Inhibition of Neutrophil Apoptosis by TLR Agonists in Whole Blood: Involvement of the Phosphoinositide 3-Kinase/Akt and NF-κB Signaling Pathways, Leading to Increased Levels of Mcl-1, A1, and Phosphorylated Bad

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Inhibition of Neutrophil Apoptosis by TLR Agonists in Whole Blood: Involvement of the Phosphoinositide 3-Kinase/Akt and NF-κB Signaling Pathways, Leading to Increased Levels of Mcl-1, A1, and Phosphorylated Bad

Stéphanie François,*† Jamel El Benna,* Pham M. C. Dang,* Eric Pedruzzi,* Marie-Anne Gougerot-Pocidalo,1*† and Carole Elbim*†

Using flow cytometry, we investigated the effect of TLR agonists on human polymorphonuclear neutrophil (PMN) apoptosis in whole blood. LPS (TLR4), peptidoglycan (TLR2), R-848 (TLR7/8), and CpG-DNA (TLR9) were equally effective at delaying spontaneous apoptosis of PMN, while PamCSK4 (TLR1/2), macrophage-activating lipopeptide-2 (TLR2/6), flagellin (TLR5), and loxoribine (TLR7) were less effective or inactive. TLR agonists found to delay apoptosis also extended the functional life span of PMN. Analysis of signaling pathways revealed that the antiapoptotic effect of TLR agonists required NF-κB and PI3K activation. Furthermore, analysis of intact cells by flow cytometry showed that TLR agonists delaying PMN apoptosis increased phosphorylation of Akt, a major target of PI3K. This effect was associated with a PI3K-dependent increase in heat shock protein 27 phosphorylation, which has been reported to play a key role in PMN survival. Finally, the TLR-induced delay in PMN apoptosis was associated with increased levels of Mcl-1 and A1, which are antiapoptotic members of the Bcl-2 family. These effects were reversed by PI3K and NF-κB inhibitors, respectively. TLR activation also led to PI3K-dependent phosphorylation of the proapoptotic protein Bad. Taken together, our results strongly suggest a role of NF-κB and PI3K in TLR-induced PMN survival, leading to modulation of Bcl-2 family molecules. The Journal of Immunology, 2005, 174: 3633–3642.

Polymorphonuclear neutrophils (PMN)2 are key components of the first line of defense against microbial pathogens (1). They contribute to the early innate response by rapidly migrating into inflamed tissues, where their activation triggers microbicidal mechanisms such as release of proteolytic enzymes and antimicrobial peptides, and rapid production of reactive oxygen species (ROS). PMN activation is initiated upon recognition of Ab- or complement-opsonized particles (2). PMN also directly recognize microbial products via pattern recognition receptors such as TLR (3). Ten human TLRs have so far been identified, mediating responses to pathogen-associated molecular patterns (PAMPs) shared by many microorganisms. Human PMN have been reported to express all TLRs except TLR3 (4). TLRs also recognize microbial products via pattern recognition receptors such as TLR (3). Ten human TLRs have so far been identified, mediating responses to pathogen-associated molecular patterns (PAMPs) shared by many microorganisms. Human PMN have been reported to express all TLRs except TLR3 (4). TLRs are members of the IL-1R superfamily and share a common activation mechanism (a TLR9 stimulus). We also examined mechanisms downstream of TLR signaling pathways leading to PMN survival by studying the involvement of the Phosphoinositide 3-Kinase/Akt and NF-κB pathways, TRX2 probably show differences in their rate, intensity, or efficiency of activation, involving unidentified mechanisms. Selective pathways are reported to be triggered by some TLRs; in particular, TLR2, TLR4, and TLR9 can activate the PI3K pathway (6, 7). Activation of cell signaling cascades triggers immune responses leading to pathogen eradication.

PMN are usually short-lived immune cells, but the prolongation of their life span is critical in their efficiency against pathogens (8). PMN activation and survival is likely to be tightly regulated, as the cytotoxic substances they release can damage adjacent healthy tissue (9). Many inflammatory mediators, including cytokines, regulate cell survival by interfering with apoptosis (10–13). Regulation of PMN survival has been widely studied but remains to be fully elucidated. In particular, the impact on PMN apoptosis of PAMPs recognized by the different TLRs has rarely been investigated. LPS, a TLR4 ligand, and peptidoglycan (PGN), a TLR2 ligand, delay PMN apoptosis through poorly known mechanisms (14, 15). No data on modulation of Bcl-2 family proteins after TLR activation have been reported.

In this study we report the first analysis of the differential effects of TLR agonists on apoptosis of human PMN studied in whole blood to minimize PMN activation during their isolation and to mimic physiological conditions. We used PGN from Staphylococcus aureus (a TLR2-selective stimulus), synthetic palmitoylated mimics of bacterial lipopeptides (PamCSK4, a TLR1/2 heterodimer stimulus), macrophage-activating lipopeptide-2 (MALP-2, a TLR2/6 heterodimer stimulus), purified LPS (a TLR4 stimulus), bacterial flagellin (a TLR5 stimulus), loxoribine (a guanosine analog and TLR7-selective stimulus), an imidazoquinoline pharmaceutical (R848, a TLR7/8 heterodimer stimulus) and unmethylated CpG-DNA (a TLR9 stimulus). We also examined mechanisms downstream of TLR signaling pathways leading to PMN survival by studying the participation of NF-κB, PI3K/Akt, and MAPK pathways, and modulation of Bcl-2 family proteins.

*Institut National de la Santé et de la Recherche Médeicale (INSERM), Unité 479, Faculté de Médecine Xavier Bichat, 16 rue Henri Huochard, 75877 Paris Cedex 18, France. E-mail address: pocidalo@bichat.inserm.fr

1 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; MALP-2, macrophage-activating lipopeptide-2; HE, hydroethidine; DPI, diphenyleneiodonium; DiOC6, 3,3′-dihexyloxacarbocyanine; 7-AAD, 7-aminomycin D; Hop, heat shock protein; FSC, forward scatter; SSC, side scatter; MFI, mean fluorescence intensity; PKB, protein kinase B; PKC, protein kinase C; IKK, IκB kinase; Δψm, mitochondrial transmembrane potential.
Materials and Methods

Reagents

The reagents used and their sources are as follows: ultrapurified LPS from Escherichia coli serotype R515 (LPS), purified flagellin from Salmonella typhimurium, and synthetic MALP-2 (Alexis); PGN from S. aureus, R-848, the guanosine analog loxoribine, and a synthetic palmitoylated nelli typhimurium (see Fig. 3634 TLR INHIBITION OF PMN APOPTOSIS IN WHOLE BLOOD

Leukocytes (obtained after red cell lysis with FACS lysing solution) were fixed with 2% paraformaldehyde-PBS for 10 min at 37°C. After one wash with PBS, leukocytes were incubated in ice-cold PBS-90% methanol in the dark for 30 min at 4°C to permeabilize the membranes as previously described (20). After one wash with PBS-human serum albumin (HSA) (2%), cells were stained with FITC-conjugated anti-active caspase-3 for 1 h at room temperature. After one wash (400 × g for 5 min), cells were resuspended in 1% paraformaldehyde-PBS and analyzed by flow cytometry.

NADPH oxidase activity

Superoxide anion (O2-) production was measured with a flow cytometric assay derived from the HE oxidation technique (21): HE diffuses into cells and, during the PMN oxidative burst, nonfluorescent intracellular HE is oxidized by O2- to highly fluorescent ethidium (E'), that is trapped in the nucleus by intercalation into DNA. After the different treatments, whole blood (1 ml) was loaded with HE (1500 ng/ml) at 37°C, followed by PBS or 10-6 M FMLP for 5 min. The reaction was stopped and RBC were lysed as described above. After one wash, leukocytes were resuspended in 1% paraformaldehyde-PBS.

Study of PMN surface CD11b expression

After the different treatments, whole blood was incubated at 37°C with PBS or 10-6 M FMLP for 5 min. Samples (100 µl) were stained at 4°C for 30 min with FITC-anti-human CD11b.

Study of intracellular Akt, Hsp27, and Bcl-2 family protein content

After incubation of whole blood with TLR agonists or PBS for various times at 37°C, leukocytes were permeabilized in 90% methanol as previously described (20). Cells were then stained with anti-Akt, anti-Akt phosphospecific, anti-Hsp27 phosphospecific, anti-Mcl-1, anti-AI/Bcl-1, anti-Bad phosphospecific Abs for 1 h at room temperature and washed once in PBS-2% HSA. Samples were then incubated for 30 min with FITC-goat anti-mouse or anti-rabbit Ab. Bad content was studied by staining with FITC-conjugated anti-Bad. After one wash, leukocytes were resuspended in 1% paraformaldehyde-PBS and analyzed by flow cytometry.

Flow cytometry

We used a BD Immunocytochemistry Systems FACSCalibur. To measure apoptosis in whole blood, PMN were identified on the CD15/SSC dot plot and 2 × 10^4 events were counted per sample. In other experiments, FSC and SSC were used to identify the PMN population and to gate out other cells and debris; 10,000 events were counted per sample. All the results were obtained with a constant photomultiplier gain. The data were analyzed using CellQuest software (BD Biosciences), and mean fluorescence intensity (MFI) was used to quantify the responses. Caspase-3 activity was expressed in relative units (percentage of caspase-3up<sup>+</sup> cells × MFI). Non-specific Ab binding was determined on cells incubated with the same concentration of the corresponding isotype control or with nonimmune serum.

Blot analysis of TLR expression

Suspensions of 40 × 10^6 PMN/ml in PBS buffer were treated with 2.7 mM diisopropylfluorophosphate for 20 min at 4°C and pelleted at 400 × g for 8 min at 4°C. The pellet was resuspended in CHAPS solubilization buffer containing 50 mM Tris, pH 7.5, 15 mM CHAPS, 1 mM EDTA and anti-proteases. The cells were incubated on ice and the suspension was then centrifuged at 1500 × g for 5 min. Following SDS-PAGE on 10% acrylamide gels, proteins were transferred to nitrocellulose filters. The filters were incubated for 1 h at room temperature in 50 mM Tris, 150 mM NaCl, 0.1%Tween 20 (TBST) containing 5% (w/v) fat-free dried milk. Nitrocellulose membranes were incubated overnight with specific Abs against TLR2, TLR3, TLR5, and TLR7 at 1/500 dilution. Following five washes with TBST, the membranes were incubated with goat anti-mouse or goat anti-rabbit Abs conjugated to HRP. After five washes with TBST, the membranes were washed three times with deionized water and exposed to X-ray film.

Statistical analysis

Data are reported as means ± SEM. Comparisons were based on ANOVA and Tukey’s posthoc test, using Prism 3.0 software (Graph Pad Software).
Results
Effects of TLR agonists on spontaneous PMN apoptosis and functions in vitro

Whole blood PMN cultured at 37°C died by apoptosis: ~26 and 70% of cells were annexin V\(^+\) after 8 and 24 h, respectively (Fig. 1D). As previously reported with isolated PMN (12, 16), apoptosis was accelerated by cycloheximide (65% of annexin V\(^+\) cells after 8 h), and delayed by GM-CSF (4% of annexin V\(^+\) cells after 8 h). As shown in Fig. 2, the percentage of total annexin V\(^+\) cells, and the percentage of annexin V\(^+\), 7-AAD\(^-\) cells, fell significantly, in a concentration-dependent manner after 8 h of treatment with all TLR agonists except flagellin (TLR5) and loxoribine (TLR7). We checked that environmental LPS did not contribute to the effect of the TLR agonists (except for the TLR4 agonist, which is purified LPS). In fact, the prolongation of PMN survival by PGN, R-848, CpG-DNA, Pam3CSK4, and MALP-2 was not modified by pre-incubation with a TLR4 neutralizing Ab, while the effect of LPS on PMN survival was completely abolished (not shown). PMN apoptosis, measured in terms of total annexin V\(^+\) PMN, fell to a similar extent after incubation for 8 h with LPS, PGN, R-848, and CpG-DNA at optimal concentrations. This inhibition was significantly stronger than that induced by Pam3CSK4 and MALP-2 (Table I). Similar levels of inhibition were found in the early stage of PMN apoptosis (annexin V\(^+\)/7-AAD\(^-\) cells; not shown). A kinetic study showed that LPS, PGN, R-848, and CpG-DNA induced ~50% inhibition of PMN apoptosis after 2 h, rising to a maximum of 80% after 8 h. In samples treated with all the TLR agonists except flagellin and loxoribine, 80% of PMN were annexin V\(^+\) after 48 h incubation, compared with 24 h in PBS-treated samples, further showing that TLR agonists delay PMN apoptosis (Fig. 3).

As cytokine production by contaminating monocytes has been implicated in TLR agonist-induced inhibition of PMN apoptosis at later times (22 h) but not at earlier times (4 h) (22), we examined the effect of 8 h of incubation with TLR agonists on highly purified PMN depleted of all monocytes as previously described (17). TLR agonist-induced inhibition of PMN apoptosis was similar whether agonist-induced inhibition of PMN apoptosis was similar whether PMN apoptosis was studied in whole blood or in highly purified preparations, suggesting a direct effect of TLR agonists on PMN apoptosis. However, apoptosis inhibition was slightly less effective with purified PMN than with whole blood: the protective agonists induced 38–69% inhibition of PMN apoptosis in purified preparations and 46–87% in whole blood (Table I). To minimize cell
production in the basal
production) (Fig. 4). This impairment was found with both annexin V
expression in response to fMLP relative to baseline (Fig. 5).

Effect of TLR agonists on caspase-3 activity and mitochondrial
membrane integrity

To identify the stage of the apoptotic program at which TLR ago-
nists act to delay apoptosis, we measured the effects of TLR ago-
nists on caspase-3 activity and Δψm. As shown in Table II, caspase-3 activity was detected during spontaneous PMN apopto-
sis after 8 h incubation with PBS at 37°C, and was significantly
reduced by the TLR agonists that delayed PMN apoptosis (LPS,
PGN, R-848, CpG-DNA, Pam3CSK4, MALP-2). In contrast,
flagellin and loxoribine did not significantly modify caspase-3 ac-
Figure 3. Kinetics of PMN apoptosis after treatment with LPS, PGN,
R-848, CpG-DNA, Pam3CSK4, and MALP-2. Whole blood samples were
incubated in 24-well tissue culture plates at 37°C with 5% CO2 for various
times with PBS or the following TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 μg/ml (TLR2); R-848, 10 μg/ml (TLR7/8); CpG-DNA, 100 μg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6); flagellin, 100 ng/ml (TLR5); or loxoribine, 100 μM (TLR7). PMN were identified by using a FITC anti-CD15 Ab. Apoptosis was quanti-
tified by staining with allophycocyanin-annexin V and 7-AAD as described in Ma-
terials and Methods.

Table I. Percentage inhibition of PMN apoptosis by TLR agonists

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<thead>
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<th>Percentage Inhibitiona</th>
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<tr>
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<tr>
<td>Whole Blood</td>
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<tr>
<td>LPS (TLR4)</td>
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<tr>
<td>PGN (TLR2)</td>
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<tr>
<td>R-848 (TLR7/8)</td>
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<tr>
<td>CpG-DNA (TLR9)</td>
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<tr>
<td>Pam3CSK4 (TLR1/2)</td>
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<tr>
<td>MALP-2 (TLR2/6)</td>
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<td>Flagellin (TLR5)</td>
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<td>Loxoribine (TLR7)</td>
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a Whole blood or isolated PMN were incubated in 24-well tissue cultures plates at 37°C with 5% CO2 for 8 h with PBS or TLR agonists at the following optimal concentrations: LPS, 10 ng/ml (TLR4); PGN, 1 μg/ml (TLR2); R-848, 10 μg/ml (TLR7/8); CpG-DNA, 100 μg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6); flagellin, 100 ng/ml (TLR5); or loxoribine, 100 μM (TLR7). PMN were identified by using a FITC anti-CD15 Ab. Apoptosis was quanti-
tified by staining with allophycocyanin-annexin V and 7-AAD as described in Ma-
terials and Methods.

b Results are expressed as the percentage inhibition of PMN apoptosis (1 - (% of total annexin V+ PMN in TLR agonist-treated sample/% of total annexin V+ PMN in PBS-treated sample)) × 100. Values are means ± SEM (n = 5).

c,d Significantly different from sample incubated with PBS (percentage inhibition of PMN apoptosis = 0) and samples incubated with flagellin or loxoribine (p < 0.05).

Role of the NF-κB signaling pathway in TLR agonist
postponement of PMN apoptosis

NF-κB activation has been implicated in PMN survival (23), and
particularly in LPS-induced PMN survival. However, the involve-
ment of the NF-κB pathway in the effects of TLR agonists that
delay PMN apoptosis has not been comparatively investigated.

To determine the involvement of the NF-κB pathway in whole
blood, we first used an inhibitor-based approach. The NF-κB in-
hibitor (SN50) had no significant effect on spontaneous apoptosis:

activation during isolation and to better mimic physiological con-
tions, the following experiments were performed with PMN in
their whole blood environment.

After 8 h of incubation, PBS-treated PMN exhibited a reduced
capacity to produce superoxide anion (O2−) and to increase surface
CD11b expression in response to fMLP relative to baseline (Fig.
4). This impairment was found with both annexin V− and annexin
V+ PMN, although annexin V− cells were less potent than an-
nexin V+ cells. In contrast, incubation for 8 h with the TLR ago-
nists found to delay apoptosis increased O2− production in the basal
state and also in response to fMLP (LPS was the most potent
agonist in terms of increasing O2− production) (Fig. 4A). This
impact of the TLR agonists on ROS production did not differ signif-
cantly according to annexin V staining, although the stimulation
index (MFI of fMLP-stimulated sample/MFI of unstimulated sam-
ple) was slightly lower in annexin V− than annexin V+ popula-
tions. Similar results were observed concerning PMN surface
CD11b expression (Fig. 4B). Flagellin and loxoribine did not signif-
icantly modify PMN responses. This increased ROS production
was not involved in the effects of TLR agonists on PMN apoptosis,
as incubation with DPI, a NADPH oxidase inhibitor, altered nei-
th other the intensity nor the kinetics of apoptosis inhibition (not
shown).

Thus, TLR agonists found to delay apoptosis also extended the
functional life span of PMN.

PMN expression of TLR5 and TLR7 by Western blotting

As flagellin (a TLR5 agonist) and loxoribine (a TLR7 agonist) did not delay PMN apoptosis, in contrast to the other TLR agonists, we checked that TLR5 and TLR7 were expressed in PMN. TLR2 and
TLR3 were used as positive and negative controls, respectively
(4). Fig. 5 shows that TLR2, TLR5, and TLR7 were expressed in
PMN, contrary to TLR3.
the percentage of annexin V+ cells was 24.2 ± 4.5 and 25.1 ± 3.8 in samples incubated with PBS and SN50, respectively. As shown in Fig. 6A, SN50 (100 μg/ml), which inhibits nuclear translocation of NF-κB, significantly reduced TLR-mediated inhibition of PMN apoptosis at 8 h, suggesting a role of NF-κB. The control peptide SN50M did not significantly alter PMN apoptosis, whatever the TLR agonist used. Another NF-κB inhibitor, the sesquiterpene lactone parthenolide (10 μM) also inhibited TLR-induced inhibition of PMN apoptosis (not shown). To confirm these findings, we used flow cytometry and a mouse anti-phospho-IKK mAb, and found that the protective agonists significantly induced IKK phosphorylation relative to PBS-treated samples (Fig. 7A).

However, the NF-κB inhibitors only partially reversed TLR agonist-induced inhibition of PMN apoptosis, suggesting the involvement of other signaling pathways.

PI3K/Akt dependence of TLR agonist action on PMN apoptosis

TLRs may activate many other signaling pathways, a number of which have already been implicated in the regulation of PMN life span, including MAPKs (24, 25), PI3K (26, 27), and tyrosine kinases (28).

To investigate the signaling pathways involved in TLR agonist-induced inhibition of apoptosis, we first examined the effects of various kinase inhibitors on PMN in whole blood incubated with TLR agonists for 8 h. We checked that kinase inhibitors alone did not alter PMN apoptosis; in particular, the percentage of annexin V+ cells was 25.3 ± 5.2 and 23.2 ± 4.7 in samples incubated with PBS and Wortmannin, respectively. As shown in Fig. 7A, only wortmannin (a PI3K inhibitor) attenuated LPS-dependent inhibition of apoptosis. Similar effect was observed with a second PI3K inhibitor, LY294002 (25 μM) (not shown). Blockade of both the NF-κB and PI3K pathways potentiated this effect (18% apoptosis inhibition with LPS + wortmannin + SN50). GI102903X (a protein kinase C (PKC) inhibitor), genistein (a broadly specific tyrosine kinase inhibitor), PD98059 (a MEK1/2 kinase inhibitor), SB203580 (a p38MAPK inhibitor), and rottlerin (a PKC inhibitor) had no effects on LPS-induced PMN survival whatever the concentrations used. Similar results were observed with the other TLR agonists that delayed neutrophil apoptosis (not shown). As shown in Fig. 7B, wortmannin has a similar inhibitory effect on LPS, PGN, R-848, Cpg-DNA, Pam3CSK4, and MALP-2-induced PMN survival. Wortmannin also inhibited the effect of TLR agonists on caspase-3 activity, and reduced the percentage of DiOC6low cells (not shown).

One mechanism involved in PI3K prevention of apoptosis is activation of the PKB/Akt pathway (29). We therefore studied the phospho-Akt content of intact PMN treated in whole blood, by means of flow cytometry with a mouse anti-human-phospho-Akt mAb. As shown in Fig. 8A, incubation of whole blood with LPS

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**Table II. Caspase-3 activity and mitochondrial membrane integrity**

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<th>Caspase-3 Activity (Relative Units)</th>
<th>DiOC6low Cells (Percentage)</th>
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<tbody>
<tr>
<td>PBS</td>
<td>290.3 ± 38.6</td>
<td>39.5 ± 4.5</td>
</tr>
<tr>
<td>LPS (TLR4)</td>
<td>66.5 ± 19.8</td>
<td>14.5 ± 1.5</td>
</tr>
<tr>
<td>PGN (TLR2)</td>
<td>73.1 ± 27.6</td>
<td>16.5 ± 2.3</td>
</tr>
<tr>
<td>R-848 (TLR7/8)</td>
<td>66.6 ± 23.4</td>
<td>11.7 ± 0.8</td>
</tr>
<tr>
<td>Cpg-DNA (TLR8)</td>
<td>97.2 ± 28.8</td>
<td>17.0 ± 1.5</td>
</tr>
<tr>
<td>Pam3CSK4 (TLR1/2)</td>
<td>123.1 ± 15.5</td>
<td>19.2 ± 2.1</td>
</tr>
<tr>
<td>MALP-2 (TLR2/6)</td>
<td>154.1 ± 12.4</td>
<td>17.0 ± 1.2</td>
</tr>
<tr>
<td>Flagellin (TLR5)</td>
<td>228.4 ± 25.6</td>
<td>31.0 ± 4.3</td>
</tr>
<tr>
<td>Loxoribine (TLR7)</td>
<td>235.2 ± 18.2</td>
<td>34.3 ± 1.8</td>
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"Whole blood samples were incubated for 8 h in 24-well tissue cultures plates at 37°C with 5% CO2, with either PBS or the following TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 μg/ml (TLR2); R-848, 10 μg/ml (TLR7/8); Cpg-DNA; 100 μg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP2, 10 ng/ml (TLR2/6); flagellin, 100 ng/ml (TLR5), or loxoribine, 100 μM (TLR7). Caspase-3 activity (*) and retention of DiOC6 (′) by PMN were measured as described in Materials and Methods. Caspase-3 activity is expressed in relative units (percentage of caspase-3high cells × MF) and retention of DiOC6 in percentage of DiOC6low cells. Values are means ± SEM (n = 3).

†, Significantly different from sample incubated with PBS and samples incubated with flagellin or loxoribine (p < 0.05).
significantly increased Akt phosphorylation after as little as 2 min, as compared with PBS. Similar results were observed with PGN, R-848, CpG-DNA, Pam3CSK4, and MALP-2 (Fig. 8f). In contrast, pretreatment with flagellin or loxoribine did not modify Akt phosphorylation. Total Akt content, measured with a mouse anti-human Akt mAb in the same conditions, was not modified by treatment with TLR agonists (not shown).

Hsp27 has been reported to associate with Akt (30). Furthermore, phosphorylation of Hsp27 by Akt results in its dissociation from Akt and may participate to the Hsp27-induced delay in PMN apoptosis (33), we found that Mcl-1 levels fell during constitutive PMN apoptosis whatever the TLR agonist used. Values are means ± SEM (n = 3). *Significantly different from sample incubated with PBS (p < 0.05). B, Effect of TLR agonists on IKK phosphorylation. Samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO2 with PBS or with TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 µg/ml (TLR2); R-848, 10 µg/ml (TLR7/8); CpG-DNA, 100 µg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6). Apoptosis was quantified as described in the legend of Fig. 1. Results are expressed as the percentage inhibition of PMN apoptosis [1 – (% of total annexin V+ PMN in TLR agonist-treated sample/% of total annexin V+ PMN in PBS-treated sample)] × 100. The SN50M control peptide did not significantly alter apoptosis whatever the TLR agonist used. Values are means ± SEM (n = 3). *Significantly different from sample incubated with PBS (p < 0.05).}

**Figure 6.** Involvement of the NF-κB signaling pathway in TLR agonist-induced PMN survival. A, Effect of an NF-κB inhibitor (SN50) on TLR agonist-induced PMN survival. Whole blood samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO2 with SN50 (100 µg/ml) or PBS for 1 h. Samples were then treated with PBS or the following TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 µg/ml (TLR2); R-848, 10 µg/ml (TLR7/8); CpG-DNA, 100 µg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6). Apoptosis was quantified as described in the legend of Fig. 1. Results are expressed as the percentage inhibition of PMN apoptosis [1 – (% of total annexin V+ PMN in TLR agonist-treated sample/% of total annexin V+ PMN in PBS-treated sample)] × 100. The SN50M control peptide did not significantly alter apoptosis whatever the TLR agonist used. Values are means ± SEM (n = 3). *Significantly different from sample incubated with PBS (p < 0.05). B, Effect of TLR agonists on IKK phosphorylation. Samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO2 with PBS or with TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 µg/ml (TLR2); R-848, 10 µg/ml (TLR7/8); CpG-DNA, 100 µg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6). Phospho-IIK content was then measured by flow cytometry on methanol-permeabilized cells as described in Materials and Methods. Results are MFI. Values obtained with an irrelevant Ab of the same isotype or with nonimmune serum were subtracted. *Significantly different from samples incubated with PBS, flagellin, or loxoribine (p < 0.05).

**Figure 7.** Effect of kinase inhibitors on TLR agonist-induced PMN survival. A, Whole blood samples were first incubated in 24-well tissue cultures plates at 37°C with 5% CO2 with PBS, PI3K inhibitor (wortmannin: 2500 nM), MEK1/2 inhibitor (PD98059: 50 µM), PKC inhibitor (GF109203X: 5 µM), tyrrosine kinase inhibitor (genistein: 100 µM), PKCα inhibitor (rottlerin: 10 µM), or p38MAPK inhibitor (SB203580: 25 µM) for 1 h and then with LPS 10 ng/ml for 8 h. Results are expressed as the percentage inhibition as described in the legend of Fig. 6A. Values are means ± SEM (n = 3). *Significantly different from sample incubated with PBS instead of kinase inhibitors (p < 0.05). B, Samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO2 with PBS or PI3K inhibitor (wortmannin: 2500 nM) for 1 h and then for 8 h with TLR agonists delaying apoptosis, namely LPS, 10 ng/ml (TLR4); PGN, 1 µg/ml (TLR2); R-848, 10 µg/ml (TLR7/8); CpG-DNA, 100 µg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); or MALP-2, 10 ng/ml (TLR2/6). Apoptosis was quantified and the results are expressed as the percentage inhibition of apoptosis as described in the legend of Fig. 6A. Values are means ± SEM (n = 3). *Significantly different from sample incubated with PBS instead of wortmannin (p < 0.05).
FIGURE 8. Effect of TLR agonists on intracellular Akt and Hsp27 phosphorylation. Whole blood samples were preincubated at 37°C in a water bath with gentle horizontal agitation for 15 min with PBS or wortmannin and then treated for 1–10 min with PBS or the following TLR agonists: LPS, 10 ng/ml (TLR4); PGN, 1 μg/ml (TLR2); R-848, 10 μg/ml (TLR7/8); CpG-DNA, 100 μg/ml (TLR9); Pam3CSK4, 500 ng/ml (TLR1/2); MALP-2, 10 ng/ml (TLR2/6); flagellin, 100 ng/ml (TLR5); or loxoribine, 100 μM (TLR7). Phospho-Akt (A and B) and phospho-Hsp27 (C and D) content was then measured by flow cytometry on methanol-permeabilized cells as described in Materials and Methods. Results are MFIs. Values obtained with an irrelevant Ab were subtracted. A and C, The time course of the LPS action on intracellular expression of phospho-Akt and phospho-Hsp27. Values are means ± SEM (n = 4). *, Significantly different from samples incubated with PBS (p < 0.05). B and D, The differential effects of TLR agonists on intracellular expression of phospho-Akt and phospho-Hsp27 at optimal incubation times (2 min for phospho-Akt and 5 min for phospho-Hsp27) and the inhibitory effect of wortmannin. Values are means ± SEM (n = 4). †, Significantly different from samples incubated with PBS, flagellin, and loxoribine (p < 0.05). ‡, Not significantly different from samples incubated with PBS, flagellin, and loxoribine (p < 0.05).

Discussion

Our results show that all TLR agonists except for flagellin (TLR5) and loxoribine (TLR7) delay spontaneous apoptosis of human PMN and extend their functional life span in whole blood. The antiapoptotic action of TLR agonists required activation of NF-κB and PI3K. Furthermore, flow cytometry of intact cells showed that the TLR agonists which delayed PMN apoptosis induced phosphorylation of Akt, a major target of PI3K, and Bad phosphorylation. As shown in Fig. 9E, levels of phospho-Bad increased significantly during incubation (30 and 60 min) with LPS, while total Bad content was unaffected (not shown). Similar results were observed with other TLR agonists that delayed apoptosis (Fig. 9F). This increase was completely reversed by preincubation with wortmannin (not shown).

One particularity of this study is that we analyzed PMN apoptosis by flow cytometry in whole blood. This avoided PMN isolation procedures, which have been shown to induce surface expression of molecules that are not detected in whole blood and may thereby alter PMN responses (36). Such artifacts might contribute to the acceleration of spontaneous apoptosis that we observed after PMN purification. In addition, analysis of TLR agonist-induced modulation of PMN survival in whole blood mimics physiological factors, such as PI3K and NF-κB, that are expressed by PMN in vivo.
conditions more closely than the use of isolated cells. In particular, interactions between cellular elements have been reported to be important in maintaining PMN viability (37). However, our data do not rule out a contribution of PBMC to the kinetics of spontaneous PMN apoptosis and to the TLR agonist-induced delay in PMN apoptosis. Release of survival factors such as cytokines by activated monocytes (22, 38) could contribute to the stronger agonist-induced inhibition of PMN apoptosis that we observed in whole blood as compared with highly purified PMN.

In keeping with previous data (12, 14, 15, 39), we found that LPS delayed PMN apoptosis in whole blood. We also extended this observation to PGN, R-848, CpG-DNA, Pam3CSK4, MALP-2, flagellin, loxoribine, or Pam3CSK4 (TLR1/2) and MALP-2 (TLR2/6) had milder effects on PMN survival. TLR1 and TLR6 act as coreceptors for TLR2 and have been reported to inhibit cellular responsiveness to activating ligands (40, 41). The lack of effect of flagellin and loxoribine could be related to a lack of triggering of their signaling pathways, itself possibly due to weak expression of TLR5 and TLR7, in keeping with previous reports that the corresponding mRNAs are weakly expressed in PMN (41, 42).

We observed, for the first time, that treatment of PMN in whole blood with TLR agonists which strongly delayed PMN apoptosis at 8 h maintained and even enhanced PMN functions, both in the basal state and in response to a bacterial product (fMLP), as compared with values obtained at baseline (T0h) and with untreated samples at 8 h. TLRs play a central role in innate immunity by mediating PAMP recognition, as reflected by the increased susceptibility to infections of children with deficient TLR transduction (43). The prolonged functional life span induced by LPS, PGN, R-848, CpG-DNA and, to a lesser degree, Pam3CSK and MALP-2 may represent a crucial enhancement of PMN defenses against microbial pathogens. Furthermore, our data, obtained with whole blood, suggest that in pathological situations such as sepsis the delayed PMN apoptosis induced by TLR agonists at the systemic level could potentiate inflammatory reactions and lead to vessel damage.

Many groups have investigated the signaling pathways involved in PMN survival, but the specific roles of individual pathways involved in the response to individual TLR ligands remain to be clarified. We first used NF-kB inhibitors commonly used to inhibit NF-kB nuclear translocation (44, 45). The NF-kB control peptide SN50M had only minor effects on TLR-induced PMN survival, whereas the active SN50 NF-kB inhibitor significantly prevented TLR agonist-induced survival. Similar effects were observed with a second NF-kB inhibitor, the sesquiterpene lactone parthenolide. In addition, using an anti-phosphorylated-IKK Ab, we found that
TLR-agonists protecting against apoptosis induced IKK phosphorylation, which permits IκB phosphorylation, leading to its degradation and NF-κB nuclear translocation (46). These data are in keeping with those previously reported on LPS-treated isolated PMN (15, 23). In this study, we extend this observation to all TLR agonists capable of delaying PMN apoptosis, pointing to a major role of NF-κB in TLR-mediated PMN survival.

Our results also demonstrate that TLR agonists delay PMN apoptosis via a PI3K-dependent pathway, although our data do not rule out the possibility that PI3K inhibitors could also act by preventing, at least in part, the generation of survival signals by other cells in whole blood. In contrast, tyrosine kinases, MAPK and PKC do not appear to be involved. The lipid products of PI3K–predominantly phosphatidylinositol 3,4,5-triphosphate–induce translocation of Akt/PKB to the plasma membrane, where it is phosphorylated and activated by phosphatidylinositol 3,4,5-phosphate–dependent protein kinase (PDK1), and this pathway has been forwarded as a major mediator downstream of PI3K (29, 47). Akt/PKB activity has been shown to prevent apoptosis induced by cytokines and growth factors, cellular stress, chemotherapeutic agents, and irradiation (48). Our data showing that TLR agonists capable of delaying PMN apoptosis increase PI3K-dependent Akt phosphorylation strongly suggest a central role of Akt in this effect. Furthermore, we show for the first time that the same TLR agonists induce a PI3K-dependent increase in Hsp27 phosphorylation. It has recently been shown that Hsp27 phosphorylation by Akt leads to disruption of the Akt-Hsp27 interaction, and it has been suggested that released Hsp27 may promote independent survival signals (31). It has also been postulated that Hsp27 inhibits apoptosis through inactivation of caspase-3, caspase-9, and inhibition of cytochrome c release (49, 50).

Akt activation could also explain, at least in part, the role of NF-κB in TLR-induced PMN survival. Indeed, it was recently reported that Akt modulates NF-κB-dependent transcription in TLR2-stimulated PMN by modifying phosphorylation of the p65 subunit (7). Nevertheless, Akt is necessary but not sufficient for NF-κB activation by TLR (51).

PI3K/Akt activation could be an alternative pathway to the IL-1R-associated kinase/IKK/NF-κB pathway, leading to independent modulation of Bcl-2 family proteins (52). Increased levels of Mcl-1 have been implicated in PMN survival induced by proinflammatory cytokines such as GM-CSF, IL-1, TNF-α, and IL-15 (12, 33, 53, 54). We found that PMN treatment for 1 h with TLR agonists capable of delaying neutrophil apoptosis prevented the loss of Mcl-1. In keeping with previous data demonstrating that increased Mcl-1 translation depends on the PI3K/Akt pathway (55), we found that the TLR-induced Mcl-1 elevation was reversed by pretreatment with wortmannin. The only other antiapoptotic gene product so far implicated in PMN survival is A1. Previous studies have been restricted to mRNA, and show that A1 transcripts are cytokine-regulated in human PMN (34). Using a novel method–flow cytometry–to investigate A1 protein expression by intact permeabilized PMN, we showed for the first time that PMN activation by TLR agonists is associated with an increased intracellular content of A1 protein. In keeping with previous data (56), this effect was regulated by NF-κB and partially inhibited by wortmannin. Finally, kinetic analysis showed an early increase in Mcl-1 and A1 levels and a return to control value by 240 min. These results strongly suggest that this early increase in Mcl-1 and A1 levels inhibits the cellular apoptosis machinery and gives an advantage to TLR agonist-stimulated cells.

Rapid regulation of PMN survival could be achieved by posttranslational protein modifications. In particular, increased Bad (Bcl-xL/Bcl-2-associated death promoter homologue) phosphorylation has been implicated in PMN survival induced by GM-CSF (13, 24). Phosphorylated Bad interacts with 14-3-3 protein, and the resulting Bad sequestration diminishes Bad binding to diverse antiapoptotic Bcl-2 proteins anchored to the mitochondrial membrane (57). Increased amounts of antiapoptotic proteins are then free to bind to Bax and to prevent its proapoptotic activity, leading to cell survival. Recently, it was reported that underphosphorylated Bad interacts with all antiapoptotic Bcl-2 family members, and particularly A1 and Mcl-1 (58). Our results demonstrating that TLR agonists which inhibit PMN apoptosis increase Bad phosphorylation from 30 to 60 min strongly suggest that this phenomenon is involved in PMN survival induced by TLR activation. In keeping with previous data showing that Bad phosphorylation on serine 136 is induced by phospho-Akt (59), we observed that TLR agonist-induced Bad phosphorylation was PI3K-dependent.

Taken together, our findings demonstrate that relatively specific agonists of TLR, namely LPS (TLR4), PGN (TLR2), R-848 (TLR7/8), CpG-DNA (TLR9), Pam3CSK4 (TLR1/2), MALP-2 (TLR2/6), with the exception of flagellin (TLR5) and loxoribine (TLR7), are able to delay PMN apoptosis and extend the PMN functional life span in whole blood. Our results also point to the involvement of the PI3K/Akt and NF-κB pathways in PMN survival induced by TLR activation. PI3K-dependent phosphorylation of Akt may be strongly involved in the increased levels of the antiapoptotic protein Mcl-1 and the increased phosphorylation of the proapoptotic protein Bad. The antiapoptotic action of TLR agonists may be facilitated by Akt-dependent phosphorylation of Hsp27. In addition, NF-κB activation may lead to increased levels of the antiapoptotic protein A1. To our knowledge, this is the first report that Bcl-2 family proteins are modulated upon TLR activation. Ongoing studies may identify new therapeutic targets for regulating TLR-induced PMN survival and functions in inflammatory disorders in which these cells contribute to bystander tissue damage.

Disclosures
The authors have no financial conflict of interest.

References


