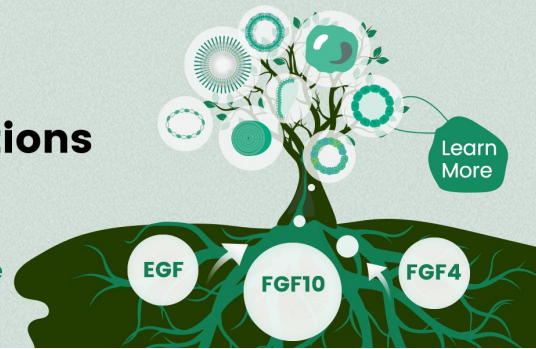


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J Immunol (2003) 170 (10): 5268–5275.

<https://doi.org/10.4049/jimmunol.170.10.5268>

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Selective Roles for Toll-Like Receptor (TLR)2 and TLR4 in the Regulation of Neutrophil Activation and Life Span¹

Ian Sabroe,^{2*} Lynne R. Prince,^{*} Elizabeth C. Jones,^{*} Malcolm J. Horsburgh,[‡] Simon J. Foster,[‡] Stefanie N. Vogel,[§] Steven K. Dower,[†] and Moira K. B. Whyte^{*}

Neutrophil responses to commercial LPS, a dual Toll-like receptor (TLR)2 and TLR4 activator, are regulated by TLR expression, but are amplified by contaminating monocytes in routine cell preparations. Therefore, we investigated the individual roles of TLR2 and TLR4 in highly purified, monocyte-depleted neutrophil preparations, using selective ligands (TLR2, Pam₃CysSerLys₄ and *Staphylococcus aureus* peptidoglycan; TLR4, purified LPS). Activation of either TLR2 or TLR4 caused changes in adhesion molecule expression, respiratory burst (alone, and synergistically with fMLP), and IL-8 generation, which was, in part, dependent upon p38 mitogen-activated protein kinase signaling. Neutrophils also responded to Pam₃CysSerLys₄ and purified LPS with down-regulation of the chemokine receptor CXCR2 and, to a lesser extent, down-regulation of CXCR1. TLR4 was the principal regulator of neutrophil survival, and TLR2 signals showed relatively less efficacy in preventing constitutive apoptosis over short time courses. TLR4-mediated neutrophil survival depended upon signaling via NF- κ B and mitogen-activated protein kinase cascades. Prolonged neutrophil survival required both TLR4 activation and the presence of monocytes. TLR4 activation of monocytes was associated with the release of neutrophil survival factors, which was not evident with TLR2 activation, and TLR2 activation in monocyte/neutrophil cocultures did not prevent late neutrophil apoptosis. Thus, TLRs are important regulators of neutrophil activation and survival, with distinct and separate roles for TLR2 and TLR4 in neutrophil responses. TLR4 signaling presents itself as a pharmacological target that may allow therapeutic modulation of neutrophil survival by direct and indirect mechanisms at sites of inflammation. *The Journal of Immunology*, 2003, 170: 5268–5275.

Neutrophils are a principal component of the innate immune system. Exposure of neutrophils to bacteria, or bacterial products, activates these cells as part of the inflammatory response resulting in the clearance of pathogens. Additionally, inappropriate or excessive neutrophil activation can cause severe tissue damage, contributing to the pathology of a range of inflammatory diseases.

Recent studies have shown that detection of the microbial environment around, upon, and within us is mediated by Toll-like receptors (TLRs).³ Ten human TLRs have been identified (1), which enable responses to a range of pathogen-associated molecules including LPS (TLR4) (2–4), lipoproteins and peptidoglycans (TLR2 in combination with TLR1 or TLR6) (4–6), flagellin (TLR5), double-stranded (viral) RNA (TLR3), and bacterial DNA

(CpG motifs, TLR9). TLR4 also permits signaling in response to noninfective inflammatory stimuli such as heat shock protein 60 (HSP60) (7) and fibrinogen peptides (8). The ability of individual TLRs to signal in response to a diverse range of agonists, which may not in themselves bind directly to the TLR, is probably explained by their incorporation upon activation into signaling complexes in lipid rafts, involving many cell surface proteins (9, 10).

TLRs are members of the IL-1R superfamily and signal via similar mechanisms, with initiation of proinflammatory gene transcription through pathways including NF- κ B and mitogen-activated protein kinase (MAPK) cascades (1). Although activation of these pathways represent core signaling mechanisms shared by all TLRs, there is increasing evidence that individual TLR activators can mediate selective responses. The mechanisms allowing selective signaling are not fully known, but clues are emerging with the identification of adapter proteins that exhibit differential association with certain TLRs, such as myeloid differentiation protein 88 adapter-like/Toll-IL-1R domain-containing adapter protein and TIR domain-containing adapter inducing IFN- β /TIR-containing adapter molecule (11–16). Probably through the use of these selective adapter proteins, TLR4 in particular can mediate the activation of IFN- β transcription, with subsequent autocrine activation of specific gene subsets (14, 17). It has also been observed that only a subgroup of TLRs, including TLR2, but not TLR4, contain typical sequences allowing signaling via p85/p110 adapter proteins (18), although both TLR2 and TLR4 can activate signaling via Akt (19).

We recently showed that human neutrophils express TLR2 and TLR4 protein on the cell surface (20). Neutrophils are short-lived, but a central component of their responses to pathogens includes the prolongation of their life span (21). Our work also showed that neutrophil responses to TLR ligands were, in part, mediated by monocytes that commonly contaminate leukocyte preparations

Academic Units of ^{*}Respiratory Medicine and [†]Cell Biology, Section of Functional Genomics, Division of Genomic Medicine, and [‡]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom; and [§]Department of Microbiology and Immunology, University of Maryland, Baltimore, MD 21201

Received for publication December 4, 2002. Accepted for publication March 13, 2003.

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¹ This work was supported by the Medical Research Council (U.K.) through a Clinician Scientist Fellowship (G108/388 to I.S.) within the Mechanisms of Cytokine Action in Chronic Inflammatory Diseases Cooperative Group (G9827663). S.N.V. is supported by National Institutes of Health Grant AI-8797.

² Address correspondence and reprint requests to Prof. Steven K. Dower, Division of Genomic Medicine, University of Sheffield, M Floor, Royal Hallamshire Hospital, Sheffield S10 2JF, U.K. E-mail address: s.dower@sheffield.ac.uk

³ Abbreviations used in this paper: TLR, Toll-like receptor; HSP60, heat shock protein 60; MAPK, mitogen-activated protein kinase; Pam₃CSK₄, Pam₃CysSerLys₄; pLPS, purified LPS; cLPS, commercial LPS; PI-3K, phosphatidylinositol 3-kinase; DCF, dichlorodihydrofluorescein diacetate.

(20). In this study, we investigated the individual functional consequences of TLR2 and TLR4 engagement in highly purified, monocyte-depleted neutrophils, and compared these with cell populations deliberately cocultured with monocytes, where TLR engagement can result in complex response patterns dependent upon molecular intercellular communication.

Materials and Methods

Reagents

General laboratory reagents were from Sigma-Aldrich (Poole, U.K.). LPS from *Escherichia coli* serotype 0111:B4 was from Sigma-Aldrich. Synthetic bacteria-like lipopeptide Pam₃CysSerLys₄ (Pam₃CSK₄) was from EMC Microcollections (Tübingen, Germany). PBS and cell culture reagents were from Invitrogen (Paisley, U.K.). FCS was from BioWhittaker (Cambrex BioScience, Wokingham, U.K.), containing <0.5 endotoxin U/ml endotoxin. Purified LPS (pLPS) from *E. coli* strain K235 was prepared as described (4). Peptidoglycans from *Staphylococcus aureus* 8325-4 and *Bacillus subtilis* 168 HR were made as previously described (22, 23), quantified by dry weight, and stored at -20°C. Pathway inhibitors were from Calbiochem (CN Biosciences, Beeston, U.K.) and comprised SB203580 (p38 MAPK inhibitor), SN50 (NF-κB inhibitor) and SN50 M control peptide, PD98059 (MAPK kinase inhibitor preventing extracellular signal-regulated kinase activation), herbimycin A (tyrosine kinase inhibitor), and LY294002 (phosphatidylinositol 3-kinase (PI-3K) inhibitor). Anti-CXCR1 mAb 5A12 (IgG2b) and anti-CXCR2 mAb 6C6 (IgG1) were generous gifts from Dr. S. Qin (Millennium Pharmaceuticals, Cambridge, MA). Matched ELISA Ab pairs were from the National Institute for Biological Standards and Controls (Potters Bar, U.K.).

Cell preparation

Peripheral venous blood was taken with informed consent from volunteers in accordance with a protocol approved by the South Sheffield Research Ethics Committee. Blood was anticoagulated with trisodium citrate, plasma and platelets were removed by centrifugation, and following dextran sedimentation, PBMC were separated from granulocytes by density either over a plasma/Percoll gradient (20), or by centrifugation over sterile, endotoxin-free Histopaque 1077 (Sigma-Aldrich, Poole, U.K.). No differences in CD11b, L-selectin expression, leukocyte function, or rates of constitutive apoptosis were observed between neutrophils prepared by either method (data not shown). Neutrophils were further purified by negative magnetic selection as described (20), using a custom mixture from StemCell Technologies (Vancouver, Canada), containing Abs to CD36, CD2, CD3, CD19, CD56, and glycophorin A.

Modulation of cell surface marker expression

Neutrophils were stimulated in assay buffer (Dulbecco's modified PBS containing Ca²⁺/Mg²⁺ plus 2% FCS plus 10 mM HEPES plus 0.18% glucose (pH 7.3–7.4)) for 1 h at 37°C, washed with ice-cold FACS buffer (PBS without Ca²⁺/Mg²⁺ plus 10 mM HEPES plus 0.25% BSA (pH 7.3–7.4)), and CD11b and L-selectin expression were determined by flow cytometry using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and quantified as described (20). Changes in expression of the chemokine receptors CXCR1 or CXCR2 were measured by flow cytometry (24). Cells were stained with anti-CXCR1 or anti-CXCR2 (both 10 μg/ml), and binding of these mAbs, compared with relevant isotype controls, was detected using FITC-conjugated goat anti-mouse F(ab')₂ (DAKO, Ely, U.K.).

Detection of respiratory burst

Dichlorodihydrofluorescein diacetate (DCF) is a cell-permeable compound, which becomes fluorescent upon oxidation by hydrogen peroxide and other reactive oxygen species and was used to measure neutrophil respiratory burst as described previously (25). Briefly, neutrophils were preincubated with 5 μM DCF for 30 min at 37°C and 5% CO₂, and then treated with pLPS, Pam₃CSK₄, or medium (RPMI 1640 plus 10% FCS plus penicillin and streptomycin) for 30 min, after which time fMLP or medium was added for an additional 15 min. Cells were then removed from culture, spun, washed in cold PBS, and resuspended in PBS to analyze immediately by flow cytometry.

Cytokine generation

Neutrophils. Highly purified neutrophils (3 × 10⁶/ml) were cultured in medium with stimuli in a final volume of 150 μl for 24 h. Cell-free supernatants were prepared and stored at -70°C. For the ELISA, a 96-well

Maxisorp immunoplate (Nunc, Roskilde, Denmark) was coated with IL-8-coating Ab diluted 1/1000. The plate was washed three times and blocked with 1% OVA. After further washing, 100 μl of standard or sample was loaded per well, and the plate incubated overnight at 4°C. The plate was washed, and bound IL-8 was detected using biotinylated anti-IL-8 Ab diluted 1/2000 in buffer containing 1% sheep serum, and incubated for 1 h at room temperature. After further washes, bound Ab was visualized using avidin-HRP (DAKO) and *o*-phenylenediamine according to the manufacturer's instructions. Absorbance was measured at 490 nm using an MRX plate reader (Thermo Labsystems, Vantaa, Finland) and Biolinx software version 2.20 (Biolinx, Frankfurt am Main, Germany).

Monocytes. PBMC purified by plasma/Percoll centrifugation were resuspended in RPMI 1640 plus 10% FCS and cultured at 1.25 × 10⁶/ml with agonists or medium alone in a final volume of 200 μl. After 22 h, cell-free supernatants were prepared and stored at -70°C. ELISAs for TNF-α, IL-1β, and GM-CSF were performed as described in *Neutrophils*, using coating Abs at 1 μg/ml (TNF-α and IL-1β) or 3 μg/ml (GM-CSF) and biotinylated detector Abs at 1/2000 dilution (1/500 for GM-CSF).

Assessment of neutrophil viability and apoptosis

Neutrophils were highly depleted of monocyte contamination by negative magnetic selection, and 100-μl aliquots (2.5 × 10⁶ or 5 × 10⁶ cells/ml) were cultured in Falcon Flexiwell plates (BD Biosciences, Mountain View, CA). Cells were pretreated (in some experiments) with drugs or medium for 30 min at 37°C and then incubated for the indicated time period with buffer or stimuli in medium at 37°C in 5% CO₂, as described previously (20). After stimulation, apoptosis was quantified by staining with annexin V PE according to the manufacturers instructions (BD Biosciences) and a vital dye (7-amino-actinomycin or To-Pro-3; Molecular Probes (Eugene, OR) (20)) and analyzed by flow cytometry, or quantified by morphology on cytopins as described previously (20). In some experiments, autologous PBMC were also added to the neutrophils during culture as described previously (20). PBMC were added back at a concentration of 1.25 × 10⁵ cells/ml which, assuming an average monocyte proportion of 10–20%, corresponded to a neutrophil:monocyte ratio of ~100:1 to 200:1 (20).

Statistics

Comparisons of more than two data sets were performed by ANOVA and a posthoc test as indicated, using the Prism 3.0 program (GraphPad Software, San Diego, CA).

Results

Stimulation of both TLR2 and TLR4 causes changes in adhesion molecule expression

We previously showed that commercial LPS (cLPS), which contains both TLR2- and TLR4-stimulating activities, caused loss of L-selectin and up-regulation of CD11b expression on neutrophils, but found that low levels of contaminating monocytes amplified these responses (20). In this study, we prepared neutrophils that had been highly purified to deplete monocytes, and treated them with cLPS, pLPS (stimulating TLR4 alone), or a palmitoylated synthetic mimic of bacterial lipopeptides (Pam₃CSK₄; stimulating TLR1 plus -2 heterodimers). Fig. 1 shows that all three agonists caused loss of L-selectin expression and up-regulation of CD11b. In comparison, we studied the actions of the cytokine IL-18, which signals via similar mechanisms to TLRs and whose receptor is expressed on human neutrophils. IL-18 induced modest increases in CD11b expression, but had no effect on L-selectin levels.

Stimulation of TLR2 and TLR4 causes respiratory burst synergistically with fMLP

Highly purified neutrophils were pretreated with pLPS or Pam₃CSK₄, and then challenged with medium or varying concentrations of the proinflammatory peptide fMLP. Both pLPS and Pam₃CSK₄ induced respiratory burst as measured by the oxidation-mediated increases in DCF fluorescence, at levels equivalent to those induced by 10 nM fMLP (Fig. 2). Pretreatment of neutrophils with TLR-stimulating activities resulted in a marked synergistic increase in cumulative oxygen free radical generation in response to a subsequent treatment with fMLP (Fig. 2).

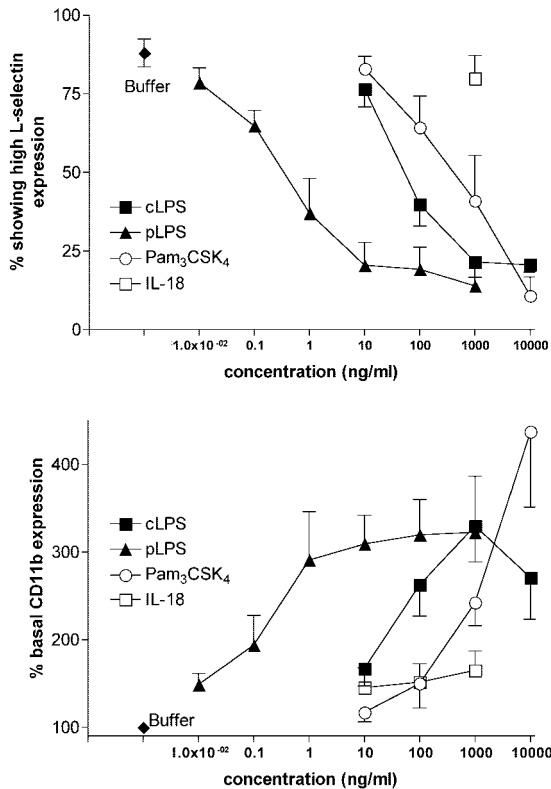


FIGURE 1. TLR stimulation modulates neutrophil adhesion molecule expression. Neutrophils were highly purified by negative magnetic selection and stimulated with cLPS (cLPS, ■), purified LPS (pLPS, ▲), Pam₃CSK₄ (○), or IL-18 (□). After 1 h, expression of L-selectin and CD11b were determined by flow cytometry and compared with buffer-stimulated cells. Data shown are mean ± SEM of four to five experiments, each from a separate donor.

Stimulation of TLR2 and TLR4 causes neutrophil IL-8 generation

Cytokine generation is a classical component of neutrophil proinflammatory responses, exemplified by the generation of IL-8 (26). We and others (20, 26) have shown that neutrophils express IL-8 mRNA in response to stimulation with cLPS, and therefore, we investigated the roles of TLR2 and TLR4 in this response. Highly purified neutrophils were treated with pLPS or Pam₃CSK₄ for 24 h, and IL-8 protein generation was measured by ELISA. Fig. 3 shows that stimulation with pLPS and Pam₃CSK₄ resulted in marked IL-8 release. Treatment with the p38 MAPK inhibitor SB203580 partially inhibited IL-8 generation in response to these ligands, particularly in response to pLPS.

TLR-mediated modulation of neutrophil chemokine receptor expression

Contradictory reports have shown that neutrophils respond to cLPS preparations with either an increase in ¹²⁵I-labeled IL-8 binding (27) or a protease-dependent shedding of the two major neutrophil chemokine receptors CXCR1 and CXCR2 (28, 29). Using flow cytometry, we investigated the roles of TLR2 and TLR4 in mediating down-regulation of these receptors. CXCR1 was relatively resistant to TLR-mediated down-regulation. Fig. 4A shows that only Pam₃CSK₄ stimulation resulted in changes in CXCR1 expression after 1 h of stimulation, but these differences did not reach statistical significance, because in only two of six donors did TLR2 stimulation cause loss of CXCR1 expression. In contrast, CXCR2 expression was more sensitive to down-regulation by TLR

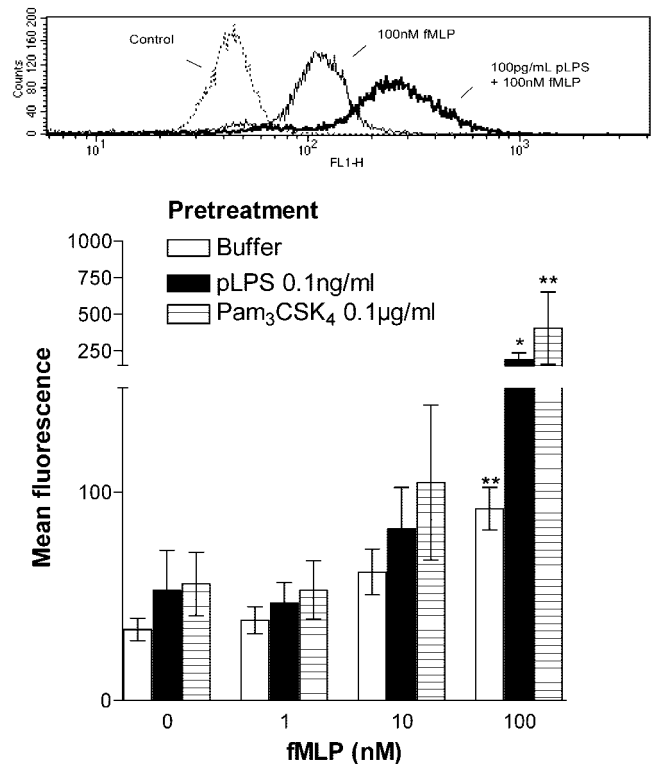


FIGURE 2. TLR agonists cause H₂O₂ generation. Neutrophils were highly purified by negative magnetic selection, loaded with DCF, and stimulated with buffer (□), pLPS (0.1 ng/ml; ■), or Pam₃CSK₄ (0.1 μg/ml; ▨). Thirty minutes later, cells were stimulated with buffer or the indicated concentrations of fMLP. After a further 15 min, respiratory burst was measured by flow cytometry. The *upper panel* shows an illustrative histogram demonstrating the synergistic effects of fMLP stimulation on pLPS-pretreated cells. Data shown in the *lower panel* are mean ± SEM of four experiments, each from a separate donor. Significant differences between samples stimulated with buffer and samples stimulated with 100 nM fMLP for each pretreatment (buffer, pLPS, and Pam₃CSK₄) are indicated as follows: *, *p* < 0.05; **, *p* < 0.01, analyzed by ANOVA and Tukey's *posttest*.

stimulation. TLR2 activation by Pam₃CSK₄ caused a concentration-dependent reduction in CXCR2 expression (Fig. 4B), evident in all donors. In contrast, pLPS failed to induce significant loss of CXCR2 expression, although there was considerable interdonor variation, with five of seven donors showing >25% reduction in CXCR2 expression after stimulation with 10 ng/ml pLPS, a concentration giving maximum responses in assays of changes in L-selectin and CD11b expression levels.

TLR4 signaling preferentially modifies neutrophil life span

We showed previously that a delay in neutrophil apoptosis in response to cLPS was evident after short (4-h) culture periods, but that in longer cultures (22 h), LPS-mediated neutrophil survival was dependent upon bystander monocytes (20). We first investigated whether the TLR-mediated neutrophil survival seen at early time points was mediated by TLR2 or TLR4, in highly purified, monocyte-depleted cell populations. After 4 h of culture, a proportion of neutrophils had undergone apoptosis (mean percentage apoptotic after culture in medium alone, 10.3 ± 3.0% (mean ± SEM, *n* = 4)). Fig. 5A shows that pLPS almost completely prevented early neutrophil apoptosis at extremely low concentrations (0.1 ng/ml) that were only minimally effective in assays investigating changes in adhesion molecule expression. In contrast,

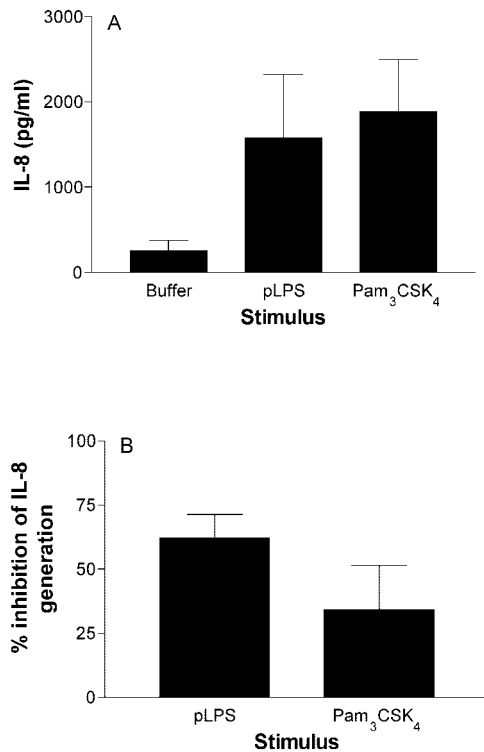


FIGURE 3. TLR agonists cause IL-8 generation. Neutrophils were highly purified by negative magnetic selection and cultured with buffer, pLPS (1 ng/ml), or Pam₃CSK₄ (10 μg/ml). IL-8 generation was measured in supernatants after 24 h of culture. In separate wells, cells were coincubated with SB203580 (10 μM), and the effect on IL-8 generation is shown as percentage inhibition. Data shown are mean ± SEM of three experiments, each from a separate donor.

Pam₃CSK₄ was markedly less efficacious in preventing neutrophil apoptosis, although at high concentrations, associated with maximal down-regulation of L-selectin expression, its effects became more evident. To confirm the observations with Pam₃CSK₄, we studied additional TLR2 ligands and found that peptidoglycan from *S. aureus* had minimal ability to modulate neutrophil apoptosis in a dose-dependent manner, and *B. subtilis* peptidoglycan inhibited neutrophil apoptosis only at high concentrations. Both *S. aureus* and *B. subtilis* peptidoglycans were able to induce marked respiratory burst as measured by DCF assay ($n = 4$, data not shown), indicating that these agonists were biologically active.

After 22 h of culture, neutrophils had undergone extensive apoptosis (mean percentage apoptotic after culture in medium alone, $80.1 \pm 5.0\%$ (mean ± SEM, $n = 4$)). The TLR agonists failed to inhibit apoptosis at this later time point in the absence of contaminating monocytes (Fig. 5B).

Signaling pathways that regulate TLR4-mediated early neutrophil survival

TLRs may activate many signaling pathways, a number of which have previously been implicated in the regulation of neutrophil life span—both constitutively and in response to various survival and prodeath stimuli—including NF-κB (30), MAPKs (31, 32), PI-3Ks (33), and tyrosine kinases (34). Therefore, we undertook a study of the roles of the candidate pathways responsible for TLR4-mediated neutrophil survival.

Pathway inhibitors had variable, nonsignificant effects on constitutive apoptosis (data not shown). When data from each of the inhibitors were expressed relative to their own controls (comparing apoptosis rates in inhibitor-pretreated cells stimulated with me-

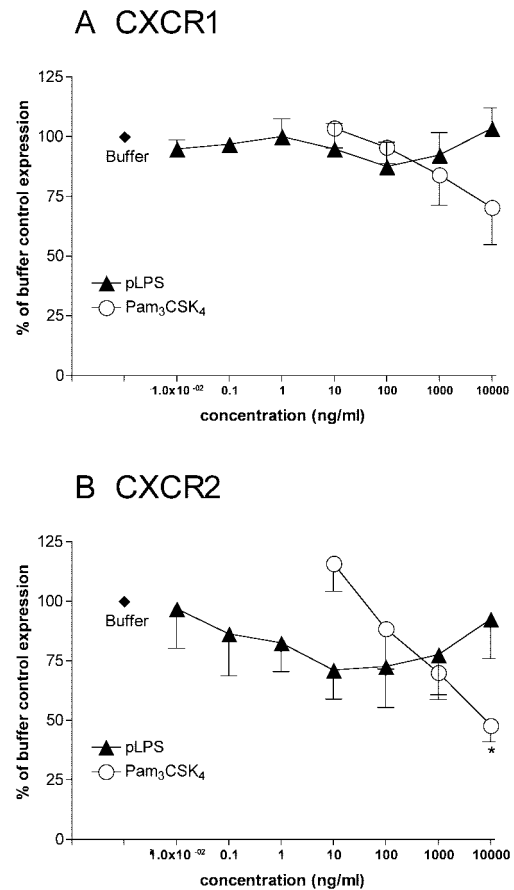


FIGURE 4. TLR agonists modulate neutrophil chemokine receptor expression. Neutrophils were highly purified by negative magnetic selection and stimulated with buffer or the indicated agonists (pLPS, ▲; Pam₃CSK₄, ○) for 1 h. Subsequently, binding of anti-CXCR1 or anti-CXCR2 mAbs was determined, and data are presented as percent specific change from cells stimulated with buffer (after subtraction of nonspecific binding of isotype-matched controls). Mean data ± SEM from three to seven experiments are shown in A (CXCR1) and B (CXCR2). *, Significant ($p < 0.05$) internalization of CXCR2 induced by Pam₃CSK₄, analyzed by ANOVA and posthoc testing.

dium vs inhibitor-pretreated cells stimulated with pLPS), it became apparent that SN50 pretreatment prevented pLPS-mediated cell survival (Fig. 6). The less active SN50 M control peptide (35) had only minimal effects on apoptosis, which were significantly different vs SN50 itself (Fig. 6). SN50 at 50 μg/ml caused a similar, significant inhibition of pLPS-mediated neutrophil survival as SN50 at 100 μg/ml (data not shown). These effects were particularly marked when SN50 (100 μg/ml) was added together with inhibitors of MAPKs. PD98059 and SB203580 when combined with NF-κB inhibition (SN50 plus SB203580, and SN50 plus SB203580 plus PD98059) effectively abolished pLPS-induced neutrophil survival. The PI-3K inhibitor LY294002, alone or in combination with SB203580, caused a small increase in constitutive apoptosis (data not shown), and did not reduce pLPS-mediated neutrophil survival (Fig. 6). The tyrosine kinase inhibitor herbimycin A caused a dramatic increase in constitutive neutrophil cell death ($77 \pm 9\%$, mean ± SEM; $n = 6$), and thus its actions on pLPS-induced neutrophil survival were not determined.

TLR4 signaling preferentially induces monocyte-mediated late neutrophil survival

After prolonged culture, neutrophils had undergone extensive apoptosis that was not rescued by TLR activation (Fig. 5B). In additional

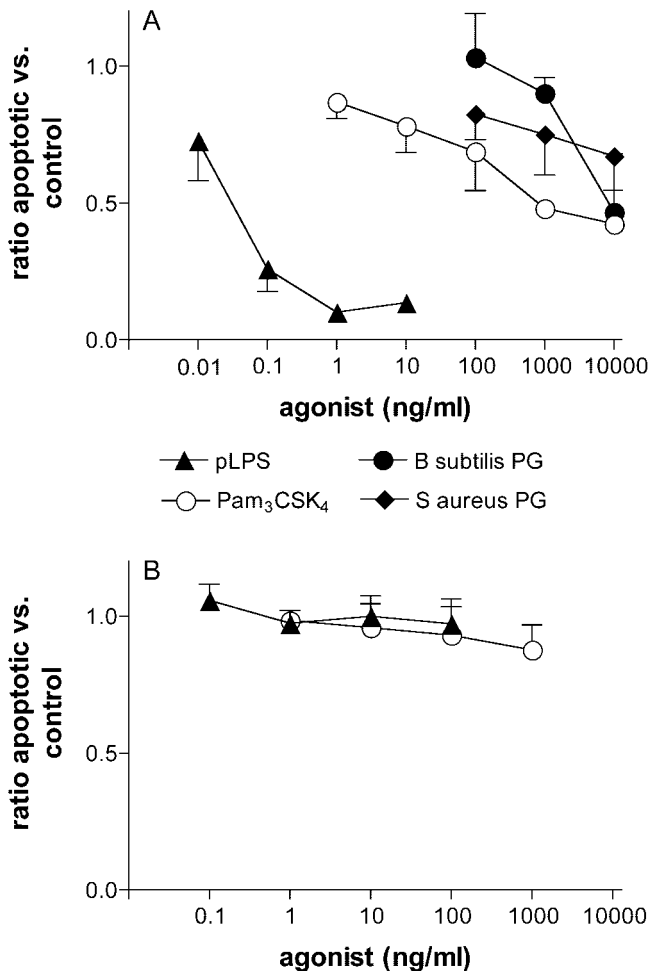


FIGURE 5. TLR4 is a more efficacious regulator of neutrophil life span than TLR2 is. Neutrophils were highly purified by negative magnetic selection and cultured with the indicated agonists for 4 h (pLPS, ▲; Pam₃CSK₄, ○; *S. aureus* peptidoglycan, ◆; *B. subtilis* peptidoglycan, ●) (A) or 22 h (pLPS, ▲; Pam₃CSK₄, ○) (B). After culture, apoptosis was assessed by cell morphology. Data in each panel are mean \pm SEM of four experiments, each from a separate donor, expressed as ratio of apoptotic cells in the treated sample vs the sample cultured in medium alone.

experiments, we added back autologous PBMC to the highly purified neutrophil cultures at a ratio of one PBMC per 20 neutrophils (corresponding to approximately one monocyte per 100–200 neutrophils) and stimulated the cocultures with the TLR agonists. Addition of cLPS and pLPS resulted in neutrophil survival, but Pam₃CSK₄ was unable to mediate PBMC-dependent neutrophil survival (Fig. 7A). We hypothesized that TLR4-driven, monocyte-mediated neutrophil survival over prolonged time courses was likely to result from the synthesis of a monocyte-derived prosurvival cytokine. In separate experiments, PBMC were cultured for 22 h with buffer, pLPS, or Pam₃CSK₄. Both TLR activators caused the generation of low levels of GM-CSF (maximum picograms per milliliter in supernatant (\pm SEM; $n = 4$) for buffer stimulation were 2.4 ± 1 , for 100 ng/ml pLPS were 17 ± 5.9 , and for 10 μ g/ml Pam₃CSK₄ were 8.5 ± 6), and higher levels of IL-8 generation (maximum picograms per milliliter in supernatant (\pm SEM; $n = 4$) for buffer stimulation were 1602 ± 565 , for 100 ng/ml pLPS were 3473 ± 62 , and for 10 μ g/ml Pam₃CSK₄ were 3301 ± 26). However, TLR4 activation caused markedly greater induction of TNF- α and IL-1 β than TLR2 activation (Fig. 7, B and C) did. Thus, both direct and indirect (monocyte-dependent) inhibition of apoptosis was mediated via TLR4.

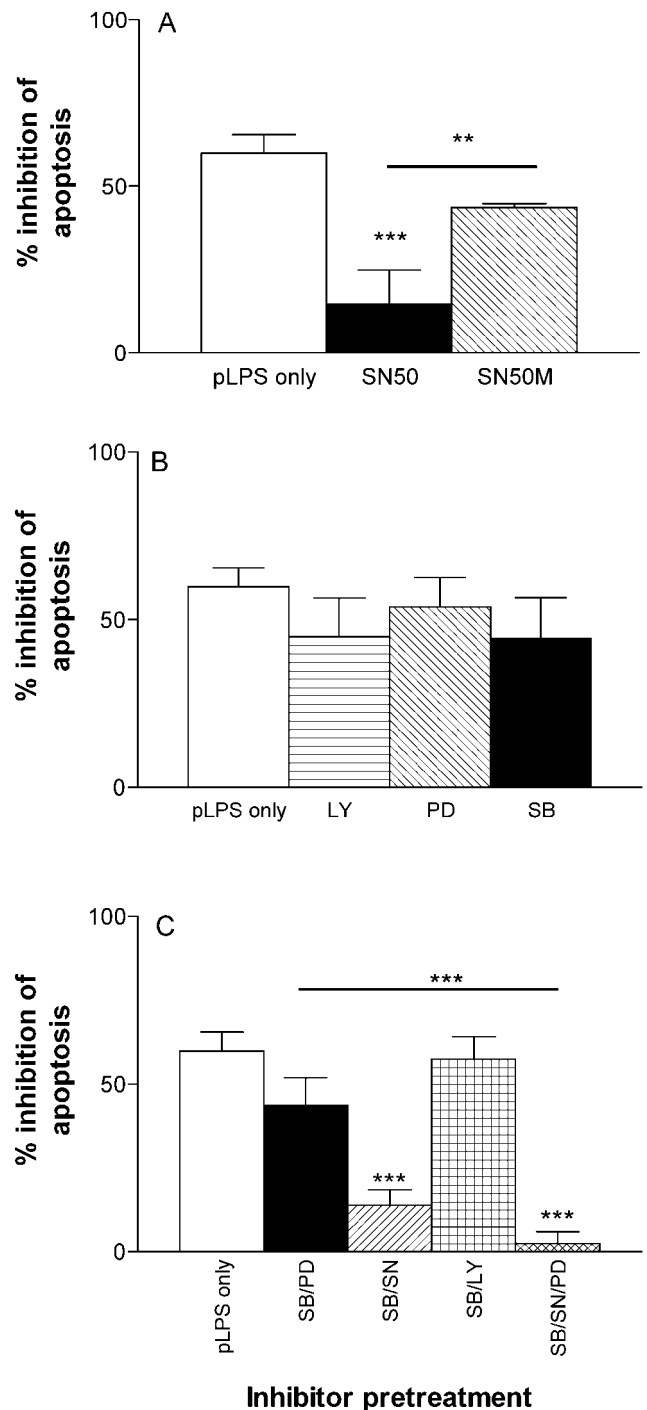


FIGURE 6. Signaling pathways regulating TLR4-mediated neutrophil survival. Neutrophils were highly purified by negative magnetic selection and pretreated with buffer or antagonists for 30 min, followed by buffer or pLPS (0.1 ng/ml) for 4 h. Apoptosis was measured by staining with annexin V as described in *Materials and Methods*. Concentrations of inhibitor used were as follows: SN50 and SN50 M control peptide, 100 μ g/ml; SB (SB203580), 10 μ M; PD (PD98059), 50 μ M; and LY (LY294002), 25 μ M. Data show the percentage inhibition of apoptosis by pLPS stimulation, in the presence of buffer or antagonist (as indicated on the x-axis). Data are expressed comparing apoptosis in cells treated with inhibitor and buffer, vs cells treated with inhibitor and pLPS. The open bars (□) in each panel show that pLPS stimulation in the presence of buffer inhibited apoptosis by $60 \pm 6\%$. Significant prevention of pLPS-mediated cell survival is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (all data analyzed by ANOVA and Tukey's posttest). Data are a mean of six to nine experiments \pm SEM, other than for SN50 M, for which data are a mean of three experiments.

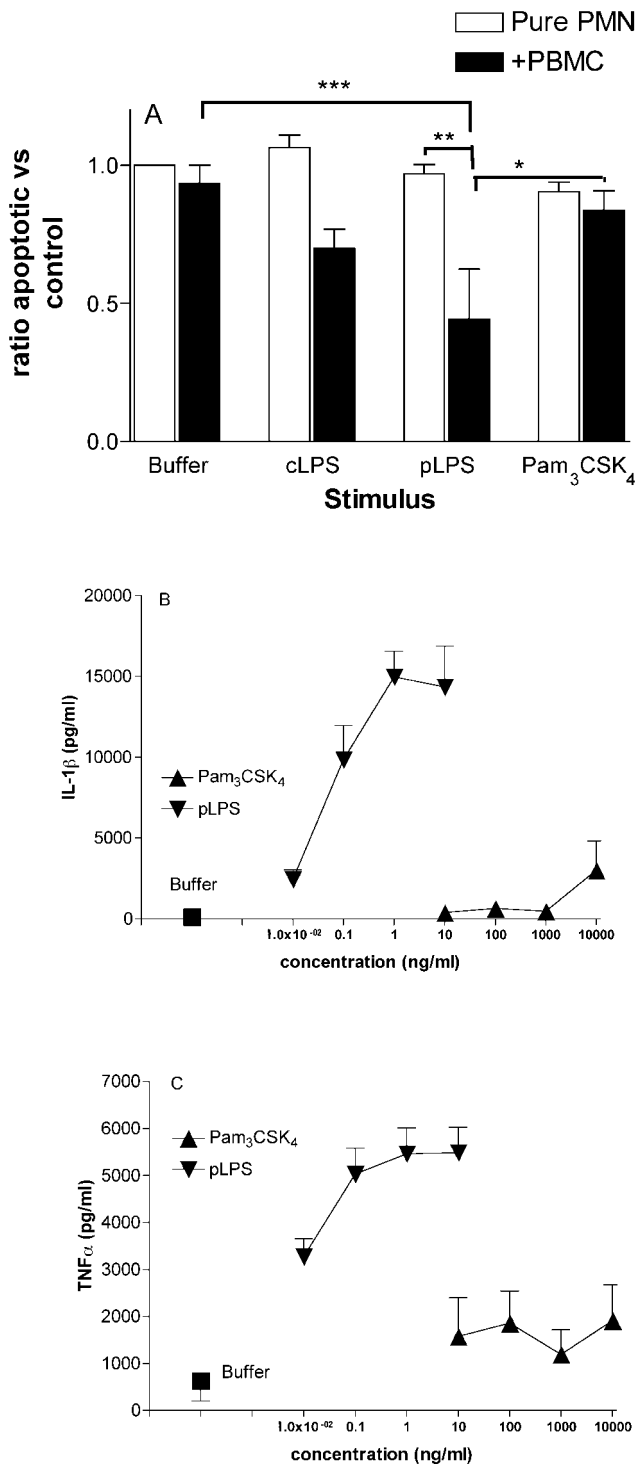


FIGURE 7. Selective TLR4-dependent pathways also regulate monocyte-mediated late neutrophil survival. *A*, Highly purified neutrophils were cultured with the indicated ligands (cLPS, 10 ng/ml; pLPS, 10 ng/ml; or Pam₃CSK₄, 10 μ g/ml) for 22 h either alone (□) or in the presence of added-back PBMC as indicated in *Materials and Methods* (■). Data are mean \pm SEM of three (pLPS, cLPS) or six (buffer, Pam₃CSK₄) experiments, each from a separate donor, expressed as the ratio of apoptotic cells in the treated sample vs the sample cultured in medium alone without added-back PBMC. Significant differences between conditions are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (ANOVA and Tukey's posttest). *B* and *C*, Cytokine generation (IL-1 β (*B*); TNF- α (*C*)) measured in the supernatant of populations of PBMC cultured for 22 h with buffer (■), Pam₃CSK₄ (▲), or pLPS (▼). Data shown are the mean \pm SEM of four experiments, each using cells from a separate donor.

Discussion

Many groups have identified roles for pathogen-associated molecules that are now known to signal via TLRs in the modulation of aspects of neutrophil function. However, most previous studies have used cLPS as a principal stimulus or comparator, which is now known to act on both TLR2 and TLR4 (4). Additionally, we have shown recently that responses to TLR activators in standard purified neutrophil preparations are partially dependent upon the presence of low numbers of contaminating monocytes (20), a problem also noted by others for cell types such as eosinophils (36). We have shown that neutrophils express both TLR2 and TLR4 protein (20), and a recent study also showed TLR2 expression on neutrophils and identified an important role for this receptor in LPS-induced neutrophil cytokine generation (37). In this study, we show the first comparative investigation of the individual roles of these receptors in highly purified, monocyte-depleted neutrophil populations, using the TLR4-selective stimulus, pLPS (4), in comparison with the TLR1/2 heterodimer stimulus, Pam₃CSK₄ (38), and purified natural peptidoglycans that activate TLR2, perhaps as TLR2/6 heterodimers (38–40). Additionally, we show selective roles for these receptors in intercellular signaling resulting in prolonged neutrophil survival.

Comparison of the potencies of purified bacterial products is hampered by the difficulties of calculating LPS molarities and the uncertainties regarding binding affinities of TLR stimuli for their receptors (indeed, it is still not certain that LPS binding to TLR4 is a crucial step for signaling, or whether intermediary protease-dependent ligand generation is involved (41)). Nonetheless, this study revealed both shared and divergent roles for TLR2 and TLR4 in neutrophil function.

Activation of either receptor caused loss of L-selectin and gain of CD11b expression (Fig. 1), in accordance with data previously shown for LPS and bacterial lipoproteins (20, 42). IL-18Rs activate similar signaling pathways to TLRs, and are expressed on neutrophils (43). In keeping with this, we observed limited responses to IL-18 in our assays, but in comparison to IL-18, TLR signaling was considerably more efficacious in modulating cell surface adhesion molecule expression.

At concentrations of agonists showing similar potencies in assays of L-selectin down-regulation, both pLPS and Pam₃CSK₄ were able to induce H₂O₂ generation alone, or synergistically with fMLP (Fig. 2). Likewise, both agonists induced IL-8 generation. Thus, both TLR2 and TLR4 mediated common neutrophil responses associated with regulation of cell recruitment and bacterial killing.

However, differences in neutrophil responses to TLR2 and TLR4 engagement were observed when investigating regulation of chemokine receptor expression, and in particular, in the control of cell survival. LPS-mediated regulation of chemokine receptor expression may provide signals causing leukocyte arrest at sites of inflammation, or prevent excessive activation at sites where inflammatory mediators are concentrated. cLPS has been shown to modulate chemokine receptor expression through autocrine ligand generation in dendritic cells (44), and in monocytes, both degradation of receptor mRNA (45) and receptor internalization have been described (46). Neutrophils express the IL-8/GCP-2 receptor CXCR1 and the more promiscuous IL-8/GCP-2/Gro α /NAP-2/ENA-78 receptor CXCR2 (47). Previous studies of neutrophils using cLPS as the stimulus have found contrasting evidence of either cLPS-mediated up-regulation of IL-8 binding (27) or cLPS-driven protease-dependent shedding of these receptors (28, 29). We observed little regulation of CXCR1 expression by TLR ligands, although this may have become evident over a longer time

course, or in the presence of small numbers of monocytes. In contrast, CXCR2 expression was down-regulated after either TLR2 or TLR4 activation, although this only showed a clear dose-response relationship with, and reached significance for, TLR2 stimulation (Fig. 4). The lack of effect of TLR ligands on CXCR1 expression argues against the hypothesis that these processes are mediated by autocrine generation of IL-8, although CXCR1 may be more resistant to internalization than CXCR2 when stimulated with IL-8 (24, 48).

Striking differences were observed between TLR2 and TLR4 in the regulation of neutrophil life span. In keeping with our previous results (20), we found that cLPS enabled prolonged neutrophil survival at early (4 h) but not late (22 h) time points in the highly purified neutrophil populations (data not shown). TLR4 stimulation by very low concentrations of pLPS almost completely prevented neutrophil apoptosis at early time points (Fig. 5). In contrast, the TLR2 stimulator Pam₃CSK₄, at 1000 ng/ml had a greater effect than 0.1 ng/ml pLPS in assays of neutrophil L-selectin and CD11b expression, yet had much more modest effects on neutrophil life span. Similarly, *S. aureus* peptidoglycan, which acts via TLR2 (39, 49, 50), had minimal effects on neutrophil survival, whereas peptidoglycan from *B. subtilis* exerted antiapoptotic actions, but only at high concentrations. In monocyte cell lines, TLR2 activation resulted in activation of both proapoptotic (dependent upon Fas-associated death domain protein and caspase 8) and proinflammatory (dependent upon NF- κ B) pathways (5, 51), and TLR2-mediated apoptosis was enhanced by NF- κ B inhibition (5, 51). In our experiments in primary human neutrophils, TLR2 activation was not associated with increased neutrophil apoptosis. The regulation of neutrophil and monocyte life span by TLR signaling is likely to be different, but it remains possible that TLR2, and not TLR4, may simultaneously activate pro- and antiapoptotic pathways in neutrophils, perhaps in part explaining the difference in regulation of neutrophil life span by these receptors.

Many groups have investigated the signaling pathways involved in neutrophil survival, but specific roles of individual pathways in response to single TLR ligands have yet to be clarified. We used the SN50 peptide inhibitor of nuclear translocation of NF- κ B to dissect these responses. SN50 was selected, because it is relatively specific for NF- κ B (35), and proteasome inhibitors, commonly used to inhibit NF- κ B, also activate proinflammatory pathways such as c-Jun N-terminal kinase and AP-1 by mechanisms that have yet to be fully characterized (52, 53). Only small effects of the less active NF- κ B control peptide SN50 M (35) were observed on pLPS-induced neutrophil survival, whereas the active SN50 inhibitor significantly prevented pLPS-induced neutrophil survival. These data are in keeping with those of Ward et al. (30), who found that NF- κ B inhibition by gliotoxin blocked cLPS-induced neutrophil survival, and although SN50 may not be entirely specific for NF- κ B inhibition (54), these data strongly support a major role for NF- κ B in TLR4-mediated neutrophil survival. In vivo, endotoxemia also results in neutrophil survival also partially dependent upon NF- κ B (55). We also identified roles for MAPKs, with evidence that combinations of NF- κ B and p38 MAPK inhibition, or NF- κ B, p38 MAPK, and extracellular signal-regulated kinase inhibition, demonstrated nearly complete ablation of TLR4-mediated neutrophil survival.

PI-3K signaling may be either antiapoptotic (in TNF- α signaling (33)) or proapoptotic (in Fas signaling (31)). We found no evidence for a role for PI-3K in TLR4-driven neutrophil survival, although inhibition of PI-3K caused a nonsignificant increase in constitutive cell death. Tyrosine kinases have been shown to play a role in neutrophil survival at late time points (34), but in our experiments herbimycin A caused a marked increase in cell death, making interpretation of the role of these pathways difficult.

Ward et al. (30) observed that NF- κ B inhibition increased constitutive neutrophil death, whereas we found that SN50 treatment nonsignificantly inhibited constitutive neutrophil apoptosis (data not shown). Apoptosis rates in SN50-treated cells were difficult to quantify morphologically, because in some experiments, the peptide caused enhanced vacuolation of the cytoplasm. Therefore, we examined the effects of inhibitors on neutrophil TLR4 responses by determination of phosphatidylserine expression (detected by annexin V binding), although this is unlikely to fully explain differences between our work and that of Ward et al. It is possible that, in other studies, NF- κ B inhibition down-regulated constitutive cytokine generation from contaminating cells, which would not be seen in our highly purified neutrophils. However, both Ward et al. (30) and our groups concur on the role of NF- κ B in LPS-induced neutrophil survival.

At the later time point in monocyte-depleted preparations, there was no evidence for direct modulation of neutrophil apoptosis by TLR signaling. Addition of PBMCs to the neutrophils restored the ability of cLPS to modulate neutrophil survival at 22 h (Fig. 7A), in keeping with our previous results (20). Dissecting the roles of individual TLRs in these responses, we observed that pLPS was able to cause monocyte-dependent neutrophil survival, whereas at the monocyte density tested, Pam₃CSK₄ was unable to do this. In additional experiments, we showed that monocytes stimulated with TLR4 activators, but not TLR2 activators, produced significant amounts of cytokines with roles in neutrophil survival, in particular TNF- α (30) and IL-1 β (56). Such divisions of cytokine secretion between TLR activators have, in the case of TNF- α , been observed by other groups (57).

In summary, we found that TLR2 and TLR4 mediated activation of neutrophils. The totality of these effects was dependent at least in part on intercellular signaling between monocytes and neutrophils, and demonstrates again that neutrophil TLR biology needs to be studied in the contexts of both highly purified and cocultured cell populations. TLR4 can also be activated by endogenous mediators of inflammation such as HSP60 (7). Different LPS preparations or endogenous ligands such as HSP60 may exert individual effects through TLR4, but our data suggests that TLR4 has a particular role to play in the regulation of neutrophil life span, via both direct and indirect pathways. Thus, TLR4 signaling, by endogenous or exogenous mediators, can regulate neutrophil survival in ways that may be amenable to pharmacological antagonism, and which may limit inappropriate or excessive neutrophilic inflammation.

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