# Gram-Positive and Gram-Negative Bacteria Synergize with Oxidants to Release CXCL8 from Innate Immune Cells

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We have recently demonstrated that oxidants can activate monocytes via an action on Toll-like receptor (TLR) 2; however, it is unclear what functional consequence this has on immune surveillance for Gram-negative and -positive bacteria. Gram-negative and -positive bacteria and their related pathogen-associated molecular patterns (PAMPs) are sensed by TLR4 and TLR2, respectively. In the current study, we used a human monocyte cell line to show that oxidants prime cells to subsequent challenge with Gram-negative or -positive bacteria as well as PAMPs specific for TLR4 (LPS), TLR2/1 ($Pam_3CSK4$), TLR2/6 (FSL-1), Nod1 (FK565), and Nod2 (MDP Lys 18). Similarly, activation of TLR4 with LPS primed for subsequent activation of cells by agonists of the TLR2/6 or TLR2/1 complex. However, no synergy was noted when cells were costimulated with $Pam_3CSK4$ and FSL-1. We then tested blood (and isolated monocytes) derived from healthy smokers, which is oxidant primed, making it more sensitive to bacterial or PAMP stimulation when compared with blood of nonsmokers. Thus an oxidant stimulation, possibly via an action on TLR2 or associated transduction pathways, provides a signal that initiates inflammatory responses and sensitizes cells to pathogenic insults.
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## INTRODUCTION

It is now widely appreciated that pattern recognition receptors (PRRs) are an integral part of the innate immune system (1). This family of receptors includes Toll-like receptors (TLRs), nuclear-binding oligomerization domain (Nod), and the recently described retinoic acid–inducible gene (RIG) (2). Pathogens have, within their structure, pathogen-associated molecular patterns (PAMPs), which are ligands for PRRs expressed on immune cells (3). Gram-negative bacteria and LPS activate, and are therefore sensed by, the PRR TLR4. Gram-positive bacteria or fungi activate TLR2. TLR2 forms heterodimers with either TLR1 or TLR6.

TLRs recognize not only pathogens, but also host-derived signals (4) as well as nonhost, nonpathogenic environmental factors (4). The emergence of a role for nonpathogen-associated sensing by TLRs has expanded their repertoire, such that TLRs may now be considered as surveillance receptors for danger signals (5), including oxidants (6–8). Oxidants are critical mediators of inflammation, but there is still considerable debate surrounding how they sense and propagate the inflammatory reaction. This is important to elucidate because the inflammation that occurs in a number of life-threatening diseases is associated with a direct increase in oxidant stress (9–12). Possibly the most obvious are those associated with smoking cigarettes. Indeed, the activation of inflammatory responses *in vitro* by cigarette smoke is entirely mediated by oxidant stress (13). Cigarette smoke evokes an inflammatory response in both humans and animal models, which is characterized by the early influx and activation of inflammatory cells together with the release of chemokines, including CXCL8 (14). In addition to effects *in vivo*, cigarette smoke can directly activate human cells *in vitro* to release mediators including CXCL8 (15–18). There is no doubt that cigarette smoke is a powerful inflammatory stimulus for the lungs and the immune system. Furthermore, smokers are more susceptible to infection, suggesting the possibility that oxidants elucidated by cigarette smoking can prime, or activate, innate immune cells. However, the potential for oxidants to interact with the signaling of PAMPs has not been fully addressed. In the current study, we investigated the interactions between oxidants, including those in cigarette smoke, bacteria, and specific PAMPs, as activators of human

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immune cells *in vitro* and human blood *ex vivo*. We have used CXCL8 as a biomarker of cell and tissue activation, as we know it to be induced by oxidants (13) and it is highly relevant to the type of inflammation induced by smoke in laboratory animals and humans.

## MATERIALS AND METHODS

### Drugs, Chemicals, Reagents, and Other Materials

All drugs were obtained from Sigma Chemical Company, unless otherwise stated.

### Cell Culture

THP-1 human monocytes were obtained from the ECACC, and cultured in RPMI 1640 containing 10 mM GlutaMAX and supplemented with 10% FCS, penicillin/streptomycin 100 U/mL, and nonessential amino acids. All cells were plated onto either 6- or 96-well plates at $5 \times 10^5$/mL in RPMI 1640 (0% FCS content) and left to equilibrate for 6 h before stimulation.

### Isolation, Morphological Examination, and FACS Analysis of Blood-Derived Monocytes

Experiments with healthy volunteers were approved by the local research ethics committee (P/00/062, Royal Brompton Hospital). Blood was drawn by venipuncture into syringes and tubes containing anticoagulant concentrations of heparin. Human peripheral blood monocytes (PBMCs) (70% lymphocytes, 30% monocytes) were isolated as described (13). Briefly, fresh citrated blood was layered on a Ficoll gradient (Histopaque 1077 and 1119) and centrifuged (400*g*; 20°C) for 30 min. PBMCs were collected, washed with prewarmed RPMI 1640, and pelleted by centrifugation (450*g*; 24°C). Cells ($10^6$/mL) were plated in 96-well culture plates. Monocytes were separated from contaminating lymphocytes by incubating them (37°C in 5% $CO_2$) for 2 h on plastic. Nonadherent cells were removed by washing, and the purity of the culture was assessed by two techniques: morphologically under light microscopy, and then by flow cytometric analysis for CD14 expression and forward- and side-scatter properties. All staining procedures were performed at 4°C using PBS containing 1.3 mM calcium chloride and 0.2% BSA as buffer. Cells were washed, then blocked for 1 h with normal human IgG (6 mg/mL) and 30 µg/mL of R-phycoerythrin (RPE)-linked isotype control (IgG2a) monoclonal antibody (clone DAK-G05) or 30 µg/mL anti-human CD14 monoclonal antibody (clone TÜK4), both purchased from DakoCytomation (Glostrup, Denmark). Cells were then washed, and FACS analysis was performed. Cell fluorescence of monocytes gated using forward and side scatter were measured in the FL2 channel using a Becton Dickinson FACScan (San Jose, CA, USA). Mean fluorescence intensity (MFI) was calculated by subtracting the MFI of the DAK-G05–stained mononuclear cells from the corresponding TÜK4-stained cells.

**Preparations of cigarette smoke extract and treatment of cells.** Four full-strength Marlboro cigarettes (filters removed) were combusted through a modified 60-mL syringe apparatus, and the smoke passed through 100 mL RPMI 1640. Each cigarette yielded five draws of the syringe, with each individual draw taking approximately 10 s to complete. Cigarette smoke extract was then passed through a 0.25-µm filter to sterilize and remove particulate matter and was used immediately, unless otherwise stated. As described (19), smoke extract "strength" was evaluated by measuring nitrite using the Griess reaction (20) to ensure continuity between batches. In all experiments, nitrite levels in 100% cigarette smoke extract were between 12 and 16 µM.

Cells were treated for 24 h with cigarette smoke extract or $H_2O_2$ and/or heat-killed *E. coli* and *S. aureus* and purified commercially available PAMPs—LPS (from strain O111.B4), FSL-1, $Pam_3CSK4$, FK565, and MDP lys18 (Bioquote, York, UK, and Calbiochem, CA, USA). After this incubation, supernatants were collected and stored at –80°C for CXCL8 determination and cells for RT-PCR.

**Determination of LPS levels in cigarette smoke extract.** Smoke extract was made as above. To test LPS contamination, a Limulus assay (Eotoxate; Sigma, Poole, UK) was performed as per the manufacturers instructions on undiluted cigarette smoke extract.

### Bacteria Preparation

All bacteria (*E. coli* strain O111.B4 and *S. aureus* strain H380) were stored as frozen glycerol stocks and streaked onto agar plates before inoculation of single colonies into RPMI medium 1640 with 10% FCS and glutamine. Cultures were incubated overnight and centrifuged at 850*g* to pellet bacteria. Bacteria were then washed twice in sterile saline, and pellets were resuspended in sterile saline. Aliquots of the bacterial suspension were serially diluted and plated onto agar to quantify the cell density. The bacterial suspensions were then heat-treated for 60 min at 80°C to kill all bacteria; sterility was confirmed by plating of the resultant suspension. Suspensions were adjusted to $10^{10}$–$10^{12}$ CFU per mL and frozen with 20% glycerol in aliquots before use in cell culture experiments.

### Assessment of Cell Respiration by MTT

The effect of IL-1β (1 ng/mL), cigarette smoke extract, or both, on THP-1 cell metabolism was assessed by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, UK) to formazan. This was performed after all treatments. None of the treatments described affected cell viability significantly.

### Measurement of CXCL8

CXCL8 levels in cell-free supernatant were determined by ELISA using commercially available matched antibody pairs, following a protocol furnished by the manufacturers (R&D Systems, Abingdon, UK). CXCL8 concentrations were

measured at 450 nm with a reference filter at 550 nm, and results were expressed as pg/mL.

### Real-Time PCR

Nuclear extracts were isolated from cultured monocytes and cDNA generated as described (13). Transcript levels were determined by real-time PCR (Rotor Gene 6, Corbet Research) using Taqman Universal PCR master mix and commercially available primers for CXCL8 (Hs00174103_m1; Applied Biosciences, CA, USA). PCR conditions were as follows: step 1, 10 min at 95°C; step 2, 15 s at 95°C; step 3, 60 s at 60°C and repeated for 40 cycles. Data from the reaction were collected and analyzed (Corbett Research, Sydney, Australia). Relative quantifications of gene expression were calculated using standard curves and normalized to GAPDH.

### Statistical Analysis

Statistical analysis was performed using a one-way ANOVA followed by a Bonferroni posttest or one-sample *t* test for normally distributed data. All data shown are mean ± SEM of $n = 4–6$ in experiments conducted on at least two or three separate experimental days.

## RESULTS

### Costimulation of Human Monocytes with TLR2 and TLR4 Agonists Results in the Synergistic Release of CXCL8

We have shown in murine macrophages that TLR2 and TLR4 ligands synergize at the level of NO and TNF-α production (21). Here we extend these observations to human monocytes. We used CXCL8 as a marker of activation, because it is highly relevant to inflammation (22,23) and is increased by oxidants (13). Under basal conditions, THP-1 cells released low to undetectable levels of CXCL8. When cells were stimulated with LPS (TLR4 agonist; 0.001 or 0.01 μg/mL), FSL-1 (TLR2/6 agonist; 0.1 μg/mL), or $Pam_3CSK4$ (TLR2/1 agonist; 0.01 μg/mL) alone, there was a moderate increase in the levels of CXCL8 produced after 24 h (Figure 1A and B). However, when cells were costimulated with LPS and either FSL-1, or $Pam_3CSK4$, there was a marked synergistic release of CXCL8 (Figure 1A and B). We next investigated whether this was also the case for monocytes costimulated with TLR2/1 and TLR2/6 ligands. It is worth noting that in all cases neither the PAMPs or oxidants alone, nor the combination of PAMPs and oxidants affected cell viability at the concentrations used.

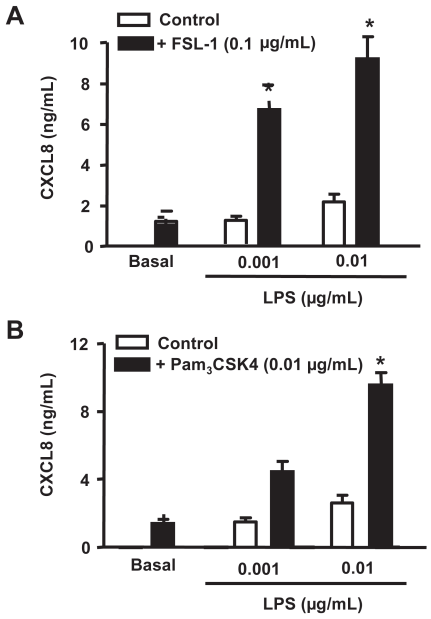


**Figure 1.** Costimulation of human monocytes with TLR2 and TLR4 agonists results in the synergistic release of CXCL8. THP-1 cells ($n = 6$) were cotreated with LPS (0.001 and 0.01 μg/mL) and (A) FSL-1 (TLR2/6 agonist; 0.1 μg/mL) or (B) $Pam_3CSK4$ (TLR2/1 agonist; 0.01 μg/mL) for 24 h, after which CXCL8 was measured in the supernatant by specific ELISA. *$P < 0.05$ between the sum of CXCL8 levels from TLR2 and TLR4 agonist together and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

### Costimulation of Human Monocytes with TLR2/1 and TLR2/6 Agonists Results in an Additive Release of CXCL8

In contrast to costimulation of cells with TLR4 and TLR2 agonists, activation of human monocytes with a combination of $Pam_3CSK4$ (TLR2/1) and FSL-1 (TLR2/6) resulted in, at low concentration of $Pam_3CSK4$ (0.001 μg/mL), less than additive levels of CXCL8. At higher concentrations of $Pam_3CSK4$, and at the lowest concentration of FSL-1 tested, the combination had a synergistic effect. However, at higher concentrations of FSL-1, the effect of cotreatment with $Pam_3CSK4$ was additive (Figure 2A–C). We have previously shown that oxidants activate human monocytes/macrophages through a TLR2-dependent mechanism (7). We therefore wanted to determine if the ability of oxidants to activate cells has implications for the sensing of pathogens *in vitro*, and if any synergy in these pathways occurs.

### CSE and $H_2O_2$ Prime Human Monocytes to Stimulation with Gram-Negative Bacteria or Selective TLR4 or Nod1 Agonists

We investigated the effects of costimulation of human monocytes with the oxidants $H_2O_2$ or CSE and whole Gram-negative *E. coli*, as well as PAMPs selective for PRRs associated with Gram-negative bacteria (LPS for TLR4 and FK565 for Nod1). Cells stimulated with either $H_2O_2$ (0.1, 1, and 10 mM) or CSE (1%, 3%, and 10%) caused a significant concentration-dependent increase in CXCL8 ($H_2O_2$, 19 ± 2, 33 ± 4, 388 ± 46 pg/mL; CSE, 19 ± 6, 26 ± 8, 105 ± 31 pg/mL) from THP-1 cells compared with baseline values (21 ± 2 pg/mL), although it is worth noting that the levels were significantly less that those evoked by PAMP stimulation alone and therefore appear absent in the figures presented. The cotreatment of monocytes for 24 h with $H_2O_2$ (0.1, 1, or 10 mM) or CSE (1%, 3%, or 10%) plus subthreshold concentrations of *E. coli*, LPS (TLR4) (Figure 3A–D), or FK565 (Nod1) (Table 1) resulted in a synergistic release of CXCL8.

### Blood from Healthy Cigarette Smokers Is Primed to Challenge with Gram-Negative Bacteria or Selective TLR4 Agonists

It is clear that smoking cigarettes is an oxidant/inflammatory challenge that goes

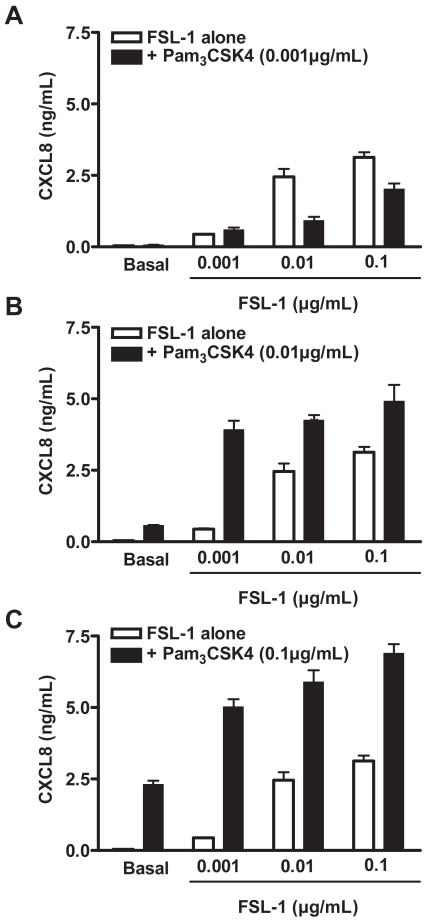


**Figure 2.** Costimulation of human monocytes with TLR2/1 or TLR2/6 agonists results in an additive release of CXCL8. THP-1 cells ($n$ = 6) were cotreated with FSL-1 (TLR2/6 agonist; 0.001, 0.01, and 0.1 µg/mL) and (A) $Pam_3CSK4$ (TLR2/1 agonist; 0.001 µg/mL), (B) $Pam_3CSK4$ (TLR2/1 agonist; 0.01 µg/mL), or (C) $Pam_3CSK4$ (TLR2/1 agonist; 0.01 µg/mL) for 24 h, after which CXCL8 was measured in the supernatant by specific ELISA. *$P$ < 0.05 between the sum of CXCL8 levels from TLR2 and TLR4 agonist together and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

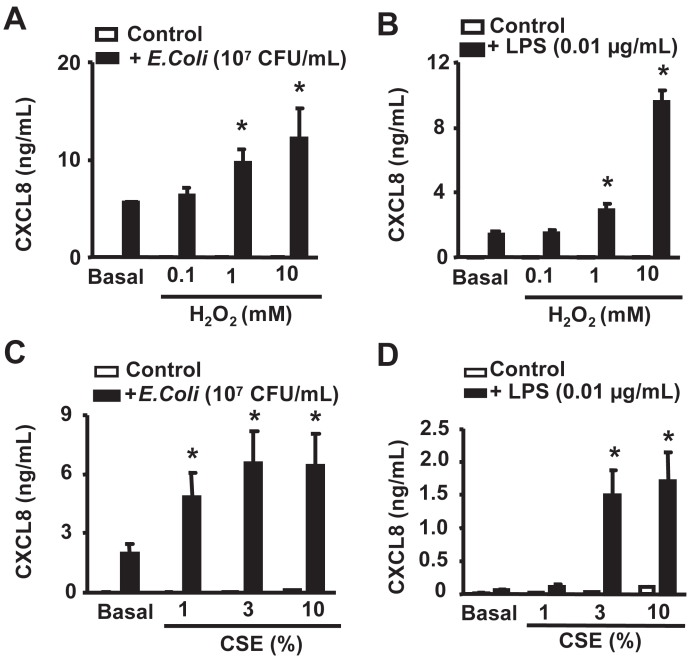


**Figure 3.** CSE and $H_2O_2$ prime human monocytes to stimulation with Gram-negative bacteria or selective TLR4 agonists. In some experiments, THP-1 cells ($n$ = 6) were treated with $H_2O_2$ (0.1, 1, and 10 mM) alone or costimulated with (A) heat-killed *E. coli* ($10^7$ CFU/mL) or (B) LPS (0.01 µg/mL). In some experiments, THP-1 cells ($n$ = 6) were treated with cigarette smoke extract (CSE; 1%, 3%, and 10%) alone or costimulated with (C) heat-killed *E. coli* ($10^7$ CFU/mL) or (D) LPS (0.01 µg/mL). In all treatments, cells were exposed to stimuli for 24 h then CXCL8 levels were measured by ELISA. *$P$ < 0.05 between the sum of CXCL8 levels from TLR2 and TLR4 agonist together and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

beyond the lungs, affecting blood-borne leukocytes (24,25). To test if the effects observed in monocytes *in vitro*, namely monocyte priming by oxidants prior to bacterial or PAMP stimulation, are echoed *in vivo*, we compared responses of blood from healthy smokers (average age 32 years) and age-matched nonsmokers (average age 31 years) who were free from medication for at least 6 weeks, to bacteria and PAMP agonists. Basal levels of CXCL8 in the blood were higher in nonsmokers (4079 ± 1637 pg/mL) than healthy smokers (752 ± 269 pg/mL); interestingly this was not the case for basal CXCL8 levels in monocytes, where the CXCL8 levels from smokers and healthy controls were similar (Figure 4). However, in direct corroboration with our monocytes *in vitro* data, we found that the ability of *E. coli* or LPS to release CXCL8 was greatly increased in blood from smokers compared with nonsmokers (Figure 5A and B). This was also true for CXCL8 levels in monocytes isolated from subjects and stimulated with LPS (Figure 4A). It is important to note that this hypersensitivity of the blood of smokers was not due to

**Table 1.** Cigarette smoke extract (CSE) primes human monocytes to stimulation with Nod1 agonists.

| | CXCL8, pg/mL | |
|---|---|---|
| | CSE alone | CSE + FK565 |
| Control | 8.5 ± 1.9 | 9.1 ± 2.3 |
| 1% CSE | 16.3 ± 0.9 | 18.5 ± 1.6 |
| 3% CSE | 33.2 ± 1.3 | 39.5 ± 1.8 |
| 10% CSE | 263.8 ± 37.7 | 441.3 ± 53.0[a] |

THP-1 cells ($n$ = 6) were treated with CSE alone or costimulated with FK565 (Nod1 agonist; 100 nM). [a]$P$ < 0.05 between the sum of CXCL8 levels from TLR2 and TLR4 agonist together and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

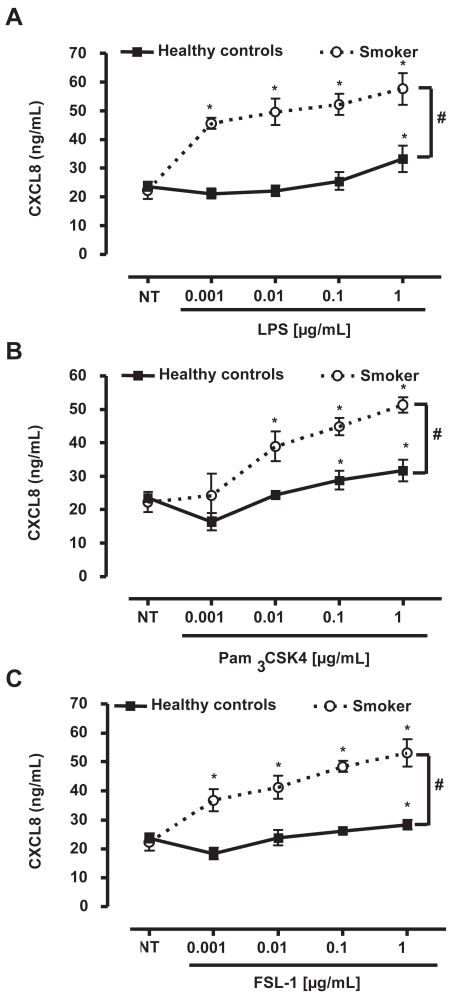


**Figure 4.** Monocytes isolated from the blood of healthy smokers are primed to challenge with selective TLR4 and TLR2 agonists. Isolated macrophages taken from whole heparinized blood from age-matched healthy smokers and nonsmokers ($n$ = 3) was stimulated with a concentration range of (A) LPS (1–0.001 µg/mL), (B) $Pam_3CSK4$ (1–0.001 µg/mL), or (C) FSL-1 (1–0.001 µg/mL). Isolated monocytes were incubated for 24 h, and then CXCL8 levels were measured by ELISA. *$P$ < 0.05 increases in CXCL8 between nontreated cells (NT) in both smokers and healthy controls by one-way ANOVA. #$P$ < 0.05 trend analysis comparing smoker and nonsmoker groups using a two-way ANOVA.

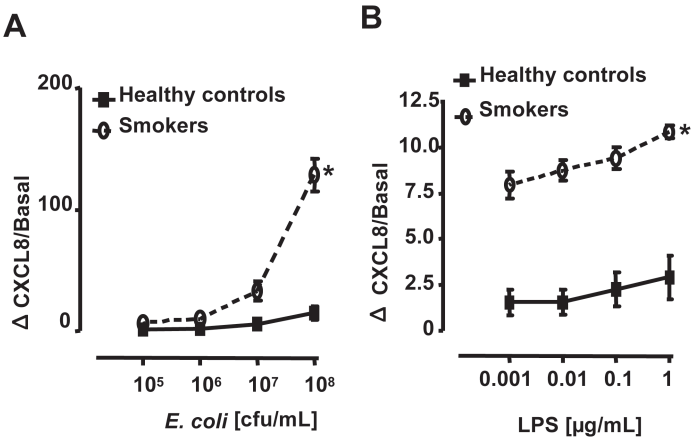


**Figure 5.** Blood from healthy cigarette smokers is primed to challenge with Gram-negative bacteria or selective TLR4 agonists. Whole heparinized blood taken from age-matched healthy smokers and nonsmokers ($n$ = 3) was stimulated with a concentration range of (A) *E. coli* (1 × $10^5$ to 1 × $10^8$ CFU/mL) or (B) LPS (1–0.001 µg/mL). Whole blood was incubated for 24 h, and then CXCL8 levels were measured by ELISA. *$P$ < 0.05 trend analysis comparing smoker and nonsmoker groups using ANOVA.

any significant differences in total white cell counts in whole blood, as nonsmokers had 6.6 × $10^6$ ± 2.0 × $10^5$ PMNs/mL, 3.5 × $10^5$ ± 1.5 × $10^4$ monocytes/mL, and 1.1 × $10^6$ ± 2.3 × $10^5$ lymphocytes/mL and healthy smokers had 6.8 × $10^6$ ± 1.8 ×$10^5$ PMNs/mL, 4.3 × $10^5$ ± 8.8 × $10^4$ monocytes/mL, and 1.2 × $10^6$ ± 5.8 × $10^5$ lymphocytes/mL. Because we have shown that oxidants activate monocytes through TLR2, we further investigated whether a synergistic or additive release of CXCL8 would be observed when human monocytes were costimulated with oxidants and Gram-positive bacteria or classic TLR2 ligands.

### $H_2O_2$ or CSE Prime Human Monocytes to Stimulation with Gram-Positive or Selective TLR2 or Nod2 Agonists

As in observations made with Gram-negative bacteria and related PAMPs, we found that a synergy was observed with oxidants and subthreshold concentrations of *S. aureus* (acts via TLR2) (21,26), FSL-1 (TLR2/TLR6) (Figure 6A–C), $Pam_3CSK4$ (TLR2/TLR1; data not shown), or MDP lys18, which activates Nod2 (Table 2).

### Blood from Healthy Cigarette Smokers Is Primed to Challenge with Gram-Positive Bacteria or Selective TLR2 Agonists

As was the case for whole Gram-negative bacteria, whole blood from healthy smokers was hypersensitive to activation by *S. aureus* or FSL-1 (Figure 7A and B) compared with age-matched controls. A similar priming of smokers' blood (compared with blood from nonsmoking controls) was seen when $Pam_3CSK4$ (TLR2/TLR1; data not shown) was used as an agonist. This was also true for monocytes isolated from similar groups of healthy smokers and nonsmokers stimulated with either $Pam_3CSK4$ or FSL-1 (Figure 4B and C).

### Synergy between Oxidants and Bacteria for CXCL8 Release Is Also Observed at the Level of Gene Induction

With respect to CXCL8 release, the synergistic priming, noted above with cell cotreatment (oxidant and pathogens), occurred at the transcription as measured by RTPCR (CXCL8/GAPDH ratio; Figure 8).

## DISCUSSION

The present study furthers our knowledge of the pathophysiological implication of oxidants interacting with TLR and other PRR pathways. We have recently demonstrated that oxidants can signal through TLR2 on innate immune cells and result in the profound release of

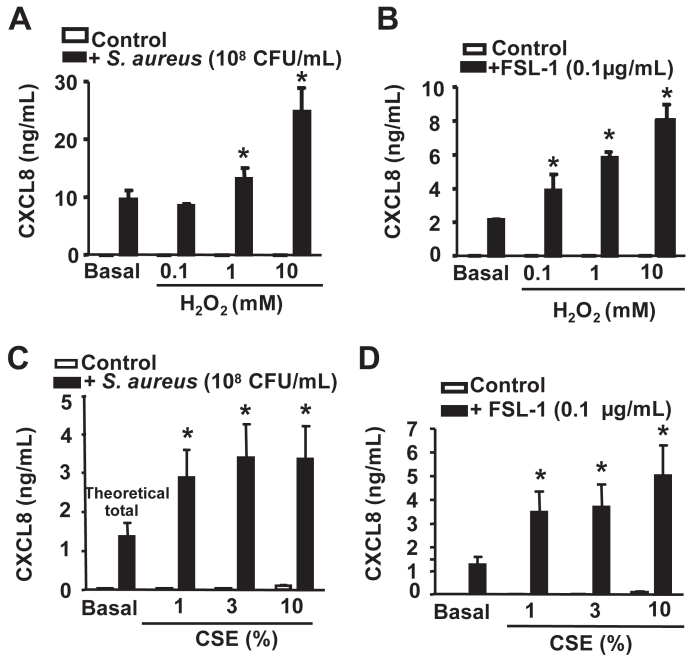


**Figure 6.** CSE and $H_2O_2$ prime human monocytes to stimulation with Gram-positive bacteria or selective TLR2 agonists. In some experiments, THP-1 cells ($n$ = 6) were treated with $H_2O_2$ (0.1, 1, and 10 mM) alone or costimulated with (A) heat-killed *S. aureus* ($10^8$ CFU/mL) or (B) LPS (0.01 µg/mL). In some experiments, THP-1 cells ($n$ = 6) were treated with cigarette smoke extract (CSE; 1%, 3%, and 10%) alone or costimulated with (C) heat-killed *S. aureus* ($10^8$ CFU/mL) or (D) LPS (0.01 µg/mL). In all treatments, cells were exposed to stimuli for 24 h then CXCL8 levels were measured by ELISA. *$P$ < 0.05 between the sum of CXCL8 levels from TLR2 and TLR4 agonist together and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

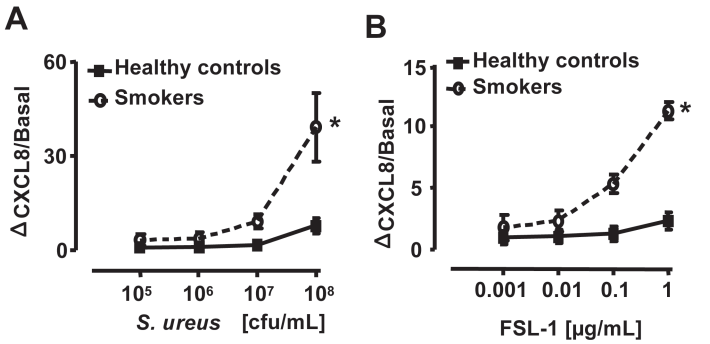


**Figure 7.** Blood from healthy cigarette smokers is primed to challenge with Gram-positive bacteria or selective TLR2 agonists. Whole heparinized blood taken from age-matched healthy smokers and nonsmokers ($n$ = 3) was stimulated with a concentration range of (A) *S. aureus* ($1 \times 10^5$ to $1 \times 10^8$ CFU/mL) or (B) FSL-1 (1–0.001 µg/mL). Whole blood was incubated for 24 h, and then CXCL8 levels were measured by ELISA. *$P$ < 0.05 trend analysis comparing smoker and nonsmoker groups using ANOVA.

CXCL8, a pivotal cytokine in directing leukocytes into inflammatory lesions (7). However, the functional consequences of TLR/oxidant interactions remain unclear. It is apparent from the data presented in this study that oxidant activation of cells results in a profound alteration in how monocytes sense both Gram-positive and -negative bacteria. More specifically, in monocytes *in vitro*, coactivation with oxidants and Gram-positive or -negative bacteria or associated PAMPs resulted in a synergistic release of CXCL8. This was directly translatable to humans because the blood or monocytes of healthy smokers (which are exposed to oxidants in smoke) were found to be exquisitely sensitive to stimulation with bacteria and TLR2 or TLR4 agonists *ex vivo*.

In some systems, ligands for TLR2 and TLR4 synergize with each other to activate cells, often monitored by the release of inflammatory cytokines (27) and/or nitric oxide (21). In the current study, we have confirmed that a similar synergy occurs for CXCL8 release by human monocytes. Specifically, we show that PAMPs for TLR4 synergize with those of TLR2/1 or TLR2/6. Interestingly, we also found evidence for a positive interaction between PAMPs for TLR2/1 and TLR2/6, although it was less robust and occurred only at some concentrations. Indeed,

**Table 2.** Cigarette smoke extract (CSE) primes human monocytes to stimulation with Nod2 agonists.

| | CXCL8, pg/mL | |
|---|---|---|
| | CSE alone | CSE + MDP lys18 |
| Control | 18.6 ± 4.7 | 15.8 ± 4.0 |
| 1% CSE | 19.2 ± 4.9 | 24.8 ± 6.3 |
| 3% CSE | 25.9 ± 6.9 | 50.2 ± 12.7 |
| 10% CSE | 105.4 ± 26.4 | 268.1 ± 68.4[a] |

THP-1 cells ($n$ = 6) were treated with CSE alone or costimulated with MDP lys18 (Nod2 agonist; 100 nM). [a]$P$ < 0.05 between the sum of CXCL8 levels from TLR2 and TLR4 agonist together, and the actual value of TLR2 and TLR4 costimulation, using ANOVA followed by a Bonferroni posttest.

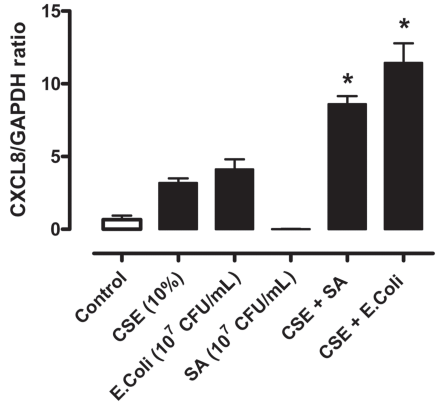


**Figure 8.** Costimulation of human monocytes with cigarette smoke extract (CSE) and Gram-positive or -negative bacteria resulted in a synergistic production of CXCL8 mRNA. THP-1 cells ($n = 6$) were treated with cigarette smoke extract (CSE; 10%), *E. coli* ($10^7$ CFU/mL), *S. aureus* (SA; $10^7$ CFU/mL) alone or costimulated with CSE (10%) and *E. coli* ($10^7$ CFU/mL) or *S. aureus* (SA; $10^7$ CFU/mL) for 8 h. *$P < 0.01$ by ANOVA followed by a Bonferroni posttest.

under some conditions we found a less than additive effect when cells were stimulated with $Pam_3CSK4$ and FSL-1. These observations clearly show that there is some level of redundancy in the TLR2 pathway when classic PAMPs are used as activators.

We were then interested to assess the effects of oxidant challenge and PAMPs in this system. We elected to use two models of oxidant stress, CSE and $H_2O_2$. Whereas CSE contains many elements, it is exclusively the oxidants within its mix that activate cells to release CXCL8 (13). Interestingly, we found that when cells were costimulated with oxidants plus bacteria or PAMPs, a significant synergy in CXCL8 release was seen. This was the case for Gram-positive or -negative bacteria. Our preliminary data suggest that, in addition to activation of AP-1 (13), oxidants can also activate cells via an action on TLR2 (7). We therefore predicted that oxidants, like classic TLR2 agonists (see above) would synergize with TLR4 agonists. In line with this, we found that oxidant stress induced by either $H_2O_2$ or CSE primed cells for stimulation with Gram-negative *E. coli* or LPS. Interestingly we also found that oxidants synergized with the Nod1 agonist FK565. Furthermore, we found that oxidants synergized with Gram-positive bacteria or selective PAMPs for TLR2 or Nod2. In other studies, we have shown a similar synergy between oxidants and IL-1β, or TNF-α and oxidants for CXCL8 release and induction in monocytes (13) or airway smooth muscle cells (28). IL-1β, like TLRs (except TLR3), signals via the adapter protein MyD88. Taken together, these observations suggest that oxidants prime cells via TLR-dependent and -independent mechanisms which converge at the downstream levels of MyD88 signaling (3). The precise signaling interactions between oxidants and PRR remain the subject of investigation.

In an attempt to extrapolate our observations *in vitro* to a human model of oxidant stress, we studied response in the blood of healthy smokers, where oxidant load is significantly increased compared with that of nonsmokers (29). It has been well recognized for several years that smoking creates an oxidant stress detectable in the blood. The level of oxidant stress is proportional to the amount of cigarettes smoked (30). Whereas oxidant markers in the blood of regular smokers are raised throughout the day, they increase further immediately after smoking (31). In direct corroboration of our *in vitro* data, we found that that the blood of smokers was more sensitive to activation by TLR ligands than the blood of nonsmokers. We then went on to compare responses of monocytes isolated from the blood of smokers and nonsmokers. Again we found that monocytes from smokers were exquisitely sensitive to bacteria and PAMPs compared with cells from nonsmokers. Our observations with monocytes show that oxidant priming persists, at least for the duration of our experiment, once the oxidant environment (in this case the blood) is removed from the cells. These observations demonstrate the principle of oxidants synergizing with PAMPs for CXCL8 release. Moreover, our observations with blood and monocytes for smokers provide further insight into the type of interaction that oxidants and PAMPs have in cells. Our *in vitro* studies were limited to looking at acute activation of cells with oxidants and PAMPs. However, others have shown that where synergy between PAMPs for TLR4 and TLR2/6 occurs when agents are added together, a cross-desensitization can result when PAMPs are added for longer times as a pretreatment (27). In the blood, monocytes of smokers are continuously exposed to elevated levels of oxidants. The fact that blood cells of smokers remain primed for subsequent challenge with bacteria or PAMPs strongly suggests that in the case of oxidants, unlike classical TLR agonists, no cross-tolerance occurs. This remains speculation at the moment, as we have yet to fully elucidate the precise role of TLRs in oxidant-mediated priming.

Interestingly, the basal levels of CXCL8 in the whole blood of smokers were significantly lower than those of nonsmokers. By contrast, the basal levels of CXCL8 made by monocytes of smokers were similar to those of nonsmokers. It is not clear why this difference existed, but it remains the subject of investigation within our laboratory. Our observations are interesting, not only because they prove the principle of our *in vitro* studies but also because they may help to explain the susceptibility of smokers to general sickness. Indeed, recent data obtained from the US government showed that smokers were far more likely to be absent due to illness at work and/or have *Mycobacterium* tuberculosis than nonsmoker colleagues (32,33). A similar mechanism driven by host-derived oxidants may make individuals with chronic inflammatory conditions more susceptible to infection, or the symptoms of infection.

In summary, we have identified a profound synergistic relationship between oxidant- and PAMP-induced cell activation at the level of CXCL8 release. The precise mechanism by which oxidants

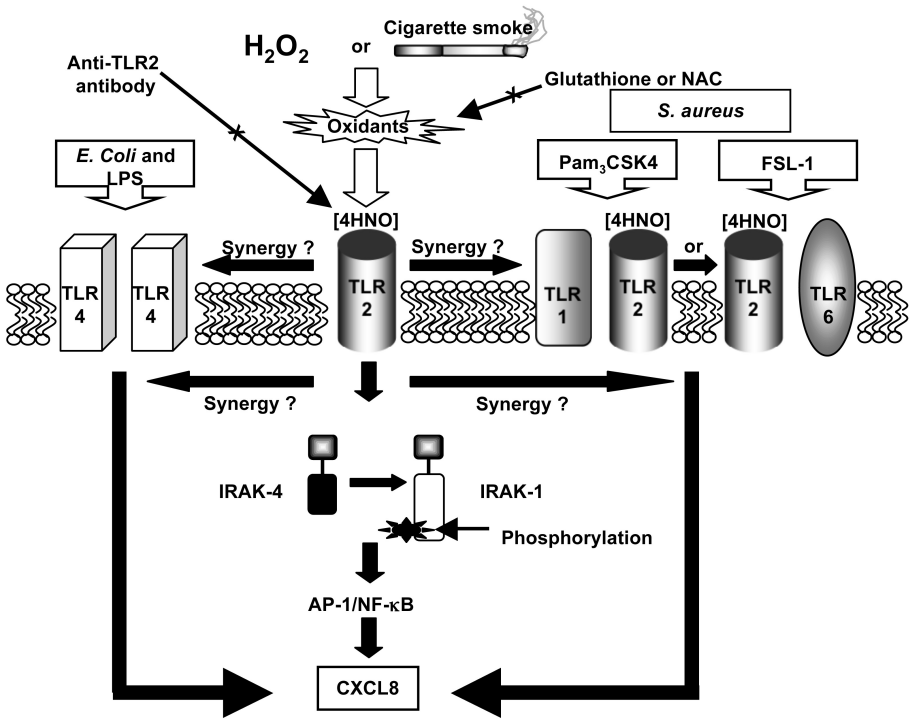


**Figure 9.** Sensing and signal transduction of oxidants through a TLR2-dependent mechanism. Oxidants present in CSE are sensed and modify TLR2, which results in the rapid phosphorylation of the recruited adaptor protein interleukin-1 receptor associated kinase-1, resulting in AP-1/NF-κB–dependent induction of the leukocyte chemokine CXCL8. CSE also synergized with the TLR4 ligands *E. coli* and LPS and the TLR2 ligands *S. aureus*, $Pam_3CSK4$, and FSL-1.

mediate these effects is beyond the scope of the current study, but as suggested by preliminary studies, may well be due to oxidation of the extracellular portion of TLR2, causing a conformational change (summarized in Figure 9), as is seen in other proteins (34,35) which link up at the level of MyD88 signaling.

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