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The mycobacterial cord factor adjuvant analogue trehalose-6,6′-dibehenate (TDB) activates the Nlrp3 inflammasome

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ABSTRACT

The success of a vaccine consists in the induction of an innate immune response and subsequent activation of the adaptive immune system. Because antigens are usually not immunogenic, the addition of adjuvants that activate innate immunity is required. The mycobacterial cord factor trehalose-6,6′-dimycolate (TDM) and its synthetic adjuvant analogue trehalose-6,6′-dibehenate (TDB) rely on the C-type lectin Mincle and the signaling molecules Syk and Card9 to trigger innate immunity. In this study, we show that stimulation of bone marrow-derived dendritic cells (BMDCs) with TDB induces Nlrp3 inflammasome-dependent IL-1β secretion. While Card9 is required for NF-κB activation by TDB, it is dispensable for TDB-induced activation of the Nlrp3 inflammasome. Additionally, efflux of intracellular potassium, lysosomal rupture, and oxygen radical (ROS) production are crucial for caspase-1 processing and IL-1β secretion by TDB. In an *in vivo* inflammation model, we demonstrate that the recruitment of neutrophils by TDB is significantly reduced in the Nlrp3-deficient mice compared to the wild-type mice, while the production of chemokines *in vitro* is not influenced by the absence of Nlrp3. These results identify the Nlrp3 inflammasome as an essential mediator for the induction of an innate immune response triggered by TDB.

Introduction

Approximately one third of the world's population is infected with tuberculosis, and the rate of new infections continues to climb (Dye et al. 2005). These statistics reveal that the sole tuberculosis vaccine used in humans, Bacille Calmette-Guérin (BCG), has limited success against this epidemic (Kaufmann 2006) and that new vaccines are required. Promising new strategies to reach this goal utilize recombinant *Mycobacterium tuberculosis* (MTB) antigens as subunit vaccines, including H1, which is a fusion of the MTB proteins Ag85B and ESAT-6 (Kaufmann 2006). Because isolated protein antigens are generally not sufficiently immunogenic on their own, adjuvants are additionally required for successful vaccination strategies. Many immunostimulatory adjuvants are pathogen-associated molecular patterns (PAMPs) or their synthetic analogues, which trigger pattern recognition receptors (PRRs) on cells of the innate immune system. These stimuli drive activation of innate immune cells of the myeloid lineage such as dendritic cells or macrophages, which, in the presence of antigen, induces T cell priming and subsequent activation of antigen-specific adaptive immune responses. Importantly, the type of adjuvant and the innate receptors and pathways that are triggered by these agents activate distinct molecular programs within innate immune cells, which shape the subsequent adaptive immune response (Singh and Srivastava 2003).

The mycobacterial cell wall component trehalose-6,6′-dimycolate (TDM), also called cord factor, is a powerful pro-inflammatory PAMP (Geisel et al. 2005). Based on this finding, a less toxic synthetic analogue, trehalose-6,6′-dibehenate (TDB) has been developed as an effective adjuvant for a tuberculosis subunit vaccine (Davidsen et al. 2005; Holten-Andersen et al. 2004). Both trehalose diesters are insoluble in water (Lemaire et al. 1986) and have a particulate appearance under cell culture conditions. Recent work demonstrated that both TDM and TDB activate innate immune cells by triggering the innate immune receptor macrophage inducible C-type lectin (Mincle) (Wells et al. 2008). Mincle signals by coupling to the immune receptor tyrosine-based

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activation motif (ITAM)-containing Fc-receptor gamma chain (FcRγ), thereby activating spleen tyrosine kinase (Syk) and the adaptor protein Card9. These events result in the activation of nuclear factor-kappaB (NF-κB) and the subsequent up-regulation of activation markers and production of cytokines including TNF-α, IL-6, and IL-1β (Werninghaus et al. 2009; Ishikawa et al. 2009; Schoenen et al. 2010). Importantly, stimulation *via* TDM in combination with vaccine antigens can trigger protective antigen specific T cell immunity against mycobacterial infection *in vivo* (Davidsen et al. 2005). In these settings, TDM is particularly potent in driving T_H-17 immune responses, which have important roles in anti-mycobacterial defense (Werninghaus et al. 2009; Khader et al. 2007).

Signaling *via* various PRRs and cytokine receptors is able to activate the transcription factor NF-κB and thereby upregulate the expression of various pro-inflammatory factors, including IL-1β and other cytokines. Unlike most other cytokines, however, the production of IL-1β is not solely controlled by gene expression. This is because IL-1β lacks a signal peptide and is therefore not secreted through the default ER-Golgi dependent mechanisms employed by other cytokines. Rather, it is produced and retained in the cytoplasm in an inactive pro-form. Maturation of the inactive precursor pro-IL-1β and subsequent secretion requires a second and in principle independent signal. This signal activates intracellular signaling complexes termed inflammasomes (Martinon et al. 2002), which contain and activate the protease caspase-1. Caspase-1 not only cleaves pro-IL-1β into bioactive IL-1β, but also orchestrates the secretion of this cytokine by an unconventional mechanism that remains poorly understood. Several types of inflammasomes have been described, each containing a specific danger sensor that mediates recognition of a distinct stimulus or set of stimuli (Martinon et al. 2009). The best-characterized inflammasome is the Nlrp3 inflammasome, which consists of the sensor molecule Nlrp3, the adaptor protein Asc, and caspase-1 (Mariathasan and Monack 2007). Numerous activators of the Nlrp3 inflammasome are known, including the endogenous danger-associated molecule ATP, monosodium urate (MSU) crystals, the adjuvant alum, as well as various bacterial products (Eisenbarth et al. 2008; Martinon et al. 2006; Perregaux and Gabel 1994). How such a multitude of chemically and structurally diverse compounds can activate the same cellular sensor, as well as the precise mechanism of Nlrp3 activation are still not known. However, several upstream events have been implicated in Nlrp3 activation. These include phagocytosis of the activating particle, phagolysosomal maturation, and subsequent lysosomal destabilization or rupture (Halle et al. 2008; Hornung et al. 2008). The ensuing release of lysosomal cathepsins into the cytoplasm has been suggested to be the mechanistic connection of these events to inflammasome activation (Hornung et al. 2008). Another event required for inflammasome activation is the generation of reactive oxygen species (ROS), as most known inflammasome stimuli trigger ROS generation and treatment with various ROS scavengers blocks Nlrp3 activation in response to the agonists (Dostert et al. 2008). Moreover, potassium efflux is thought to be a prerequisite in Nlrp3 inflammasome activation (Petrilli et al. 2007).

As TDM and TDB are potent inducers of IL-1β production (Werninghaus et al. 2009) we here investigated the specific mechanisms by which TDB triggers IL-1β production. We report that the activation of the Nlrp3 inflammasome is required for the inflammatory response induced by TDB.

Materials and methods

Mice

Mice genetically deficient for Nlrp3, Asc, caspase-1, and Card9 were described previously (Martinon et al. 2006; Kuida et al. 1995; Mariathasan et al. 2004; Gross et al. 2006; LeibundGut-Landmann et al. 2007). Mice were 6–12 weeks of age at the onset of experiments and were housed and used according to local guidelines.

Media and reagents

Cell culture reagents were obtained from Invitrogen. FCS was from HyClone. TDB was purchased from Avanti Polar Lipids. Ultrapure LPS (*Escherichia coli* K12) was from Invivogen. TDM, Adenosine 5′-triphosphate (ATP), z-VAD-FMK, glibenclamide, ebselen, cytochalasin-D, CA-074Me, APDC, and bafilomycin A1 were obtained from Sigma–Aldrich. KCl was purchased from Roth. Imject alum was from Pierce. MSU was prepared as described (Martinon et al. 2006).

Generation of dendritic cells

BMDCs were generated as previously described (Gross et al. 2006; Gross 2012). In short, DCs were differentiated from freshly isolated murine bone marrow cells on Petri dishes at a density of 1×10^6 cells ml^{-1} in complete RPMI containing 20 ng ml^{-1} of recombinant murine GM-CSF (Peprotech) for 7 days.

Cell stimulation

Inflammasome assays were performed as previously reported (Gross 2012). For stimulation, cells were seeded in RPMI medium containing 10% FCS at 1×10^6 cells ml^{-1}. Where indicated, cells were primed with ultrapure LPS (50 ng ml^{-1}) for 2 h prior to addition of inflammasome activators. Cells were stimulated for 6 h (LPS-primed cells) or 24 h (unprimed cells) with TDB (5–300 μg ml^{-1}), TDM (5–300 μg ml^{-1}), alum (500 μg ml^{-1}), or MSU (50 μg ml^{-1}), and for 2 h with ATP (5 mM). Chemical inhibitors were added 1 h prior to cell stimulation with inflammasome activators. Cell-free supernatants were collected for detection of cytokine production. Where indicated, cells were seeded in OptiMEM serum free media for Western blot analysis of the supernatants for the presence of cleaved caspase-1 as described below.

Quantification of cytokines and chemokines

Levels of cytokines were analyzed by enzyme-linked immunosorbent assay (ELISA, BD). Production of chemokines was detected by FlowCytomix (Bender MedSystems).

Western blot

Detection of caspase-1 cleavage and secretion by Western blot was performed as described (Gross 2012). Briefly, cell-free supernatants were well mixed with methanol and chloroform at a ratio of 5:5:1 in 1.5 ml tubes and centrifuged 5 min with full speed in a table-top centrifuge at room temperature. The supernatant above the interphase was discarded and the interphase was washed with methanol, mixed, and centrifuged. The supernatant was discarded and the pellet was dried for 20 min at 37 °C. The pellet was resuspended in Lämmli sample buffer (50 mM Tris/HCl pH 6.8, 5% Glycerol, 2% β-Mercaptoethanol, 1% SDS, 0.01 g Bromphenol blue), heated for 5 min at 95 °C, and directly analyzed or stored at −20 °C until analysis. Electrophoresis of proteins was performed with the NuPAGE system (Invitrogen) according to the manufacturer's protocol. 5% milk in 50 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Trition-X100 was used as blocking and wash buffer. As primary antibody, polyclonal rabbit anti-mouse caspase-1 p10 (M-20) (Santa Cruz, sc-514) antibody was used.

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In vivo peritonitis model

Mice were injected *i.p.* either with 100 μg TDB in 200 μl PBS or with PBS alone. Mice were sacrificed 6 h later and peritoneal cavity washed with PBS. Total cell number was determined and recruitment of neutrophils ($CD11b^+$ $Ly\text{-}6Gr^+$ 7/4-$Neutrophil^+$, all antibodies from eBioscience) was analyzed by FACS.

Statistical analysis

Differences between experimental groups were analyzed using the Prism software and Student's *t* test. Differences were considered significant when $p < 0.05$.

Results

TDB activates the inflammasome

To test whether TDB activates the inflammasome, we first stimulated LPS-primed bone marrow-derived dendritic cells (BMDCs) from wild-type mice with increasing amounts of TDB. The LPS priming allowed us to separate NF-κB activation and pro-IL-1β production from inflammasome activation. While LPS priming alone was insufficient to cause IL-1β secretion, co-treatment with TDB was able to induce the secretion of IL-1β after a 6 h stimulation period in a dose-dependent manner, suggesting inflammasome activation (Fig. 1A). Similarly, TDM induced the secretion of IL-1β, indicating that this ability of TDB resembles that of the naturally occurring cord factor. The amount of IL-1β secreted after stimulation with TDB was comparable to that induced by established particulate inflammasome activators like MSU (Fig. 1B). Because the maturation of IL-1β is dependent on the autocatalytic processing of caspase-1, we analyzed the appearance of the active p10 subunit of caspase-1 in the supernatant upon cell stimulation with TDB (Fig. 1C). Caspase-1 was robustly activated and secreted upon TDB stimulation. Blocking of caspase-1 activity with the pan-Caspase-Inhibitor z-VAD-FMK strongly reduced IL-1β secretion (Fig. 1D, left), while production of TNF-α was largely unaffected (Fig. 1D, right). These data indicate that TDB activates IL-1β production in a caspase activity-dependent manner in BMDCs.

TDB-induced processing of IL-1β requires Nlrp3, Asc, and caspase-1

To analyze which signaling molecules of the inflammasome complex are necessary for the processing of IL-1β, we generated BMDCs from mice deficient in the components of the Nlrp3 inflammasome and measured IL-1β after stimulation with TDB. Stimulating the BMDCs with the optimal TDB dose of 100 μg ml^{-1} showed that the Nlrp3-deficient BMDCs had a marked defect in the secretion of IL-1β as compared to the wildtype BMDCs (Fig. 2A, left). To exclude a general defect of Nlrp3-deficient cells in the production of inflammatory cytokines, we also measured the production of TNF-α (Fig. 2A, right). Both cells from wildtype and from Nlrp3-deficient mice secreted comparable amounts of TNF-α. Consistent with the lack of IL-1β secretion, the activation of caspase-1 was absent in BMDCs deficient in Nlrp3 (Fig. 2B). Moreover, deficiency in the adaptor protein Asc or in caspase-1 caused a failure of IL-1β processing in BMDCs, while the production of TNF-α was not inhibited or even somewhat increased in inflammasome-deficient BMDCs (Fig. 2C and D). These data indicate that TDB activates the Nlrp3 inflammasome for IL-1β secretion.

TDB activates the inflammasome independently of Card9

We have previously demonstrated that TDB activates Card9 signaling in myeloid cells (Werninghaus et al. 2009). We therefore tested whether this pathway is also involved in inflammasome activation. To this end, we first treated unprimed Card9-deficient BMDCs with TDB for 24 h and compared IL-1β and TNF-α secretion from these cells to that from unprimed wildtype and Nlrp3-deficient cells. Card9-deficient cells showed a marked reduction in IL-1β secretion that was comparable to that seen in Nlrp3-deficient cells (Fig. 3A, left). However, while TNF-α production from Nlrp3-deficient cells under these conditions was normal, secretion of the cytokine from Card9-deficient cells was likewise defective (Fig. 3A, right). This indicates that the reduced IL-1β production might be due to a defect in TBD-induced priming rather than in inflammasome activation, which would be in line with the role of Card9 in TDB-induced NF-κB signaling (Werninghaus et al. 2009). To test this, we induced Card9-independent pro-IL-1β synthesis, by priming BMDCs from Card9-deficient mice with LPS before TDB stimulation (Fig. 3B and D). Interestingly, under these conditions, Card9 was not required for TDB-induced secretion of mature IL-1β (Fig. 3B and C). Consistently, LPS-primed Card9-deficient BMDCs showed normal caspase-1 activation (Fig. 3D). Thus, although Card9 is required for TDB induced priming, it is dispensable for the actual inflammasome activation induced by this stimulus.

Inflammasome activation by TDB requires phagocytosis, lysosomal acidification, and cathepsin activity

Nlrp3 activation by crystalline or particulate structures involves phagocytosis and subsequent lysosomal destabilization, rupture, and release of phagolysosomal contents including cathepsins into the cytoplasm (Halle et al. 2008; Hornung et al. 2008). We therefore investigated next whether this pathway is involved in TDB-induced inflammasome activation. First, we blocked phagocytosis by treating BMDCs with cytochalasin D, an inhibitor of the actin polymerization (Hornung et al. 2008). IL-1β secretion upon TDB stimulation was strongly reduced by cytochalasin D pretreatment (Fig. 4A). In contrast, ATP, a soluble activator which is known to trigger Nlrp3 through binding to and activation of the purinoreceptor P2X7 (Martinon et al. 2009), induced normal amounts of IL-1β in the presence of cytochalasin D (Fig. 4A). Next, we blocked lysosomal acidification with bafilomycin A1. Again, this treatment inhibited IL-1β production upon TDB stimulation, but did not affect ATP-induced IL-1β secretion (Fig. 4B). Finally, we used the cathepsin inhibitor CA-074 Me. This treatment also significantly affected the TDB induced inflammasome activation (Fig. 4C). Together, these results indicate that the activation of the Nlrp3 inflammasome by TDB involves the phagolysosomal pathway and cathepsin activity.

K^+ efflux and production of ROS are required for inflammasome activation by TDB

The efflux of potassium from the cytosol is required for Nlrp3 activation (Petrilli et al. 2007; Gross et al. 2011). To investigate whether inflammasome activation by TDB requires reduction of intracellular potassium, we prevented efflux of potassium by adding glibenclamide, a selective inhibitor of ATP-sensitive K^+ channels (Chrabi and Horisberger 1999). Glibenclamide reduced processing of IL-1β in BMDCs after exposition to TDB or alum (Fig. 5A, left). However, production of TNF-α was unaffected by glibenclamide (Fig. 5A, right). Since the inhibition of TDB induced IL-1β release was incomplete, we performed Western blots for caspase-1 as an additional readout. Consistent with the impaired release of IL-1β, the activation of caspase-1 was blocked by glibenclamide in a dose-dependent manner (Fig. 5A, bottom). In further

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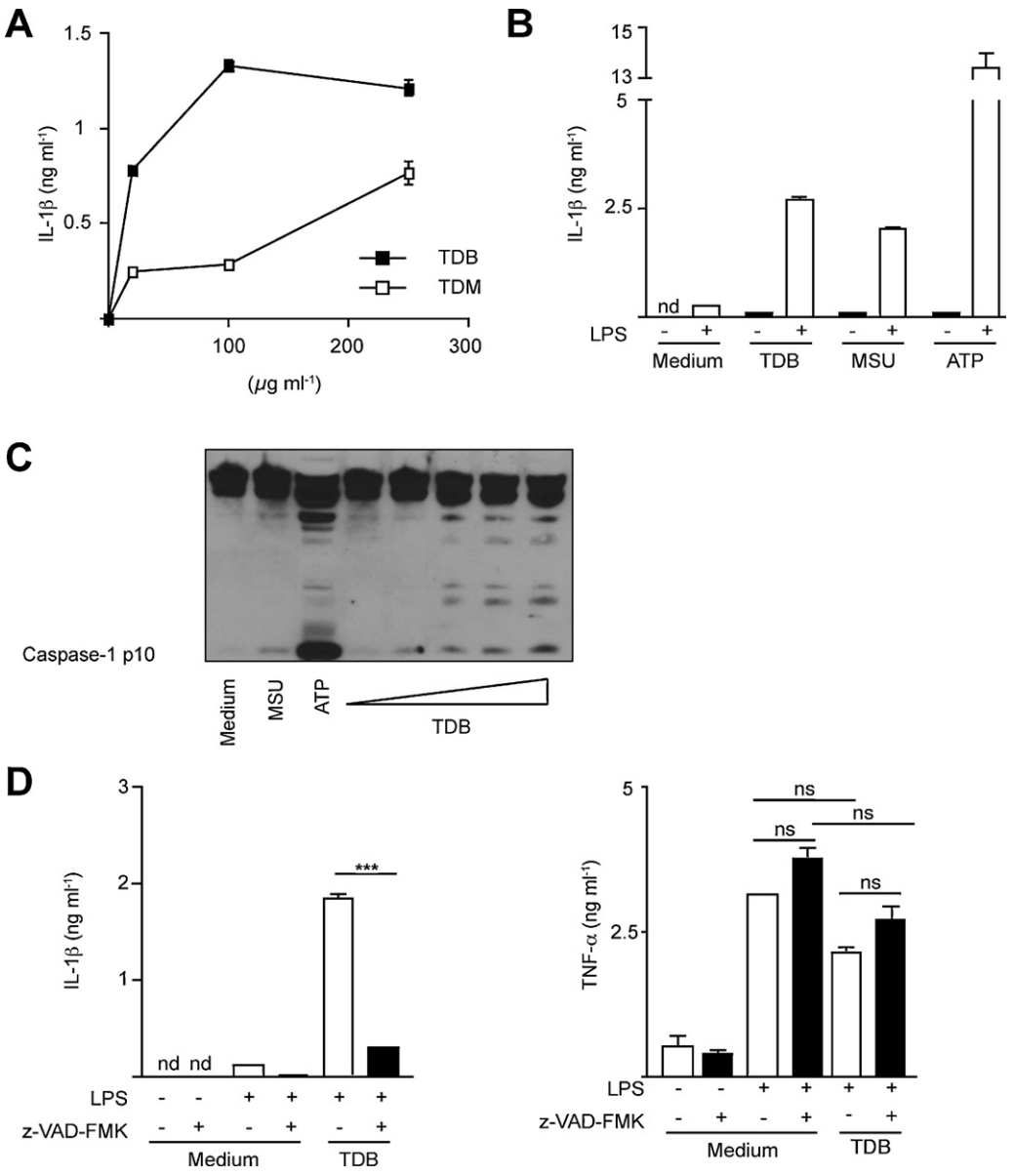

Fig. 1. TDB activates IL-1β secretion and the processing of caspase-1 in a caspase activity-dependent manner. (A) BMDCs were primed for 2 h with LPS (50 ng ml^{-1}) and stimulated with the indicated amounts of TDB or TDM. IL-1β release into the culture supernatant was determined by ELISA. (B) BMDCs were left unprimed or primed with LPS (50 ng ml^{-1}) for 2 h and then stimulated for 6 h with TDB (100 μg ml^{-1}), MSU (50 μg ml^{-1}), or for 2 h with ATP (5 mM) and analyzed as in (A). (C) LPS-primed BMDCs were left unstimulated (medium) or treated with MSU (50 μg ml^{-1}), ATP (5 mM), or TDB (5-10-50-150-300 μg ml^{-1}) and the processing of caspase-1 (p10 subunit) was analyzed in cell culture supernatants by Western blot. (D) Primed BMDCs were pretreated for 1 h with 2 μg ml^{-1} of the pan-Caspase-Inhibitor z-VAD-FMK and stimulated with TDB (100 μg ml^{-1}). IL-1β (left) and TNF-α (right) were measured by ELISA. All values in (A, B, and D) are means ± s.d.; all results are representative of at least three independent experiments. ***$p < 0.001$; ns, not significant; nd, not detected.

experiments we increased the amount of extracellular KCl as a second method to block the efflux of potassium and observed again that the production of IL-1β is significantly reduced after stimulation with TDB (Fig. 5B, left), while the production of TNF-α is not influenced by extracellular potassium (Fig. 5B, right). Production of reactive oxygen species has also been implicated as an event required of Nlrp3 inflammasome activation (Petrilli et al. 2007; Gross et al. 2011; Zhou et al. 2009). Inactivation of ROS by treating the BMDCs with the ROS scavenger (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) diminished the IL-1β production (Fig. 5C, left), but not the production of TNF-α (Fig. 5C, right). Ebselen is described as a glutathione mimetic and a potent scavenger of oxygen radicals (Ledesma et al. 2012) and treatment of BMDCs with ebselen reduced the processing of IL-1β, while TNF-α production was only slightly influenced by this compound (Fig. 5D, right). These results indicate that activation of the Nlrp3 inflammasome by TDB involves potassium efflux and the generation of elevated levels of ROS.

TDB-induced neutrophil recruitment to the peritoneal cavity depends on the Nlrp3-inflammasome

Neutrophils are a key component of the inflammatory response. They are recruited from the bloodstream to the inflamed tissue by secreted factors including IL-1 and various chemokines that are generated by tissue-resident immune cells. To investigate whether the Nlrp3 inflammasome also plays a role in the production of chemokines, we stimulated unprimed BMDCs from wildtype and Nlrp3-deficient mice with TDB for 6 h and detected the secreted amount of various chemokines (Fig. 6A). Chemokine release was not

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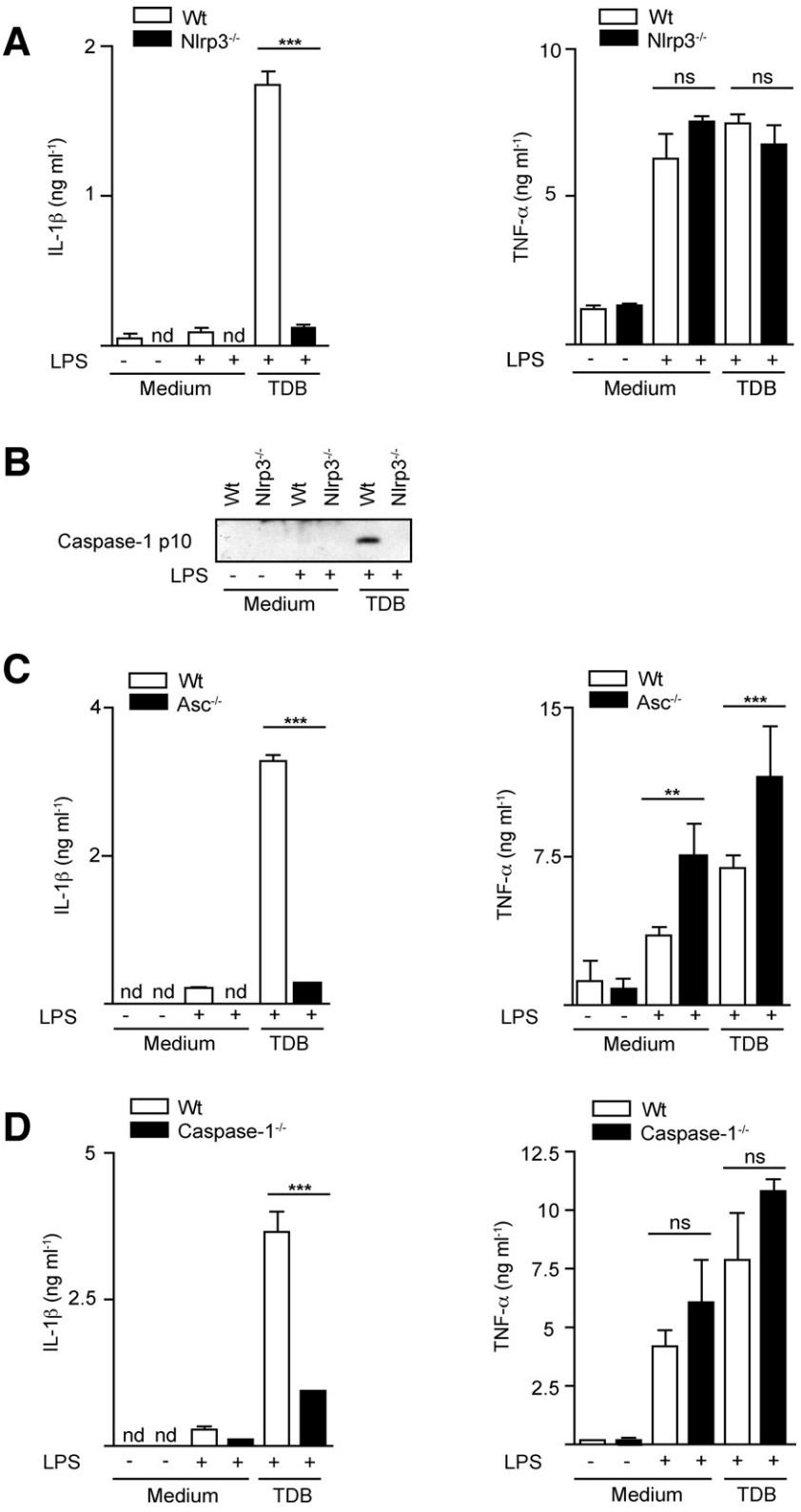

Fig. 2. IL-1β activation by TDB requires Nlrp3, Asc, and caspase-1. (A) Unprimed or LPS-primed BMDCs from wildtype or Nlrp3-deficient mice were stimulated with the optimal TDB dose of 100 μg ml^{-1}. Release of IL-1β (left) and TNF-α (right) into culture supernatant was determined by ELISA. (B) The presence of the caspase-1 p10 subunit in supernatants from unprimed or LPS-primed BMDCs from wildtype or Nlrp3-deficient mice after stimulation with TDB (100 μg ml^{-1}) was detected by Western blot. (C) Unprimed or LPS-primed BMDCs from wildtype or Asc-deficient mice were left in medium or stimulated with TDB (100 μg ml^{-1}) and analyzed as in (A). (D) Unprimed or LPS-primed BMDCs from wildtype or caspase-1-deficient mice were stimulated as in (C) and analyzed as in (A). All values in (A, C, and D) are means ± s.d.; all results are representative of at least three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant; nd, not detected.

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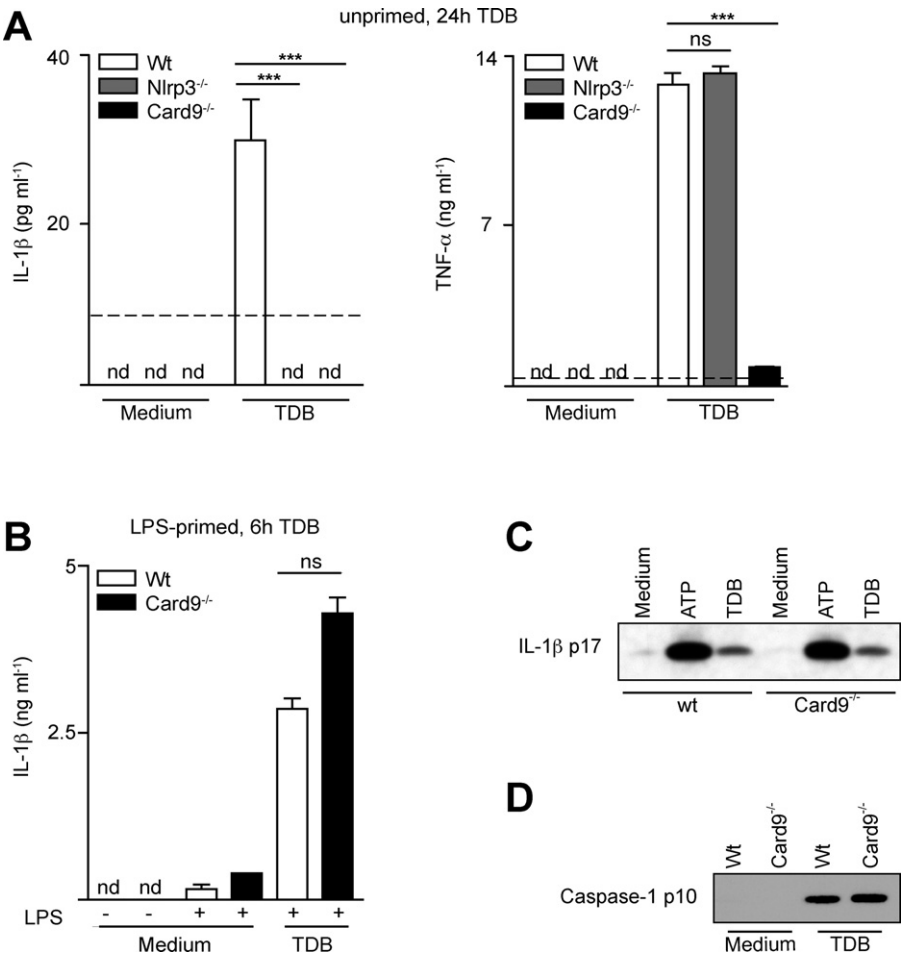

Fig. 3. TDB induces the inflammasome independent of Card9. (A) BMDCs from wildtype, Card9-deficient, or Nlrp3-deficient mice were left unprimed and left unstimulated (medium) or stimulated with TDB (100 μg ml^{-1}) for 24 h. The release of IL-1β (left) and TNF-α (right) into the supernatant was detected by ELISA. (B) BMDCs from wildtype or Card9-deficient mice were left unprimed or were LPS-primed and left unstimulated (medium) or stimulated with TDB (100 μg ml^{-1}) and release of IL-1β into the supernatant was detected by ELISA. (C) BMDCs from wildtype or Card9-deficient mice were LPS-primed and left unstimulated (medium) or stimulated with ATP (5 mM for 1 h) or TDB (100 μg ml^{-1} for 6 h) and release of mature IL-1β into the supernatant was detected by Western blot. (D) Processing and release of caspase-1 p10 after stimulation with TDB was detected by Western blot in supernatants from LPS-primed wildtype and Card9-deficient BMDCs. All values in (A and B) are means ± s.d.; all results are representative of at least four independent experiments. ***$p < 0.001$; ns, not significant; nd, not detected; dashed line, ELISA detection threshold.

affected by the deficiency of Nlrp3, indicating that the regulation of chemokine production is independent of Nlrp3 or an autocrine activity of IL-1β in these cells. To evaluate the impact of Nlrp3 in the induction of inflammatory responses by TDB *in vivo*, we injected TDB into the peritoneum of wildtype and Nlrp3-deficient mice and analyzed the number of neutrophils recruited to the inflamed peritoneum. The mice treated with PBS did not recruit detectable numbers of neutrophils, while the TDB-treated wildtype mice presented with a strong migration of neutrophils. This neutrophil recruitment was significantly reduced in the Nlrp3-deficient mice as compared to the wildtype animals (Fig. 6B). Taken together these results indicate that TDB-induced activation of NF-κB and production of chemokines does not suffice to trigger a full inflammatory response but in addition, requires the Nlrp3 inflammasome for the initiation of an inflammatory response *in vivo*.

Discussion

Our results provide new insight into the molecular mechanisms through which TDB activates innate immunity. It has been reported that the signaling axis Mincle-FcRγ-Syk-Card9 mediates the TDB-induced signaling for NF-κB activation, which in turn induces the production of pro-inflammatory cytokines (Werninghaus et al. 2009; Ishikawa et al. 2009; Schoenen et al. 2010). Furthermore, the particulate adjuvant alum has been shown to be a strong inflammasome activator and this activity contributes to its adjuvanticity (Eisenbarth et al. 2008). Therefore, the aim of this work was to analyze whether the particulate adjuvant TDB can, in addition to Mincle signaling, also activate the inflammasome for the initiation of an inflammatory response. We found that TDB induces IL-1β secretion in BMDCs in a dose-dependent manner by activating the Nlrp3 inflammasome, which contributes to inflammation *in vivo*.

Maturation and secretion of IL-1β requires both a priming stimulus to induce the expression of pro-IL-1β, Nlrp3, and potentially other factors involved in inflammasome activation, as well as a second stimulus that induces the assembly and activation of the inflammasome (Bauernfeind et al. 2009). The translocation of NF-κB from the cytoplasm into the nucleus is detectable 12 h after treatment with TDB and the expression of NF-κB-regulated genes peaks at about 72 h of stimulation (Werninghaus et al. 2009). In line with this, substantial amounts of cytokines like TNF or IL-6 are released only several days after addition of TDB to the cells. The molecular basis for this delayed response remains unclear. In contrast, stimulation with inflammasome activators is usually performed for no more than 6 h in order to minimize side effects like autocrine activity of IL-1β and cell death (Gross et al. 2011). Since we wanted to directly investigate the primary effects of TDB on inflammasome activation, we applied the commonly used *in vitro*

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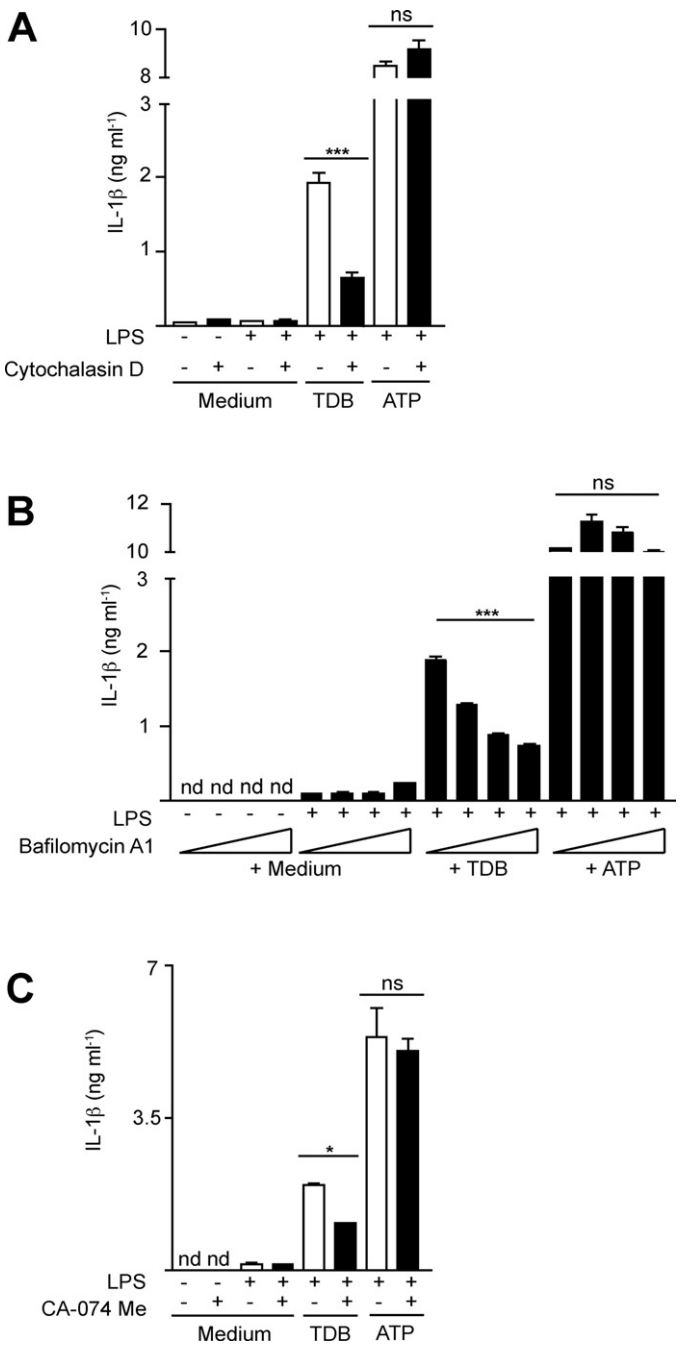

Fig. 4. TDB-induced activation of Nlrp3 relies on phagocytosis, lysosomal acidification, and cathepsin activity. (A) Unprimed or LPS-primed BMDCs were left untreated or cultured with cytochalasin D (100 nM) 2 h prior to stimulation with the indicated stimuli. Release of IL-1β into the cell culture supernatant was measured by ELISA. (B) Unprimed or LPS-primed BMDCs were exposed to increasing amounts of bafilomycin A1 (100-200-400 nM) for 2 h before adding the indicated inflammasome stimuli and IL-1β secretion was analyzed as in (A). (C) Unprimed or LPS-primed BMDCs were left untreated or treated for 2 h with CA-074-Me (10 μM) and stimulated as indicated. IL-1β secretion was detected by ELISA. All values are means ± s.d.; all results are representative of at least three independent experiments. $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant; nd, not detected.

protocol of priming the cells with LPS prior to stimulation with TDB for 6 h. This experimental approach allowed the identification of TDB as a potent activator of IL-1β processing and secretion. The use of cells derived from various mouse knockout lines demonstrates that this ability relies on the activation of the Nlrp3 inflammasome, since IL-1β release is dependent on the presence of Nlrp3, Asc, and caspase-1. Therefore, TDB can simultaneously provide both a priming signal and an inflammasome-activating signal. This dual ability is common for live pathogens, while being rare for pure, synthetic compounds (Gross et al. 2012), and might account for the good adjuvanticity of TDB. However, while in an *in vitro* setting, priming is crucial for full inflammasome activity, *in vivo*, the addition of a second stimulus for priming is dispensable, even for stimuli that do not provide a simultaneous priming signal (Eisenbarth et al. 2008). This has first been observed for alum, which has long been known to be a potent adjuvant on its own, while for inflammasome activation *in vitro*, it requires priming (Eisenbarth et al. 2008). Similarly, the addition of a TLR-ligand like LPS or any other priming additive is dispensable for the *in vivo* use of TDB as shown before by different immunizations (Davidsen et al. 2005; Pimm et al. 1979). Possible explanations for the differential requirement of priming could be the presence of pre-primed (*i.e.*, pro-IL-1β-producing) cells at the site of adjuvant injection, and the action of autocrine feedback loops, as well as the contribution of other cell types in the tissue.

We have previously reported that the Card9 signaling pathway is crucial for the production of pro-inflammatory cytokines in response to TDB *in vitro* by coupling ITAM-coupled receptor stimulation to NF-κB activation (Werninghaus et al. 2009), and could show that this signaling pathway is crucial for TDB adjuvant activity *in vivo*. Here, we found that the priming signal for inflammasome activation, leading to pro-IL-1β production, is likewise Card9-dependent for TDB, while the actual inflammasome activation is Card9-independent. These findings are reminiscent of the inflammasome-activating potential of the opportunistically pathogenic fungus *Candida albicans* (Gross et al. 2009). If and how TDB-induced Card9-dependent priming and Nlrp3-dependent inflammasome activation interact *in vivo* remains to be shown.

Interestingly, we detected an enhanced TNF-α secretion in the Asc- and caspase-1-deficient BMDCs compared to the wild-type BMDCs. This observation might merely reflect pyroptotic cell death in the wildtype cells, leading to a higher number of viable cells producing TNF in the knockout background. However, the finding is also consistent with recent studies reporting that Nlrp3 or Asc activity may dampen NF-κB-dependent pro-inflammatory signals, possibly in order to avoid an inappropriately strong immune response (O'Connor et al. 2003; Jeru et al. 2006; Bedoya et al. 2007). Until now, it remains unclear what mechanisms are used by the inflammasome to affect the NF-κB signaling. In contrast, it has been published that NF-κB regulates the expression of Nlrp3 and potentially other, unknown players, enhancing inflammasome activation (Bauernfeind et al. 2009). TDB, being a rare synthetic compound that can activate both NF-κB and inflammasome signaling might be useful in order to study the poorly understood relations between these pathways.

In order to gain an insight into the mechanisms of TDB-induced inflammasome activation, we performed inhibitors studies. Consistent with previous publications on other particulate inflammasome activators (Hornung et al. 2008; Sharp et al. 2009), we found that blocking of actin polymerization by treating the BMDCs with cytochalasin D prevented inflammasome activation by TDB, implicating phagocytosis as a crucial event. Recently, it has been shown that TDM and TDB are recognized by the C-type lectin-like receptor Mincle, which transduces signals through the common, ITAM containing FcRγ chain (Werninghaus et al. 2009; Ishikawa et al. 2009; Schoenen et al. 2010). ITAM-coupled receptor signaling can directly engage phagocytosis of the recognized particle (Herre et al. 2004). It remains to be investigated whether a deficiency in Mincle or FcRγ is sufficient to prevent phagocytosis of, and thereby potentially inflammasome activation by TDB.

The current model of inflammasome activation by particulates suggests that after phagocytosis, lysosomal rupture and the action of lysosomal cathepsins in the cytoplasm are important events in

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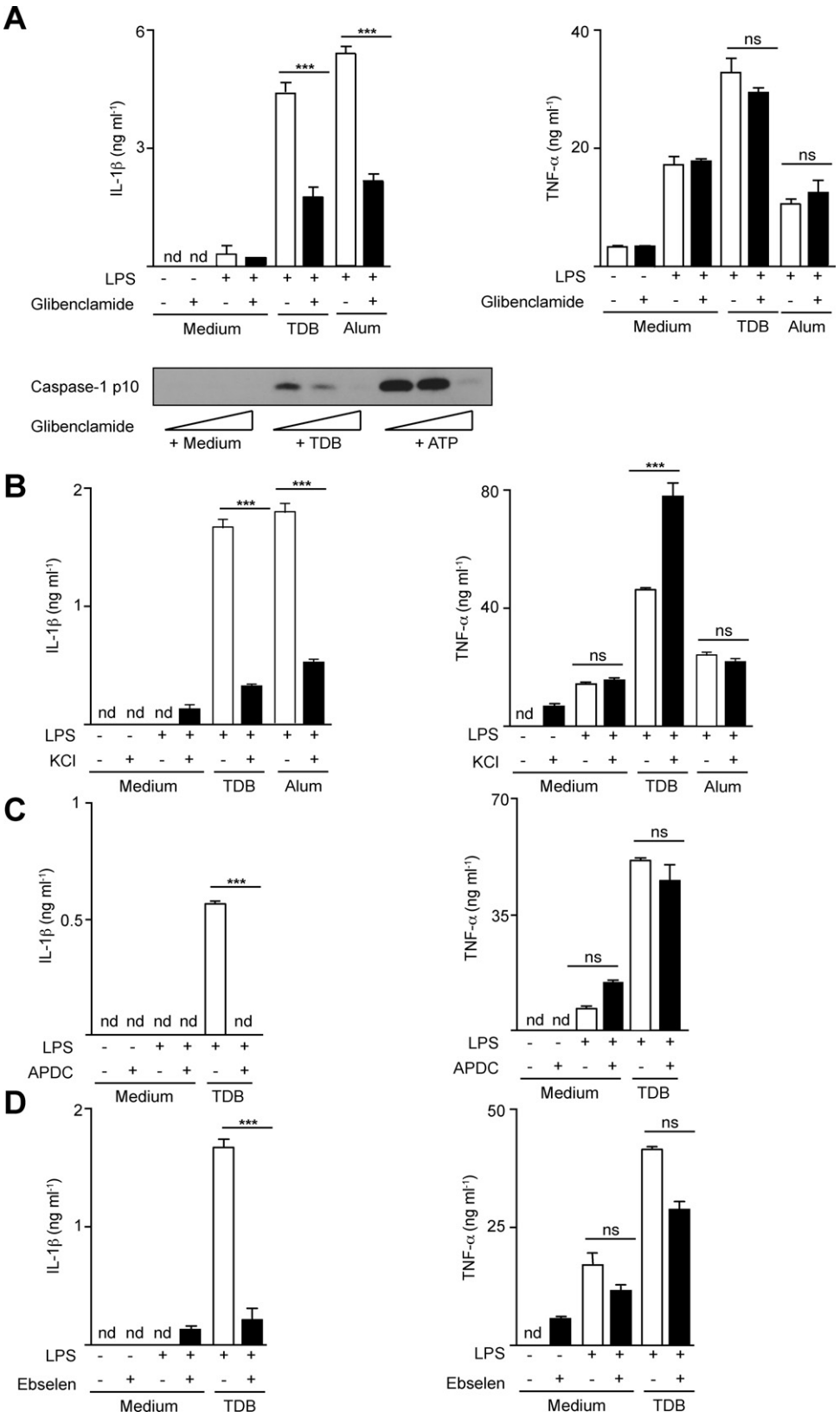

Fig. 5. TDB induced Nlrp3 inflammasome activation depends on efflux of K^+ and elevated levels of ROS. (A) Unprimed or LPS-primed BMDCs were treated with glibenclamide (50 μM) 1 h prior to stimulation with stated inflammasome activators. Imject alum ("Alum") was used in a dose of 500 μg ml^{-1}. Supernatants were collected and IL-1β (top, left) and TNF-α (top, right) were measured by ELISA or caspase-1 p10 was detected by Western blot (bottom). (B) 50 mM KCl was added to unprimed or LPS-primed BMDCs 1 h before stimulation with TDB or Alum. IL-1β (left) and TNF-α (right) were detected by ELISA. (C) Unprimed or primed BMDCs were left untreated or treated with 500 μM APCD for 1 h before addition of TDB and IL-1β secretion was analyzed as in (B). (D) Unprimed or LPS-primed BMDCs were treated with ebselen (2 μM) 1 h prior to stimulation with TDB and IL-1β secretion was analyzed as in (B). All values are means ± s.d.; all results are representative of at least three independent experiments. ***$p < 0.001$; ns, not significant; nd, not detected.

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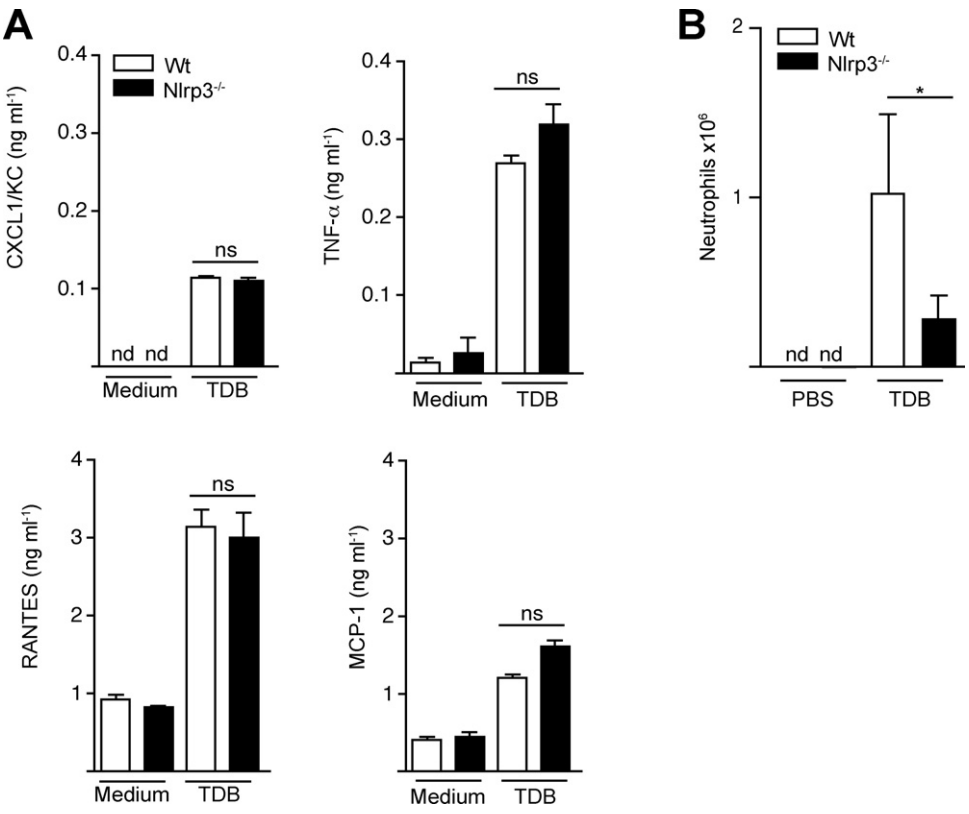

Fig. 6. Neutrophil recruitment to the TDB-inflamed peritoneum depends on Nlrp3. (A) Unprimed BMDCs from wildtype and Nlrp3-deficient mice were left unstimulated or stimulated with TDB (100 μg ml^{-1}) for 6 h. Supernatants were collected and chemokine production detected by CBA. (B) Wildtype and Nlrp3-deficient mice were challenged intraperitoneally with TDB (100 μg ml^{-1}) or PBS and absolute numbers of neutrophils recruited to the peritoneum 6 h later were determined by FACS analysis. All values in (A) and (B) are means ± s.d.; all results are representative of at least three independent experiments. $^{*}p < 0.05$; ns, not significant; nd, not detected.

this process (Tschopp and Schroder 2010). Indeed, we find that inhibitors of lysosomal acidification as well as of cathepsin activity both significantly reduced TDB-induced inflammasome activation. Interestingly, cells deficient in cathepsin B, the main target of the cathepsin inhibitor used, show no phenotype in particle-induced inflammasome activation (Dostert et al. 2009). Therefore, there might be redundancy between several lysosomal cathepsins. Independent of the question which cathepsins are involved, TDB appears to use the same common pathway for inflammasome activation implicated for all other particulate activators.

The two upstream events that are mechanistically involved in Nlrp3 inflammasome activation by all known activators are the reduction of the intracellular potassium concentration and increased ROS levels (Gross et al. 2011). As for TDB, inhibiting potassium efflux efficiently blocked caspase-1 processing and IL-1β secretion. Likewise, ROS inhibitors efficiently blocked TDB-induced inflammasome activation. There is some debate as to whether ROS inhibitors actually block inflammasome activation or merely interfere with priming, since many ROS inhibitors have been shown to reduce NF-κB activity. In order to avoid the contribution of such an effect, we primed our cells for 2 h before adding the inhibitor. The fact that TNF-α production is not reduced by ROS inhibitor treatment implicates that NF-κB activity over the course of the experiment was largely normal. We therefore conclude that both, potassium efflux and elevated ROS levels are common events upstream of inflammasome activation induced by TDB as they are for all other known inflammasome activators.

The combination of TDB and the cationic, micelle-forming surfactant dimethyl dioctadecyl ammonium bromide (DDA) was found to be an effective adjuvant for tuberculosis subunit vaccination, with the ability to raise a high level of protective memory immunity (Holten-Andersen et al. 2004). The efficacy of an adjuvant to induce a protective immunological memory relies on its ability to trigger an innate immune response. Injection of vaccines causes a sterile inflammation with recruitment of neutrophils that are typically guided to the site of inflammation by chemokines. In previous publications, it has been shown that the Nlrp3 inflammasome is the crucial signaling complex in an *in vivo* model of crystal-induced peritonitis (Martinon et al. 2006). Since TDB, in contrast to many pure inflammasome activators, can also activate NF-κB, there might be some redundancy between the two pathways in *in vivo* responses. In line with this, and presumably because of the intact production of chemokines, we detected a basal level of neutrophil influx in the Nlrp3-deficient mice after injection of TDB. As is the case for cytokines like TNF-α, chemokine production in response to TDB might very well depend on Mincle/Syk/Card9 signaling. Indeed neutrophil influx to the lung in response to TDM has been shown to rely on the presence of Mincle (Lee et al. 2012). In our experimental system, the Nlrp3 inflammasome was required to induce a full inflammatory response to TDB. This reduced inflammatory response might well translate into a weakened adjuvant efficacy.

Taken together, our data demonstrate that the ability of TDB to induce an inflammatory response both *in vitro* and *in vivo* involves not only the activation of NF-κB, but also the activation of the Nlrp3 inflammasome. Further studies are required in order to evaluate the relative contribution of the Card9/NF-κB and Nlrp3 inflammasome pathways to DDA + TDB vaccine efficacy. Since the surfactant DDA has an adjuvant activity on its own, it will be important to evaluate whether its activity might also, at least in part, rely on

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inflammasome activation. However, it is likely that many factors, including activation of NF-κB and the inflammasome, contribute to the ability of DDA and TBD to promote immunity to tuberculosis.

Conflict of interest

The authors declare no competing financial interests.

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