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Tobacco Smoking Inhibits Expression of Proinflammatory Cytokines and Activation of IL-1R-Associated Kinase, p38, and NF-κB in Alveolar Macrophages Stimulated with TLR2 and TLR4 Agonists[1]

Haiyan Chen,* Mark J. Cowan,† Jeffrey D. Hasday,† Stefanie N. Vogel,* and Andrei E. Medvedev[2]*

Tobacco smoking has been associated with impaired pulmonary functions and increased incidence of infections; however, mechanisms that underlie these phenomena are poorly understood. In this study, we examined whether smokers' alveolar macrophages (AM) exhibit impaired sensing of bacterial components via TLR2 and TLR4 and determined the effect of smoking on expression levels of TLR2, TLR4 and coreceptors, and activation of signaling intermediates. Smokers' AMs exhibited reduced gene expression and secretion of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (RANTES and IL-8) upon stimulation with TLR2 and TLR4 agonists, *S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-Lys4-OH trihydrochloride (Pam₃Cys), and LPS, whereas expression of anti-inflammatory cytokines (IL-10 and IL-1 receptor antagonist) was not affected. TLR3 activation with polyinosinic-polycytidylic acid led to comparable or even higher cytokine responses in smokers' AMs, indicating that smoking-induced suppression does not affect all TLRs. Comparable expression of cytokines and chemokines was detected in PBMC and purified monocytes obtained from smokers and nonsmokers, demonstrating that the suppressive effect of smoking is restricted to the lung. TLR2/4-inducible IL-1R-associated kinase-1 (IRAK-1) and p38 phosphorylation and NF-κB activation was suppressed in smokers' AMs, whereas TLR2, TLR4, CD14, MD-2 mRNA levels, and TLR4 protein expression were not altered. These data suggest that changes in expression and/or activities of signaling intermediates at the postreceptor level account for smoking-induced immunosuppression. Thus, exposure of AMs to tobacco smoke induces a hyporesponsive state similar to endotoxin tolerance as manifested by inhibited TLR2/4-induced expression of proinflammatory cytokines, chemokines, and impaired activation of IRAK-1, p38, and NF-κB, resulting in suppressed expression of proinflammatory mediators. *The Journal of Immunology,* 2007, 179: 6097–6106.

T obacco smoking, a major risk factor for development of cancer (1), has also been associated with an increased incidence of bacterial infections, chronic pulmonary obstructive disease (COPD),[3] asthma and bronchitis (2–4). Smoking-induced immunosuppression has been implicated in the pathogenesis of these diseases (5), as evidenced by suppres-

sion of NK cytotoxicity (6, 7), inhibition of B cell proliferation and Ab production (1, 8), and impaired Ag- and mitogen-mediated T cell responses (1, 9, 10). Smoking severely impairs functions of alveolar macrophages (AM) and airway epithelial cells, including inhibited LPS-induced expression of TNF-α, IL-1β, and IL-6 (6, 11–16), NO secretion (13), microbicidal activity, and phagocytosis (14). This suggests that smoking suppresses the ability of the host to develop the innate immune response to infection.

AMs and epithelial cells provide the first line defense against infection in the lung by recognizing conserved pathogen-associated molecular patterns (PAMP) expressed by bacteria, fungi, and viruses through TLRs. TLRs share a similar structural organization, expressing an ectodomain with leucine-rich repeats, a transmembrane region, and an intracellular tail containing a conserved signaling Toll-IL-1R (TIR) domain (reviewed in Ref. 17). TLRs exhibit different recognition patterns, enabling the host immune cells to sense a variety of bacterial and viral PAMPs. TLR2, in cooperation with TLR1 or TLR6, recognizes lipoproteins and lipopeptides from Gram-positive bacteria, *Mycoplasma,* and mycobacteria (18–20). TLR4 is the principal signaling receptor for LPS from Gram-negative bacteria (21) but also responds to many other structurally unrelated agonists (reviewed in Ref. 22), and TLR5 senses bacterial flagellin (23). TLRs 1, 2, and 4–6 are expressed primarily on the cell surface, whereas TLRs 3, 7, and 8 (recognizing viral nucleic acids) and TLR9 (sensing unmethylated CpG motifs in bacterial and viral

FIGURE 1. Tobacco smoking inhibits expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in AMs stimulated with TLR4 (LPS) and TLR2 (Pam3Cys)-agonists. AMs obtained from nonsmokers (□) and smokers (■) were stimulated for 4 h (cytokine mRNA) (*A*) or 6 h (cytokine release) (*B*) with medium, LPS, or Pam3Cys (1 μg/ml each). Total RNA was isolated and cell-free supernatants were collected to analyze levels of cytokine gene expression and secretion by real-time PCR and ELISA, respectively. Data were collected from 15 nonsmokers and 15 smokers (cytokine mRNA results) and 23 nonsmokers and 23 smokers (cytokine secretion data). *, $p < 0.05$.
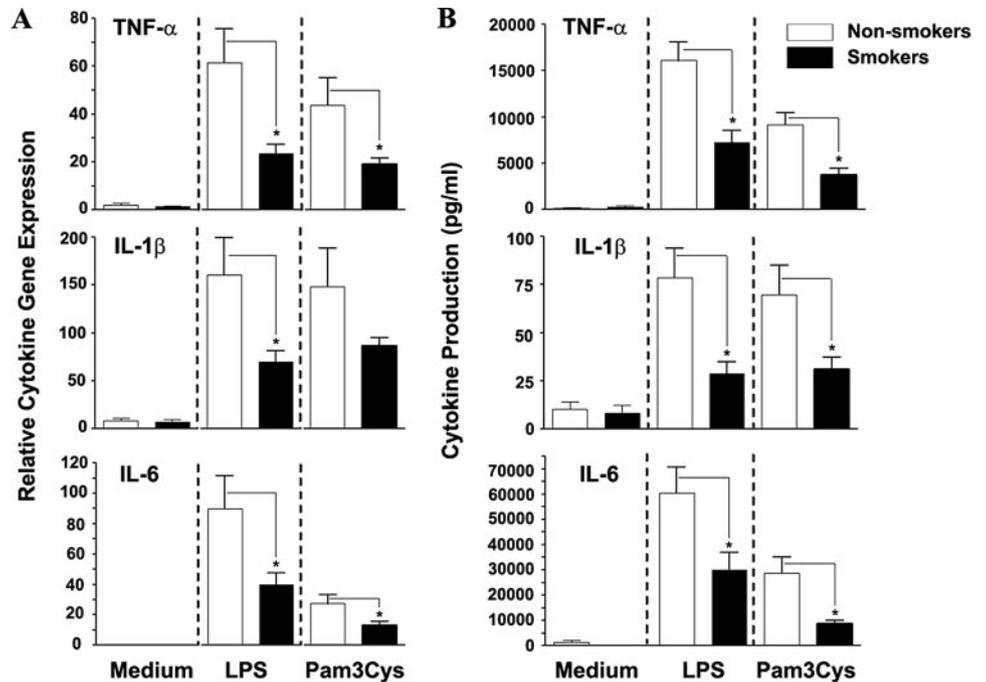
DNA) are largely confined to the endosomes (reviewed in Refs. 22 and 24). Recognition of PAMPs by TLRs may be facilitated by a number of coreceptors and accessory proteins, such as CD14, CD11b/CD18 (TLR2 and TLR4), CD36, and dectin-1 (TLR2; reviewed in Refs. 22 and 24). MD-2, an extracellular protein that binds LPS and presents it to TLR4, is necessary for TLR4 sensing of LPS, as evidenced by the lack of LPS responsiveness and the failure to clear bacterial infections in MD-2$^{-/-}$ mice (25, 26). Recognition of PAMPs by TLRs initiates receptor oligomerization, conformation changes, and TIR-TIR domain interactions (27), creating docking platforms for various adapter molecules and kinases (reviewed in Ref. 28). As a result, adapter proteins are recruited to their TLRs: MyD88 (all TLRs except TLR3), TIR domain-containing adapter protein (TLR2 and TLR4), TIR domain-containing adapter inducing IFN-β (TRIF; TLR3, TLR4), and TRIF-related adapter molecule (TLR4 only; Refs. 22, 24, 29, and 30). Concurrently, a series of kinases (e.g., IL-1R-associated kinase (IRAK)-4-IRAK-1, TRIF-TANK-binding kinase-1/IκB kinase-ε are recruited to the TLRs. They become activated and interact with downstream adapters (e.g., TNFR-associated factor-6), leading to activation of cytokines, chemokines, IFNs, and up-regulation of MHC and costimulatory molecules (reviewed in Refs. 22, 24, 27, and 28).

Endotoxin tolerance is a state of refractoriness to LPS challenge following prior exposure to LPS, and it is thought to limit excessive inflammatory responses (22). Whereas activation of ERK, JNK, p38 MAPK, and induction of NF-κB and proinflammatory cytokines (e.g., TNF-α and IL-12) are inhibited in LPS-tolerant cells, other responses (e.g., IL-10 and IL-1R antagonist (IL-1RA) expression) are not affected (reviewed in Ref. 22). This led to the concept that endotoxin tolerance represents cellular reprogramming to adapt to the persisting infection (31). However, this suppresses the ability of monocytes and macrophages to respond to secondary infections as evidenced by the finding that septic shock survivors exhibit decreased monocyte responsiveness to LPS and increased susceptibility to bacterial infections (32, 33). Molecular mechanisms responsible for induction and maintenance of endotoxin tolerance remain poorly

understood. Although LPS tolerance has been suggested to occur due to decreased numbers of LPS receptors on the cell surface (34, 35), other studies indicate dysregulation of signaling downstream of TLR4 as a primary mechanism. These include suppressed LPS-induced MyD88/TLR4 and IRAK-1/MyD88 interactions, inhibited IRAK-1 activation (36, 37), increased expression of negative regulators of LPS signaling (e.g., suppressor of cytokine signaling-1, IRAK-M; Refs. 38–40), and accumulation of transcription-deficient p50 NF-κB (41, 42).

Previous studies showed that tobacco and tobacco smoke contain bioactive LPS (43) at concentrations sufficient for the induction of endotoxin tolerance (31–33, 36, 37, 44). Thus, repeated exposure of smokers' AM to LPS contained in cigarette smoke may induce macrophage refractoriness to TLR2- and TLR4-inducible activation by mechanisms resembling those that operate in endotoxin tolerance. Eventually, these may contribute to the pathogenesis of chronic bronchitis that develops in smokers. To delineate these mechanisms, we compared gene expression and production of cytokines, chemokines, and IFNs in AMs and PBMCs obtained from smokers and healthy nonsmoking volunteers in response to defined TLR2 and TLR4 agonists. In addition, expression of TLR2 and TLR4, coreceptors CD14 and MD-2, and TLR2/4-inducible activation of IRAK-1, p38 MAPK, and NF-κB were examined. Our data support the hypothesis that induction of tolerance in smokers' AMs underlies suppressed proinflammatory responses to TLR2 and TLR4 agonists and may contribute to smokers' increased susceptibility to pulmonary infection.

Materials and Methods

Human subjects

The study population consisted of 25 healthy smokers with ages between 21 and 55 years having a >5-pack-year smoking history and currently smoking ≥1 pack per day, and 27 healthy age- and gender-matched non-smoking individuals. Human subjects with known lung disease, allergy to lidocaine, infection within past 3 wk, forced expiratory volume1/forced vital capacity of < 0.7, history of adverse reaction during blood drawing, history of anemia, chronic immune suppression (HIV, rheumatologic, organ transplantation), currently pregnant, or using corticosteroids, anticoagulants, or anti-platelet drugs were excluded. Recruitment, investigative
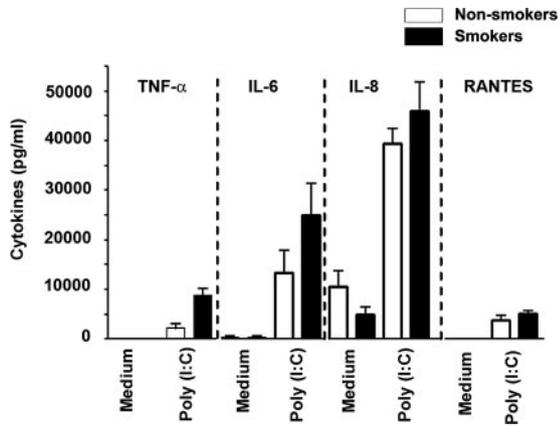
FIGURE 2. Effect of tobacco smoking on production of cytokines and chemokines by alveolar macrophages stimulated with poly (I:C). Non-smokers' (□) and smokers' (■) AMs were stimulated with 100 μg/ml poly (I:C) for 24 h, followed by analyses of cytokine and chemokine levels in cell-free culture supernatants by ELISA. Data (mean ± SD) are shown for 3 nonsmokers and 3 smokers.

bronchoscopy, blood drawing, and other procedures involving human subjects were conducted under the University of Maryland, Baltimore Institute Review Board-approved protocol.

Reagents and cell culture

Cells were cultured in RPMI 1640 (Mediatech.) supplemented with 1 mM L-glutamine, 100 μg/ml penicillin/streptomycin, (Invitrogen Life Technologies), 5×10^{-5} M 2-ME (Sigma-Aldrich), and 5% FBS (HyClone). Highly purified LPS from *Escherichia coli* K235 was prepared as described (45) and used as a TLR4 agonist. The synthetic lipopeptide *S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-Lys4-OH trihydrochloride (Pam3Cys) was from EMC Microcollections and used as a TLR2 agonist. Polyinosine-polycytidylic acid (poly(1:C)) was obtained from Invivogen and used as a TLR3 agonist when added to cells without liposomal cytoplasmic delivery (to avoid additional activation of RIG-I/MDA-5). Cells were stimulated with LPS, Pam3Cys (1 μg/ml each) or poly(1:C) (100 μg/ml) for 15 min (for cellular extract preparation), 4 h (for RNA isolation), or 6 h (for cytokine assays). Abs against IκB-α, TLR4 (H80), β-actin, lamin B, and IRAK-1 were from Santa Cruz Biotechnology; anti-phospho-p38 Ab was from Promega; and anti-p50, p-IRAK-1, p-p65, and p65 Abs were from Cell Signaling. Dynal Monocyte Negative Isolation Kit and a Dynal MPC-15 magnet were from Invitrogen Life Technologies, and anti-CD14-FITC- and anti-CD45-PE-conjugated Abs were from BD Pharmingen.

Isolation of AMs, PBMCs, and monocytes

Bronchoalveolar lavage fluid was obtained by investigative bronchoscopy and bronchoalveolar lavage as described previously (11). In brief, after topical 2% lidocaine anesthesia, a fiberoptic bronchoscope was inserted into the airways and wedged in a segment of the right middle lobe. Bronchoalveolar lavage was performed by instilling four 50-ml aliquots of saline (0.9%), followed by gentle suction. Bronchoalveolar lavage fluid was filtered through sterile gauze to remove mucus, and cells were collected by centrifugation. Macrophages were obtained by adherence to sterile 24-well (5×10^5 cells per well) or 6-well (3×10^6 cells per well) polystyrene culture plates at 37°C for 20 h, nonadherent cells were removed by washing with warm culture medium, and macrophages were resuspended in culture medium. Heparinized blood was centrifuged in Ficoll-Paque PLUS (<0.12 endotoxin units/ml; Amersham Biosciences) and interphase layers containing PBMCs were collected and plated as indicated for AM cultures. Monocytes were isolated from PBMC using a Dynal Monocyte Negative Isolation Kit according to the manufacturer's recommendations. Briefly, PBMCs were incubated with the Ab mix containing Abs for CD2, CD7, CD16, CD19, CD56, and CD235a, followed by incubation with Dynabeads and immunomagnetic removal of T and B lymphocytes, NK cells, and granulocytes on a Dynal MPC-50 magnet. The purity of the resulting population of monocytes was assessed by staining with anti-CD14-FITC (that stains CD14+ monocytes) and anti-CD45-PE (that stains CD45, a transmembrane glycoprotein broadly expressed by hematopoietic cells including monocytes, granulocytes, and lymphocytes)-conjugated Abs (BD Pharmingen). FACS analysis was conducted on a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Isolation of RNA, real-time PCR, and analysis of cytokine production

Total RNA was isolated with RNeasy kits (Invitrogen Life Technologies), followed by DNase digestion and repurification as recommended by the manufacturer. cDNA was prepared from 1 μg of RNA using a Reverse Transcription System (Promega) and subjected to quantitative real-time PCR analysis with gene-specific primers for human hypoxanthine-guanine phosphoribosyltransferase, IL-8, IL-1β, IL-6, TNF-α, IFN-γ, RANTES, and IFN-β on a MyIQ minicycler with optical detection system (Bio-Rad). Real-time PCR data were processed using the $-2^{\Delta\Delta CT}$ method as described (46). Levels of cytokines and chemokines in cell-free supernatants were determined by ELISA in the University of Maryland, Baltimore Cytokine Core Laboratory using commercially paired Abs and recombinant standards obtained from R&D Systems (TNF-α, IL-1β, IFN-γ, and RANTES), Biosource (IL-1RA and IL-8), and Pierce Biotech (IL-6). The lower detection limits for these assays were (picograms per milliliter) 3.9, 3.12, 6.25, 3.9, 9.3, 15.6, and 31.25 for TNF-α, IL-1β, IL-6, IL-8, IL-10, IFN-γ, and RANTES, and IL-1RA, respectively.

Immunoblotting and immunoprecipitation

Cell extracts were prepared as described (37, 47) using a lysis buffer containing 20 mM HEPES (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, 12.5

FIGURE 3. Impaired expression of chemokines IL-8 and RANTES in smokers' AM stimulated with TLR2 and TLR4 agonists. Nonsmokers' (□) and smokers' (■) AMs were treated for 4 h (chemokine mRNA expression) (*A*) or 6 h (chemokine secretion) (*B*) with medium, LPS, or Pam3Cys (1 μg/ml each). IL-8 and RANTES mRNA levels and secretion were assessed in samples of controls' and smokers' RNA and cell-free supernatants by real-time PCR and ELISA, respectively. Results are presented for 13 nonsmoking volunteers and 13 smokers (gene expression) and 23 nonsmokers and 23 smokers (cytokine secretion data), respectively. *, $p < 0.05$.
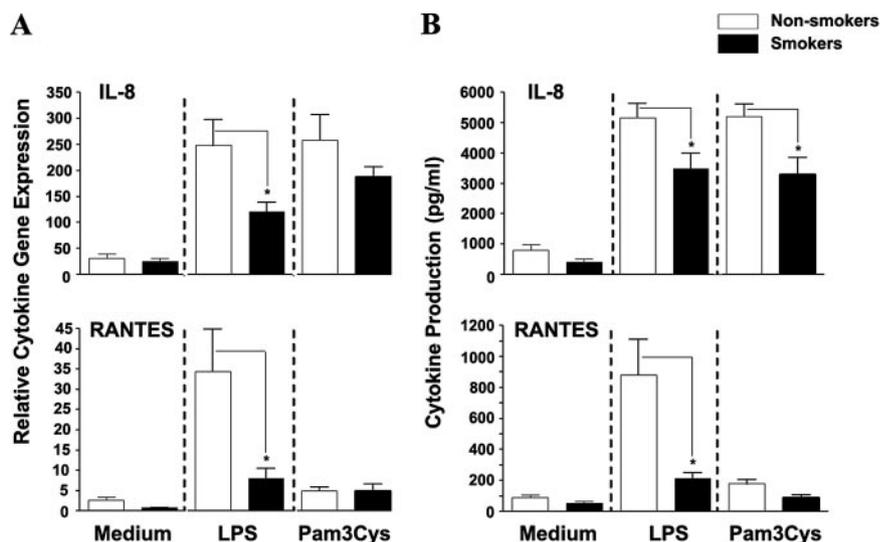
FIGURE 4. Tobacco smoking does not affect LPS- and Pam3Cys-inducible expression of anti-inflammatory cytokines IL-10 and IL-1RA in AMs. Pulmonary macrophages obtained from nonsmokers (□) and smokers (■) were stimulated for 4 h (cytokine mRNA) (*A*) or 6 h (cytokine release) (*B*) with medium, LPS, or Pam3Cys (1 μg/ml each). After stimulation, RNA (six subjects in each group) was prepared, reverse-transcribed, and subjected to real-time PCR to analyze mRNA levels of IL-10 and IL-1RA. Cell-free supernatants (23 subjects in each group) were collected and analyzed by ELISA to measure cytokine secretion levels.
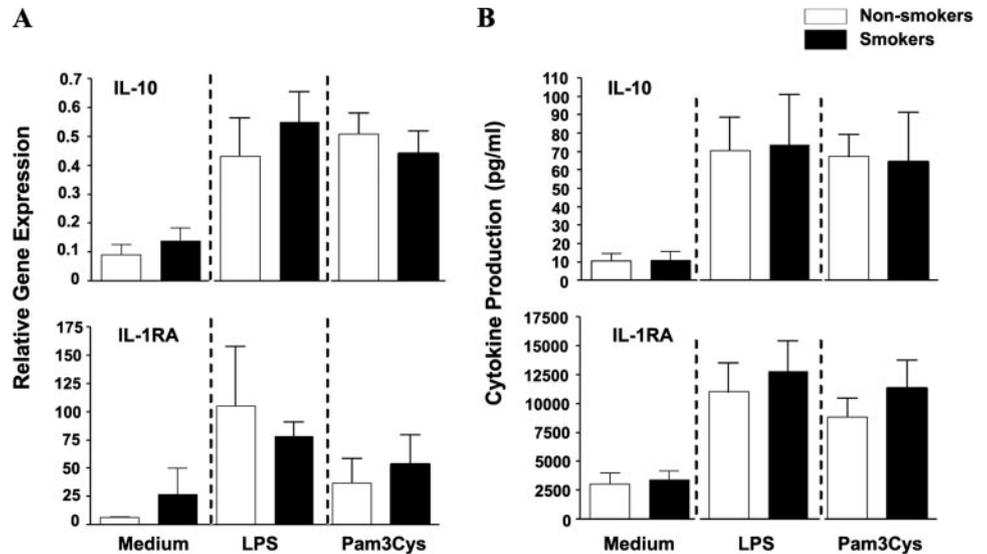
mM β-glycerophosphate, 50 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Roche). Nuclear extracts were prepared using a Nuclear Extract kit (Active Motif) according to the manufacturer's recommendations. Cell and nuclear extracts were subjected to immunoblot analysis by separating proteins on 4–20% PAGE minigels (Invitrogen Life Technologies), transferring to Immobilon-P membranes (Millipore), and probing with corresponding Abs as described previously (47). Densitometric analysis of intensities of the corresponding bands was performed using the Quantity One software (Bio-Rad) and were normalized for β-actin expression.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4 program for Windows (Graphpad Software). Statistical differences among experimental groups were evaluated by Student's *t* test with the level of significance set at $p < 0.05$. Values are expressed as mean ± SD.

Results

Smoking inhibits expression of proinflammatory, but not anti-inflammatory, cytokines, and chemokines in AMs stimulated with TLR2 and TLR4 agonists while not affecting TLR3 responses

Smokers' AMs were previously reported to secrete reduced levels of TNF-α, IL-1β, and IL-6 upon stimulation with LPS (6, 11–15). We were interested to extend these observations to a broader panel of cytokines and chemokines and to examine whether TLR2- and TLR3-inducible responses were similarly suppressed in smokers' AMs. Hence, we examined the effect of smoking on gene expression and production of pro- and anti-inflammatory cytokines in AMs activated in vitro via TLR2, TLR3, and TLR4. LPS stimulation of nonsmokers' AMs induced marked up-regulation of TNF-α, IL-1β, and IL-6 mRNA levels and a potent increase in secretion of these three proinflammatory cytokines (Fig. 1). The TLR2 agonist Pam3Cys elicited similar levels of IL-1β mRNA

and protein expression, but it was ~20–60% less potent than LPS in triggering TNF-α and IL-6 mRNA expression and production (Fig. 1). Compared with nonsmokers' macrophages, LPS- and Pam3Cys-mediated gene expression and production of these cytokines in smokers' AM were inhibited by ~50–70% (Fig. 1). In contrast, smokers' AMs exhibited comparable or higher secretion of proinflammatory cytokines TNF-α and IL-6 after stimulation with a TLR3 agonist, poly(I:C), for 6 h (data not shown) and 24 h (Fig. 2), compared with the cytokine levels produced by nonsmokers' cells. Similar results were obtained when cytokine mRNA levels were determined by real-time PCR in poly(I:C)-stimulated smokers' AMs (results not shown).

To further characterize the defect in the response of smokers' AMs to TLR2, TLR3, and TLR4 agonists, we compared chemokine expression (IL-8 and RANTES) in cells isolated from nonsmokers and smokers after treatment with Pam3Cys, poly(I:C), and LPS. Stimulation of nonsmokers' AMs with LPS or Pam3Cys led to robust IL-8 gene expression and secretion (Fig. 3, *tops*). LPS markedly increased RANTES mRNA expression and secretion in nonsmokers' AMs (Fig. 3), whereas Pam3Cys failed to induce these responses, consistent with the inability of TLR2 agonists to activate the MyD88-independent pathway that activates RANTES expression (48). As was the case for IL-8, smoker's AM showed reduced expression of RANTES mRNA and protein compared with the responses observed in nonsmokers' cells (Fig. 3). In contrast with TLR2/4-induced chemokine responses, activation of TLR3 with poly(I:C) led to comparable gene expression (data not shown) and production (Fig. 2) of IL-8 and RANTES from nonsmokers' and smokers' AMs.

In contrast to proinflammatory cytokine expression, similar levels of anti-inflammatory IL-10 and IL-1RA mRNA (Fig. 4*A*) and

Table I. *Gene expression levels of pro- and anti-inflammatory cytokines in normal and smokers' PBMCs*

Cytokine Gene Expression	Nonsmokers (*n* = 22)			Smokers (*n* = 24)		
	Medium	LPS	Pam3Cys	Medium	LPS	Pam3Cys
TNF-α	1.002 ± 0.3370	5.356 ± 1.582	4.173 ± 1.170	0.8050 ± 0.2419	3.975 ± 0.8900	2.952 ± 0.6337
IL-1β	3.906 ± 2.153	55.69 ± 14.74	99.50 ± 18.43	4.037 ± 3.254	52.10 ± 16.98	67.29 ± 19.20
IL-6	0.1091 ± 0.07035	6.038 ± 1.478	5.168 ± 1.422	0.3177 ± 0.2509	5.076 ± 1.301	4.129 ± 1.920
IL-8	3.788 ± 1.084	41.42 ± 8.528	87.29 ± 12.56	5.771 ± 2.213	51.99 ± 16.65	72.32 ± 17.43
RANTES	18.40 ± 3.889	43.15 ± 12.26	26.21 ± 9.057	24.26 ± 5.023	43.95 ± 11.80	25.48 ± 6.212

Table II. *Production of pro- and anti-inflammatory cytokines in nonsmokers' and smokers' PBMCs*

Cytokine Production (pg/ml)	Nonsmokers (n = 22)			Smokers (n = 24)		
	Medium	LPS	Pam3Cys	Medium	LPS	Pam3Cys
TNF-α	109.9 ± 34.3	1,485 ± 303.0	680.5 ± 186.9	34.3 ± 17.3	1,520 ± 381.3	861.8 ± 365.1
IL-1β	4.72 ± 2.2	69.3 ± 13.22	38.9 ± 7.7	3.5 ± 1.5	66.7 ± 9.8	44.7 ± 8.8
IL-6	741.9 ± 351.9	5,216 ± 970.1	2,161 ± 456.7	477.0 ± 372.8	4,038 ± 1,613	1,639 ± 474.5
IL-8	2,023 ± 742.5	13,480 ± 3,393	14,900 ± 2,127	2,757 ± 798.2	13,140 ± 2,869	14,000 ± 3,095
RANTES	636.0 ± 143.9	1,781 ± 281.3	941.0 ± 199.3	697.1 ± 121.0	1,570 ± 339.4	909.7 ± 199.1
IL-10	54.4 ± 22.6	505.6 ± 84.6	324.7 ± 67.5	33.2 ± 12.9	440.2 ± 80.7	281.5 ± 77.8
IL-1RA	453 ± 149.7	1,421 ± 306.9	874.5 ± 309.7	448.7 ± 163.6	1,542 ± 439.0	777.0 ± 248.5

secreted protein (Fig. 4B) were detected in smokers' and nonsmokers' AMs stimulated with LPS and Pam3Cys. Taken collectively, these data indicate severe suppressive effects of tobacco smoking on TLR2- and TLR4-initiated expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (IL-8 and RANTES) in AM, whereas anti-inflammatory IL-10 and IL-1RA gene expression and secretion are not influenced by smoking. Our results also indicate that smoking-mediated immunosuppression of the cytokine responses of AMs targets TLR2 and TLR4 signaling but does not affect TLR3-mediated responses.

PBMCs and monocytes from nonsmokers and smokers exhibit comparable expression of cytokines and chemokines in response to stimulation with TLR2 and TLR4 agonists

Next, we analyzed whether smoking-mediated immunosuppression is localized to the lung or exerts systemic effects on the response of circulating white blood cells. Therefore, mRNA expression and secretion of various pro- and anti-inflammatory cytokines and chemokines were analyzed in PBMCs and monocytes isolated from smokers and nonsmoking volunteers. In contrast to the inhibited responsiveness of smokers' AMs, similar patterns of constitutive and LPS- or Pam3Cys-induced mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (IL-8 and RANTES) were observed in control and smokers' PBMC (Table I). Consistent with gene expression data, similar secretion levels of proinflammatory (TNF-α, IL-1β, and IL-6) or anti-inflammatory (IL-10 and IL-1RA) cytokines and chemokines (IL-8 and RANTES) were observed in nonsmokers' and smokers' PBMC stimulated with LPS or Pam3Cys (Table II).

To extend these results, production of cytokines and chemokines was compared in nonsmokers' and smokers' monocytes isolated from PBMC by depleting T and B lymphocytes, NK cells, and granulocytes with the Dynal Monocyte Negative Isolation Kit. PBMCs were incubated with Abs for CD2, CD7, CD16, CD19, CD56, and CD235a, followed by incubation with Dynabeads and immunomagnetic removal of T and B lymphocytes, NK cells, and granulocytes. FACS analyses confirmed the enrichment of CD14^{+high}CD45^{+high} monocytes from ~35–41% in the PBMC population to ~88–93% in isolated monocytes. Similar levels of LPS- and Pam3Cys-induced TNF-α, IL-6, and IL-1β were pro-

duced by PBMCs and monocytes isolated from the same donors, as determined by ELISA (data not shown). These data suggest that monocytes represent the main cytokine-secreting cells in the PBMC population responding to TLR2 and TLR4 agonists. Stimulation of smokers' and nonsmokers' purified monocytes with LPS and Pam3Cys resulted in similar levels of production of IL-8, RANTES, IL-10; and IL-1RA (Table III). Likewise, no statistically significant differences were observed between cytokine-secreting capacities of nonsmokers' and smokers' monocytes when LPS- and Pam3Cys-inducible production of TNF-α and IL-6 was examined (data not shown). These results indicate that the suppressive effect of chronic cigarette smoke exposure on macrophage capacity for TLR2/4-dependent cytokine expression is regional, being confined to the bronchoalveolar compartment.

Effect of smoking on expression of TLR2, TLR4, CD14, and MD-2 in AMs

To examine whether down-regulation of receptors and coreceptor molecules is responsible for smoking-mediated immunosuppression, basal and TLR2/4-inducible expression of genes that encode TLR2, TLR4, and coreceptors CD14 and MD-2 was compared in nonsmokers' and smokers' AMs. Real-time PCR revealed similar levels of CD14 and MD-2 mRNA in nonsmokers' and smokers' AMs, with no statistically significant modulation caused by LPS or Pam3Cys (Fig. 5A). LPS stimulation of both nonsmokers' and smokers' AMs for 4 h resulted in a ~2.5-fold increase in TLR2 mRNA levels, whereas TLR4 gene expression was slightly down-regulated (Fig. 5A). Similarly, Pam3Cys treatment of AMs caused comparable regulatory effects on TLR2 (an increase) and TLR4 (a decrease) mRNA expression levels (Fig. 5A). Nonsmokers' and smokers' AMs showed comparable constitutive and LPS- and Pam3Cys-induced TLR2 and TLR4 gene expression (Fig. 5A). To substantiate the gene expression data, we also assessed TLR4 protein expression in cellular extracts obtained from control and smokers' AMs incubated with medium, LPS, or Pam3Cys. Results of representative immunoblots and densitometric quantification of all five experiments, normalized to β-actin expression (loading control), revealed no significant changes in TLR4 protein expression in control vs smokers' AMs (Fig. 5B). Taken collectively,

Table III. *Production of pro- and anti-inflammatory cytokines in nonsmokers' and smokers' monocytes*

Cytokines (pg/ml)	Nonsmokers (n = 3)			Smokers (n = 3)		
	Medium	LPS	Pam3Cys	Medium	LPS	Pam3Cys
IL-8	1,356.4 ± 115.8	35,591.3 ± 3,318.8	46,395.4 ± 2,121.7	3,179.0 ± 2,559.2	38,687.3 ± 8,189.5	40,719.4 ± 1,325.7
RANTES	72.1 ± 53.1	318.5 ± 61.9	120.2 ± 38.8	83.9 ± 55.9	296.2 ± 32.9	128.1 ± 12.0
IL-10	14.5 ± 9.6	187.0 ± 43.2	89.2 ± 11.5	3.0 ± 1.5	146.8 ± 33.1	65.5 ± 21.5
IL-1RA	940.7 ± 249.2	2,812.3 ± 476.3	2,816.0 ± 420.4	925.6 ± 391.6	2,457.9 ± 573.9	2,450.9 ± 234.3

FIGURE 5. Comparative analysis of basal and LPS/Pam3Cys-modulated CD14, MD-2, TLR2, and TLR4 mRNA expression and TLR4 protein expression in control and smokers' AMs. Control and smokers' AMs were treated with medium, LPS, or Pam3Cys (1 μg/ml each) for 4 h (gene expression studies, *A*) or 30 min (Western blot analysis of TLR4 protein expression, *B*). *A*, RNA samples were prepared from nonsmokers' AMs (□) and smokers' cells (■) (13 subjects in each group), and CD14, MD-2, TLR2, and TLR4 mRNA levels were analyzed by real-time RT-PCR. *B*, *Top*, After treatment, cellular extracts were prepared and TLR4 protein expression was assessed by immunoblot analysis with anti-TLR4 Ab. β-Actin immunoblot was used to control for protein loading. *Bottom*, Densitometric quantification of TLR4 protein expression in cellular extracts prepared from nonsmokers' and smokers' AM (*n* = 5). TLR4 and β-actin bands were subjected to densitometric analysis using Quantity One software (Bio-Rad). Values are presented as TLR4 protein expression normalized to β-actin levels (arbitrary units).
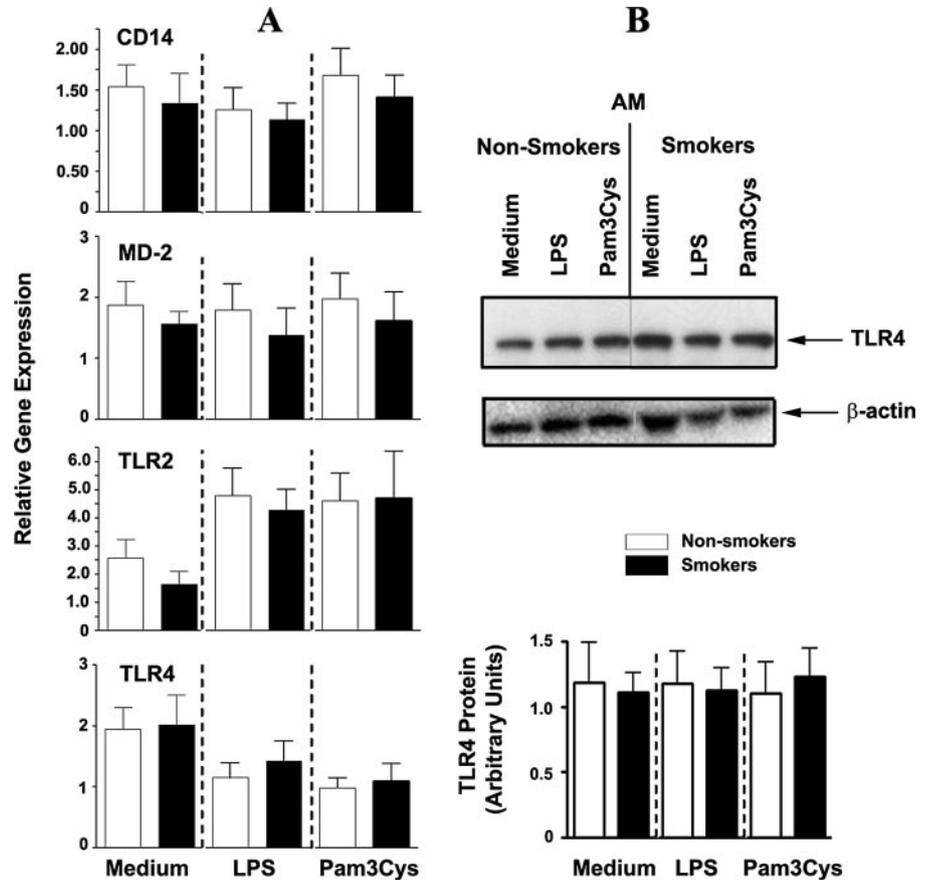
these data indicate that the impaired TLR2/4-induced cytokine expression exhibited by smokers' AMs is not due to reduced expression levels of TLR2, TLR4, or coreceptors CD14 and MD-2.

Smokers' AMs show suppressed phosphorylation of IRAK-1 and p38 and impaired NF-κB activation in response to TLR2 and TLR4 agonists

Having observed similar expression levels of TLR2, TLR4, CD14 and MD-2 in smokers' and nonsmokers' AMs, we hypothesized

that smoking inhibits TLR2/4-induced AM proinflammatory responses by suppressing activation of downstream signaling intermediates. To address this possibility, we analyzed the effect of smoking on LPS- and Pam3Cys-inducible activation of IRAK-1, a key kinase involved in activation of MAP kinases and transcription factors (reviewed in Ref. 49). Phosphorylation correlates with induction of IRAK-1 kinase activity, and is widely used to assess IRAK-1 activation (36–47, 50, 51). IRAK-1 was immunoprecipitated from cellular extracts obtained from control and smokers'
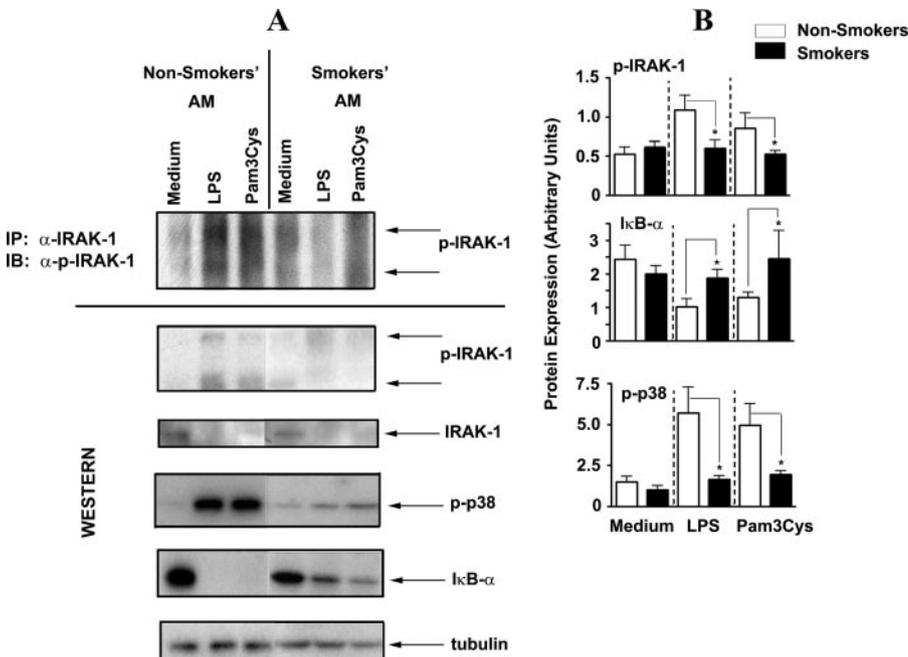
FIGURE 6. Smokers' AMs exhibit markedly impaired activation of IRAK-1, p38, and degradation of IκB-α in response to stimulation with TLR2 and TLR4 agonists. AMs from nonsmokers and smokers were stimulated for 30 min with medium, LPS, or Pam3Cys (1 μg/ml each). Thereafter, cellular extract were prepared, immunoprecipitated with α-IRAK-1 Ab, followed by immunoblot analyses of IRAK-1 phosphorylation, using anti (α)-p-IRAK-1 Ab (*top*). In addition, cell lysates were directly subjected to immunoblot analyses to determine phosphorylation/degradation of IRAK-1(*second and third panels from the top*), phosphorylation (p) of p38 MAPK, and degradation of IκB-α analyzed by immunoblotting (*A*). Tubulin immunoblot was used to estimate protein loading. *B*, Densitometric quantification of immunoblot data obtained from three nonsmokers and three smokers. The p-IRAK-1, IRAK-1, p-p38, and IκB-α bands were quantified using Quantity One software (Bio-Rad) and normalized to tubulin values. *, *p* < 0.05.
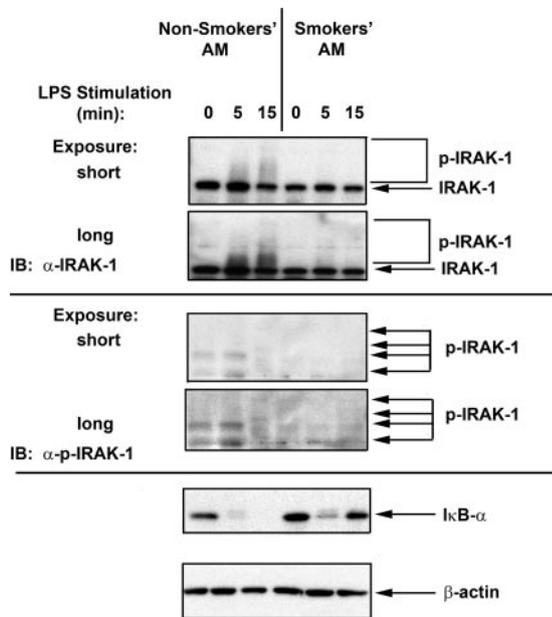
FIGURE 7. Kinetics of LPS-inducible IRAK-1 phosphorylation in non-smokers' and smokers' alveolar macrophages. Nonsmokers' and smokers' AMs were treated with medium or LPS for 0, 5, and 15 min; cellular extracts were prepared and analyzed for IRAK-1 phosphorylation by immunoblotting, using anti-pIRAK-1 and anti-IRAK-1 Abs. IκB-α immunoblot was run to control for LPS-induced tolerance/activation, and β-actin immunoblot was used to control for equal protein expression. Shown are the data of a representative ($n = 4$) experiment.

AMs treated with medium, LPS, and Pam3Cys and subjected to immunoblot analysis of IRAK-1 phosphorylation, using an Ab specific for phosphorylated species of IRAK-1. In addition, phosphorylation was assessed by immunoblotting of cellular extracts with anti-IRAK-1 Ab based on the appearance of IRAK-1 variants with slower electrophoretic mobilities (>80 kDa). Whereas LPS and Pam3Cys stimulation of nonsmokers' AM resulted in robust phosphorylation of IRAK-1, IRAK-1 failed to be phosphorylated to any appreciable extent in smokers' AMs (Fig. 6A, top two panels, and B). Stimulation with LPS or Pam3Cys also caused marked degradation of the unmodified (80 kDa) IRAK-1 band; however, no measurable differences in degradation patterns of unmodified IRAK-1 were noted between smokers' and nonsmokers' AM (Fig. 6A). LPS-inducible IRAK-1 phosphorylation was also inhibited at earlier time points (5 and 15 min) in smokers' AMs compared with nonsmokers' cells, as measured by immunoblot analyses with both anti-phospho-IRAK-1 and anti-IRAK-1 Abs (Fig. 7, top two panels).

To assess how smoking affects activation of intracellular signaling intermediates downstream of IRAK-1, LPS- and Pam3Cys-induced phosphorylation of p38 MAP kinase and degradation of IκB-α were analyzed. LPS- and Pam3Cys induced robust p38 phosphorylation and almost complete degradation of IκB-α in AMs obtained from nonsmoking volunteers, whereas these responses were significantly reduced in smokers' AMs (Fig. 6A). The densitometric quantification showed that LPS and Pam3Cys caused a 50% vs only ~5% decrease in the IκB-α levels and 4.5-fold vs ~1.3–1.5-fold induction of p38 phosphorylation observed in control cells and smokers' AMs, respectively (Fig. 6B). Similar to IRAK-1 results, time course experiments showed the inhibited capacity of LPS and Pam3Cys to induce IκB-α degradation also at 5 and 15 min poststimulation (Fig. 7). Immunoblot analyses of nuclear extracts showed that while nonsmokers' AMs respond to
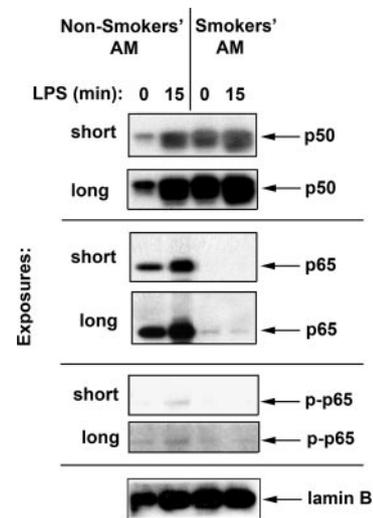
FIGURE 8. LPS-mediated nuclear translocation of the p50 and p65 NF-κB subunits in nonsmokers' and smokers' AMs. Pulmonary macrophages obtained from nonsmokers and smokers were treated with medium or LPS for 15 min. Thereafter, nuclear extracts were prepared and analyzed by immunoblotting with Abs against p50 and p65 subunits and anti-phospho-p65 Ab. Lamin B immunoblot was run to control for equal loading of nuclear extracts. Data of a representative experiment ($n = 3$) are depicted.

LPS with translocation of p50 and p65 and p65 phosphorylation, higher constitutive levels of p50, and the lack of LPS-inducible translocation and phosphorylation of p65 were observed in smokers' AMs (Fig. 8). These data demonstrate high levels of the transcriptionally inactive p50 NF-κB subunit and impaired TLR2/4-inducible phosphorylation of IRAK-1 and p38, degradation of IκB-α, and nuclear translocation and phosphorylation of the p65 NF-κB subunit in smokers' AMs.

Discussion

Tobacco smoking is associated with increased incidence of infections, bronchiolitis, and COPD (5–8). However, the molecular mechanisms underlying impaired host immune defense in smokers remain poorly understood. Previous studies (43) demonstrated that cigarette smoke contains biologically active endotoxin, and Karimi et al. (52) showed that in vitro exposure of PBMC to cigarette smoke induces production of cytokines in a TLR4-dependent manner. Therefore, we hypothesized that chronic exposure of AM to bioactive LPS present in cigarette smoke induces macrophage reprogramming that alters their responses to TLR2 and TLR4 agonists, similar to that seen in endotoxin tolerance. One feature of such reprogramming of cellular responses to microbial PAMPs observed in endotoxin-tolerant cells is down-regulation of expression of proinflammatory cytokines and chemokines, but preserved or even enhanced expression of anti-inflammatory cytokines (reviewed in Ref. 22). The data presented herein show that smoking exerts regional immunosuppression of AM reactivity to TLR2 and TLR4 agonists manifested by impaired gene expression and secretion of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (IL-8 and RANTES). In contrast, comparable levels of LPS- and Pam3Cys-induced expression of anti-inflammatory cytokines IL-10 and IL-1RA were found in control and smokers' AMs, indicating that, similar to LPS tolerance, smoking does not influence anti-inflammatory AM cytokine responses. These data extend previous observations on suppressed cytokine secretion by smokers' AMs stimulated with LPS (6, 11–15) and demonstrate: 1) the defect in cytokine expression occurs both at

the level of steady-state mRNA and protein secretion; 2) smokers' AMs exhibit impaired TLR-inducible expression of chemokines IL-8 and RANTES; 3) smoking inhibits AM responses to both TLR4 (LPS) and TLR2 (Pam3Cys) agonists; and 4) TLR-dependent expression of the anti-inflammatory cytokines IL-1RA and IL-10 are preserved in smokers' AMs. Impairment of TLR-dependent expression of proinflammatory cytokines with preservation of anti-inflammatory cytokine responses is a central feature of macrophage reprogramming occurring in endotoxin tolerance and septic shock survivors (reviewed in Ref. 22). Interestingly, smoking-mediated suppression of AM cytokine and chemokine responses was restricted to TLR2 and TLR4 signaling, whereas activation of AMs via TLR3 (by poly(I:C)) led to comparable, or even elevated, cytokine and chemokine production in smokers' AMs. Similar results have been recently reported in mouse macrophage cell lines tolerized with LPS where no inhibition of poly(I:C)-induced cytokine production was observed (53). Our data, combined with these results, further stress functional similarities between LPS-tolerized cells and smokers' AMs.

Our study further shows that smokers' AMs exhibited inhibition of both MyD88-dependent (e.g., TNF-α, IL-1β, IL-8) and MyD88-independent (e.g., RANTES) signaling pathways in response to LPS. This is reminiscent of our previous finding that endotoxin-tolerant phenotype induced in murine macrophages by prior LPS exposure is manifested by decreased LPS-inducible activation of both MyD88-dependent (TNF-α mRNA) and independent (IFN-β gene expression) signaling (54). We and others (54, 55) previously found that prior engagement of one TLR could diminish subsequent responses via activated not only the same TLR (homotolerance) but also through a different TLR (heterotolerance). Our results demonstrate that both TLR2 (Pam3Cys) and TLR4 (LPS) agonists elicited significantly suppressed expression of proinflammatory cytokines and chemokines in smokers' AMs, a reprogramming phenomenon resembling heterotolerance (54, 55). Because extracts from cigarette smoke stimulate cells via TLR4 (52), and cigarette smoke contains bioactive LPS (43), repeated exposure of AM to LPS (or other TLR4 agonists) present in cigarette smoke leads to homo- and heterotolerization of AM and diminished responses to Pam3Cys and LPS. However, prior LPS exposure does not heterotolerize mouse macrophages to TLR3 activation (53), consistent with our observation of similar or even higher TLR3-activated cytokine production capacities observed in smokers' AMs.

Relative expression of TLR2 and TLR4, as well as coreceptors CD14 and MD-2, is known to regulate the magnitude of TLR responsiveness to microbial PAMPs (17, 22, 24–27). In addition, MD-2 potentiates both TLR2- and TLR4-mediated signaling (56) and is prerequisite for TLR4 responsiveness to LPS (24–27). Thus, we determined whether smoking-mediated suppression of AM proinflammatory cytokine and chemokine responses to TLR2 and TLR4 agonists could be linked to modulation in expression of TLRs, CD14, and MD-2. Real-time PCR analysis revealed similar basal and LPS- or Pam3Cys-modulated expression levels of CD14 and MD-2 in AMs obtained from nonsmokers and smokers. Consistent with our previous findings in mouse macrophages and human PBMCs (37, 47), LPS stimulation down-regulated TLR4 mRNA expression but increased TLR2 gene expression by ~2-fold, a pattern also induced by Pam3Cys. However, similar levels of TLR2/4 mRNA expression were observed in smokers' and nonsmokers' AMs treated with medium or stimulated with TLR2 or TLR4 agonists. Similar to our results, Droemann et al. (57) found no differences in TLR4 expression between smokers' and nonsmokers AM. However, in contrast to our data, these authors demonstrated a decreased basal and LPS-inducible expression of TLR2

mRNA and protein in AM from smokers and patients with COPD (57). The reason for these differences is unclear but may be related to different methods used for detection of TLR expression (real-time PCR and Western blot analyses in our study vs FACS and immunohistochemistry in Ref. 57), differences in exposure levels to cigarette smoke within the two study populations, or ethnicity/ancestry of subjects analyzed in the two studies. Reprogramming in murine macrophages tolerized with TLR4 or TLR2 agonists was originally correlated with down-regulation in TLR2/4 and MD-2 expression (35, 58). However, these findings were not confirmed by other, more recent studies that have implicated, instead, suppressed expression or functions of downstream signaling intermediates rather than levels of TLRs and coreceptors as the primary mechanisms of TLR tolerance (36–42, 47, 59). Such a mechanism is more consistent with the observed selective defect in TLR-inducible proinflammatory cytokine expression and preservation of anti-inflammatory cytokine expression, all of which are activated via the same TLR complexes. The data presented in this paper support the conclusion that smoking-mediated inhibition of expression of proinflammatory cytokines and chemokines in AM operates at the postreceptor level by targeting downstream signaling intermediates. If decreased TLR expression were the principal mechanism of tolerance in smokers' AM, then total suppression of TLR-inducible production of both pro- and anti-inflammatory cytokines would be observed. However, this is not the case, in that exposure of AMs to tobacco smoke suppresses only TLR2/4-inducible proinflammatory cytokine responses while not affecting expression of anti-inflammatory cytokines IL-10 and IL-1RA.

Thus, we reasoned that the suppressive effect of smoking on proinflammatory cytokine and chemokine expression is likely to affect intracellular signaling intermediates required for proinflammatory gene expression. Because of functional similarities in the manifestation of reprogramming upon tolerization and in smokers' AMs, and because suppressed activation of IRAK-1, p38 MAPK, and NF-κB are molecular hallmarks of LPS tolerance, activation of these signaling intermediates was next compared in smokers' vs nonsmokers AMs. The functional importance of our studies addressing the effect of smoking on activation of signaling intermediates downstream of TLR4 was also stressed by a recent report showing that exposure of human monocytes to cigarette smoke results in phosphorylation of IRAK-1 and IκB-α (52). Because phosphorylation of IRAK-1 is necessary for IRAK-1 activation (60) and, similarly, because phosphorylation of MAPK correlates with kinase activity (61), phosphorylation of IRAK-1 and p38 was measured as indicators of TLR-inducible kinase activation. To judge activation of NF-κB, we measured LPS- and Pam3Cys-inducible degradation of IκB-a, a prerequisite for NF-κB nuclear translocation in the classical pathway of NF-κB activation (62) and LPS-mediated nuclear translocation of the NF-κB subunits p50 and p65. Similar to LPS-tolerant cells that were reported to accumulate transcriptionally incompetent p50 in the nucleus and to have decreased proportions of p65 (41, 42, 47), smokers' AMs also showed increased basal levels of p50 and deficient nuclear translocation and phosphorylation of p65 in response to LPS. Because phosphorylation of p65 regulates its transcriptional activity (62), low amounts of nuclear p65 in smokers' AMs exhibited deficient LPS-induced phosphorylation and therefore were transcriptionally inactive. To the best of our knowledge, our results represent the first report revealing that smoking is associated with impaired TLR2/4-inducible activation of IRAK-1, p38 MAPK, degradation of IκB-α, deficient nuclear translocation and phosphorylation of transcriptionally competent p65, and increased nuclear localization of transcription-deficient p50 NF-κB subunit in AMs. Thus, impaired activation of IRAK-1, p38, and NF-κB is likely to represent

a common mechanism accounting for suppressed expression of proinflammatory cytokines and chemokines in tolerance and smoking.

Although LPS is one component of tobacco smoke that could induce reprogramming via mechanisms similar to those associated with heterotolerance, we cannot exclude a potential contribution of other factors in suppressing AM functions. Indeed, acrolein, nicotine, and tar components, hydroquinone and catechol, have been shown to repress immune responses (63–65). Further studies using TLR-specific antagonists and LPS-neutralizing Abs will be required to delineate the relative contribution of LPS component of cigarette smoke to macrophage reprogramming that, in turn, impairs host immune defenses against respiratory infections in smokers.

Disclosures

The authors have no financial conflict of interest.

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