shown).

Although the upstream mechanisms involved in NLRP3 inflammasome activation by various stimuli remain unclear certain cellular processes and factors have been characterized. In the subsequent series of experiments, we pre-treated macrophages with chemical inhibitors targeting these factors and processes prior to MTB infection, but post-LPS priming, to determine their involvement: cytochalasin D (phagocytosis), glibenclamide (K^+ channels), piceatannol (spleen tyrosine kinase), bafilomycin (vacuolar ATPase), DPI and APDC (reactive oxygen species, ROS). In this system, production of mature IL-1β was significantly altered when bacterial internalization was impaired and K^+ channels were blocked, but unaffected by other treatments including inhibition of ROS (Fig. 2E and F). Thus, MTB and BCG::RD1 specifically activated the NLRP3 inflammasome depending on a functional ESX-1 secretion system. Moreover, intracellular persistence of MTB and K^+ efflux were necessary for IL-1β release.

Role of NLRP3 in pulmonary TB

To determine the relevance of NLRP3 during pulmonary TB, WT and *Nlrp3*-deficient mice were infected with a low dose (200 CFU) of virulent MTB via aerosol. Intriguingly, infection did not result in different bacterial burdens in lungs and spleen (Fig. 3A). Moreover, the size, morphology and cellular composition of the lung lesions were not affected by NLRP3 absence (Fig. 3B). Granulomatous infiltrates with peri-vascular and -bronchiolar localization were detected in both WT and KO mice at 30 days

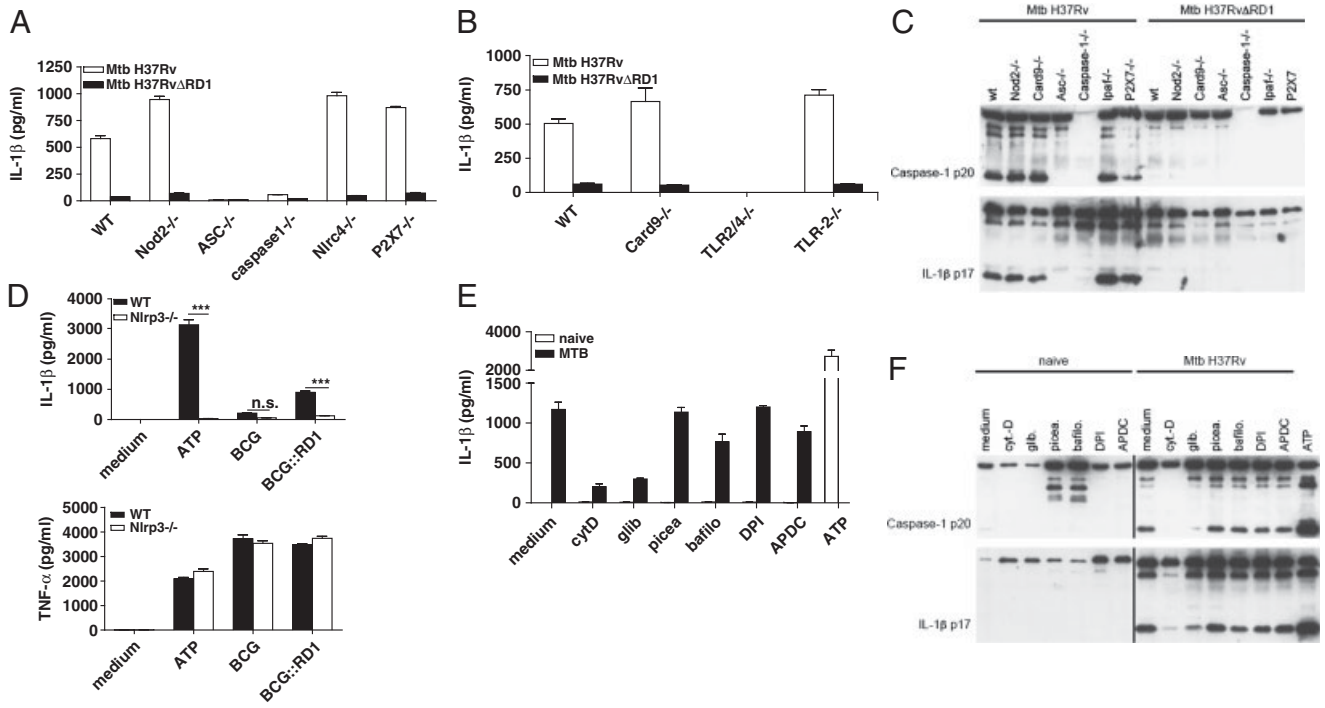

Figure 2. MTB activates the NLRP3 inflammasome via RD1 gene products. (A and B) LPS-primed WT BMDMs cultured in OptiMEM were infected with MTB H37Rv or H37RvΔRD1 at MOI 5 and IL-1β was measured 6 h p.i. by ELISA. (C) western blots of cell-free supernatants from LPS-primed BMDMs isolated from the indicated mice and infected with MTB H37Rv or H37RvΔRD1 at MOI 5 to detect cleaved caspase-1 and cleaved IL-1β. (D) LPS-primed WT and Nlrp3$^{-/-}$ BMDMs cultured in OptiMEM were either infected with BCG or BCG::RD1 at MOI 10 or cultured with LPS plus ATP and IL-1β and TNF-α were determined 6 h p.i. by ELISA. Cell-free supernatants from BMDMs pre-treated with cytochalasin D (cyt), glibenclamide (glib), piceatannol (picea), bafilomycin (bafilo), DPI and APDC and infected with MTB at MOI 5 were (E) analyzed by ELISA to detect IL-1β levels and (F) by western blots to detect cleaved caspase-1 and cleaved IL-1β. Data are (C, F) representative or (A, B, D, E) mean+SEM of one representative of two independent experiments with three biological replicates per experiment. ***p<0.001, two-way ANOVA with Bonferroni's post-test.

post-infection (p.i.). TB progression resulted in lesions consisting mostly of mononuclear cells, which were extended to the whole parenchyma by day 120 p.i. Due to the relevance of inflammatory cytokines, including IL-1β, which are involved in the regulation of leukocyte trafficking to the site of infection, APC populations recruited to the lung and secretory properties of T cells were analyzed by flow cytometry. Details on markers and gating strategies are depicted in Supporting Information Fig. 2. *Nlrp3*-deficient mice were not impaired in the recruitment of granulocytes, inflammatory macrophages and DCs into lung tissue (Fig. 3C). Moreover, the numbers of CD4$^+$ and CD8$^+$ pulmonary T lymphocytes did not differ between both groups. Finally, functions of these T-lymphocyte subsets were apparently not altered. Secretion of IFN-γ by T cells following ex vivo stimulation with MTB peptides, were unchanged in the absence of NLRP3 (Fig. 3C). KO mice mounted delayed-type hypersensitivity responses following BCG vaccination similar in magnitude to WT controls. Finally, course and severity of TB disease in immunized animals did not differ between the mouse strains (Supporting Information Fig. 3). To evaluate the impact of the site of infection in the context of NLRP3 deficiency, we used the dermal infection model [18]. KO and WT mice showed similar kinetics of bacterial growth in both skin dermis and draining lymph nodes (Fig. 3D). These data suggest that irrespective of the site of infection, lung versus dermis, the pattern of response was conserved. We conclude that NLRP3 is not essential for control of pulmonary TB.

Potential mechanisms for NLRP3-independent IL-1β release

In an attempt to define compensatory mechanisms active in the face of NLRP3 deficiency during pulmonary TB, we first measured IL-1β and IL-1α concentrations in lung homogenates of MTB-infected NLRP3 KO and WT mice. Abundance of these cytokines in the lungs remained unaltered over 28 days p.i. in the absence of NLRP3 (Fig. 4A), suggesting NLRP3-independent production of IL-1β during TB. We verified by western blot that indeed NLRP3 deficiency did not affect release of cleaved IL-1β during TB (Fig. 4B). Intriguingly, the size of the mature IL-1β detected in infected lungs differed from the 17 kDa product which results upon caspase-1 cleavage (Fig. 4C). This difference was verified by spiking lung homogenate samples with recombinant mouse IL-1β. Notably, we identified the processed IL-1β at the peak of inflammation, at 3–4 wks p.i. The lower abundance of IL-1α did not allow identification of the cytokine by western blot (data not shown). Our results are suggestive for caspase-1-independent

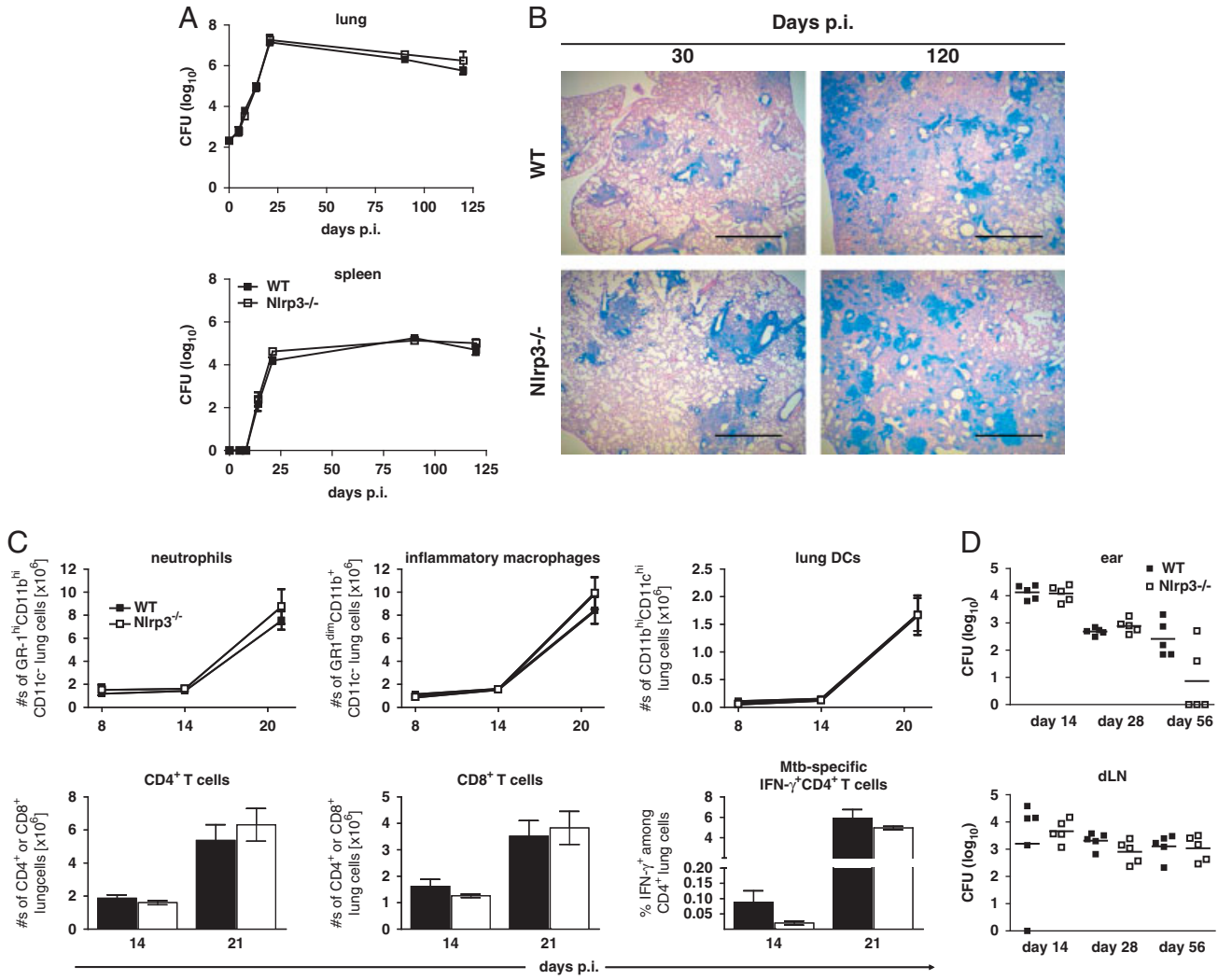

Figure 3. *Nlrp3*-deficient mice efficiently control TB. (A–C) WT and *Nlrp3*-deficient mice were infected via aerosol with virulent MTB H37Rv (200–400 CFU). (A) Bacterial burdens were determined over a period of 3 months p.i. (B), Giemsa staining of lung tissue collected during disease progression, scale bar 1 mm, magnification 50×. Data are (A) mean ± SEM or (B) representative of two independent experiments ($n = 4$–6 per experiment). (C) Single-cell lung suspensions prepared at the indicated times p.i. were either analyzed by flow cytometry using the indicated Abs to detect the frequency of the indicated immune cells or simulated with MTB peptides and the percentage of IFN-γ secreting $CD4^+$ T cells measured by intracellular cytokine staining. Date are mean ± SEM of two independent experiments ($n = 4$–5 per experiment) (D) WT and *Nlrp3*-deficient mice were infected via the skin dermis with virulent MTB H37Rv (10^4 CFU) and bacterial burdens estimated over 2 months p.i. Data are mean ± SEM of one experiment ($n = 5$).

processing of pro-IL-1β. Thus, we aimed at identifying the cellular source of the IL-1β in lung tissue. To this end, we performed immunohistochemical analysis of lung tissue. The staining patterns for IL-1β in infected parenchyma did not differ between WT and KO (Fig. 5A) and suggested similar IL-1β abundance. Cells recruited to inflammatory foci, rather than tissue-resident epithelial cells, were positive for IL-1β staining. We detected positive signals in both MTB-infected and non-infected cells (Fig. 5B). To define more precisely the source and the mechanisms underlying IL-1β production, we analyzed DCs and polymorphonuclear granulocytes (PNGs) as these cells are infected with high frequency by MTB upon aerogenic infection. $CD11c^+$ purified LPS-primed BM-derived dendritic cells (BMDCs) released low IL-1β when NLRP3 was absent, albeit residual cytokine levels were secreted in caspase-1 KO cells (Fig. 5C). PNGs, however, produced IL-1β at low abundance, independent of both NLRP3 and caspase-1 (Fig. 5D). The cell death patterns of the infected PNGs were similar in all tested mouse strains (Supporting Information Fig. 4). Abundance of secreted IL-1α was low in infected PNGs (data not shown), while for BMDCs the secretion pattern was very similar to IL-1β (Supporting Information Fig. 5). We consider PNGs and DCs as likely sources of NLRP3-independent IL-1β production in mice suffering from TB. We cannot, however, formally exclude tissue proteases or macrophages at different activation stages as producers of alternatively cleaved IL-1β.

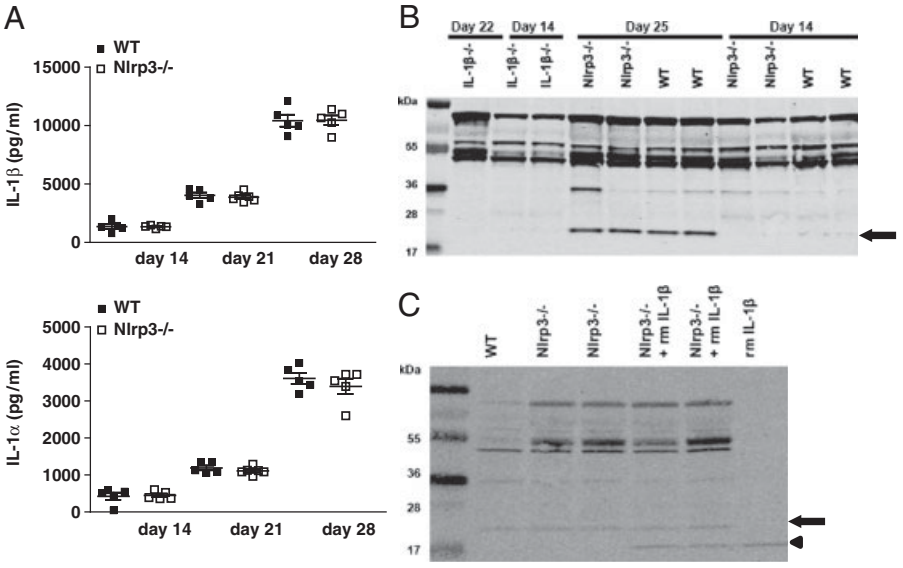

Figure 4. NLRP3-independent release of IL-1β during lung TB. (A) IL-1β and IL-1α in the lung were measured over 28 days p.i. by bead-based immunoassays using samples from WT and NLRP3 KO mice infected via aerosol with virulent MTB H37Rv (200–400 CFU). Each symbol represents data from an individual mouse and the horizontal line and error bars indicate the mean ± SEM. (B and C) Cleaved IL-1β in lung homogenates collected from WT, NLRP3 KO and IL-1β KO mice at different time-points upon infection was determined by western blot. Arrow indicates observed bands in lung homogenates, arrow head indicates band of rmIL-1β. Data are representative of two independent experiments ($n = 5$).

Discussion

Our work shows that IL-1β release during pulmonary TB was NLRP3-independent, while IL-1β production by MTB-infected BMDMs was strictly NLRP3-dependent. MTB induces chronic, nonresolving inflammation of the target organ as a consequence of insufficient clearance of the pathogen [19]. However, despite its chronicity, inflammation in TB undergoes continuous fine-tuning. As long as inflammation is restricted to circumscribed lesions, solid granulomas are beneficial for the host, but once control wanes, lesions caseate and become detrimental. The lung is the main port of entry and prime site of disease manifestation in TB, which is generally transmitted via the aerogenic route. An instructive experiment has recently emphasized the critical role of proper balancing between pro- and anti-inflammatory responses for control of TB [20]. Yet, precise information about how MTB modulates the inflammatory status of the host in molecular terms is scarce. We show that MTB gene products encoded in the virulence-associated gene cluster RD1 specifically trigger NLRP3 inflammasome activation in macrophages. This genetic region is essential and sufficient for NLRP3 activation as demonstrated by experiments with MTB RD1 deletion mutants and *M. bovis* BCG RD1 reconstituted mutants. Yet, during pulmonary TB, alternative mechanisms for NLRP3-independent IL-1β release become operative and it is likely that enzymes other than caspase-1 promote alternative IL-1β processing during active TB.

MTB primarily persist in macrophages. Accordingly, most studies aiming at deciphering the molecular cross-talk between pathogen and host focus on these phagocytes. We found that MTB induces mature IL-1β release in mammalian macrophages similar to *M. marinum* in zebrafish [11]. Both pathogens harness molecules encoded by the RD1 gene complex to activate the NLRP3 inflammasome. Interestingly, the RD1-encoded secretion system was reported to mediate translocation from the phagosome into the cytosol in both species [21, 22]. As lysosomal rupture and release of cathepsins were implicated in NLRP3 inflammasome activation, such mechanisms might also be involved in the case of MTB. Similarly, both MTB and *M. marinum*, but not *M. avium*, utilize a conserved nonlytic spreading mechanism that requires an intact secretion system [23]. In the case of *M. marinum*, cytolysis and phagosome destabilization by ESX-1 is directly linked to specific activation of the NLRP3 inflammasome [11].

Previously, the major RD1 gene product ESAT-6 was considered necessary and sufficient for release of cleaved IL-1β [14]. Our results confirm the crucial role of RD1/ESX-1, but not ESAT-6 alone, in NLRP3 inflammasome activation because addition of the ESAT-6/CFP-10 proteins failed to induce release of IL-1β. In addition to the ESX-1 secretion apparatus of MTB, intracellular persistence of MTB was found critical. Secretion of these proteins with membranolytic activity within the phagosome was necessary for inflammasome activation. Recently, a link between the PhoP regulator and ESAT-6 secretion by MTB was established [24]. However, the propensity of attenuated MTB strains, such as PhoP-deficient H37Ra, to induce inflammasome activation has been demonstrated [25]. Consistent with our results, RD1 was recently reported necessary for caspase-1 activation by MTB [12]. Moreover, in agreement with our experiments this mechanism was found to be independent of P2X7 receptor, but controlled by K^+ efflux. Inflammasome activation by low K^+ concentration is unique for NLRP1 and NLRP3 [26].

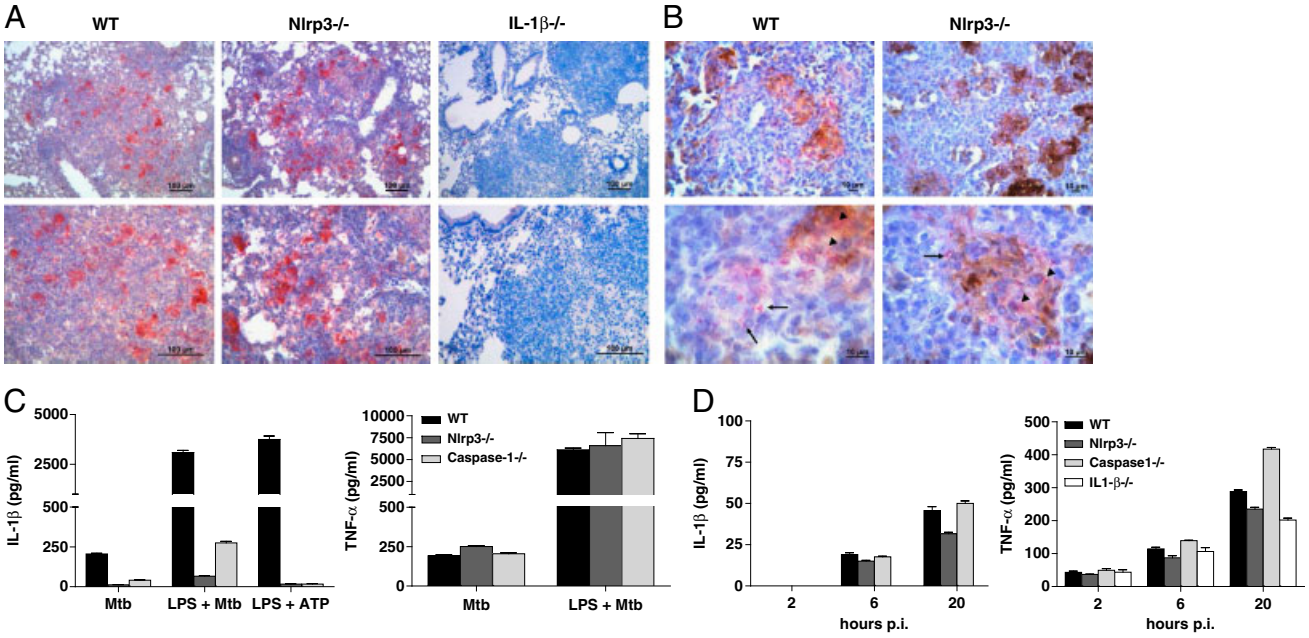

Figure 5. NLRP3-independent production of cleaved IL-1β upon MTB infection. (A) Immunohistochemistry for IL-1β in lung tissue from MTB-infected mice collected at 28 days p.i., scale bar 100 μm, magnification 100 × upper panel, 200 × lower panel. (B) Immunohistochemistry for IL-1β and Ellis staining for MTB in lung tissue from MTB-infected mice collected at 28 days p.i., scale bar 100 μm, magnification 400 × upper panel, scale bar 10 μm and magnification 1000 × lower panel. Arrows indicate MTB in IL-1β negative cells, arrow heads point to MTB co-localized with IL-1β positive cells. (C) IL-1β and TNF-α in supernatants of LPS-primed BMDCs infected with MTB at MOI 5 was measured by ELISA 6 h p.i. (D) IL-1β and TNF-α in supernatants of PNGs infected with MTB at MOI 10 was measured by ELISA at the indicated time-points p.i. Data are (A, B) representative of two independent experiments ($n = 4–6$) or (C, D) the mean + SEM of one representative of three experiments with three to six biological replicates per experiment.

Notably, NLRP3 dependency of IL-1β release was described for other bacterial species, which express cytolysins [27].

Others have associated the mycobacterial *zmp1* gene, which encodes a putative $Zn^{(2+)}$ metalloprotease, with caspase-1-dependent inhibition of the inflammasome [10]. These studies describe a similar phenotype for both MTB and BCG, ruling out RD1 dependency of *zmp1*-related effects. These effects are likely related to caspase-1-dependent, inflammasome-independent mechanisms. In contrast, we found that MTB actively induces cleavage of pro-IL-1β. Moreover, we determined that NLRP3, but not NLRC4 [10], is involved in the formation of an MTB-triggered inflammasome platform. *Zmp1*-interference with phagosome maturation and MTB clearance is potentially due to caspase-1-dependent unconventional protein secretion [28], interference with glycolysis pathways [29], and lipid metabolism [30].

Our experiments and a recent report by others [14] reveal regulation of the inflammasome during MTB infection in resting macrophages. Further investigations in polarized macrophages, which bear resemblance to in vivo infection, remain to be done. Recent findings suggest distinct responses of classical or alternatively activated macrophages to NRLP3-dependent inflammation [31]. These include differences in ROS abundance and actin polymerization (i.e. edge versus intracellular clustering). Intriguingly, molecules involved in inflammasome platforms, such as ASC, co-localize to cellular sites rich in polymerized actin [32]. Thus, impact of the polarized macrophage phenotype on IL-1β release during TB remains to be established.

At first sight, resistance of *Nlrp3*-deficient mice to TB as well as the presence of IL-1β in lungs of these mice might be surprising. However, recent observations, consistent with our in vivo findings [25, 33], emphasize the complexity of the inflammatory responses triggered by MTB during pulmonary infection [15]. A role for necrosis and solely compensatory signaling by IL-1α in this scenario appears unlikely. Concentrations of IL-1α in the lung were similar in both mouse strains. IL-1β production independent from NLRP3 could be caused by variable inflammasome composition in vivo. This could be due to redundancy of different NLRs or involvement of sensors for DNA (AIM2) as reported recently for cytosolic bacteria [34, 35]. Moreover, signals transmitted by PRRs or TNFR regulate the composition of the inflammasome [36]. Alternatively, secretion of IL-1β in the absence of NLRP3 could be governed by cell type-specific regulation. Probably inflammatory monocytes/macrophages, which are actively recruited to MTB-infected tissue sites, differ in caspase-1 activation or show release of IL-1β in a caspase-1-independent fashion. Finally, granulocytes are recruited in high numbers to the MTB-infected lung (Fig. 3C), which can produce IL-1β in a caspase-1-independent manner [37]. We provide evidence that PNGs and DCs release IL-1β, independent of NLRP3 and caspase-1, probably relying on protease activity of other enzymes [38, 39]. Matrix metalloproteases, can cleave pro-IL-1β [40] and are abundantly expressed in the MTB-infected lung. Their role in generation of mature IL-1β will be clarified in future experiments. We cannot exclude occurrence of NLRP3-dependent

p17kDa IL-1β release in lung tissue upon TB, as described for BMDMs. However, secretion via other mechanisms seems to prevail. The alternative cleavage of IL-1β suggests activity of other proteases than caspase-1 [41]. Another possible alternative for release of a higher molecular weight IL-1β could be explained by massive cell destruction. Recognition of damaged tissue rather than sensing of bacilli could induce this form of cleaved-IL-1β. Several studies focused on IL-1β release upon sterile inflammation [42–44] support this notion. Lastly, microorganisms have been claimed to harness self-proteases for pro-IL-1β cleavage [45], and therefore the propensity of MTB to process IL-1β needs to be considered.

In contrast to our findings in TB, resistance against influenza virus [46, 47], *Klebsiella pneumoniae* [48] and *Streptococcus pneumoniae* [49] are markedly reduced in *Nlrp3*-deficient animals. These respiratory infections are characterized by acute progression whereas TB takes a chronic course. Susceptibility of *Nlrp3*-deficient mice to these lung infections has been linked to differential cell death patterns, inflammatory cytokine production and consequently exacerbated lung pathology. In response to MTB infection, in vitro IL-1β production was strictly NLRP3-dependent whilst during pulmonary TB compensatory mechanisms arose, which overcame NLRP3 dependency. We conclude that the persistence of MTB requires continuous fine-tuning of local inflammation and thus host backup systems have evolved that effectively guarantee availability of the central mediator IL-1β.

Materials and methods

Reagents

Ultrapure LPS (5 ng/mL) was purchased from Invivogen; cytochalasin (1 μg/mL), ATP (5 mM), DPI and APDC (50 μM) from Sigma; piceatannol (1 μM); bafilomycin (100 nM) and glibenclamide (25 μM) from Axxora.

MTB and BCG culture

MTB strains H37Rv, H37RvΔRD1 and *M. bovis* BCG strain Danish SSI 1331 were grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.05% glycerol and Tween 80, and 10% ADC enrichment (BD Biosciences) to an early log phase. The BCG strain BCG::RD1 and the control BCG Pasteur strain with the empty vector were grown in the same media, but supplemented with hygromycin (50 μg/mL).

Macrophage, DC and neutrophilic granulocyte cultures and infection

BMDMs were generated from the following mouse strains: C57BL/6, *Nlrp3*$^{-/-}$ [50], *TLR2/4*$^{-/-}$, *TLR2*$^{-/-}$ [51], *Card9*$^{-/-}$ [52], P2X7$^{-/-}$ [53], *Caspase1*$^{-/-}$ [54], *IL-1β*$^{-/-}$, *ASC*$^{-/-}$, *Nlrc4*$^{-/-}$ [55]. Tibial and femural bones were aseptically removed and flushed with DMEM (Invitrogen) medium with 10% FCS (Gibco). BM cells were differentiated to BMDMs in DMEM containing 20% L929-cell supernatant, 10% heat-inactivated FCS, 5% heat-inactivated horse serum, 2 mM glutamine for 5–7 days. Differentiated resting cells and cells pre-treated with ultrapure LPS (5 ng/mL), 10^6 cells per well in a six-well plate in 1 mL media, were infected with mycobacteria in DMEM 10% FCS or OptiMEM (Invitrogen). Alternatively, differentiated BMDMs were seeded at 10^5 cells per well in a 96-well plate in 0.1 mL of media. BMDCs were obtained by culturing BM cells in RPMI 1640 10% FCS supplemented with 200 U/mL recombinant GM-CSF (Strathmann Biotech AG) for 6 days. CD11c$^+$ BMDCs were sorted with magnetic beads (Miltenyi Biotech), seeded at 5×10^5 cells per well in a 24-well plate in 1 mL medium and rested overnight before infection. PNGs were isolated from BM cells with Ly6G magnetic beads (Miltenyi Biotech) and seeded at $1–5 \times 10^6$ cells per well in a 24-well plate in 1 mL media. Single cell bacterial suspensions were prepared prior to infection from log-phase cultures after washing in PBS. Infection was performed 3 h post-LPS stimulation and supernatants were collected at 6 h p.i. or 24–48 h p.i., as described in figure legends. Abundance of IL-1β and TNF-α was determined at various time-points by commercial ELISA (R&D).

MTB infection

WT (C57BL/6) and NLRP3 KO mice were bred at the Max Planck Institute for Infection Biology, Berlin, Germany. Mice were 8–12 wks of age at the beginning of experiments, matched for age and sex, and kept under specific pathogen-free (SPF) conditions. The Glas-Col inhalation exposure system was used for aerosol infection of mice. The challenge dose was determined 24 h p.i. by plating complete lung homogenates onto Middlebrook 7H11 agar plates supplemented with ampicillin (25 μg/mL). To estimate bacterial burdens, at different time points organs were collected in PBS 0.05% v/v Tween 80 followed by mechanical disruption, dilution and plating onto Middlebrook 7H11 agar plates. MTB colonies were counted 3 wks after plating. Ear dermis infections were performed as described [18]. All experiments were performed in accordance with German Animal Protection Law.

Lung cell isolation and flow cytometry

Single-cell suspensions from lungs were prepared as described [56]. For intracellular cytokine staining (ICS), $2–4 \times 10^6$ cells were cultured for 6 h in the presence of BrefeldinA in culture medium either alone or supplemented with the following MTB-derived peptides at 10^{-4} M concentrations: MHC class II: $Ag85A_{241–260}$ (QDAYNAGGGHNGVFDFPDSG), $Ag85B_{240–260}$ (FQDAYNAAGGHNAVFNFPPNG), $ESAT6_{1–20}$ (MTEQQWNFAG IEAAASAIQG); MHC class I: PepA (GAPINSATAM; derived from MTB32A protein) or supplemented with αCD3 and αCD28 mAbs

(10 μg/mL). Cells were blocked with rat serum and αCD16/αCD32 mAb, surface-stained with αCD4-Pacific Blue (RM4-5; BD), αCD8-PerCP (53–6.7, BD) mAb, fixed with 2% paraformaldehyde, permeabilized with saponin buffer, and stained intracellularly with αIFN-γ-PE-Cy7 (XMG1.2, BD) and αTNF-α-PE (TN3-19.2; BD).

For innate immune cell staining, 2–4 × 10^6 cells were blocked with rat serum and αCD16/αCD32 mAb, and stained with αGr-1-PacificBlue (RB6-8C5; ATCC), CD11c-Cy5 (N418; ATCC) and CD11b-PE-Cy7 (M1/70; BD). All stained cells were acquired using a BD FACS Canto II and analyzed with BD FACSDiva and FACS Analyzer software.

Histology

Organs were fixed with 4% w/v paraformadehyde for 24 h and embedded in paraffin. Two-μm sections were stained with Giemsa reagent and assessed for pathology. Immunostaining for IL-1β was performed following heat-mediated antigen retrieval. An affinity purified goat anti-mouse-IL-1β antibody (R&D) was used at 10 μg/mL, followed by staining with a secondary donkey anti-goat antibody (Dako) at 1:200 dilution. Fuchsin (Dako) was used for immunohistochemical visualization. For the double immunostaining we used POD/DAB reagent (Dako) with the antibodies mentioned above to visualize IL-1β and Ellis staining to visualize MTB [57].

Western Blot

Cell culture supernatants were subjected to standard western blot techniques as described [58]. Proteins from cell-free supernatants from experiments done in serum-free OptiMEM medium (Invitrogen) were extracted by methanol/chloroform precipitation. Sheep anti-mouse IL-1β and rabbit anti-mouse Caspase-1 p20 primary antibodies were used. IL-1β in lung homogenates was determined using a goat anti-mouse antibody (R&D), which was previously adsorbed on BCG to minimize cross-reactions.

Multiplex cytokine assays

Lung homogenates were prepared by disrupting PBS-perfused lung tissue in PBS supplemented with a protease inhibitor cocktail (Roche). Cell-free suspensions of lung homogenates and sera were assayed for cytokine and chemokine concentrations using bead-based immunoassay kits (Bio-Plex Cytokine Assay, Bio-Rad or Millipex, Millipore) according to manufacturer's instructions. A Bio-Rad instrument was used for measurements.

Statistical analysis

PRISM Graphpad software was used for statistical analysis. Bacterial titers were analyzed by the Mann-Whitney test. Frequencies and numbers of APCs, cytokine-positive T cells, concentrations of cytokines and chemokines were compared using Student's *t* test or a two-way ANOVA followed by Bonferoni's post-test. Only *P* values ≤ 0.05 were considered statistically significant.

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Abbreviations: **BMDC:** BM-derived DC · **BMDM:** BM-derived macrophage · **MTB:** *Mycobacterium tuberculosis* · **p.i.:** post infection · **PNG:** polymorphonuclear granulocyte · **TB:** tuberculosis

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