The Nucleotide-Binding Domain and Leucine-Rich Repeat Protein–3 Inflammasome Is Not Activated in Airway Smooth Muscle Upon Toll-Like Receptor–2 Ligation

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Inflammasomes have emerged as playing key roles in inflammation and innate immunity. A growing body of evidence has suggested that the nucleotide-binding domain and leucine-rich repeat protein–3 (NLRP3) inflammasome is important in chronic airway diseases such as asthma and chronic obstructive pulmonary disease. Inflammasome activation results, in part, in pro–IL-1β processing and the secretion of the proinflammatory cytokine IL-1β. Because asthma exacerbations are associated with elevated concentrations of secreted IL-1β, we addressed whether the NLRP3 inflammasome is activated under *in vitro* conditions that mimic infectious exacerbations in asthma. Primary cultures of airway smooth muscle (ASM) cells were treated with infectious stimuli (mimicked using the Toll-like receptor–2 agonist Pam3CSK4, a synthetic bacterial lipopeptide). Whereas Pam3CSK4 robustly up-regulated ASM cytokine expression in response to TNF-α and significantly enhanced IL-1β mRNA expression, we were unable to detect IL-1β in the cell supernatants. Thus, IL-1β was not secreted and therefore was unable to act in an autocrine manner to promote the amplification of ASM inflammatory responses. Moreover, Toll-like receptor–2 ligation did not enhance NLRP3 or caspase-1 expression in ASM cells, and NLRP3 and caspase-1 protein were not present in the ASM layer of tracheal sections from human donors. In conclusion, these data demonstrate that the enhanced synthetic function of ASM cells, induced by infectious exacerbations of airway inflammation, is NLRP3 inflammasome–independent and IL-1β–independent. Activation of the NLRP3 inflammasome by invading pathogens may prove cell type–specific in exacerbations of airway inflammation in asthma.

Keywords: interleukin-1β; NLRP3; caspase-1; inflammasome; TLR2

Inflammasomes control innate immunity and inflammation. By regulating the processing and release of proinflammatory cytokines (predominantly IL-1β), burgeoning evidence supports a key role for inflammasome activation in lung diseases such as asthma and chronic obstructive pulmonary disease, as recently reviewed (1, 2). The nucleotide-binding domain and leucine-rich repeat protein–3 (NLRP3) inflammasome has been best characterized, and both *in vivo* and *in vitro* models of allergic asthma and asthmatic inflammation have demonstrated an important role for the NLRP3 inflammasome in the amplification and potentiation of inflammation during airway disease (3–5). Because elevated IL-1β protein secretion was suggested to play a role in asthma exacerbation (5, 6), we were interested in exploring whether the NLRP3 inflammasome is activated under *in vitro* conditions that mimic infectious exacerbations in asthma.

The NLRP3 inflammasome is a multimeric intracellular protein complex consisting of three domains: NLRP3, the apoptosis-associated speck-like protein containing the caspase activation and recruitment domain, and caspase-1 (7). The sensor protein NLRP3 regulates inflammasome assembly in a temporal and spatial manner (8). NLRP3 expression is inducible, and mRNA regulation can take place at the transcriptional and posttranscriptional levels (9, 10). Recognition of invading pathogens by the NLRP3 domain results in the assembly of three components to form a stable inflammasome, which in turn activates pro–caspase-1 into active caspase-1 autocatalytically (1). Caspase-1 is a cysteine protease that cleaves inactive precursors of IL-1β into bioactive cytokine, which is then competent to be secreted out of the cell.

During the past decade, the ability of airway smooth muscle (ASM) cells to produce and secrete proinflammatory mediators, including cytokines and chemokines, has been established by many studies, as reviewed elsewhere (11, 12). Furthermore, accumulating evidence has shown that the treatment of ASM cells with IL-1β *in vitro* augments the production of numerous proinflammatory mediators, including IL-6, IL-8 (13), IL-17A (14), prostaglandin E_2 (15), and matrix metalloproteinase–9 (16), thus serving to amplify airway inflammation. The enhanced synthetic function of ASM may play a pivotal role, leading to acute exacerbations of asthma, by provoking and amplifying airway inflammatory responses (17, 18). To date, however, most NLRP3 inflammasome studies have been limited to airway epithelium and macrophages (19). The possible involvement of the NLRP3 inflammasome and IL-1β activation in the enhanced synthetic function of ASM cells in response to invading pathogens remains largely unexplored.

Intriguingly, our previous studies revealed an important cell-type difference in human airways. Whereas NLRP3 and caspase-1 were present in human lung epithelium, as shown by Hirota and colleagues (19) and confirmed here, these proteins were not present in dissected ASM strips, and neither were they detected by immunohistochemistry in human tracheal sections. These *in situ* data support our *in vitro* studies, where the engagement of Toll-like receptor 2 (TLR2) with the TLR2 agonist Pam3CSK4 exerted no effect on NLRP3 and caspase-1 expression. These

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TABLE 1. SUBJECT CHARACTERISTICS

Immunohistochemistry Samples	Age (*yr*)	Sex	Status	Weight (*kg*)	Height (*cm*)	Cause of Death
Donor 1	19	F	Not asthmatic	87	165	Head trauma, MVA
Donor 2	20	F	Not asthmatic	72.2	165	Head trauma
Donor 3	25	M	Asthmatic	103	185	Anoxia
Immunoblot Samples						
Donor 4	11	M	Not asthmatic	49.9	157	CVA
Donor 5	26	F	Asthmatic	91.6	168	Anoxia, probable asthma attack
Donor 6	20	M	Asthmatic	81.2	172.7	Head trauma

Definition of abbreviations: CVA, cardiovascular attack; MVA, motor vehicle accident.

results suggest that because of the absence of these key components, NLRP3 inflammasome activation will not be possible, and consequently IL-1β will not be processed for secretion. Our data support this assertion, because we show that although TLR2 ligation increased TNF-α–induced IL-1β mRNA expression, IL-1β is not secreted from ASM cells. Thus, the NLRP3 inflammasome is not activated in ASM upon TLR2 ligation.

MATERIALS AND METHODS

Human ASM Cell Culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or from lung transplant donors, in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee at the University of Sydney. A minimum of three different ASM primary cell cultures established from individual patients was used for each experiment, in accordance with established procedures (20). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Doncaster, Victoria, Australia), and reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with IL-1β (TaqMan ID: Hs01555410_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), NLRP3 (Hs00918082_m1), and 18S ribosomal RNA measured with TaqMan probes (Applied Biosystems)

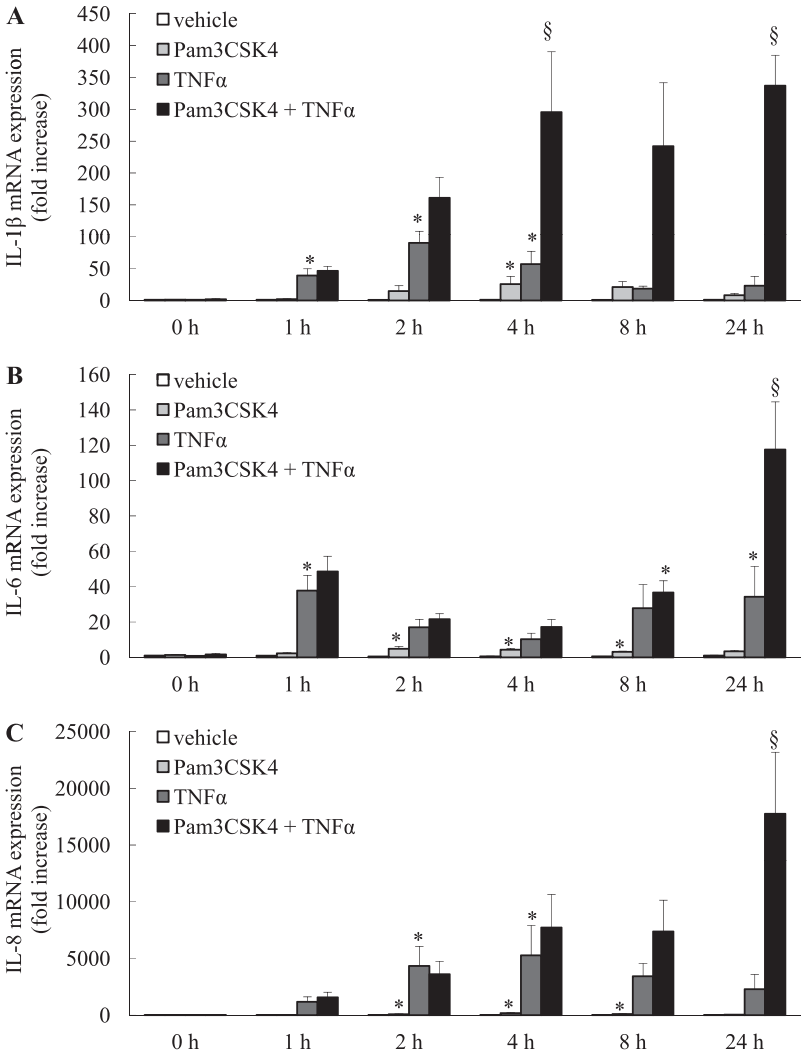

Figure 1. Time course of TNF-α–induced IL-1β mRNA expression and augmentation by Pam3CSK4 (in comparison with IL-6 and IL-8 mRNA temporal kinetics). Growth-arrested airway smooth muscle (ASM) cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 hour, followed by treatment with vehicle or TNF-α (10 ng/ml) for 0, 1, 2, 4, 8, and 24 hours. (*A*) IL-1β, (*B*) IL-6, and (*C*) IL-8 mRNA expression was quantified by real-time RT-PCR, and the results are expressed as fold increase compared with vehicle-treated cells at 0 hour (mean ± SEM values from $n = 7$ primary ASM cell cultures). Statistical analysis was performed using two-way ANOVA, and then the Bonferroni *post hoc* test (where an *asterisk* denotes a significant effect of treatment on mRNA expression, compared with vehicle-treated cells; §significant effect of Pam3CSK4 on TNF-α–induced mRNA expression at the same time point; $P < 0.05$). h, hours.

or caspase-1 (PPH00105B) and 18S (PPH0566E) measured by SYBR Green RT^2 qPCR Primer Assays (Qiagen).

ELISAs

IL-1β (DuoSet DY201; R&D Systems, Minneapolis, MN), IL-6 (BD OptEIA 555220; BD Biosciences, San Diego, CA), and IL-8 (BD OptEIA 555244; BD Biosciences) ELISAs were performed according to the manufacturers' instructions.

IL-1β Neutralization

To neutralize IL-1β, conditioned media from treated ASM cells or IL-1β recombinant protein (R&D Systems) were incubated for 1 hour at 37°C with 2 μg/ml of either vehicle, normal mouse IgG_1 (Santa Cruz Biotechnology, Santa Cruz, CA) as an isotype control, or a monoclonal mouse IgG_1 antibody to human recombinant IL-1β (clone 8516; R&D Systems).

Immunoblotting of ASM Cell Lysates

NLRP3 or caspase-1 in ASM cells was detected by immunoblotting with rabbit anti-human NLRP3 (HPA012878; Sigma-Aldrich) or caspase-1 polyclonal antibodies (catalogue number 2225; Cell Signaling Technology, Danvers, MA), compared with α-tubulin as the loading control (mouse monoclonal IgG_1, DM1A; Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology), and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). Lysates from THP-1 monocyte cells treated with and without phorbol myristate acetate (50 ng/ml) served as positive controls (19).

Human Lung Tissue

Human tracheas were obtained from lungs donated for medical research at the International Institute for the Advancement of Medicine (Edison, NJ) (19). Tracheas from three individual patients were used for each experiment. For the immunohistochemical analysis of NLRP3 and caspase-1 in human tracheas, tissue was fixed in 10% formalin and processed for immunohistochemistry (*see* the online supplement). For the immunoblotting of NLRP3 and caspase-1, tracheas were stored in Krebs solution, and then ASM was isolated and protein was extracted for immunoblotting (see the online supplement). For subject characteristics, please see Table 1.

Statistical Analysis

Statistical analysis was performed using either the Student unpaired *t* test or two-way ANOVA, followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered sufficient to reject the null hypothesis for all analyses. Data represent means ± SEMs.

RESULTS

Time Course of TNF-α–Induced IL-1β mRNA Expression and Augmentation by Pam3CSK4

We measured the temporal kinetics of TNF-α–induced IL-1β mRNA expression in ASM cells, and examined whether the TLR2 agonist, Pam3CSK4, up-regulated IL-1β concentrations. As shown in Figure 1A, TNF-α significantly enhanced IL-1β mRNA expression over time, with significant expression observed

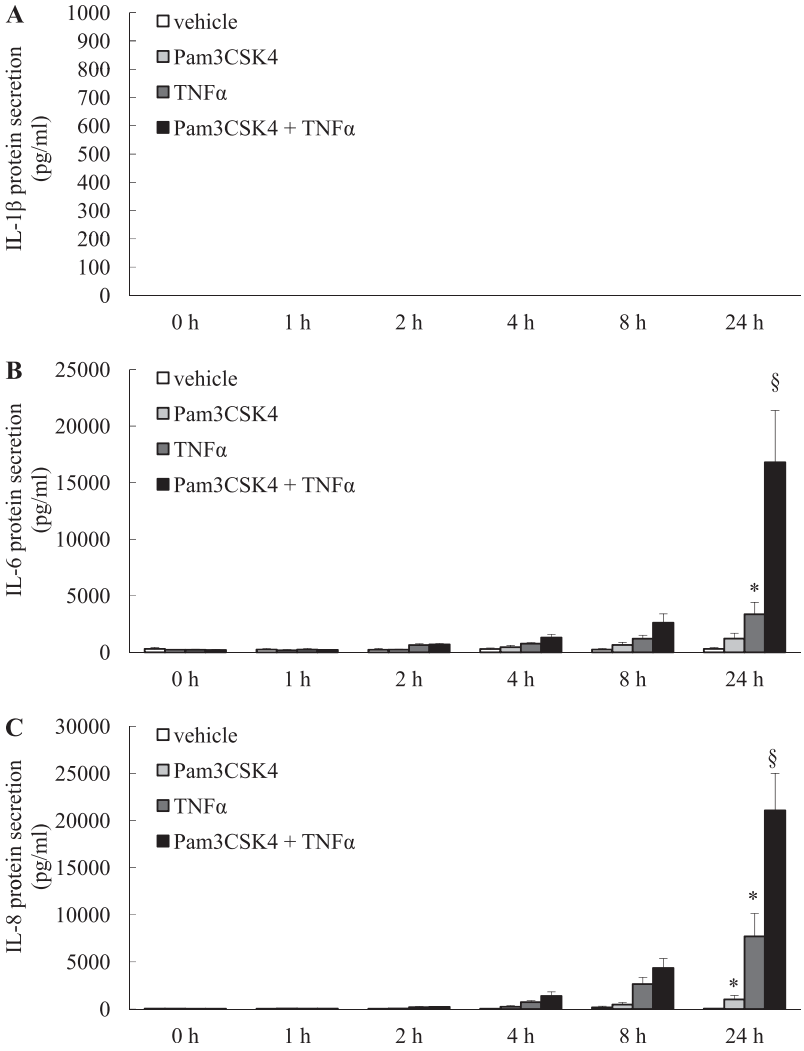

Figure 2. Pam3CSK4 potentiates TNF-α–induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells. Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 hour, followed by treatment with vehicle or TNF-α (10 ng/ml) for 0, 1, 2, 4, 8, and 24 hours. ELISAs were used to measure (*A*) IL-1β, (*B*) IL-8, and (*C*) IL-6 protein secretion. Note that IL-1β secretion from ASM cells (*A*) was not detected. Statistical analysis was performed using two-way ANOVA and then the Bonferroni *post hoc* test (where an *asterisk* denotes a significant effect of treatment on protein secretion, compared with vehicle-treated cells; §significant effect of Pam3CSK4 on TNF-α–induced protein secretion at the same time point; $P < 0.05$). Data represent the mean ± SEM values from $n = 4$ primary ASM cell cultures.

as early as 1 hour, and a peak of 90.4- ± 18.4-fold at 2 hours, before subsiding to concentrations that were not significantly different from those of vehicle controls by 8 hours ($P < 0.05$). Pam3CSK4 alone also significantly enhanced IL-1β mRNA expression, with a significant 14.8- ± 8.4-fold increase observed at 4 hours ($P < 0.05$). Importantly, TNF-α–induced IL-1β up-regulation was significantly potentiated by Pam3CSK4 ($P < 0.05$). As shown in Figure 1A, the effect of Pam3CSK4 on TNF-α–induced IL-1β mRNA was first observed at 2 hours (although not significantly), and this effect increased to significant levels at 4 hours, and by 24 hours was measured at 337.0- ± 48.0-fold, compared with 23.0- ± 14.9-fold using TNF-α alone ($P < 0.05$).

Interestingly, when we compared the temporal kinetics of IL-1β mRNA expression with those of IL-6 (Figure 1B) and IL-8 (Figure 1C), we also observed a later significant up-regulation of TNF-α–induced cytokine secretion at 24 hours ($P < 0.05$). This raises the intriguing possibility that the early phase of IL-1β mRNA may undergo translation, to result in IL-1β protein secretion that can then act back on ASM cells in an autocrine manner to stimulate the cytokine mRNA expression observed at 24 hours. Thus, we hypothesize that the TLR2 agonist, Pam3CSK4, activates the inflammasome to result in IL-1β secretion from ASM cells and the potentiation of cytokine secretion from ASM cells.

Pam3CSK4 Potentiates TNF-α–Induced IL-6 and IL-8 Protein Secretion, but IL-1β Is Not Secreted from ASM cells

To address this hypothesis, we measured concentrations of IL-1β, IL-6, and IL-8, as secreted from ASM cells over 0 to 24 hours. As shown in Figures 2B and 2C, respectively, Pam3CSK4 pretreatment significantly enhanced TNF-α–induced IL-6 and IL-8 at 24 hours (Figures 2A and 2B; $P < 0.05$), in confirmation of our earlier report (18). We then attempted to measure IL-1β in the same supernatants, using an ELISA that had been confirmed able to measure secreted IL-1β that resulted from inflammasome activation (21). Importantly, IL-1β was not detected (Figure 2A), suggesting that even though IL-1β mRNA was up-regulated, the inflammasome was not activated and IL-1β was not secreted from ASM cells.

Neutralization of IL-1β in Conditioned Media Exerts No Effect on Cytokine Secretion

To confirm that IL-1β was not secreted from ASM cells, we used an IL-1β–neutralizing antibody. We initially validated IL-1β neutralization by incubating a range of concentrations of IL-1β recombinant protein (1–500 pg/ml) with IL-1β–neutralizing antibody, and measured its inhibitory effect on IL-1β–induced IL-6 and IL-8 protein secretion. As shown in Figure 3, IL-1β induced a concentration-dependent augmentation of IL-6 and IL-8 protein secretion in vehicle-treated or isotype control–treated ASM cells, with no significant difference between cells treated with vehicle or the isotype control. Importantly, the neutralization of IL-1β reduced IL-6 protein secretion from ASM cells in response to a range of IL-1β concentrations (1–500 pg/ml; Figure 3A), with only 79.5 ± 39.8 pg/ml IL-6 secreted in response to 500 ng/ml IL-1β preincubated with the IL-1β–neutralizing antibody, as opposed to approximately 150-fold greater amounts of IL-6 secretion after preincubation with the isotype control ($P < 0.05$). Similarly, this down-regulation by IL-1β neutralization was also observed in IL-8 protein secretion, as depicted in Figure 3B.

We next examined whether the neutralization of IL-1β in conditioned media exerted any effect on IL-6 and IL-8 protein secretion by ASM cells. As shown in Figure 4A, the removal of IL-1β by the neutralizing antibody exerted no effect on the amount of IL-6 secreted in response to conditioned media from cells stimulated with TNF-α alone or Pam3CSK4 + TNF-α. Similarly, IL-8 secretion in response to conditioned media preincubated with IL-1β–neutralizing antibody was not significantly different from that preincubated with the isotype control (Figure 4B). As a positive control, we confirmed IL-1β neutralization

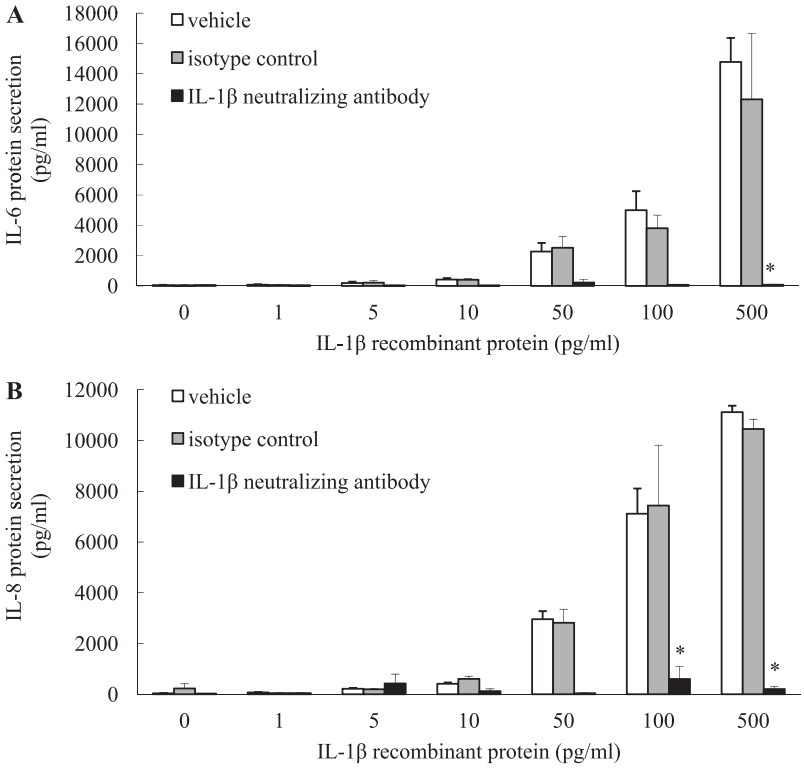

Figure 3. Effect of IL-1β–neutralizing antibody on IL-6 and IL-8 protein secretion induced by IL-1β recombinant protein. To validate the effect of neutralization antibody on IL-6 (*A*) and IL-8 (*B*) protein secretion, vehicle or increasing concentrations of IL-1β recombinant protein (1–500 pg/ml) were incubated for 1 hour at 37°C with 2 μg/ml of vehicle, isotype control, or IL-1β–neutralizing antibody, and then added to growth-arrested ASM cells for 24 hours. IL-6 and IL-8 protein secretion was measured by ELISA. Statistical analysis was performed using two-way ANOVA and then the Bonferroni *post hoc* test (where an *asterisk* denotes a significant effect of IL-1β–neutralizing antibody on protein secretion, compared with the isotype control; $P < 0.05$). Data represent the mean ± SEM values from $n = 3$ primary ASM cell cultures.

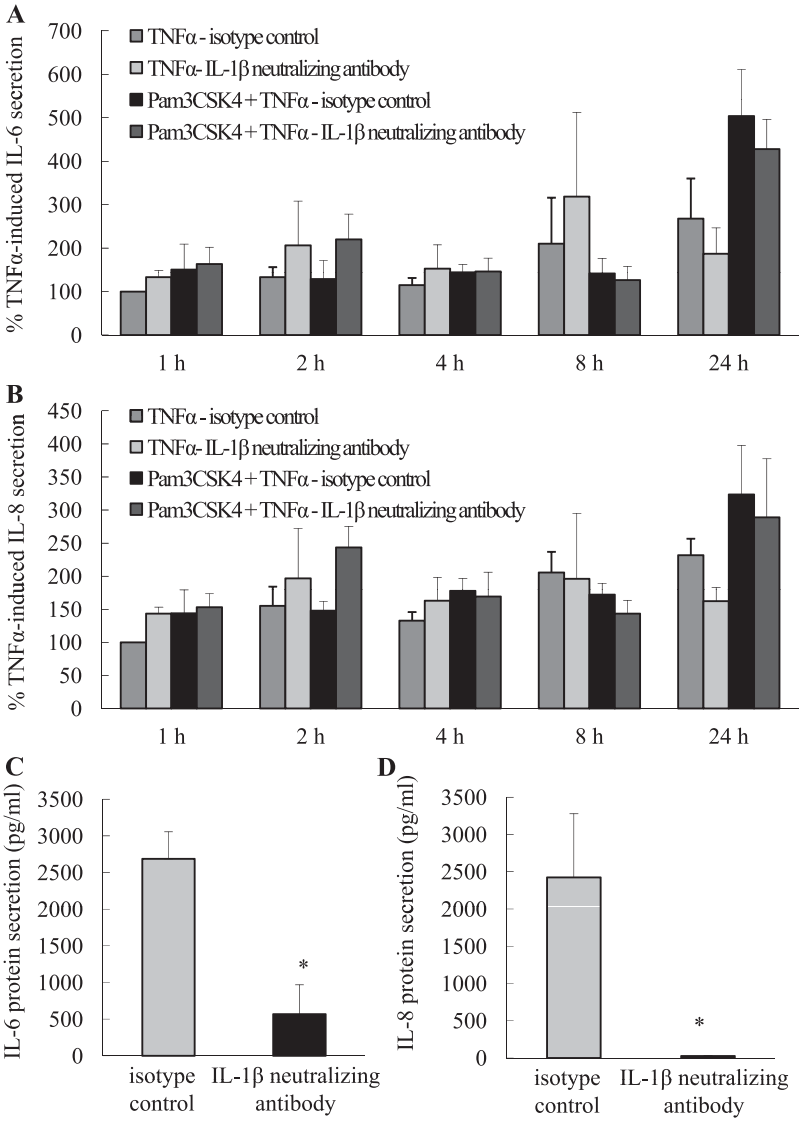

Figure 4. The neutralization of IL-1β in conditioned media exerts no effect on cytokine secretion. Conditioned media were prepared by removing supernatants from ASM cells treated with TNF-α or Pam3CSK4 + TNF-α for 1, 2, 4, 8, and 24 hours. Conditioned media were incubated at 37°C for 1 hour with 2 μg/ml of either IL-1β–neutralizing antibody or isotype control, and then incubated with ASM cells for 24 hours. The resultant (*A*) IL-6 and (*B*) IL-8 protein in the supernatants was measured by ELISA. Results were expressed as a percentage of TNF-α–induced cytokine in isotype control at 1 hour. To confirm IL-1β neutralization, parallel experiments were conducted with vehicle or IL-1β recombinant protein (100 pg/ml) treated under identical conditions, and (*C*) IL-6 and (*D*) IL-8 were measured by ELISA. Statistical analysis was performed using the Student unpaired *t* test (where an *asterisk* denotes a significant effect of IL-1β–neutralizing antibody on protein secretion, compared with isotype control). Data represent mean ± SEM values from $n = 3$ primary ASM cell cultures.

with ASM cells treated with 100 ng/ml IL-1β recombinant protein in parallel experiments. As shown in Figures 4C and 4D, IL-1β neutralization significantly inhibited IL-6 and IL-8 secretion from ASM cells, compared with the isotype control ($P < 0.05$).

Pam3CSK4 Does Not Affect TNF-α–Induced NLRP3 mRNA and Protein Expression in ASM Cells

NLRP3 is an important component of the inflammasome, and has been shown to be up-regulated under inflammatory conditions. Caspase-1 also plays an important role in inflammasome function, to cleave precursor pro–IL-1β into mature IL-1β. Collectively, our data thus far demonstrate that the Pam3CSK4-mediated augmentation of TNF-α–induced IL-6 and IL-8 mRNA and protein concentrations is not mediated by IL-1β secretion. Thus, these results indicate that components of the NLRP3 inflammasome may not be up-regulated in ASM cells upon TLR2 activation. To address this, we measured NLRP3 and caspase-1 mRNA expression in response to Pam3CSK4 and TNF-α, alone and in combination, over time (0–24 h). As shown in Figure 5A, neither Pam3CSK4 nor TNF-α exerted a significant effect on NLRP3 mRNA expression over 24 hours. Moreover, the temporal kinetics of NLRP3 mRNA expression was not affected in response to Pam3CSK4 + TNF-α in combination. Importantly, we were unable to detect NLRP3 protein in the lysates of human ASM cells, in confirmation of our mRNA results. As shown in Figure 5C, NLRP3 protein was not detected, or up-regulated, by treatment with Pam3CSK4 or TNF-α, alone or in combination. We also measured the temporal kinetics of caspase-1 mRNA expression in response to Pam3CSK4 + TNF-α, alone and in combination (Figure 5B). Whereas TNF-α induced a small (~ 3-fold) but significant effect on caspase-1 mRNA expression at 8 hours ($P < 0.05$), this did not result in changes in caspase-1 protein (Figure 5D). Importantly, Pam3CSK4 did not up-regulate TNF-α–induced caspase-1 mRNA and protein expression. Taken together, these results suggest that because NLRP3 and caspase-1 protein are not present in ASM cells, the NLRP3 inflammasome is not activated upon TLR2 ligation. Thus, IL-1β is not secreted from ASM cells.

NLRP3 and Caspase-1 Are Not Expressed in ASM from Human Tracheal Tissue

We then investigated whether NLRP3 and caspase-1 are present in human tracheas *in situ*, using immunohistochemistry and immunoblot analyses of protein isolated from ASM strips freshly dissected from human tracheas. As shown via immunohistochemistry, whereas the epithelial layer is positive

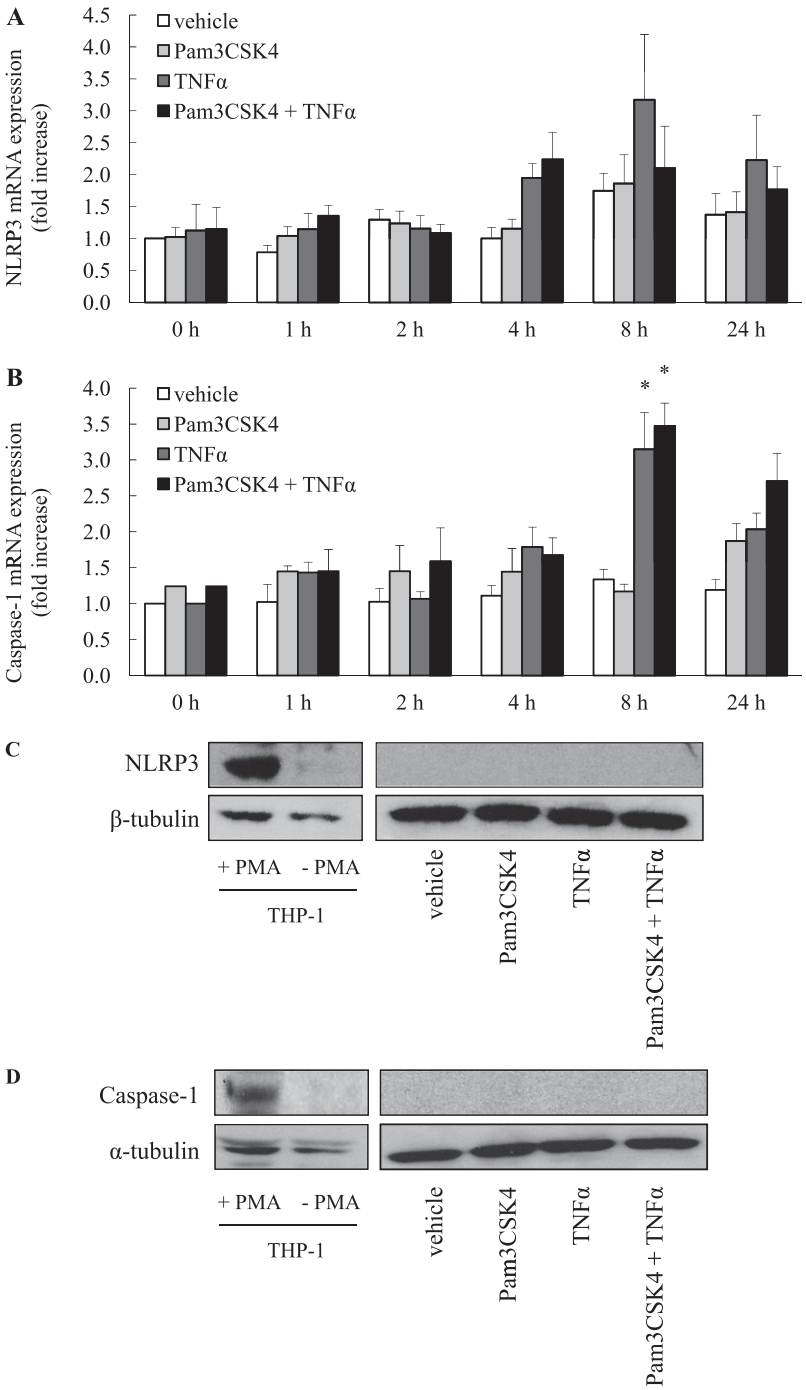

Figure 5. Pam3CSK4 does not affect TNF-α–induced nucleotide-binding domain and leucine-rich repeat protein–3 (NLRP3) mRNA and protein expression in ASM cells. (*A* and *B*) Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 hour, followed by treatment with vehicle or TNF-α (10 ng/ml) for 0, 1, 2, 4, 8, and 24 hours. (*A*) NLRP3 and (*B*) caspase-1 mRNA expression was quantified by real-time RT-PCR, and results are expressed as fold increase compared with vehicle-treated cells at 0 hour, expressed as mean ± SEM values from (*A*) $n = 6$ or (*B*) $n = 4$ primary ASM cell cultures. Statistical analysis was performed using two-way ANOVA and then the Bonferroni *post hoc* test (where an *asterisk* denotes a significant effect of treatment on caspase-1 mRNA expression, compared with vehicle-treated cells; $P < 0.05$). (*C* and *D*) Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 hour, followed by treatment with vehicle or TNF-α (10 ng/ml) for 24 hours. Whole cell lysates were prepared and immunoblotted for (*C*) NLRP3 and (*D*) caspase-1 protein, using α-tubulin as the loading control. (*C* and *D*) Representative blots of $n = 3$ primary ASM cell cultures and lysates from THP-1 monocytes, treated with or without phorbol myristate acetate (PMA; 50 ng/ml), served as positive controls.

for NLRP3 (Figures 6A–6F) and caspase-1 protein (Figures 6G–6L), in accordance with the findings of Hirota and colleagues (19), these proteins are not detected in ASM bundles *in situ*. Moreover, using protein homogenates from freshly isolated ASM strips of human tracheas from three donors, NLRP3 and caspase-1 were not detected by immunoblotting, as shown in Figure 6M. In contrast, a clear band for NLRP3 and caspase-1 protein was evident in lysates from stimulated THP-1 macrophages, run in parallel as a positive control, whereas these proteins were expressed at lower concentrations in unstimulated cells. Collectively, our results demonstrate that NLRP3 and caspase-1 are not expressed in ASM from human tracheal tissue.

DISCUSSION

Our data demonstrate that the Pam3CSK4-mediated augmentation of TNF-α–induced IL-6 and IL-8 mRNA and protein concentrations is not mediated by IL-1β secretion via NLRP3 inflammasome activation in ASM cells. NLRP3 and caspase-1 are not detected immunohistochemically *in situ*, they are not present in dissected ASM strips from human tracheas, and these proteins are not expressed in ASM cells *in vitro*. Thus, the NLRP3 inflammasome is not activated in ASM upon TLR2 ligation.

Recent *in vivo* and *in vitro* studies have implicated the NLRP3 inflammasome as a potential therapeutic target in asthma (1–4), although debate continues (22, 23). To date,

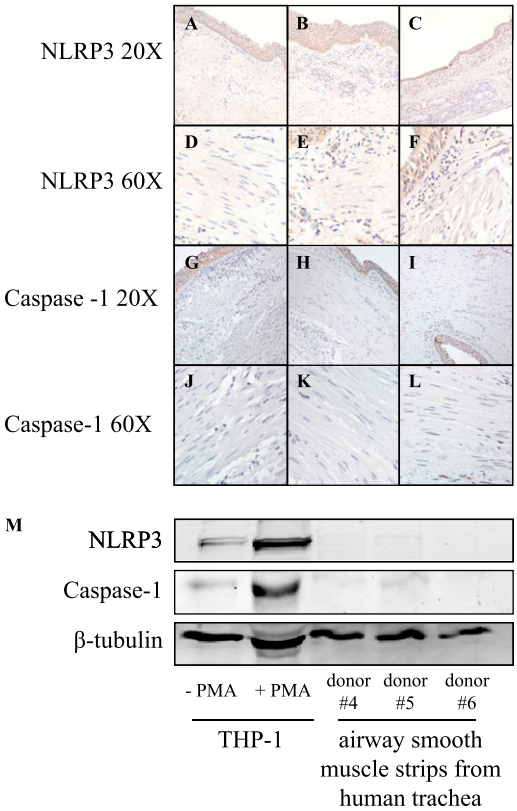

Figure 6. NLRP3 and caspase-1 are not expressed in human ASM from tracheal tissue. (*A–F*) *In situ* immunohistochemistry of three human tracheas donated for medical research (*see* Table 1 for subject characteristics, i.e., Donors 1–3) for (*A–F*) NLRP3 and (*G–L*) caspase-1 protein (*brown* = positive stain) at ×20 and ×60 magnification. (*M*) Immunoblots of NLRP3 and caspase-1 in isolated airway smooth muscle strips from three human tracheas (*see* Table 1 for subject characteristics, i.e., Donors 4–6). Lysates from THP-1 monocytes treated without or with PMA (50 ng/ml) served as positive controls, and β-tubulin was used as a loading control.

several reports demonstrated the presence of the functional NLRP3 inflammasome in asthmatic airway epithelia (19, 24, 25). The NLRP3 inflammasome–mediated production of IL-1β in airway epithelial cells leads to airway neutrophilic inflammation (19), which is the predominant phenotype of airway inflammation in acute exacerbations (26, 27). Allen and colleagues in 2009 (24) demonstrated that the NLRP3 inflammasome expressed in airway epithelial cells and macrophages is required for inflammation after influenza infection. Thus, a growing body of research suggests that the NLRP3 inflammasome may be activated in the airways to promote increased cytokine expression and a proinflammatory milieu during infections.

Increasing evidence demonstrates that ASM cells play a role in the amplification of inflammatory responses during infectious exacerbations in asthma via augmented cytokine production (18, 28–31). Our study is the first to examine the involvement of the NLRP3 inflammasome and possible IL-1β secretion in Pam3CSK4 and TNF-α–induced cytokine up-regulation in ASM cells. Although Pam3CSK4 is primarily known as a TLR2 agonist, its ability to activate the NLRP3 inflammasome to release IL-1β has been demonstrated in macrophages (32). This led us to examine the effects of IL-1β neutralization on Pam3CSK4 and TNF-α–induced cytokine up-regulation. We first demonstrated that IL-1β induces a significant increase in IL-6 and IL-8 protein secretion by ASM cells in a concentration-dependent manner. We also demonstrated that the IL-1β–induced up-regulation of IL-6 and IL-8 secretion is completely inhibited by IL-1β–neutralizing antibody. These results show that the IL-1β–neutralizing antibody can successfully neutralize the effects of IL-1β on cytokine up-regulation in ASM cells. However, the Pam3CSK4-induced up-regulation of IL-6 and IL-8 protein secretion was not inhibited by the neutralization of IL-1β. This finding suggests that IL-1β secretion is not involved in TLR2 ligand–induced cytokine up-regulation in ASM cells.

In support of this, we also demonstrated that ASM cells treated with Pam3CSK4 and TNF-α do not secrete detectable concentations of IL-1β protein over 24 hours, although gene expression is significantly augmented. For the NLRP3 inflammasome in ASM cells to be active in a classic manner, the production and secretion of mature IL-1β should be observed in response to Pam3CSK4 (33), because IL-1β secretion is widely accepted as a surrogate marker for NLRP3 inflammasome activation. However, TLR2 ligation by Pam3CSK4 appears unable to induce assembly of the NLRP3 inflammasome and the processing of IL-1β secretion. We were unable to detect the expression of NLRP3 and caspase-1 protein. Together, our results provide evidence that the NLRP3 inflammasome is not activated to promote IL-1β maturation and secretion in response to Pam3CSK4 and TNF-α in ASM cells. This rules out the possibility that IL-1β secreted as a result of Pam3CSK4-enhanced, TNF-α–induced IL-1β mRNA expression observed at an early time point (4 h) acts in an autocrine manner to boost the further augmentation of IL-6, IL-8, and IL-1β gene expression at later time points (24 h) via a positive feedback loop.

The results presented here indicate important cell-type specificity in airway inflammation, because we demonstrate that the robust up-regulation of proinflammatory cytokines by invading pathogens may not be mediated by activation of the NLRP3 inflammasome and IL-1β secretion in ASM. Using primary cultures of human ASM cells, we demonstrated that Pam3CSK4 robustly up-regulates TNF-α–induced IL-1β mRNA expression, but does not significantly change the expression of NLRP3 and caspase-1. Importantly, IL-1β is not secreted from ASM cells. Thus IL-1β is unable to act in an autocrine manner to induce an inflammatory milieu that mimics exacerbation in ASM cells by promoting IL-6 secretion and up-regulation of the neutrophil chemoattractant chemokine, IL-8. Moreover, although NLRP3 and caspase-1 are present in human airway epithelium (corroborating Hirota and colleagues) (19), these proteins are not detected in the nearby ASM bundles in immunohistochemical analyses of human tracheas *in situ.*

Respiratory infections are key factors underlying asthma exacerbations. By propagating and amplifying inflammation in response to bacterial and viral pathogens, airway structural cells play an important immunomodulatory role in asthma. We explored the role of the NLRP3 inflammasome and the secretion of IL-1β from ASM cells in response to Pam3CSK4 activation, and although TLR2 ligation robustly enhanced cytokine expression, this up-regulation was IL-1β–independent. Exacerbated asthmatic symptoms are not effectively attenuated by current pharmacological management. By addressing molecular mechanisms of exacerbation, our experiments have revealed a potentially important cell type difference, that is, whereas the NLRP3 inflammasome is activated in human airway epithelium (19), the TLR2-mediated enhancement of proinflammatory cytokine secretion in the underlying ASM layer is NLRP3 inflammasome–independent. Although the functional relevance of the restricted localization of the NLRP3 inflammasome in the airways is unclear at present, crosstalk between airway structural cells (and

inflammatory cells) remains a possibility. Thus, further studies are warranted to develop new therapeutic strategies to combat asthma exacerbations in the future.

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ONLINE DATA SUPPLEMENT

The NLRP3 inflammasome is not activated in airway smooth muscle upon TLR2 ligation

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ONLINE SUPPLEMENT

Materials and Methods

Immunohistochemical analysis of NLRP3 and caspase-1 in human trachea

Formalin fixed and paraffin embedded human tracheal sections were cut at 4 micron thickness on poly-l-lysine coated slides, baked for 35 min at 65°C, and allowed to dry. Tissues were de-waxed and hydrated through xylene and decreasing grades of ethanol, followed by tris-buffered saline (TBS) wash for 5 minutes. Tissue sections were blocked with 10% normal goat serum for 2 h at room temperature followed by overnight incubation at 4 ^{o}C with primary rabbit anti-human NLRP3 (HPA012878: Sigma) 1:100 or primary rabbit anti-human caspase-1 polyclonal antibody (2225: Cell Signaling Technology) 1:500 in blocking buffer. Primary antibody was removed with TBS washes and tissue sections were incubated with biotinylated secondary goat anti-rabbit antibody incubated for 2 h at room temperature (1:200) (BA-1000: Vector Laboratories, Burlingame, CA). A streptavidin-linked horseradish peroxidase (K0675) and diaminobenzene (K3467) colour development was performed following manufacturer's directions (Dako, Glostrup, Denmark). Counterstaining was completed using a Mayer's Hematoxylin followed by blueing in TBS. Slides were dehydrated, mounted in permount, and images collected under 20X and 60X magnification using a Nikon A20 microscope with no colour adjustments performed.

Isolation of airway smooth muscle and NLRP3 and caspase-1 immunoblot analysis

Tracheal rings containing airway smooth muscle bundles were placed into a waxed-floor dissecting bath with Krebs solution and opened by cutting the cartilage side. The open ring was pinned with the epithelial side down so that the adventitia and the cartilage could be cleared. Adventitia was removed until the bundles of smooth muscle underneath could be seen through a dissecting microscope. The tracheal segment was then flipped over and the

epithelium was removed with care taken to not damage the underlying airway smooth muscle. Isolated airway smooth muscle strips were then collected for immunoblot protein analysis. Protein isolation from airway smooth muscle bundles was performed using a radio-immuno precipitation assay lysis buffer with protease and phosphatase inhibitors {Hirota, 2009 #3133} and a Qiagen TissueLyser LT operating at 50 Hz for 3 cycles of 2 minutes separated by 1 minute on ice. Protein quantification was performed using a Nanodrop spectrophotometer and a total of 35 μg of protein was added to each lane for each sample on a 7.5% polyacrylamide gel run at 120 V constant. Proteins were transferred to nitrocellulose membrane (Biotrace NT 0.2 micron pore size: Pall, Port Washington, NY) for 2.5 h at 200 mA constant. Membranes were blocked, then incubated overnight at $4^{o}C$ with primary rabbit anti-human NLRP3 or capase-1 antibody in blocking buffer. Membranes were washed and incubated with IR-680 labeled goat anti-rabbit antibody for imaging on a Li-Cor System (Lincoln, NE). Positive control samples included unstimulated THP-1 monocyte cell protein and phorbol myristate acetate (PMA: 50 ng/ml)-stimulated THP-1 monocyte cell protein {Hirota, 2012 #327}. Protein loading was verified by probing membranes for β-tubulin (Clone AA2: Millipore, Billerica, MA).