TLR2 ligand engagement upregulates airway smooth muscle TNF$\alpha$-induced cytokine production

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Manetsch M, Seidel P, Heintz U, Che W, Hughes JM, Ge Q, Sukkar MB, Ammit AJ. TLR2 ligand engagement upregulates airway smooth muscle TNF$\alpha$-induced cytokine production. Am J Physiol Lung Cell Mol Physiol 302: L838–L845, 2012. First published January 13, 2012; doi:10.1152/ajplung.00317.2011.—Airway inflammation and respiratory infections are important factors contributing to disease exacerbation in chronic airway diseases such as asthma and chronic obstructive pulmonary disease. Airway smooth muscle (ASM) cells express Toll-like receptors (TLRs) and may be involved in the amplification of airway inflammatory responses during infectious exacerbations. We determined whether infectious stimuli (mimicked using Pam3CSK4, a synthetic bacterial lipopeptide that binds to TLR2/TLR1) further enhance ASM cell inflammatory responses to TNF$\alpha$ in vitro and the signaling pathways involved. Human ASM cells were pretreated for 1 h with Pam3CSK4 (1 $\mu$g/ml) in the presence or absence of TNF$\alpha$ (10 ng/ml), and IL-6 and IL-8 release was measured after 24 h. As expected, stimulation with Pam3CSK4 or TNF$\alpha$ alone induced significant IL-6 and IL-8 release. Furthermore, Pam3CSK4 significantly increased TNF$\alpha$-induced IL-6 and IL-8 mRNA expression and protein release and neutrophil chemotactic activity. The potentiating effect of Pam3CSK4 on TNF$\alpha$-induced inflammatory responses was not due to enhanced TLR2 expression or NF-$\kappa$B activation of NF-$\kappa$B- and MAPK signaling pathways. Rather, Pam3CSK4 induced cAMP response element (CRE) binding protein phosphorylation and induced CRE-mediated transcriptional regulation, suggesting that Pam3CSK4 and TNF$\alpha$ are acting in concert to enhance ASM cytokine secretion via parallel transcriptional pathways. Our findings suggest that ASM cells may be involved in the amplification of airway inflammatory responses during infectious exacerbations in chronic airway disease. interleukin-6 and -8; adenosine 3’,5’-cyclic monophosphate response element binding; nuclear factor-$\kappa$B; neutrophil chemotaxis; Toll-like receptors; tumor necrosis factor-$\alpha$—

INFLAMMATORY AIRWAY DISORDERS, such as asthma and chronic obstructive pulmonary disease (COPD), bacterial and viral infections are important factors contributing to exacerbation in respiratory diseases (28). Pathogens interact with airway cells via Toll-like receptors (TLRs), a family of pattern-recognition receptors that recognize pathogen-associated molecular patterns. The airway smooth muscle (ASM) plays an important immunomodulatory function in chronic inflammatory airways disease, contributing to the perpetuation and amplification of airway inflammation (9). Recent in vitro evidence demonstrates that ASM cells can also contribute via TLRs to the inflammatory milieu during exacerbations (reviewed in Ref. 17); therefore, investigation of the molecular mechanisms downstream of TLR signaling may identify new approaches to restrain infectious exacerbations in chronic airways disease.

Human ASM cells express mRNA for a number of TLRs (30). Engagement of TLR2 and -4 by bacterial cell wall components such as lipopolysaccharide and lipopeptides and TLR3 by viral RNA has been shown to modulate ASM synthetic functions such as cytokine secretion (6–7, 13, 19, 30) and cell adhesion molecule upregulation (20). These studies support the involvement of ASM cells in the amplification of airway inflammatory responses during infectious exacerbations in asthma and COPD via augmented cytokine production.

To address this, we performed in vitro study examining the hypothesis that infectious stimuli may potentiate ASM inflammatory responses and contribute to neutrophilic inflammation. Neutrophils play a pivotal role in host defense against pathogens, but when their accumulation is excessive, such as in bacterial exacerbations in asthma and COPD, airway damage can ensue (reviewed in Ref. 29). TLR2 has been implicated as a key contributor in neutrophilic inflammation (29); importantly, the TLR2 receptor is functional on ASM cells (19, 30) and TLR2 ligation has been shown to induce ASM secretion of the neutrophil chemoattractant chemokine IL-8 (30) and IL-6 (7), a cytokine that promotes neutrophil survival (5). Thus, in this study, we determine whether ligation of TLR2 by the synthetic bacterial lipoprotein Pam3CSK4 augments the expression of TNF$\alpha$-induced IL-6 and IL-8 in primary cultures of human ASM cells and potentiates neutrophil chemotaxis.

We also aim to uncover the cellular signaling pathways activated by TLR2 ligation in human ASM cells. TLRs recognize pathogens and initiate diverse responses through distinct cellular signaling pathways. Ligation of TLR2 common activates MyD88/TRAF6-dependent pathways to induce proinflammatory cytokine expression via MAPK- and NF-$\kappa$B-mediated gene expression (16). However, cell and species differences are known to exist (15), and the role of other transcription factors in TLR2-mediated gene expression, such as cAMP response element binding (CREB) protein, has recently emerged (18, 21). To date, only a few studies have investigated TLR2 signaling in ASM cells. Lee et al. (19) reported that the MyD88/TRAF6-pathway is responsible for TLR2 signaling induced in human ASM cells by lipoteichoic acid, a component of Gram-positive bacteria cell wall. In ASM cells from the Brown Norway rat, Chiuo and Lin (7) demonstrated that Pam3CSK4 induces cytokine secretion (including IL-6) and activates NF-$\kappa$B-mediated signaling. Further investigations in primary ASM cultures of human origin are warranted, as understanding and targeting these pathways may
allow regulation of the excessive proinflammatory response during infectious exacerbation.

Here we show that Pam3CSK4 robustly amplifies TNFα-induced IL-6 and IL-8 expression in ASM cells and enhances neutrophil chemotaxis. We demonstrate that the effect of Pam3CSK4 occurs via phosphorylation of CREB protein at Ser133 and CRE-mediated transcription, rather than via enhanced NF-κB- or MAPK-mediated signaling. Our findings suggest that ligation of TLR2 may enhance airway smooth muscle inflammatory responses during infectious exacerbations of chronic airway diseases such as asthma and COPD.

MATERIALS AND METHODS

Cell culture. Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified and cultured as previously described by Johnson et al. (14). A minimum of three different ASM primary cell lines were used for each experiment. 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, VIC, Australia) and reverse transcription performed by using the RevertAid first strand cDNA synthesis kit (Fermentas Life Sciences, Hanover, MD) as per the manufacturer’s protocol. IL-6, IL-8, and TLR2 mRNA levels were measured using real-time RT-PCR on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with IL-6 (HS00174131_m1), IL-8 (HS00174103_m1), or TLR2 (HS00610101_m1) TaqMan gene expression assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles.

ELISAs. Cell supernatants were collected and stored at −20°C for later analysis by ELISA or cytokine array. IL-6 and IL-8 ELISAs were performed according to the manufacturer’s instructions (BD Biosciences Pharmingen, San Diego, CA).

Western blotting. Western blotting was performed using mouse monoclonal antibodies or rabbit polyclonal antibodies against TLR2 (Invivogen, San Diego, CA), IκBα (C-21: Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated (Thr180/Tyr182) and total p38 MAPK, phosphorylated (Thr183/Tyr185) and total JNK, and phosphorylated (Ser133) and total CREB. MAPK and CREB antibodies were from Cell Signaling Technology (Danvers, MA), and α-tubulin was used as the loading control (mouse monoclonal IgG1, 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).

NF-κB translocation. Cytoplasmic and nuclear protein extraction was performed using NE-PER nuclear and cytosolic extraction kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Rockford, IL). p50 or p65 NF-κB was quantified by Western blotting using rabbit polyclonal IgG antibodies (C-20 (p65) or H-119 (p50); Santa Cruz Biotechnology) compared with α-tubulin and lamin A/C (rabbit polyclonal IgG antibodies; Cell Signaling Technology) as loading controls for the cytoplasmic and nuclear fractions, respectively. Translocation of p65 NF-κB to the nucleus was detected by immunofluorescence as previously published (27), with the rabbit polyclonal antibody against NF-κB p65 (C-20) detected by an Alexa488 conjugated donkey anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA). Nuclei were visualized using DAPI (Molecular Probes, Eugene, OR).

Transfection. ASM cells were transiently transfected with either pIL-6-luc 651 or pIL-6-luc 158 as previously described (2, 11). The full-length IL-6 promoter construct pIL-6-luc 651 is a pGL3 luciferase reporter vector (Promega, Madison, WI) incorporating a 651-bp fragment of the human IL-6 gene promoter containing transcription factor consensus binding elements for the glucocorticoid receptor, activator protein-1 (AP-1), CCAAT enhancer-binding protein-β, NF-κB, and CRE (kindly provided by Dr. Oliver Eickelberg, Ludwig-Maximilians-Universität and Helmholtz Zentrum, München, Germany; Ref. 10). The pIL-6-luc 158 construct is a truncated version of the IL-6 promoter without the AP-1 and CREB elements (described in ref. 2 as pIL-6-luc 160). To exclude the involvement of the AP-1 element in Pam3CSK4-induced luciferase activity, parallel studies were performed using a pIL-6-luc 651 construct where the AP-1 site had been deleted (pIL-6-luc 651 ΔAP-1: described in Refs. 2, 10). NF-κB activity assays were performed with the NF-κB reporter vector pNF-kB-Luc, according to our previously published methods (12, 23).

Neutrophil chemotaxis. Neutrophils were purified from anticoagulated venous blood of a healthy volunteer by discontinuous density gradient centrifugation with monolayer resolving medium (MP Biologicals, Solon, OH; Ref. 31). Neutrophil chemotaxis towards conditioned media from treated ASM cells was quantified microscopically using a 96-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) as previously published (12). Parallel studies were conducted by incubating conditioned media from treated ASM cells for 1 h with neutralizing antibody (2 μg/ml) against human IL-8 (mouse monoclonal IgG1) or isotype control (R&D Systems: Minneapolis, MN) before measurement of neutrophil chemotaxis.

Statistical analysis. Statistical analysis was performed using either the Student’s unpaired t-test or two-way ANOVA followed by Bonferroni’s posttest. P values <0.05 were sufficient to reject the null hypothesis for all analyses.

RESULTS

Pam3CSK4 augments TNFα-induced IL-6 and IL-8 expression in ASM cells. Stimulation of ASM cells with Pam3CSK4 (1 μg/ml) or TNFα (10 ng/ml) alone for 24 h significantly increased IL-6 and IL-8 protein secretion (Fig. 1, A and B). Furthermore, Pam3CSK4 significantly augmented TNFα-induced IL-6 and IL-8 protein secretion by ~3.7 ± 0.8- and 2.6 ± 0.4-fold, respectively (P < 0.05). This upregulation was also observed at the mRNA level where Pam3CSK4 significantly augmented TNFα-induced IL-6 and IL-8 mRNA expression by 4.6 ± 1.2- and 6.9 ± 1.8-fold, respectively, as demonstrated in Fig. 1, C and D (P < 0.05).

Pam3CSK4-mediated potentiation of ASM inflammatory responses is not due to enhanced TLR2 expression. TLR ligands induce the expression of their cognate receptors in ASM cells (30). Thus we determined whether Pam3CSK4-mediated potentiation of ASM cell cytokine production occurs as a result of increased TLR2 expression. Stimulation of ASM cells with Pam3CSK4 or TNFα alone induced a time-dependent increase in TLR2 mRNA expression (Fig. 2A). However, when ASM cells were treated with Pam3CSK4 and TNFα in combination, there was no significant difference in the temporal kinetics of TLR2 mRNA expression in cells stimulated with TNFα alone compared with treatment with Pam3CSK4 + TNFα (Fig. 2A). Moreover, TNFα-induced protein was similarly unaffected by Pam3CSK4 pretreatment (Fig. 2B), whereas densitometric analysis showed that Pam3CSK4 had no significant effect on TLR2 protein upregulation induced after 24-h stimulation with TNFα (Fig. 2C).
Pam3CSK4-mediated potentiation of ASM inflammatory responses is not due to enhanced NF-κB signaling. Because TLR2 activation has been shown to lead to the activation of NF-κB in cell types apart from ASM (16), and in ASM cells of rat origin (7), we examined whether Pam3CSK4 activates NF-κB signaling in ASM cells from humans. NF-κB is usually held in an inactive state in association with inhibitory IκBα. Signals that induce NF-κB activity cause the phosphorylation of IκBα, their dissociation, and subsequent degradation by the proteasome that allows NF-κB proteins (p50 and p65 subunits) to enter the nucleus and induce gene expression. We examined the effect of Pam3CSK4 on TNFα-induced IκBα degradation and, as shown in Fig. 3A, TNFα stimulation of ASM cells caused a rapid degradation of IκBα after only 10 min of treatment. Importantly, the levels of IκBα were unchanged by Pam3CSK4 and there was no effect on TNFα-induced IκBα degradation when ASM cells were treated with Pam3CSK4 and TNFα in combination.

We then investigated the effects of Pam3CSK4 on TNFα-induced NF-κB nuclear translocation. As evident from Fig. 3B, the amount of NF-κB in the nuclear fraction of the cell lysates was unchanged after treatment with Pam3CSK4 alone. While there was an expected increase in the amount of both p50 and p65 NF-κB in the nuclear fraction after TNFα treatment, there was no augmentation when ASM cells were pretreated with Pam3CSK4. The lack of effect of Pam3CSK4 on TNFα-induced NF-κB nuclear translocation was further confirmed using immunofluorescence microscopy. As shown in Fig. 3C, after a 30-min stimulation with TNFα, p65 NF-κB was found in the nucleus of ASM cells. Pam3CSK4 alone did not induce translocation of p65 NF-κB into the nucleus of ASM cells and had no additional effect on TNFα-induced NF-κB nuclear translocation.

Finally, we performed NF-κB luciferase reporter assays and while we demonstrated that TNFα robustly enhances NF-κB activity, in corroboration of our previous studies (12, 23), there was no potentiation by Pam3CSK4 (Fig. 3D). Taken together,
these data suggest that Pam3CSK4-mediated potentiation of ASM inflammatory responses is not due to enhanced NF-κB signaling. A: to examine the effects of Pam3CSK4 on TNFα-induced IκBα degradation, growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 h, followed by treatment with vehicle or TNFα (10 ng/ml) for 0, 10, 30, and 60 min. Cells were then lysed, and IκBα was analyzed by Western blotting, compared with α-tubulin as a loading control. B: to investigate the effects on p50/p65 NF-κB nuclear translocation, growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 h, followed by treatment with vehicle or TNFα (10 ng/ml) for 30 min. Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of NF-κB p50/p65 quantified by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. C: immunofluorescence microscopy was used to confirm p65 NF-κB nuclear translocation in ASM cells treated as in B. NF-κB translocation was determined using an antibody against NF-κB p65, and nuclei were visualized with DAPI. Results represent n = 3 primary ASM cell lines. D: ASM cells transiently transfected with a NF-κB reporter vector, pNF-κB-Luc, were growth arrested and then pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 h, followed by treatment with vehicle or TNFα (10 ng/ml) for 6 h. Data represent normalized luciferase activity, relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student’s unpaired t-test: *significant effect of treatment on NF-κB activity (means ± SE values from n = 3 primary ASM cell lines; P < 0.05).

Pam3CSK4 does not activate MAPKs, or enhance TNFα-induced MAPK phosphorylation, but does activate CREB and induce CRE-mediated transcriptional regulation.

Because TLR2 activation had been shown to lead to the activation of MAPK-mediated signaling pathways (7, 16), we now examined the effect of Pam3CSK4 alone, or in combination with TNFα, on phosphorylation of the MAPK family members, ERK, p38 MAPK, and JNK, in human ASM cells. As demonstrated in Fig. 4A, we confirmed that TNFα induced activation of all MAPKs in a temporally distinct pattern, as previously described (24, 26). Notably, Pam3CSK4 added alone did not activate MAPK nor augment TNFα-induced MAPK signaling when ASM cells were treated with Pam3CSK4 and TNFα in combination (Fig. 4A).

Activation of the transcription factor CREB by Pam3CSK4 has recently been shown to be responsible for regulation of TLR2-mediated responses (18), and the role of CREB/CRE-
mediated gene expression pathways upon TLR2 activation has emerged (21). The treatment conditions used throughout this study use a 1-h period of pretreatment with Pam3CSK4 (1 μg/ml) before stimulation with TNFα (10 ng/ml), compared with vehicle control. It is possible that Pam3CSK4 activates CREB phosphorylation at the end of the pretreatment period, i.e., before the addition of TNFα. This was examined in Fig. 4, B and C, where we addressed whether Pam3CSK4 activates CREB in ASM after a 60-min stimulation with Pam3CSK4. We found that the TLR2 ligand activated CREB at this time.
point. This is demonstrated by Western blotting in Fig. 4B, where there were greater levels of phospho-CREB (Ser133) after treatment with Pam3CSK4 and in Fig. 4C where densitometric analysis revealed that Pam3CSK4 significantly increased CREB phosphorylation by 2.0 ± 0.5-fold, compared with vehicle-treated cells (P < 0.05).

Moreover, as the 5'-promoter for IL-6 contains a CRE consensus sequence (2), we confirmed the role of CREB/CRE pathway by transient transfection of ASM cells with either the full-length IL-6 promoter (pIL-6-luc 651) or an IL-6 promoter construct where the CREB element had been removed (pIL-6-luc 158; represented graphically as Fig. 4D). As shown in Fig. 4E, Pam3CSK4 or TNFα added individually to ASM cells significantly increased full-length IL-6 promoter activity by 2.5 ± 0.6- or 2.5 ± 0.3-fold, respectively, compared with vehicle-treated cells (P < 0.05). Importantly, IL-6 promoter activity in response to Pam3CSK4 was lost when the CREB element was removed. This is shown in Fig. 4E, where Pam3CSK4-induced luciferase activity in cells transfected with the pIL-6-luc 158 truncated promoter was reduced to a level that was not significantly different from vehicle-treated cells (1.1 ± 0.2-fold). As the pIL-6-luc 158 construct is a truncated version of the IL-6 promoter missing both the AP-1 and CREB elements (Fig. 4D), we performed parallel studies to exclude the involvement of AP-1. As shown in Fig. 4F, there was no significant difference in Pam3CSK4-induced luciferase activity using a pIL-6-luc 651 construct where the AP-1 site had been deleted, compared with the full-length IL-6 promoter. In accordance with our previous report (2) that TNFα-induced IL-6 transcriptional regulation is NF-κB-, not CRE-mediated, the extent of TNFα-induced promoter activity was similar in cells transfected with the full-length or truncated construct. Finally, when added in combination, Pam3CSK4 significantly increased TNFα-induced pIL-6-luc 651 luciferase activity (from 2.5 ± 0.3- to 3.6 ± 0.4-fold), and this activity was reduced when the CREB element was removed (1.6 ± 0.2-fold; P < 0.05). Thus Pam3CSK4 and TNFα appear to act in concert to enhance ASM cytokine secretion via parallel transcriptional pathways.

Pam3CSK4 and TNFα enhance human neutrophil chemotaxis in vitro. Pam3CSK4 and TNFα alone or in combination act to augment the synthetic function of ASMs to stimulate the levels of the potent neutrophil chemoattractant chemokine, IL-8, and the neutrophil prosurvival cytokine IL-6. In Fig. 5A, we show that human neutrophils undergo significantly more migration toward conditioned media from ASM cells treated with Pam3CSK4 (28.4 ± 2.5 cells/high-power field) or TNFα (98.9 ± 8.1 cells/high power field), compared with vehicle-treated cells (9.7 ± 1.9 cells/high power field; P < 0.05). Notably, chemotaxis of human neutrophils was significantly enhanced toward supernatants from ASM cells treated with Pam3CSK4 and TNFα in combination (171.1 ± 8.0 cells/high-power field) (P < 0.05). In parallel studies, conditioned media from treated ASM cells were preincubated with neutralizing antibodies towards human IL-8 and compared with isotype control. As shown in Fig. 5B, there was significantly less neutrophil chemotaxis towards conditioned media from cells stimulated with Pam3CSK4, TNFα, or both when IL-8 had been neutralized, demonstrating the potential role in neutrophil chemotaxis played by IL-8 secreted from ASM cells measured in vitro following TLR2 activation.

DISCUSSION

In this study, we showed that engagement of TLR2 by the synthetic bacterial lipopeptide Pam3CSK4 robustly upregulated TNFα-induced IL-6 and IL-8 expression from ASM cells. Interestingly, the mechanism underlying the augmentation of TNFα-induced cytokine production by Pam3CSK4 in ASM differs from other cell types, in that activation of NF-κB- or MAPK-mediated pathways was not observed. Enhanced mRNA expression for TLR2 receptor was also not responsible. Instead, we showed that Pam3CSK4 induces CREB phosphorylation and CRE-mediated transcriptional regulation. We suggest that Pam3CSK4 and TNFα are acting in concert to enhance ASM cell inflammatory responses via parallel transcriptional pathways. Our findings indicate that ASM cells may be involved in the amplification of airway inflammatory responses during infectious exacerbations in asthma and COPD via augmented cytokine production. In support of this, we demonstrated that Pam3CSK4 potentiated ASM inflammatory responses and enhanced neutrophil chemotaxis measured in vitro.

Sukkar et al. (30) examined the expression of TLR2 (among other TLRs) by human ASM cells. A possible explanation for
the robust upregulation of cytokine secretion that occurred when ASM cells were stimulated with TLR2 ligand in combination with TNFα was that there was a corresponding increase in levels of the TLR2 receptor. To address this, we examined the temporal kinetics of TLR2 mRNA expression after treatment of ASM cells with Pam3CSK4 and TNFα, alone or in combination. TLR2 expression after Pam3CSK4 treatment was significantly increased over time and TNFα induced a comparatively greater increase of TLR2 mRNA expression. This is in accord with Sukkar et al. (30) where Pam3CSK4 and TNFα added individually to ASM cells were shown to induce TLR2 receptor expression after 24 h. However, there was no significant difference between the temporal kinetics of TLR2 mRNA expression in cells stimulated with TNFα alone compared with treatment with Pam3CSK4 + TNFα. Moreover, TNFα-induced TLR2 protein was not upregulated by Pam3CSK4.

In cell types apart from ASM, stimulation of the TLRs leads, through several activator proteins, to the activation of downstream signaling pathways such as NF-κB and MAPKs and subsequently to increased production of proinflammatory cytokines, chemokines, and other inflammatory mediators (8, 16). To date, there are limited studies elucidating the signaling pathways activated by TLR2 ligands in ASM cells (7, 19). We (1, 3–4, 23) and others (24) have established that TNFα leads to activation of NF-κB and MAPKs, and this is corroborated in our current study. Somewhat unexpectedly, however, there was no effect of Pam3CSK4 alone on NF-κB or MAPK activation nor was TNFα-induced NF-κB- or MAPK-signaling enhanced by pretreatment with Pam3CSK4. Our results in ASM are consistent with a recent study in primary cultures of human airway epithelial cells (32) where NF-κB and MAPKs pathways do not account for the observed augmented inflammatory gene expression observed after treatment with the TLR2 ligand Pam3CSK4.

TLRs can trigger activation of other transcription factors apart from NF-κB and contribution of CREB has been recently recognized (21). This led us to examine CREB activation as a putative pathway leading to the enhanced secretion of proinflammatory cytokines from ASM cells mediated by TLR2 engagement. In cell types apart from ASM, Pam3CSK4-induced phosphorylation of CREB has been shown to be involved in regulation of TLR2-mediated immune response (18). Importantly, both IL-6 and IL-8 are CREB-responsive genes as the 5′-promoter for IL-6 (2) and IL-8 (25) contain CRE consensus sequences and are induced by CREB-mediated pathways. To confirm the role of CREB/CRE pathway in Pam3CSK4-induced upregulation of TNFα-mediated cytokine production from ASM cells, we performed transient transfection of ASM cells with either the full-length IL-6 promoter or an IL-6 promoter construct where the CREB element had been removed (2). When added individually to ASM cells transfected with pIL-6-luc 651, Pam3CSK4 or TNFα enhanced full-length IL-6 promoter activity. Importantly, IL-6 promoter activity in response to Pam3CSK4 was lost using the pIL-6-luc 158-truncated promoter, suggesting that Pam3CSK4 transcriptional regulation may be CRE mediated. As pIL-6 158 lacks both CRE and AP-1 sites, we excluded the involvement of AP-1 by determining that Pam3CSK4-induced luciferase activity in full-length IL-6 promoter (pIL-6-luc 651) did not appear different from that in a pIL-6-luc 651 construct where the AP-1 site had been deleted. This suggests that effects observed are due to CRE truncation and not AP-1 site truncation. In contrast, TNFα-induced promoter activity was equivalent in cells transfected with the full-length or truncated construct, confirming that TNFα-induced IL-6 transcriptional regulation is NF-κB mediated (2). Together, Pam3CSK4 significantly increased TNFα-induced IL-6 promoter activity, and our results are consistent with Pam3CSK4 and TNFα acting in concert to enhance ASM cytokine secretion via parallel transcriptional pathways.

ASM cells play an important immunomodulatory role, and in this study we provide further evidence to support a role for the involvement of ASM in the amplification of airway inflammatory responses during infectious exacerbations via augmented cytokine production. TLR receptors are expressed on ASM cells (22, 30), and TLR engagement with synthetic ligands or bacterial products enhance TLR function and induce proinflammatory cytokine secretion from human ASM cells, including eotaxin and IL-8 (30). Our current report extends these studies to demonstrate that Pam3CSK4 also increases secretion of IL-6 and confirms the effect of TLR2 ligand on IL-8 secretion (30). Secretion of IL-6 upon stimulation with Pam3CSK4 has been previously reported in ASM cells of rat origin (7). We show a substantial potentiation of IL-6 and IL-8 expression when ASM cells are stimulated by inflammatory stimuli in the presence of a synthetic bacterial ligand for TLR2. The augmented effect on cytokine secretion by ASM may have important consequences in vivo, as was highlighted by the in vitro demonstration of enhanced neutrophil chemotaxis towards conditioned media from ASM cells that had been stimulated with Pam3CSK4 in combination TNFα, as opposed to TNFα alone. These findings may have implications for our understanding of neutrophilic inflammation in asthma and COPD and bacterial exacerbations. We demonstrate that potentiation of ASM inflammatory responses by infectious stimuli was CREB- not NF-κB mediated, thus targeting these pathways may allow regulation of the excessive proinflammatory response.

In summary, we have obtained greater insight into how respiratory infections in combination with inflammatory conditions in the airways are important factors that may contribute to disease exacerbations and identified key molecules regulating the ASM cells proinflammatory response.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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