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Cigarette Smoke-Induced Pulmonary Inflammation Is TLR4/MyD88 and IL-1R1/MyD88 Signaling Dependent[1]

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Acute cigarette smoke exposure of the airways (two cigarettes twice daily for three days) induces acute inflammation in mice. In this study, we show that airway inflammation is dependent on Toll-like receptor 4 and IL-1R1 signaling. Cigarette smoke induced a significant recruitment of neutrophils in the bronchoalveolar space and pulmonary parenchyma, which was reduced in TLR4-, MyD88-, and IL-1R1-deficient mice. Diminished neutrophil influx was associated with reduced IL-1, IL-6, and keratinocyte-derived chemokine levels and matrix metalloproteinase-9 activity in the bronchoalveolar space. Further, cigarette smoke condensate (CSC) induced a macrophage proinflammatory response in vitro, which was dependent on MyD88, IL-1R1, and TLR4 signaling, but not attributable to LPS. Heat shock protein 70, a known TLR4 agonist, was induced in the airways upon smoke exposure, which probably activates the innate immune system via TLR4/MyD88, resulting in airway inflammation. CSC-activated macrophages released mature IL-1β only in presence of ATP, whereas CSC alone promoted the TLR4/MyD88 signaling dependent production of IL-1α and pro-IL-1β implicating cooperation between TLRs and the inflammasome. In conclusion, acute cigarette exposure results in LPS-independent TLR4 activation, leading to IL-1 production and IL-1R1 signaling, which is crucial for cigarette smoke induced inflammation leading to chronic obstructive pulmonary disease with emphysema. *The Journal of Immunology,* **2008, 180: 1169–1178.**

C hronic obstructive pulmonary disease (COPD)[4] is a major cause of morbidity among pulmonary diseases with high mortality (1). COPD is defined as a disease state characterized by poorly reversible chronic inflammatory response with progressive loss of lung function commonly as a result of cigarette smoking (2). In the bronchoalveolar lavage (BAL) fluid from COPD patients, an increase of proinflammatory cytokines and chemokines including TNF-α and IL-8 has been reported, and these mediators may play an important role in establishing and maintaining the inflammatory condition, characterized by high local neutrophilia (3). Cigarette smoke-induced chronic inflammation leads to the destruction of alveolar septae, and to the loss of surface area for gas exchange and to loss of elasticity known as

emphysema (4). Cigarette smoke exposure rapidly induces production of reactive oxygen species impairing endothelial functions (5). The mechanisms leading to these changes after lung exposure to cigarette smoke are not completely understood. Emphysema may be due to a relative excess of cell-derived proteases, mainly serine proteases such as neutrophil elastase and matrix metalloproteinases (MMPs), that degrade the connective tissue of the lung, and to a relative paucity of antiproteolytic defenses (3). Among the MMPs, MMP-9 and MMP-12 produced by inflammatory cells or tissue cells seem to play a predominant role in the pathogenesis of emphysema. Indeed, increased concentrations of MMP-12 have been reported in the BAL fluid from COPD patients (6). In this study, using an acute model of cigarette smoke-induced inflammation in mice (7), we asked whether cigarette smoke components may be recognized by molecular pattern recognition receptors of the innate immune system such as TLRs, which sense not only microorganism associated molecular patterns but also environmental agents (8) or danger signals like heat shock proteins (hsp) (9–12) or high mobility group box 1 protein (13, 14).

The endotoxin that activates TLR4 is a component of pollution and smoke (15). Therefore, we addressed the role of TLR4 recognition and signaling in cigarette smoke-induced airway inflammation. Using mice deficient for TLR4, the adaptor protein MyD88 (16), or IL-1R1 (17), we report in this study for the first time that TLR4 is involved in the inflammatory response to cigarette smoke both in vitro and in vivo, and that MyD88/IL-1R1 signaling is central to this response.

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[4] Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage; MMP, matrix metalloproteinase; Penh, enhanced pause as a sign of respiratory dysfunction; TTBS, Tween 20 Tris Buffer Saline; MPO, myeloperoxidase; BMDM, bone marrow-derived macrophages; CSC, cigarette smoke condensate; 7-AAD, 7-aminoactinomycin D; hsp, heat shock protein; WT, wild type.

Materials and Methods

Animals

Mice deficient for MyD88 (18), TLR4 (19), or IL-1R1 (17) were used at backcross 10 on C57BL/6 genetic background. All mice, including wild type (WT) control C57BL/6, were bred in our animal facility at the Transgenose Institute (Centre National de la Recherche Scientifique, Orleans,

France). For experiments, adult (8–10-wk-old) animals were kept in ventilated cages. All animal experiments complied with the French government's ethical and animal experiment regulations. All animals had access to water and food ad libitum before and after exposure.

Cigarette smoke and endotoxin exposure

Mice were placed in a plexiglass chamber (volume of 17L) covered by a disposable filter. The smoke produced by cigarette burning was introduced at a rate of 25 ml/min into the chamber with the continuous airflow generated by a mechanical ventilator (Heidolph PD 5101), with no influence on the chamber temperature ($<0.1°C$ variation). The animals received smoke of two references 1R3 cigarettes (Tobacco Health Research; University of Kentucky, Lexington, KY) per exposure, 2 exposures a day during 3 days. After the last exposure, the airway resistance was measured for 15 h. Sixteen hours after exposure, BAL was performed.

For comparison, mice were exposed to endotoxin as described before (20). In brief, LPS (0.01 to 0.1 μg) from *Escherichia coli* (serotype 055: B5, Sigma-Aldrich) in saline or saline alone was given by intranasal instillation (50 μl) under light ketamine-xylasine anesthesia. After challenge, respiratory function was measured. Sixteen hours after LPS administration, BAL fluid was collected.

Measurement of respiratory dysfunction

The airway resistance was evaluated by whole-body plethysmography (21). Unrestrained conscious mice were placed in whole-body plethysmography chambers (EMKA Technologies). Enhanced Respiratory Pause (Penh) was recorded over a period of 3 to 15 h. Penh can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves. Increased phase shift correlates with increased respiratory system resistance. Penh is calculated by the formula Penh = (Te/RT-1) × PEF/PIF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow (21). Data are analyzed using Datanalyst Software (EMKA Technologies) and expressed as mean ± SEM of Penh per group.

Bronchoalveolar lavage

Sixteen hours after LPS challenge or after the last cigarette smoke exposure, mice were sacrificed and BAL was performed. The BAL fluid was collected by canulating the trachea under deep ketamine-xylasine anesthesia and washing the lung four times with 0.5 ml of saline at room temperature as described before (21).

Total cell count was determined in BAL fluid using a Malassez chamber. Differential cell counts were performed on cytospin preparations (Cytospin 3, Thermo-Shandon) after May-Grünwald/Giemsa staining (DiffQuick, Medion Diagnostics). Differential cell counts were made on >200 cells using standard morphological criteria.

Western blotting for hsp70 expression

Proteins contained in BAL samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (5 μg of total protein per lane). The membrane was blocked for 1h at room temperature with 10% nonfat dry milk in Tween 20 Tris Buffer Saline (TTBS). The blot was then incubated for 1 h at room temperature with the primary Ab (anti hsp70, clone 7; BD Biosciences) in blocking buffer as above. After two washes in TTBS, the bands were visualized with HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) via an ECL detection system (Amersham Biosciences).

Western blotting for IL-1β expression

Proteins contained in cell culture supernatants were precipitated by addition of 0.2 volume of chloroform and 1 volume of methanol. Protein precipitates were resolubilized in 0.5 M Tris-HCl (pH 6.8). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (2 μg of total protein per lane). The membrane was blocked for 1 h at room temperature with 10% nonfat dry milk in Tween 20 TTBS. The blot was then incubated for 2 h at room temperature with the primary Ab (anti IL-1β, clone B122, Santa Cruz Biotechnology) in blocking buffer (TTBS with 5% nonfat dry milk). After two washes in TTBS, the bands were visualized with HRP-conjugated goat anti-Armenian hamster IgG (Santa Cruz Biotechnology) via an ECL detection system (Amersham Biosciences).

Lung homogenization

After BAL, lungs were removed and placed inside a microtube (lysing matrix D, Q Bio Gene) with 1 ml of PBS (Invitrogen Life Technologies), total lung tissue extract was prepared using a Fastprep system (FP120, Q Bio Gene); the extract was then centrifuged and the supernatant stored at $-80°C$.

Mediator measurements

TNF, IL-1β, IL-6, and keratinocyte-derived chemokine (KC) levels in BAL fluid, lung homogenate, or cell culture supernatants were determined using ELISA kits (Duoset R&D Systems) according to the manufacturer's instructions. MMP-9 levels in BAL fluid or lung homogenate were determined by gelatin zymography. In brief, nonreduced supernatant samples of BAL fluid (15 μl) were loaded onto 7% polyacrylamide gels (wt/vol) incorporating 0.1% (wt/vol) gelatin substrate. Proteins were subjected to electrophoresis at 30 mA for 3 h. The gel was then washed twice in 2.5% Triton X-100 (vol/vol; ICN Pharmaceuticals), rinsed three times quickly with distilled water, and placed two times for 20 min in distilled water. Each different wash was made under gentle stirring. Gels were incubated 20 h at 37°C in 50 mM Tris buffer (containing CaCl$_2$ 5 mM and ZnCl$_2$ 2 μM). Finally, gels were stained in Coomassie Blue and then destained progressively until bands of lysis (enzyme activity) in the gels showed up as regions of negative staining. The areas of lysis in the gels were analyzed using a densitometric analyzer (BIO1D, Vilbert-Lourmot), images were taken, and band densities were measured.

### Histological investigation

For histological analysis, the lung vascular system was perfused through the right heart ventricle with ISOTON II. After complete perfusion, the lungs were excised and fixed in 4% buffered formaldehyde and processed for microscopic examination using H&E (22). The lesions were assessed semiquantitatively using a severity score ranging from 0 to 5 (from no lesion to severe lesions). Lungs from other animals in the experimental groups were frozen in isopentane at $-40°C$ and stored at $-80°C$. Cryostat sections (8 μm) of the median region of the right lobe were fixed in acetone and stored at $-80°C$. The number of neutrophils present in the lung slides was evaluated in situ using a myeloperoxidase activity detection. In brief, myeloperoxidase in neutrophils reacts with H$_2$O$_2$ and O-dianisidine added to the section, giving a brown-black staining color that is detected by microscopy. Myeloperoxidase (MPO) positive cells were scored in 20 microscopy fields per mouse and given in arbitrary units.

Cigarette smoke condensate

Cigarette smoke from 1R4F reference cigarette (University of Kentucky, Louisville, KY) was collected on Cambridge filter pads as described (23). The mass of particulate matter was determined by weighing the dried filter pads before and after cigarette smoke filtration. The particulate phase was then dissolved in DMSO at a final concentration of 10 mg/ml. The CSC concentration used in the in vitro experiments corresponded to <1% of pure DMSO (vol/vol) in the final volume of cell cultures in the presence of stimuli and in controls.

BMDM culture and stimulation in vitro

Primary bone marrow-derived macrophages (BMDM) were obtained from femoral bone marrow as described (24). In brief, cells from the femur of mice deficient for TLR4, MyD88 or IL-1R1 and C57BL/6 mice were isolated and cultured at 10^6 cells/ml for 7 days in DMEM (Sigma-Aldrich) supplemented with 20% horse serum and 30% L929 cell conditioned medium as a source of M-CSF. Three days after washing and reculturing in fresh medium, the cell preparation contained a homogenous population of >95% macrophages. The BMDM were plated in 96-well microculture plates (at 10^5 cells/well) and stimulated with LPS (*Escherichia coli*, serotype 055:B5, at 100 ng/ml), a synthetic bacterial lipopeptide Pam$_3$CSK$_4$ ([S-[2,3-bis-(palmitoy-loxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys$_4$-OH], trihydro-chloride, EMC Microcollections at 0.5 μg/ml), or cigarette smoke condensate (CSC, 1–25 μg/ml). To neutralize endotoxins, samples were treated with polymyxin (10 μg/ml) at a concentration pretitrated for neutralizing 100 ng/ml LPS. For IL-1β maturation, the activated macrophages were incubated in the presence of 5 mM ATP 20 min before harvesting. Alternatively, CSC (25 μg/ml) was added for 6 h onto activated cells before cell harvest. Cell supernatants were collected after 20 h of stimulation and analyzed immediately for cytokine content or stored at $-80°C$. Absence of cytotoxicity of the different stimuli used was verified by MTT using standard protocol. Briefly thiazollyl blue tetrazolium bromide solution was added onto the cells after supernatant collection and incubated for 2 h at 37°C. 10% SDS acetic acid solution is then added. MTT reduction to formazan was quantified by the absorbance at 610 nm. Necrosis and apoptosis were also monitored by flow cytometry using 7-aminoactinomycin D (7-AAD) and Annexin V staining. Although 1 mg/ml CSC induced necrosis with 93% of 7-AAD and Annexin V double positive cells, at concentrations of CSC up to 25 μg/ml >94% of cells were viable,
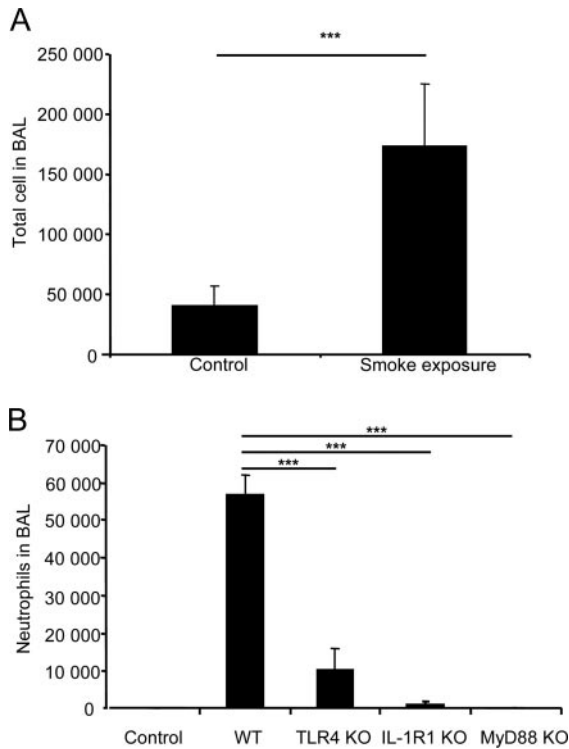
FIGURE 1. Cell recruitment in the airways after smoke exposure mediated by TLR4, MyD88, and IL-1R1. Cell recruitment into the BAL after two cigarettes twice a day for three consecutive days was analyzed. *A*, Total cell counts in the BAL of C57BL/6 (WT). *B*, Neutrophil in the BAL of WT, TLR4, MyD88 and IL-1R1 deficient mice. Data are representative of three independent experiments and are expressed as mean values ± SD ($n = 4$ mice per group). Some macrophages, but no neutrophils, were detected in the BAL of wild-type and IL-1R1-deficient mice not exposed to smoke ($n = 3$), and <1400–3600 neutrophils were detected in MyD88- and TLR4-deficient mice, respectively ($n = $ 2–3). (∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

double negative for Annexin V and 7-AAD, similar results were obtained in wild-type, TLR4-, MyD88-, or IL-1R1-deficient macrophages, and the viability was not affected in the presence of polymyxin or ATP.

Statistical Analysis

Data are presented as mean values and SD indicated by error bars, if not otherwise indicated. Statistical significance was determined by ANOVA analysis with Bonferroni's multiple comparison test, or by Student's *t* test for two group comparison. *p* values of <0.05 were considered statistically significant.

Results

Cigarette smoke exposure induces TLR4, MyD88, and IL-1R1-dependent cellular inflammation in the airways

C57BL/6 mice exposed to two cigarettes twice a day for three consecutive days developed a marked inflammatory cell recruitment into the bronchoalveolar space (Fig. 1*A*), consisting of neutrophils, and to a lesser extent of macrophages and lymphocytes, as previously described (7). To assess the potential contribution of endotoxins contained in cigarette smoke, we first asked whether the cellular influx was TLR4 dependent. Cigarette smoke-exposed TLR4-deficient mice displayed reduced neutrophil recruitment in BAL (Fig. 1*B*), whereas macrophages and lymphocytes were essentially unchanged (data not shown). Furthermore, the neutrophil inflammatory response was ablated in MyD88-deficient mice, in which most TLR dependent signals are impaired (Fig. 1*B*). Because MyD88 is also involved in IL-1R1/IL-1R-associated kinase
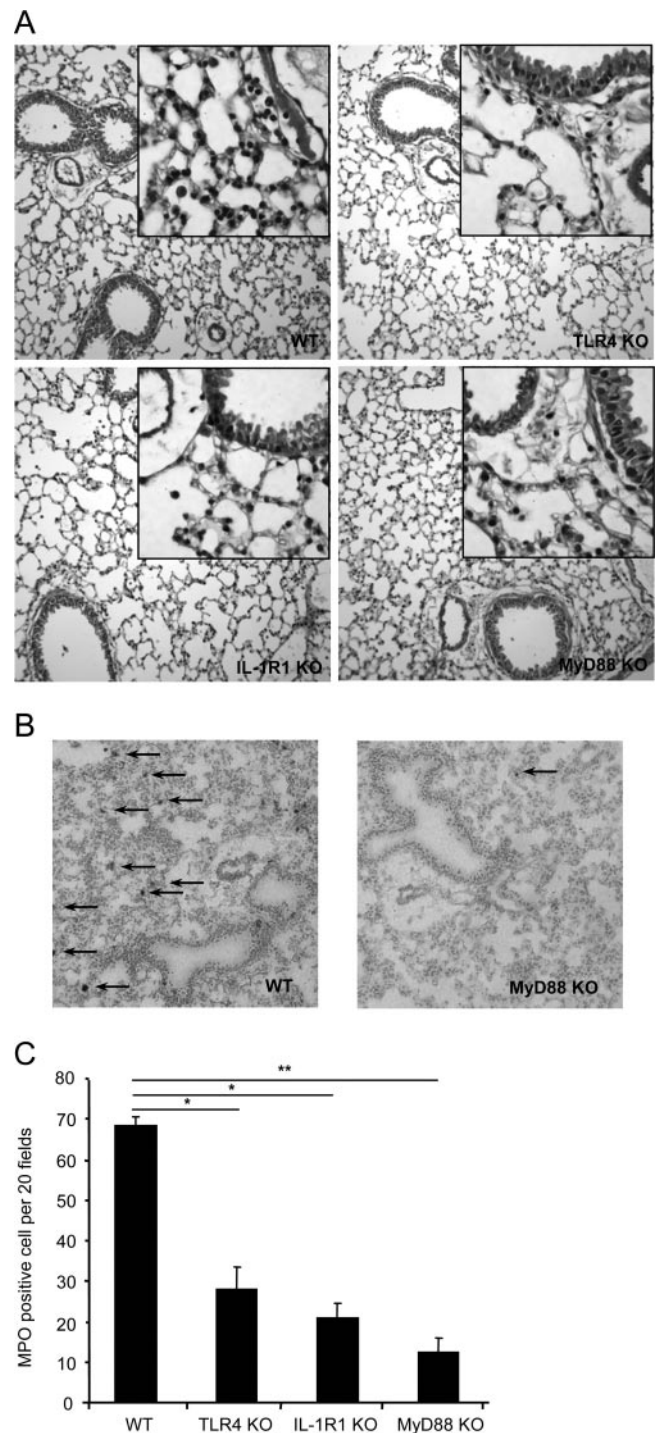
FIGURE 2. Microscopic analysis of the presence of neutrophils in lung tissues after smoke exposure in C57BL/6, TLR4, MyD88, and IL-1R1-deficient mice. *A*, Micrographs of the lungs with reduced neutrophils in TLR4-, MyD88-, and IL-1R1-deficient mice, H&E staining (×20, ×100). *B*, Myeloperoxidase staining of frozen sections as described in methods in WT and MyD88-deficient mice (×20). *C*, MPO activity score in all groups. The number of MPO positive cells was determined in 20 fields per mouse. Mean values ± SD ($n = 4$; ∗, $p < 0.05$; ∗∗, $p < 0.01$) from one of two representative experiments are shown.

signaling, we investigated the response of IL-1R1-deficient mice to smoke exposure. We show that in the absence of IL-1R1, neutrophil recruitment in response to smoke exposure was dramatically decreased (Fig. 1*B*).
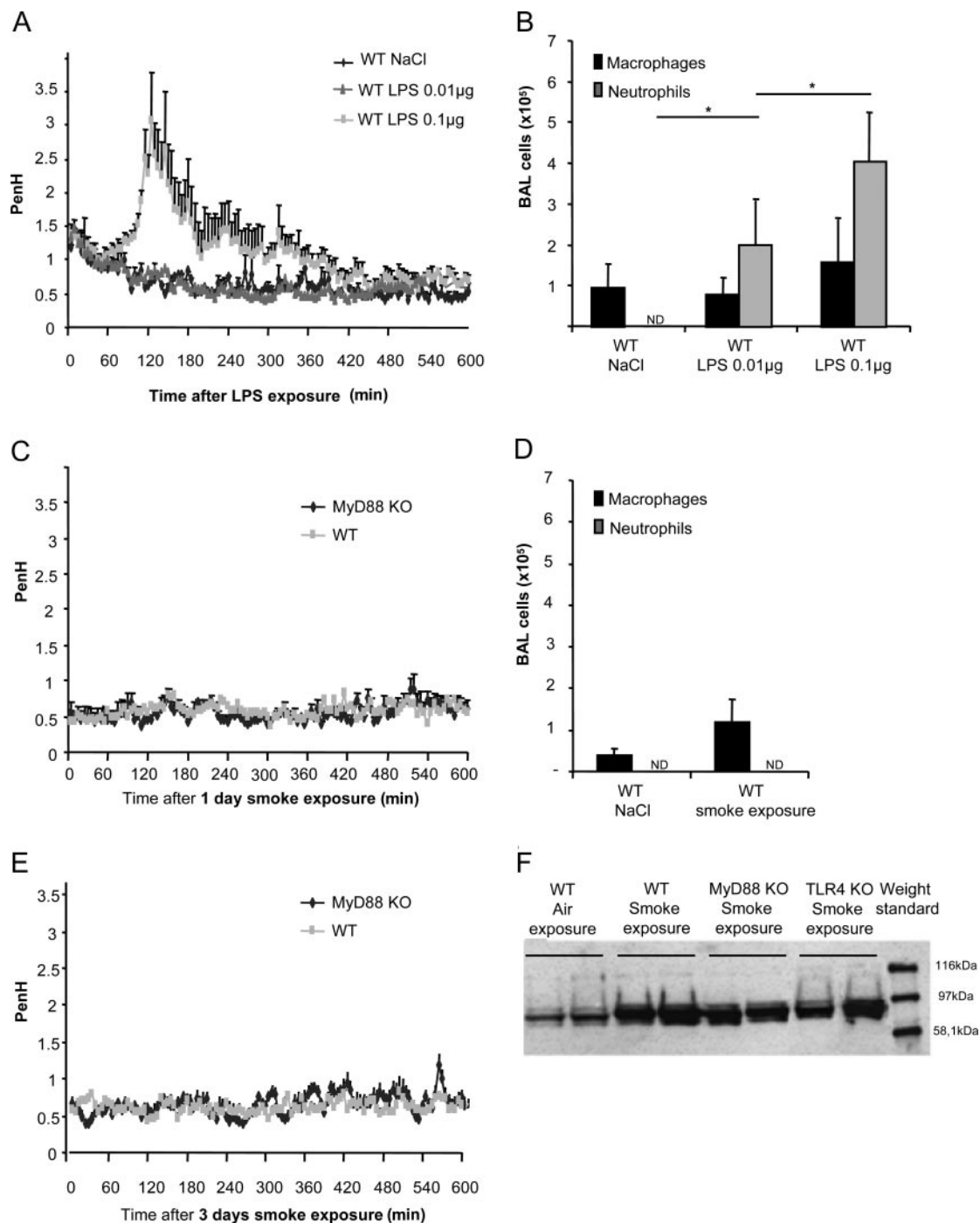
FIGURE 3. Comparison of low-dose intranasal LPS and cigarette smoke exposure effects on respiratory dysfunction in C57BL/6 and MyD88 deficient mice. *A*, LPS given intranasally at a dose of 0.1 μg induces an augmentation of Penh measured by plethysmography, whereas 0.01 μg LPS behaved as saline control. Macrophage and neutrophil recruitments into BAL, 24 h after challenge with LPS (0.01 μg or 0.1 μg) (*B*) or after a single day cigarette smoke exposure (*D*) are shown. Cigarette smoke exposure, either on a single day (*C*) or on three consecutive days (*E*) had no effect on Penh, neither in WT nor in MyD88-deficient mice. *F*, Western blot analysis revealed an induction of Hsp70 in BAL fluid after 3-day cigarette smoke exposure in WT, but also in MyD88- and TLR4-deficient mice, as compared with air-exposed control mice (results from two individual mice per genotype are shown). Mean values ± SD (*n* = 4) or blots from individual mice from one of three representative experiments are shown.

Investigations of the lung parenchyma confirmed the presence of increased numbers of neutrophils in lung tissue sections of mice exposed to cigarette smoke (Fig. 2*A*), which was less pronounced in TLR4- and MyD88-deficient mice. Neutrophil recruitment in the lung was quantified by assessing MPO activity in frozen section. The MPO activity induced after cigarette smoke exposure was found to depend on TLR4 and MyD88, but also IL-1R1. Staining of MPO activity revealed increased neutrophil recruitment in

the lung parenchyma of wild-type mice and a clear reduction of MPO positive cells in the other groups (Fig. 2, *B* and *C*). The reduced lung inflammation in IL-1R1-deficient mice suggests that IL-1 production probably induced after TLR4/MyD88 signaling is central for lung inflammation in response to cigarette smoke. Therefore, neutrophil recruitment in the airway and lung parenchyma is TLR4 dependent and mediated by MyD88/IL-1R1 signaling.
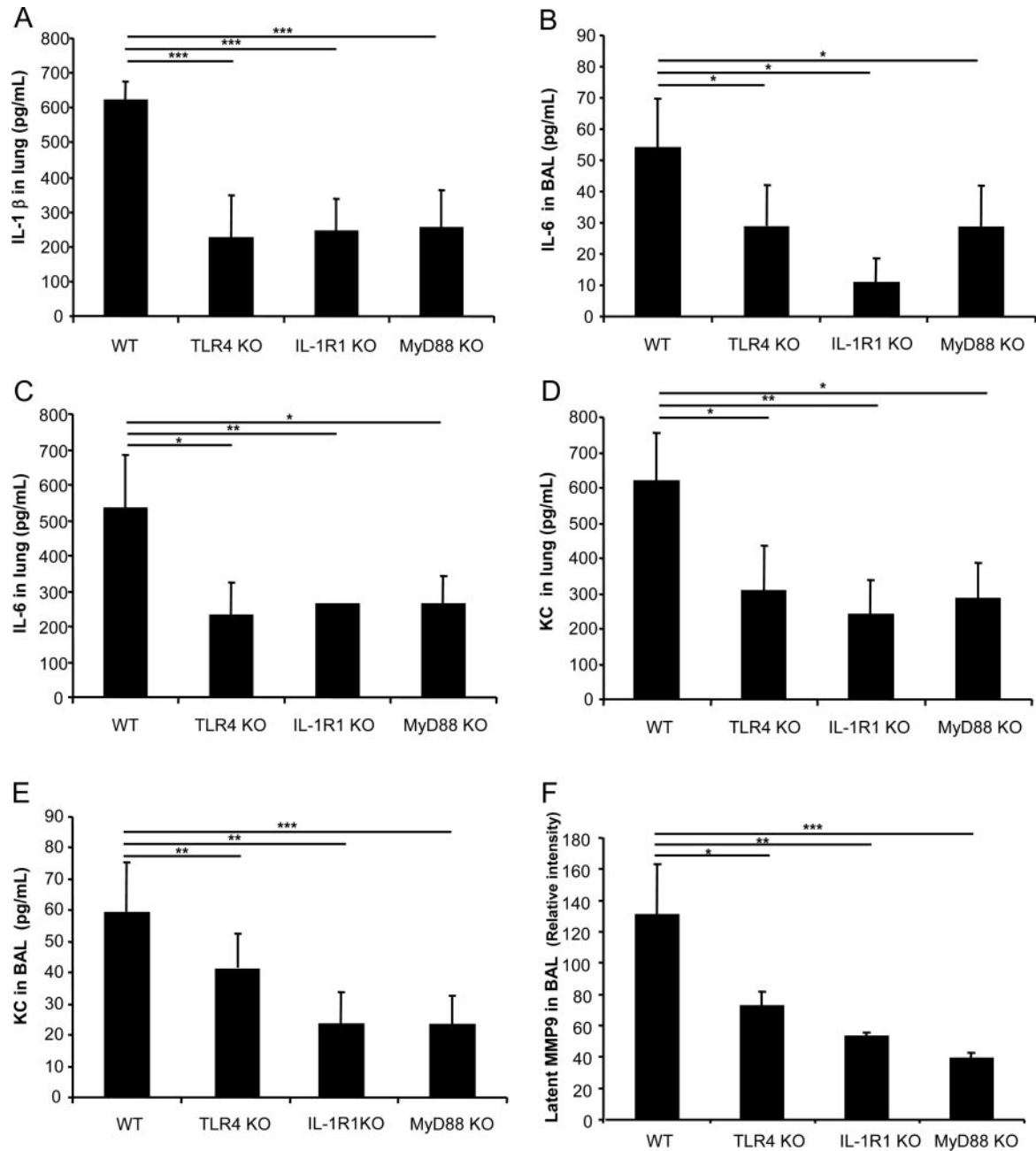
FIGURE 4. Cytokine and chemokine release in the airways upon cigarette smoke exposure. IL-1β (*A*), IL-6 (*B*), and KC (*D*) levels in lung homogenates and IL-6 (*C*), KC (*E*), and MMP-9 activity (*F*) in BAL fluids after 3 days of cigarette smoke exposure twice a day in C57BL/6 (WT), TLR4-, MyD88-, and IL-1R1-deficient mice. Data from one of two representative experiments are shown; means $\pm$ SD ($n = 4$). Baseline levels in non smoke-exposed WT mice were 142 ± 101 pg/ml IL-1β, 223 ± 74 pg/ml IL-6, and 42 ± 15 pg/ml KC in lung homogenates, and <15 pg/ml IL-6, 32 ± 46 pg/ml KC, and 16 ± 1 RI latent MMP9 in BAL ($n = 2$). (*, $p < 0.05$ vs control; **, $p < 0.01$; ***, $p < 0.001$).

Because TLR4 was identified as a critical component of the LPS receptor, we asked whether the response observed by cigarette smoke was due to endotoxin contamination. We investigated the direct bronchoconstrictive effect of LPS in comparison to cigarette smoke. Intranasal LPS at 10 μg is known to induce a rapid increase of respiratory pause or Penh (Pause enhanced), a measure of respiratory dysfunction and inflammation obtained by noninvasive plethysmography (20, 22). We determined the threshold dose of LPS eliciting significant respiratory dysfunction, 0.01 or 0.1 μg LPS intranasally per mouse (Fig. 3*A*). Twenty-four hours later BAL analysis revealed inflammatory cells recruitment (Fig. 3*B*). By contrast, twice daily cigarette smoke exposure on a single or three consecutive days did not cause any detectable Penh increase

(Fig. 3, *C* and *E*). Compared with LPS 0.01 or 0.1 μg, twice daily cigarette smoke exposure was not sufficient to induce neutrophils recruitment (Fig. 3*D*). The LPS content in smoke is difficult to assess. Endotoxin content in cigarettes was reported to be around 18 μg LPS per cigarette before combustion, with 1% remaining after combustion (15). The absence of relevant bronchoconstriction after single or repeated exposure to cigarette smoke makes endotoxin an unlikely factor contributing to smoke activation of TLR4/MyD88, and suggests that other components in the cigarette smoke may directly or indirectly trigger TLR4.

In an attempt to identify a mechanistic explanation for the TLR4 dependent inflammatory response, we searched for known endogenous TLR4 agonists, such as heat shock protein (hsp) 70 (9, 10,
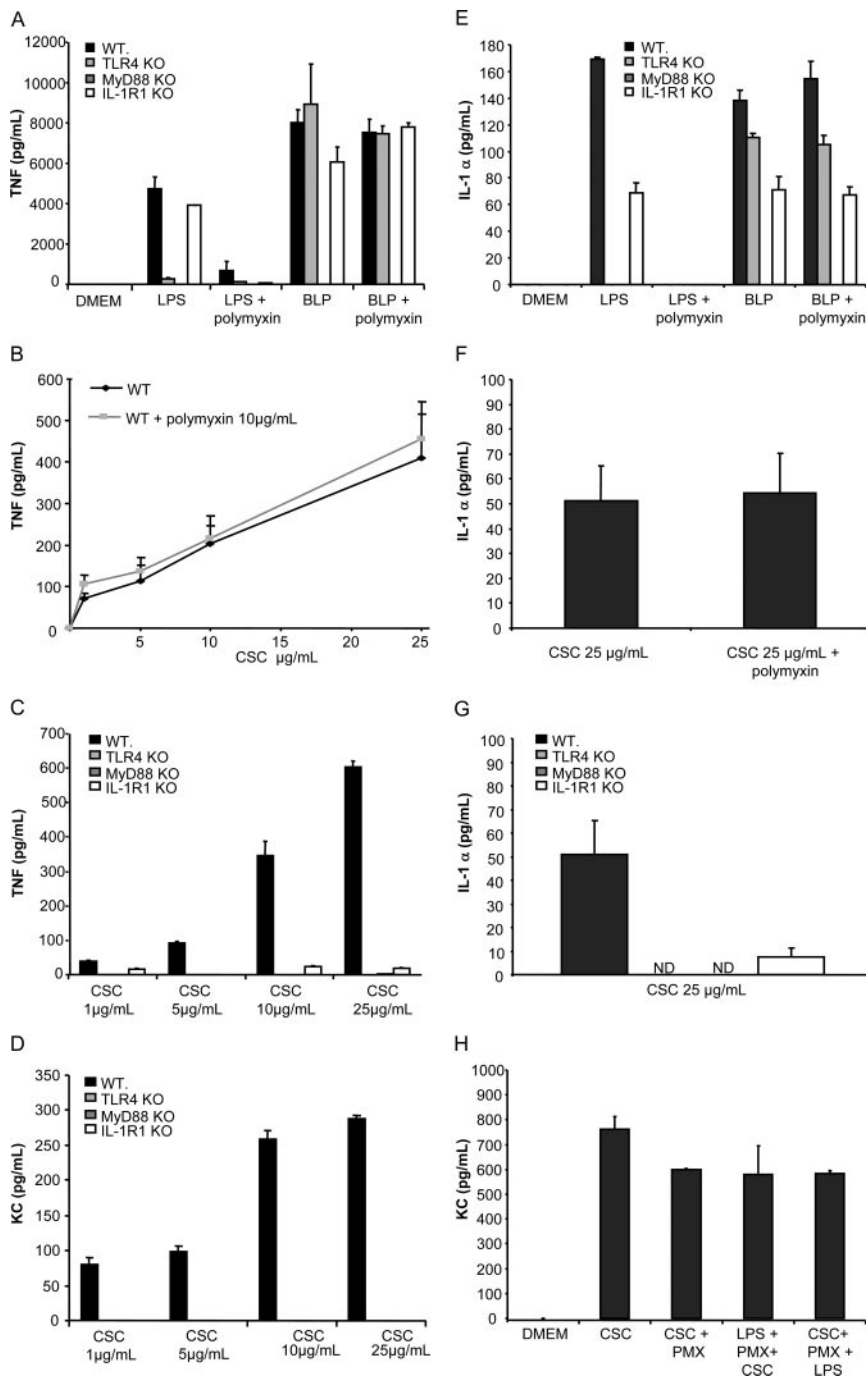
FIGURE 5. TLR4, MyD88, and IL-1R1-dependent cell activation by CSC. Macrophages from C57BL/6 (WT), TLR4, MyD88, or IL-1R1 deficient (KO) mice were stimulated with the TLR4 agonist LPS 100 ng/ml and the TLR2 agonist bacterial lipopeptide (0.5 μg/ml) alone or in presence of polymyxin (10 μg/ml) (*A* and *E*). Addition of polymyxin to increasing concentrations of CSC (1–25 μg/ml) did not impair WT macrophage stimulation (*B* and *F*). CSC-induced release of TNF (*C*), KC (*D*), and IL-1α (*G*) was tested in WT, TLR4-, MyD88-, or IL-1R1-deficient macrophages. Polymyxin neutralizing capacity was verified by adding sequentially polymyxin (10 μg/ml) plus LPS (100 ng/ml) followed 2 h later by CSC (25 μg/ml), or conversely polymyxin plus CSC followed by LPS, on WT macrophages (*H*). The concentration of TNF (*A*, *B*, and *C*), KC (*D* and *H*), and IL-1α (*E*, *F*, and *G*) was quantified in cell culture supernatants 20 h after stimulation. Results are mean ± SD of two mice per genotype and are from one representative experiment of three independent experiments.

12). Indeed, we identified a marked induction of hsp70 in the BAL fluid upon smoke exposure (Fig. 3*F*). The induction of hsp70 itself, after 3-day smoke exposure, was independent of the presence of its receptor TLR4 or the MyD88 adaptor (Fig. 3*F*). Therefore, the data suggest that yet undefined components in the smoke induce hsp, and potentially other "stress markers" release in the airways, and that these stress proteins may contribute to the TLR4-dependent lung inflammation induced by cigarette smoke.

Cigarette smoke induces TLR4, MyD88, and IL-1R1 dependent IL-1β, KC, IL-6, and MMP-9 in the airways

We then asked whether soluble factors implicated in neutrophil recruitment are induced in the lung upon smoke exposure. We showed that cigarette smoke exposure induced IL-1β production in lung homogenate, which was markedly reduced in TLR4-,

MyD88-, or IL-1R1-deficient mice (Fig. 4*A*). IL-1β was undetectable in the BAL fluids (data not shown). The production of another inflammatory cytokine, IL-6 (Fig. 4, *B* and *C*) and the neutrophil chemotactic factor KC (Fig. 4, *D* and *E*) were elevated in the BAL fluids and in the lung tissue homogenates after 3 days of smoke exposure, but were reduced in mice deficient for TLR4, MyD88, or IL-1R1. MIP-1α expression was also diminished in these deficient mice, but these trends did not reach statistical significance (data not shown). To assess the effect of MyD88 pathways on MMPs expression, we quantified the gelatinase activity of latent MMP-9 (96 kDa) produced by inflammatory cells such as neutrophils using zymography, as described (7). Densitometry analysis of the band showed that the latent MMP-9 induced after cigarette smoke exposure in the BAL fluid was significantly reduced in mice deficient for TLR4, MyD88, and IL-1R1 (Fig. 4*F*). In contrast, no marked
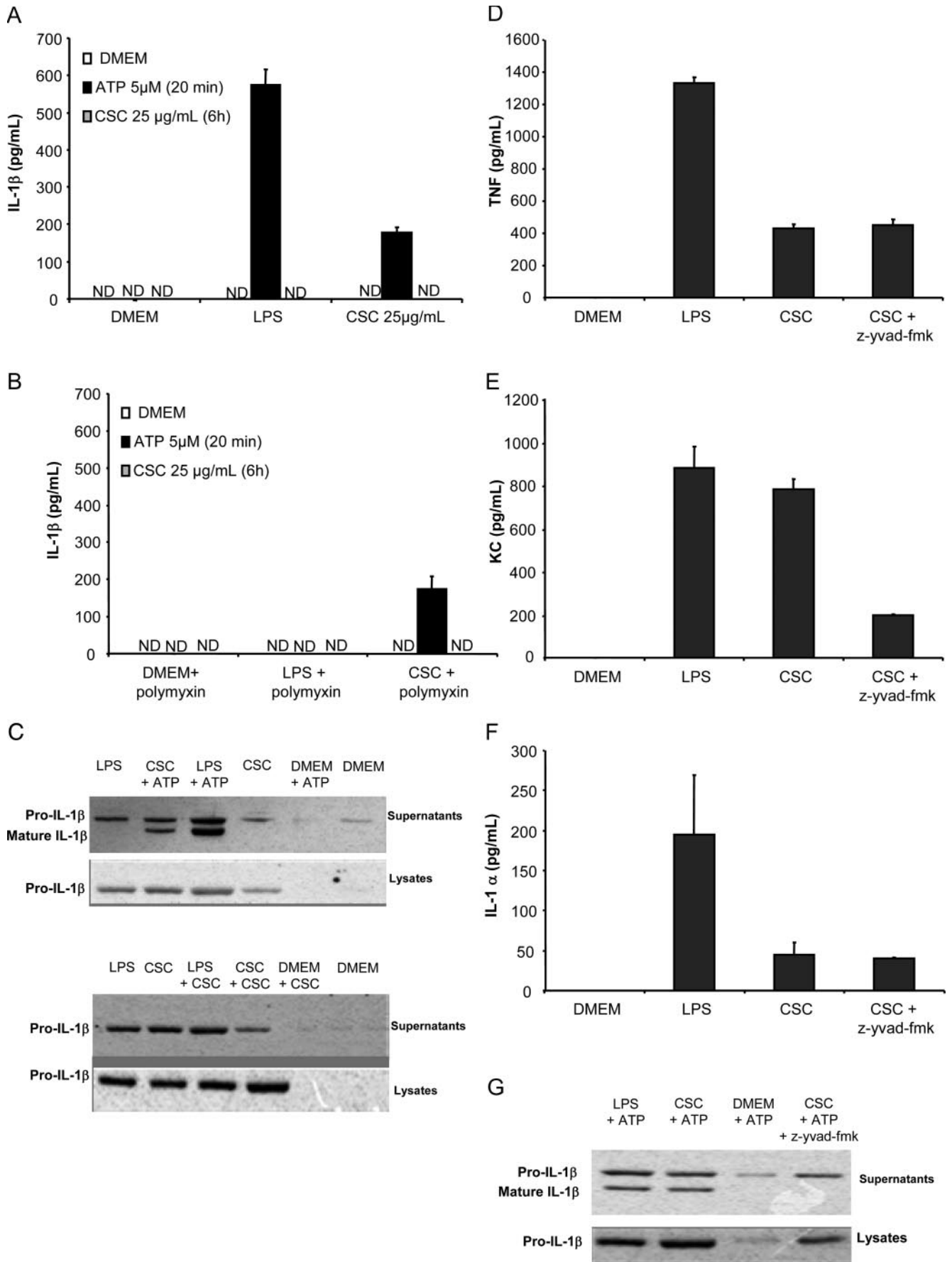
difference of latent or active MMP-2 between the different deficient mice was found (data not shown). Thus, cigarette smoke-induced expression of proinflammatory cytokine and chemokine and production of latent MMP-9 depend both on TLR4/MyD88 and IL-1R1/MyD88 signaling pathways.

TLR4-, MyD88-, and IL-1R1-dependent in vitro activation of macrophages by CSC

To test the direct effect of smoke on target cells, we investigated the effects of CSC on in vitro cultured BMDM. Macrophages from C57BL/6 and TLR4-, MyD88-, or IL-1R1-deficient mice were incubated with increasing concentrations of CSC from 1–25 μg/ml. CSC effects were compared with those of the TLR4 and TLR2 agonists, LPS and bacterial lipopeptide, respectively. We first confirmed the MyD88 dependence of TNF (Fig. 5A) and KC (data not shown) production from macrophages in response to both TLR2 and TLR4 agonists and the absence of response to LPS of TLR4-deficient macrophages. The LPS-induced TNF production (100 ng/ml) was fully inhibited by addition of polymyxin (10 μg/ml), while the response to bacterial lipopeptide was unaffected (Fig. 5A). CSC induced a dose-dependent production of TNF by wild-type macrophages. This response was not affected by polymyxin addition (Fig. 5B). In vitro TNF and KC production by wild-type macrophages after CSC stimulation in presence of polymyxin was clearly dose-dependent (Fig. 5, C and D). We verified that CSC is not cytotoxic for cells in our culture conditions at concentrations up to 25 μg/ml (as measured by MTT assay but also by Annexin V and 7-AAD staining, with >94% of double negative cells; data not shown). CSC-induced TNF and KC release were strongly inhibited in IL-1R1-deficient cells and completely absent in TLR4 and MyD88 deficient macrophages (Fig. 5, C, D, and G). To address the contribution of IL-1 in this response, we first confirmed the MyD88 dependence of IL-1α release after macrophage stimulation by TLR2 and TLR4 agonists (Fig. 5E), the absence of IL-1α response to LPS in TLR4-deficient macrophages or after addition of polymyxin (Fig. 5E), and showed that the IL-1α produced after exposure to CSC was not affected by polymyxin addition (Fig. 5F). Interestingly, CSC-induced IL-1α was strongly inhibited in IL-1R1 deficient cells and absent in TLR4 or MyD88 deficient macrophages (Fig. 5G). We verified that endotoxin potentially present in the CSC preparation did not block polymyxin activity by adding sequentially polymyxin plus CSC then LPS, or conversely polymyxin plus LPS, then CSC on wild-type and TLR4-deficient BMDM. In both cases polymyxin spared the response to CSC stimulation, in terms of TNF, IL-1α, or KC release (Fig. 5H and data not shown), whereas this concentration of polymyxin efficiently neutralized the LPS stimulation (Fig. 5, A and E).

Therefore, the data show that CSC activation of macrophages is TLR4 dependent and not attributable to a potential endotoxin contamination. IL-1R1 signaling is necessary to TNF and KC production, and therefore both IL-1 production and TLR activation seem to be required for optimal TNF and KC production upon CSC stimulation of macrophages. In conclusion, CSC-induced production of TNF and KC by macrophages is strictly dependent on TLR4/MyD88 and IL-1R1/MyD88 pathways.

Contribution of the inflammasome/IL-1β pathways in vitro

Cigarette smoke-induced inflammation is significantly decreased in the absence of IL-1R1 which highlights the importance of IL-1 in this process. Because that cigarette exposure induced IL-1β production in the lungs (Fig. 4A), we further investigated whether IL-1β was also produced by macrophages in response to cigarette smoke condensate. Processing and release of IL-1β by macrophages ex vivo require two distinct stimuli: first, an inflammatory one such as LPS to prime pro-IL-1β transcription and a second, stimulus such as ATP for pro-IL-1β maturation and release (25). We tested the potential effect of CSC on both steps. Wild-type macrophages were stimulated with CSC or LPS as control overnight and then pulsed with either 25 μg/ml CSC for 6 h or 5 mM ATP for 20 min. The production of mature IL-1β in culture supernatant was analyzed. CSC alone did not activate macrophages to produce mature IL-1β whereas addition of ATP to CSC-activated macrophages led to IL-1β release (Fig. 6A) that was not reduced in the presence of polymyxin (Fig. 6B). ATP alone did not trigger IL-1β production in non stimulated cells. Conversely, incubation with CSC for 6 h after macrophage stimulation by LPS did not lead to release of mature IL-1β (Fig. 6B). We therefore performed Western blot analysis to evaluate pro-IL-1β vs IL-1β production in cell lysates and macrophage culture supernatants after CSC stimulation. We showed that CSC activated macrophages produced intracellular pro-IL-1β but did not produce mature IL-1β (Fig. 6C). Addition of ATP to CSC-activated macrophages led to release of both pro- and mature IL-1β (Fig. 6C). Similarly, LPS alone triggered intracellular pro-IL-1β production, whereas addition of ATP allowed pro-IL-1β and IL-1β release as described (25). CSC incubation for 6 h did not promote the maturation of LPS-induced pro-IL-1β, because no mature IL-1β could be detected (LPS plus CSC; Fig. 6C). The release of pro-IL-1β and IL-1β after CSC or LPS stimulation and addition of ATP was abrogated in TLR4-deficient mice (undetectable by ELISA or Western blot; data not shown). These results demonstrate that CSC induced the production of pro-IL-1β and that a second signal, such as ATP, is needed to activate the inflammasome complex leading to caspase-1 activation and maturation of pro-IL-1β into IL-1β.

To further address the potential involvement of the inflammasome in this pathway, we stimulated macrophages with CSC in the presence of the caspase-1 inhibitor, z-yvad-fmk. z-yvad-fmk had no influence on TNF (Fig. 6D) and IL-1α (Fig. 6F) release, but it decreased significantly KC in CSC-activated wild-type macrophages (Fig. 6E), suggesting that KC production is dependent on mature IL-1β. We confirmed by Western blot analysis that mature IL-1β released after stimulation with CSC plus addition of ATP was indeed inhibited in presence of z-yvad-fmk, while pro-IL-1β release was unimpaired (Fig. 6G).

Therefore, we document that CSC triggers the production of pro-IL-1β but does not induce the inflammasome activation resulting in maturation of pro-IL-1β into mature IL-1β.

FIGURE 6. Inflammasome implication in pro-IL-1β, IL-1β, TNF, KC, and IL-1α production upon activation with CSC. Macrophages were stimulated overnight with LPS (100 ng/ml) or cigarette smoke condensate (CSC 25 μg/ml). The induction of pro-IL-1β maturation after addition of ATP (5 mM, 20 min) or CSC (25 μg/ml, 6 h) was analyzed by ELISA in cell culture supernatants in the absence (A) or in presence of polymyxin (10 μg/ml) (B). The production of pro-IL-1β and IL-1β upon CSC stimulation was analyzed by Western blot in C57BL/6 macrophage cell lysates or culture supernatants (C). The release of TNF (D), KC (E), and IL-1α (F) upon LPS and CSC stimulation and in presence of the caspase-1 inhibitor z-yvad-fmk (20 μM) was analyzed by ELISA. The production of pro-IL-1β and IL-1β after LPS or CSC stimulation and addition of ATP (5 mM, 20 min) was analyzed by Western blot analysis in the absence or in presence of z-yvad-fmk (20 μM) (G). Results are represented as bar graphs are mean $\pm$ SD of two mice per genotype and are from one representative of three independent experiments.

Discussion

The present study clearly demonstrates that acute lung inflammation induced by 3 days of exposure to cigarette smoke is TLR4, IL-1R1, and MyD88 dependent. Absence of TLR4, MyD88, or IL-1R1 signaling abrogated neutrophil recruitment to the lung parenchyma and BAL with low IL-1β, KC, and IL-6 as well as MMP-9 levels. Therefore, TLR4 activation by cigarette smoke causes an inflammatory response with IL-1β production. Interaction of IL-1β with its receptor, IL-1R1, is then leading to pulmonary inflammation. MyD88, which is both necessary for TLR4 and IL-1R1 signaling, is a crucial adaptor protein in this response.

The molecular mechanisms by which cigarette smoke causes the inflammatory process and pathology of COPD are not fully understood and, therefore, we believe that experimental models may help to elucidate the pathogenesis. In the acute model, cigarette smoke (two cigarettes twice per day for three consecutive days) causes a pronounced influx of neutrophils in the lung tissue and in the bronchoalveolar space with increased levels of KC and IL-6 (7). Chronic exposure to cigarette smoke leads to development of chronic pulmonary inflammation with increased recruitment of macrophages and neutrophils as well as production of inflammatory mediators (4), reduced in the tlr4 mutant C3H/HeJ mice after 5 wk of extensive exposure (26).

Endotoxin/LPS, a potential contaminant of cigarette smoke, given intranasally causes rapid respiratory dysfunction and inflammation with increased cytokine production and recruitment of neutrophils to the lung and BAL. This process occurs in a TLR4-dependent manner (27) and requires MyD88, TIR-domain containing adaptor protein, and p38 MAPK pathways (20, 22), but is independent of IL-1R1 and IL-18R signaling (22).

The development of cigarette smoke-induced acute pulmonary inflammation is also TLR4 dependent. Indeed, we show in this study that neutrophil recruitment, cytokine, and chemokine production (IL-1β, IL-6, KC) and MMP-9 activation upon smoke exposure were greatly reduced in TLR4-deficient mice. MMP-9 expression was shown to be associated with neutrophil recruitment and to potentiate the action of neutrophil chemokines (28). Therefore, cigarette smoke triggered inflammation bears similarities with LPS-induced airway pathology. However, we demonstrate in this study that cigarette smoke-induced inflammation required in addition IL-1R1 signaling, whereas the LPS response is IL-1R1 independent (22). Therefore, cigarette smoke activates TLR4, leading to IL-1 production and amplification of the response by IL-1/IL-1R1 engagement. MyD88, which is an adaptor for both TLR4 and IL-1R1 signaling pathways, is a crucial component of cigarette smoke-induced lung inflammation. Cigarette smoke exposure was previously associated with early IL-1β production, alveolar macrophage activation, and recruitment, and treatment with an anti-IL-1β Ab significantly reduced both IL-1β levels and alveolar macrophage influx, suggesting amplification loop of IL-1β production in vivo (29).

The present data suggest that LPS is not responsible for TLR4/MyD88-dependent inflammation upon exposure to cigarette smoke, because the airway responses to LPS and cigarette smoke are qualitatively and quantitatively different. The 1R4F cigarettes used in this study reportedly contain a LPS bioactivity equivalent to 18 μg LPS/cigarette (15). However, only 1% of bioactive LPS remains after combustion, so such a low LPS contamination is unlikely responsible for the TLR4-dependent inflammation observed. Indeed, the titration and direct comparison of LPS and cigarette smoke revealed that LPS at 0.1 μg, but not cigarette smoke, caused bronchoconstriction with respiratory dysfunction. Moreover, LPS at 0.01 and 0.1 μg induced a recruitment of neu-

trophils respectively 6- to 10-fold more important than what is seen after 3-day cigarette smoke exposure. Therefore, the data support the notion that TLR4 sensing is essential for the inflammatory response to cigarette smoke and that IL-1 acts as a central mediator, contributing to the amplification of the response. CSC should thus contain molecular species that may act as TLR4 ligands, and we cannot exclude that it contains also IL-1R1 ligands. Moreover, several endogenous activators of TLR4 have been described recently, including the proteins Mrp8 and MrP14 that are released during phagocyte activation and contribute to amplify endotoxin-triggered inflammatory responses (30). However, the nature of endotoxin-independent, TLR4-activating cigarette smoke constituents requires further investigations.

We tested possible involvement of alternative TLR4 signal induced by smoke. Reactive oxidant species from inhaled cigarette smoke cause cell necrosis or apoptosis of both parenchyma and alveolar septae (31). Dying cells produce danger signals such as hsp (11), or high mobility group box 1 protein (13), which may activate TLR2 and TLR4 and/or the receptor for advanced glycation end products (9, 14) and cause apoptosis (32, 33). We show a marked expression of hsp70 in the BAL fluid after cigarette smoke exposure, which is independent of TLR4 and MyD88 signaling. Therefore, hsp70 is likely the candidate activating TLR4 in our model. As yet undefined smoke components induce the release of hsp and potentially other "stress markers" in the airways, and these stress proteins rather than endotoxins mediate lung inflammation through TLR4 and MyD88 pathway.

Further, cell injury may also activate proteases with degradation of collagen and matrix proteins, which may cause inflammation (34). Dying cells further generate danger signals resulting in inflammasome activation in macrophage, allowing maturation of pro-IL-1β to IL-1β by caspase-1 (35). Our in vitro data demonstrate that TLR4 activation of macrophages by CSC promotes pro-IL-1β production, while the maturation of IL-1β requires a second danger signal, probably provided by in vivo damaged cells that may depend on inflammasome activation (35). CSC also promotes a TLR4-dependent production of IL-1α independent of inflammasome activation. Other important players in the immune response to cigarette smoke are epithelial cells (36–38), and the role of TLR4, IL-1R1 and MyD88 in these cells requires further investigations.

In conclusion, acute cigarette exposure results in LPS independent TLR4 activation, leading to IL-1β production and IL-1R1 signaling, which is crucial for cigarette smoke-induced inflammation in vivo. MyD88 plays a central role in this inflammatory response as it is at the crossroad of a TLR4 activation pathway and the regulatory/amplification inflammatory loop involving IL-1β and IL-1α secretion and response through IL-1R1. Therefore, inhibitors of TLR4 and IL-1/IL-1R1 may prove useful in diminishing cigarette smoke induced pulmonary inflammation and chronic pathology.

Acknowledgments

Disclosures

References

1. Pauwels, R. 2000. COPD: the scope of the problem in Europe. *Chest* 117: 332S–335S.
2. Shapiro, S. D. 2001. End-stage chronic obstructive pulmonary disease: the cigarette is burned out but inflammation rages on. *Am. J. Respir. Crit. Care Med.* 164: 339–340.

3. Barnes, P. J. 2004. Mediators of chronic obstructive pulmonary disease. *Pharmacol. Rev.* 56: 515–548.

4. Barnes, P. J., S. D. Shapiro, and R. A. Pauwels. 2003. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur. Respir. J.* 22: 672–688.

5. Michaud, S. E., S. Dussault, J. Groleau, P. Haddad, and A. Rivard. 2006. Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species. *J. Mol. Cell. Cardiol.* 4: 275–284.

6. Molet, S., C. Belleguic, H. Lena, N. Germain, C. P. Bertrand, S. D. Shapiro, J. M. Planquois, P. Delaval, and V. Lagente. 2005. Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease. *Inflamm. Res.* 54: 31–36.

7. Leclerc, O., V. Lagente, J. M. Planquois, C. Berthelier, M. Artola, T. Eichholtz, C. P. Bertrand, and F. Schmidlin. 2006. Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. *Eur. Respir. J.* 27: 1102–1109.

8. Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* 24: 353–389.

9. Asea, A., M. Rehli, E. Kabingu, J. A. Boch, O. Bare, P. E. Auron, M. A. Stevenson, and S. K. Calderwood. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem.* 277: 15028–15034.

10. Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and H. Wagner. 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J. Biol. Chem.* 277: 15107–15112.

11. Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker, and H. Wagner. 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem.* 276: 31332–31339.

12. Dybdahl, B., A. Wahba, E. Lien, T. H. Flo, A. Waage, N. Qureshi, O. F. Sellevold, T. Espevik, and A. Sundan. 2002. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation* 105: 685–690.

13. Lotze, M. T., and K. J. Tracey. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* 5: 331–342.

14. Park, J. S., F. Gamboni-Robertson, Q. He, D. Svetkauskaite, J. Y. Kim, D. Strassheim, J. W. Sohn, S. Yamada, I. Maruyama, A. Banerjee, et al. 2006. High mobility group box 1 protein interacts with multiple toll-like receptors. *Am. J. Physiol.* 290: C917–C924.

15. Hasday, J. D., R. Bascom, J. J. Costa, T. Fitzgerald, and W. Dubin. 1999. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 115: 829–835.

16. Akira, S. 2003. Mammalian toll-like receptors. *Curr. Opin. Immunol.* 15: 5–11.

17. Labow, M., D. Shuster, M. Zetterstrom, P. Nunes, R. Terry, E. B. Cullinan, T. Bartfai, C. Solorzano, L. L. Moldawer, R. Chizzonite, and K. W. McIntyre. 1997. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. *J. Immunol.* 159: 2452–2461.

18. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.

19. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162: 3749–3752.

20. Schnyder-Candrian, S., V. F. Quesniaux, F. Di Padova, I. Maillet, N. Noulin, I. Couillin, R. Moser, F. Erard, B. B. Vargaftig, B. Ryffel, and B. Schnyder. 2005. Dual effects of p38 MAPK on TNF-dependent bronchoconstriction and TNF-independent neutrophil recruitment in lipopolysaccharide-induced acute respiratory distress syndrome. *J. Immunol.* 175: 262–269.

21. Couillin, I., I. Maillet, B. B. Vargaftig, M. Jacobs, G. C. Paesen, P. A. Nuttall, J. Lefort, R. Moser, W. Weston-Davies, and B. Ryffel. 2004. Arthropod-derived histamine-binding protein prevents murine allergic asthma. *J. Immunol.* 173: 3281–3286.

22. Noulin, N., V. F. Quesniaux, S. Schnyder-Candrian, B. Schnyder, I. Maillet, T. Robert, B. B. Vargaftig, B. Ryffel, and I. Couillin. 2005. Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. *J. Immunol.* 175: 6861–6869.

23. Narayan, S., A. S. Jaiswal, D. Kang, P. Srivastava, G. M. Das, and C. G. Gairola. 2004. Cigarette smoke condensate-induced transformation of normal human breast epithelial cells in vitro. *Oncogene* 23: 5880–5889.

24. Muller, M., H. P. Eugster, M. Le Hir, A. Shakhov, F. Di Padova, C. Maurer, V. F. Quesniaux, and B. Ryffel. 1996. Correction or transfer of immunodeficiency due to TNF-LT α deletion by bone marrow transplantation. *Mol. Med.* 2: 247–255.

25. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228–232.

26. Maes, T., K. R. Bracke, K. Y. Vermaelen, I. K. Demedts, G. F. Joos, R. A. Pauwels, and G. G. Brusselle. 2006. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int. Arch. Allergy Immunol.* 141: 354–368.

27. Togbe, D., S. Schnyder-Candrian, B. Schnyder, I. Couillin, I. Maillet, F. Bihl, D. Malo, B. Ryffel, and V. F. Quesniaux. 2006. TLR4 gene dosage contributes to endotoxin-induced acute respiratory inflammation. *J. Leukocyte Biol.* 80: 451–457.

28. Van den Steen, P. E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-α and leaves RANTES and MCP-2 intact. *Blood* 96: 2673–2681.

29. Castro, P., A. Legora-Machado, L. Cardilo-Reis, S. Valenca, L. C. Porto, C. Walker, C. Zuany-Amorim, and V. L. Koatz. 2004. Inhibition of interleukin-1β reduces mouse lung inflammation induced by exposure to cigarette smoke. *Eur. J. Pharmacol.* 498: 279–286.

30. Vogl, T., K. Tenbrock, S. Ludwig, N. Leukert, C. Ehrhardt, M. A. van Zoelen, W. Nacken, D. Foell, T. van der Poll, C. Sorg, and J. Roth. 2007. Mrp8 and Mrp14 are endogenous activators of toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat. Med.* 13: 1042–1049.

31. Dekhuijzen, P. N. 2004. Antioxidant properties of N-acetylcysteine: their relevance in relation to chronic obstructive pulmonary disease. *Eur. Respir. J.* 23: 629–636.

32. Vayssier, M., N. Banzet, D. Francois, K. Bellmann, and B. S. Polla. 1998. Tobacco smoke induces both apoptosis and necrosis in mammalian cells: differential effects of HSP70. *Am. J. Physiol.* 275: L771–L779.

33. Anbarasi, K., G. Kathirvel, G. Vani, G. Jayaraman, and C. S. Shyamala Devi. 2006. Cigarette smoking induces heat shock protein 70 kDa expression and apoptosis in rat brain: modulation by bacoside A. *Neuroscience* 138: 1127–1135.

34. Jiang, D., J. Liang, J. Fan, S. Yu, S. Chen, Y. Luo, G. D. Prestwich, M. M. Mascarenhas, H. G. Garg, D. A. Quinn, et al. 2005. Regulation of lung injury and repair by toll-like receptors and hyaluronan. *Nat. Med.* 11: 1173–1179.

35. Martinon, F., and J. Tschopp. 2005. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* 26: 447–454.

36. Gelbman, B. D., A. Heguy, T. P. O'Connor, J. Zabner, and R. G. Crystal. 2007. Upregulation of pirin expression by chronic cigarette smoking is associated with bronchial epithelial cell apoptosis. *Respir. Res.* 8: 8–10.

37. Luppi, F., J. Aarbiou, S. van Wetering, I. Rahman, W. I. de Boer, K. F. Rabe, and P. S. Hiemstra. 2005. Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione. *Respir. Res.* 6: 140–152.

38. Liu, X. 2007. STAT3 activation inhibits human bronchial epithelial cell apoptosis in response to cigarette smoke exposure. *Biochem. Biophys. Res. Commun.* 353: 121–126.