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*J Immunol* 2006; 176:1195-1202; doi: 10.4049/jimmunol.176.2.1195
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Activation of TLR-9 Induces IL-8 Secretion through Peroxynitrite Signaling in Human Neutrophils

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Bacterial DNA containing unmethylated CpG motifs is emerging as an important regulator of functions of human neutrophil granulocytes (polymorphonuclear leukocytes (PMN)). These motifs are recognized by TLR-9. Recent studies indicate that peroxynitrite (ONOO⁻) may function as an intracellular messenger for the production of IL-8, one of the key regulators of leukocyte trafficking in inflammation. In this study we investigated whether bacterial DNA (CpG-DNA) could induce ONOO⁻ signaling in human PMN. Human whole blood, isolated PMN (purity, >95%), and high purity (>99%) PMN respond to CpG-DNA, but not to calf thymus DNA, with secretion of IL-8 and, to a lesser extent, IL-6 and TNF. Methylation of cytosines in CpG-DNA resulted in a complete loss of activity. The endosomal acidification inhibitors, baflomycin A and chloroquine, inhibited CpG-DNA-induced cytokine release from PMN. CpG-DNA-induced IL-8 mRNA expression and release was also blocked by the NO synthase inhibitor N⁴-nitro-L-arginine methyl ester. CpG-DNA evoked concomitant increases in intracellular superoxide and NO levels, leading to enhanced ONOO⁻ formation and, consequently, nuclear accumulation of c-Fos and NF-κB. Pharmacological inhibition of NF-κB activation attenuated ~75% of CpG-DNA-evoked IL-8 release. These results identify ONOO⁻-dependent activation of NF-κB and c-Fos as an important mechanism that mediates PMN responses, including IL-8 gene expression and release, to bacterial DNA and unmethylated CpG motifs in particular. Enhanced ONOO⁻ formation represents a mechanism by which bacterial DNA may contribute to prolongation and amplification of the inflammatory response.


Mammalian innate immune cells detect the presence of bacteria through TLR that recognize pathogen-associated molecular patterns (1). Bacterial DNA contains short sequences of unmethylated CpG dinucleotides that are at least 20-fold more common in bacterial than in vertebrate DNA (2). In eukaryotic DNA these CpG motifs are suppressed and mostly methylated (2). Bacterial DNA (CpG-DNA)³ is recognized by TLR-9 expressed in both immune and nonimmune cells (3, 4). Although human neutrophils (polymorphonuclear leukocytes (PMN)) express TLR-9 mRNA and protein (5–7), surprisingly little is known about the impact of TLR-9 signaling on PMN functions. Intratracheal instillation of CpG-DNA results in PMN accumulation in the lower airways in mice (8). Consistent with enhanced PMN trafficking, CpG-DNA induces IL-8 release (5), triggers L-selectin shedding and up-regulation of CD11b/CD18 on the surface of isolated PMN (6), enhances PMN phagocyte activity (5), and rescues PMN from constitutive apoptosis (7). CpG-DNA was detected in the absence of bacteria in tissues of patients with chronic inflammatory conditions, including cystic fibrosis (8) and unstable coronary angina (9). Such pathological conditions are also characterized by increased nitrotyrosine formation (10–12), widespread PMN activation (13), and persisting PMN trafficking into inflamed tissues (14). The elevation in nitrotyrosine may reflect local formation of reactive nitrogen species, in particular peroxynitrite (ONOO⁻) (15). High micromolar concentrations of ONOO⁻ are cytotoxic (16). However, at much lower concentrations, ONOO⁻ may activate intracellular signaling pathways that mediate PMN responses. Indeed, ONOO⁻ promotes PMN adhesion to endothelial cells through ERK-dependent up-regulation of the surface expression of CD11b/CD18 on PMN (17). ONOO⁻ functions as an intracellular messenger to induce activation of the transcription factors NF-κB and AP-1, leading to enhanced expression and production of cytokines, including IL-8, in human leukocytes challenged with LPS, TNF-α, or IL-1β (18–20). IL-8 plays a pivotal role in the recruitment and activation of neutrophils and monocytes in various experimental models of inflammation (21–23).

In this study we investigated whether CpG-DNA and unmethylated CpG motifs in particular induce the formation of ONOO⁻ in human PMN, and whether ONOO⁻ signaling events mediate primary cytokine responses in PMN.

Materials and Methods

Materials

Escherichia coli DNA (strain B) and calf thymus DNA (Sigma-Aldrich) were purified by extraction with phenol/chloroform/isooxylen alcohol (25/24/1, v/v/v) and ethanol precipitation. Heat-denatured genomic ssDNA was used in all experiments. For some experiments, E. coli DNA was treated for 16 h at 37°C with CpG methylase SsI (2 U/μg DNA) in NE Buffer 2 supplemented with 160 μM S-adenosylmethionine (New England Biolabs). Met-CpG-DNA was purified as described above. All DNA preparations contained <5 ng LPS/μg DNA by Limulus assay.

Cell isolation and stimulation

Venous blood (anticoagulated with sodium heparin, 50 U/ml) was obtained from healthy volunteers who had denied taking any medication for at least 18 U.S.C. Section 1734 solely to indicate this fact.

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Received for publication May 5, 2005. Accepted for publication November 2, 2005.

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Abbreviations used in this paper: CpG-DNA, bacterial DNA; DAF, 4,5-diamino-oxofluorescein diacetate; DHR 123, dihydrorhodamine 123; eNOS, endothelial NO synthase; met-CpG-DNA, methylated bacterial DNA; NO synthase; ONOO⁻, peroxynitrite; PDTC, pyrrolidine dithiocarbamate; PMN, polymorphonuclear leukocyte; iNOS, inducible NO synthase; N⁴-nitro-L-arginine methyl ester.
2 wk. The clinical research committee approved the experimental protocols. PMN were isolated as described previously (17). The PMN preparations (purity and viability routinely >95%) contained <1.2% monocytes (neutral red-positive or CD14bright cells). In some experiments, high purity PMN were prepared by immunomagnetic depletion of monocytes, T cells, and B cells using mouse anti-human CD14 mAb, anti-CD3 mAb, anti-CD19 mAb, and Dynabeads pan mouse IgG (Dynal Biotech) according to the manufacturer’s instructions. This procedure yielded PMN with a purity >99%, with <0.4% cells staining positively for HLA-DR (assessed by flow cytometry), without affecting cell viability.

Whole blood aliquots, isolated PMN, or high purity PMN (5 × 10^6 cells/ml) in sterile microcentrifuge tubes were placed on a rotator, preincubated for 20–30 min with inhibitors of endosomal acidification, nitrogen synthesis, or NF-κB activation as indicated, and then challenged with CpG-DNA, met-CpG-DNA, or calf thymus DNA at 37°C in 5% CO₂ atmosphere. At the designated time points, plasma was harvested and stored at −20°C for later cytokine analysis. Cells were processed as described below.

**TLR-9 expression**

Freshly isolated PMN were permeabilized with permeabilization buffer (eBioscience) and stained with an R-PE-conjugated anti-human TLR-9 Ab eB72-1665 or a class-matched irrelevant Ab (eBioscience). Neutrophil TLR-9 expression was assessed on a FACScan flow cytometer (BD Biosciences). PMN, defined as CD16⁺ CD3⁺ CD19⁻ HLA-DR⁻ cells, were gated at a purity of >99.9%.

**Measurements of cytokine secretion and transcripts**

Levels of IL-8, TNF-α, and IL-6 in plasma or culture medium were determined by selective ELAs (OptEIA; BD Biosciences). Intra- and interassay coefficients of variation were typically <4 and <6%, respectively. Transcripts for IL-8 and GAPDH were assessed with the Direct Protect kit (Ambion) as described previously (18).

**Measurement of NO and superoxide**

Intracellular formations of NO and superoxide were monitored simultaneously by flow cytometry after incorporation of 4,5-diaminofluorescein diacetate (DAF; 5 μM; BD Clontech) and dihydroethidium (5 μM; Molecular Probes) into PMN as previously described (24).

**Expression of NO synthase (NOS) isoforms**

Total RNA was isolated from 1 × 10⁷ PMN using TRIzol reagent, and cDNA was prepared with SuperScript reverse transcriptase (Invitrogen Life Technologies). The following primers were used for subsequent PCR analysis: human inducible NOS (iNOS): sense, 5'-TCCTCTGCGCACC TTCGATGAG-3'; antisense, 5'-CTTCTGGCAACAGCGGCAATTTG-3'; human neuronal NOS: sense, 5'-CTTCTGGCAACAGCGGCAATTTG-3'; antisense, 5'-CCGAGCCCCGAACACACGAAC-3'; human endothelial NOS (eNOS): sense, 5'-GGTGGCGAAGCGGCAATGGA-3'; antisense, 5'-CTCTGGCAACAGCGGCAATTTG-3'; and human inducible NOS (iNOS): sense, 5'-CTTCTGGCAACAGCGGCAATTTG-3'; antisense, 5'-CCGAGCCCCGAACACACGAAC-3'. cDNA was synthesized with SuperScript reverse transcriptase (Invitrogen Life Technologies), and PCR products were sequenced using BigDye Terminator v3.1 and analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems). Primers were used as follows: human inducible NOS (iNOS): sense, 5'-CTTCTGGCAACAGCGGCAATTTG-3'; antisense, 5'-CCGAGCCCCGAACACACGAAC-3'. cDNA was synthesized with SuperScript reverse transcriptase (Invitrogen Life Technologies), and PCR products were sequenced using BigDye Terminator v3.1 and analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems).

**Detection of peroxynitrite**

Intracellular ONOO⁻ formation was monitored as NO-dependent fluorescence of rhodamine, an oxidation product of dihydrorhodamine 123 (DHR 123) (26). The NOS blocker-inhibitable proportion of DHR 123 oxidation can be attributed to ONOO⁻, because ONOO⁻ readily oxidizes DHR 123, whereas NO does not (26). DHR 123 (20 μM; Molecular Probes) was added to some samples during the last 60 min of incubation in the presence or the absence of N⁵-nitro-l-arginine methyl ester (l-NAME; 1 mM), an inhibitor of NOSs, and fluorescence was analyzed with a flow cytometer (FACScan; BD Biosciences) (18). In additional experiments, PMN extracts (prepared as described in Ref. 17) were analyzed for the presence of nitrotyrosine, a fingerprint of ONOO⁻ (15), by an enzyme immunoassay (Cayman Chemicals), using nitrotyrosine as standard (24). The detection limit of the assay was 2 ng/ml. Intra- and interassay coefficients of variation were typically <6%.

**NF-κB and c-Fos**

Intraneuronal DNA-bound NF-κB/p65 and c-Fos were measured with a flow cytometric assay (19, 24) and were used as an estimate of transcription factor activity in the cell. In brief, leukocyte nuclei prepared with the Cyto-Select Plus DNA reagent kit (BD Biosciences) were stained with rabbit polyclonal anti-human NF-κB/p65, NF-κB/p50, or c-Fos Abs or with normal rabbit IgG (to assess nonspecific binding of IgG to nuclei) and then with FITC-conjugated anti-rabbit IgG Ab (all from Santa Cruz Biotechnology) and propidium iodide. Single PMN nuclei were gated using the doublet discrimination module, and fluorescence intensity was analyzed with a FACScan flow cytometer using CellQuest Pro software (BD Biosciences).

In additional experiments, PMN nuclear extracts were prepared with the TransFactor Extraction kit (BD Biosciences). Binding of NF-κB/p65 to the immobilized κB consensus sequence GGTTATTTCC was quantified by ELISA (Mercury TransFactor NF-κB p65 kit; BD Biosciences) using 15 μg of nuclear extracts. Binding is expressed as OD after correction for binding to the mutant sequence GGCTAGGTCC.

**Neutrophil viability**

PMN viability was assessed by flow cytometry immediately after staining with propidium iodide (0.5 μg/ml).

**Statistical analysis**

Results are expressed as the mean ± SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test), followed by Dunn’s multiple contrast hypothesis, tests to identify differences between various treatments. A value of p < 0.05 was considered significant.

**Results**

**TLR-9 expression in human neutrophils**

Intracellular flow cytometry on high purity (>99.9%) PMN confirmed the presence of TLR-9 (Fig. 1). In this assay, PMN were first permeabilized, because TLR-9 is predominantly expressed in intracellular vesicles (27), and stained with an R-PE-tagged anti-TLR-9 Ab.

**CpG-DNA induces IL-8, IL-6, and TNF-α secretion**

Whole blood samples or isolated PMN were challenged with CpG-DNA, and the concentrations of selected cytokines in plasma or culture medium were measured. To minimize the autocrine and paracrine stimulations that contribute to elevated cytokine levels (28), the duration of the study was limited to 4 h. In whole blood, CpG-DNA induced IL-8 release in a concentration-dependent fashion (Fig. 2A). CpG-DNA also stimulated, albeit to a considerably lesser extent, IL-6 and TNF-α production. CpG-DNA evoked potent IL-8 production by isolated PMN, but induced only minimal secretion of IL-8 and almost no detectable release of TNF-α (Fig. 2A). To address the potentially confounding influence of contaminating mononuclear cells, monocytes in particular, in standard PMN preparations, the experiments were repeated using...
high purity PMN (containing <0.4% HLA-DR-positive cells). High purity PMN released comparable amounts of IL-8, but markedly less IL-6 in response to CpG-DNA, as reflected by a marked increase in the IL-8 to IL-6 ratio (Fig. 2B).

Neither calf thymus nor met-CpG-DNA produced detectable increases in IL-8 and IL-6 production (Fig. 3). Stimulation of PMN with 0.02 ng/ml LPS (the maximum concentration equivalent to that detected in our DNA preparations) for 4 h failed to evoke significant cytokine release (data not shown).

Pretreatment of PMN with the endosomal acidification inhibitors, bafilomycin A (29) or chloroquine (30), almost completely inhibited CpG-DNA-induced IL-8 and IL-6 production (Fig. 3), indicating that endosomal acidification of CpG-DNA is a necessary step for initiating intracellular signaling for cytokine production. Inhibition of NO synthesis with L-NAME effectively suppressed the production of IL-8 without affecting IL-6 release (Fig. 3).

The inhibitory actions of bafilomycin A and L-NAME were concentration dependent (Fig. 3).

We next investigated whether CpG-DNA induction of IL-8 production and its inhibition by l-NAME occur at the level of transcription. The results shown in Fig. 4 confirmed those obtained for protein synthesis, with CpG-DNA inducing concentration-dependent increases in IL-8 mRNA, with a 1.8-fold maximum increase observed at 1.6 μg/ml. Although l-NAME alone had no detectable effect on IL-8 mRNA expression (data not shown), it attenuated ~77% of the CpG-DNA (1.6 μg/ml)-induced increase (Fig. 4B).

Induction of O$_2^-$, NO, and peroxynitrite formation in PMN

We used flow cytometry to monitor simultaneously intracellular formation of O$_2^-$ and NO in PMN 4 h after CpG-DNA administration. CpG-DNA induced concentration-dependent increases in dihydroethydine and DAF fluorescence, indicating enhanced formation of O$_2^-$ and NO, respectively (Fig. 5). These changes occurred in parallel with marked increases in l-NAME-inhibitable rhodamine fluorescence (Fig. 5), indicating ONOO$^-$-mediated oxidation of DHR123. l-NAME (1 mM) reduced 98% of NO formation detected by DAF fluorescence (data not shown). Addition of the NO donor spermine NONOate (0.5 mM) to l-NAME-treated PMN restored DHR123 oxidation (rhodamine fluorescence in relative fluorescence units: control, 13 ± 2; CpG-DNA, 44 ± 3; l-NAME and CpG-DNA, 19 ± 2; spermine NONOate, l-NAME, and CpG-DNA, 41 ± 3; n = 5; p > 0.1 compared with CpG-DNA). To provide additional evidence for ONOO$^-$ formation, we quantified the PMN nitrotyrosine content. PMN exposed to CpG-DNA contained significantly higher amounts of nitrotyrosine than unchallenged cells, and the increases coincided with increases in NO-dependent DHR123 oxidation (Fig. 5).

To identify the NOS isofrom(s) responsible for increased NO production, we performed RT-PCR on cDNA prepared from freshly isolated PMN and from PMN challenged with CpG-DNA for 2 or 4 h. These assays resulted in amplification of eNOS.
CpG-DNA induces the expression of eNOS mRNA, but not neuronal NOS or iNOS (Fig. 6). CpG-DNA did not appear to increase the expression of eNOS mRNA (Fig. 6).

**CpG-DNA induces nuclear accumulation of c-Fos and NF-κB in PMN**

CpG-DNA mobilized NF-κB/p65 and c-Fos to the nucleus (i.e., the increased fluorescence represents increased amounts of NF-κB or c-Fos bound to DNA), and this mobilization was attenuated by L-NAME (Fig. 7, A and B). Immunostaining was completely prevented by preincubation of the Abs with the appropriate blocking peptides (Fig. 7A). The effect of CpG-DNA on the nuclear translocation of NF-κB/p65 and c-Fos proteins was rapid; near-maximum changes were detectable within 10 min (Fig. 7, C and E). Nuclear accumulation of NF-κB/p65 peaked ∼30 min after addition of CpG-DNA, then staining with the Abs gradually decreased (Fig. 7B). The actions of CpG-DNA were concentration dependent (Fig. 7B). Similar changes were observed when staining PMN nuclei with NF-κB/p50 (data not shown). Cycloheximide (35 μM) did not affect the nuclear accumulation of NF-κB/p65 and c-Fos (data not shown). To confirm these observations, we prepared PMN nuclear extracts and assessed DNA binding by NF-κB/p65 with an ELISA. CpG-DNA evoked time- and concentration-dependent increases in DNA binding by NF-κB/p65 that coincided with the changes observed in the flow cytometry assay (Fig. 7D).

We next examined whether the increased nuclear accumulation of NF-κB correlates with the induction of IL-8 production. Preincubation of PMN with pyrrolidine dithiocarbamate (PDTC) or gliotoxin, inhibitors of NF-κB activation (31, 32), attenuated up to ∼75% of CpG-DNA-induced nuclear accumulation of NF-κB/p65 and IL-8 production in a concentration-dependent fashion (Fig. 8). None of these inhibitors significantly affected CpG-DNA-induced DHR 123 oxidation (data not shown), indicating that they did not scavenge peroxynitrite.

**CpG-DNA and neutrophil viability**

Because increased NO and ONOO− formation may be associated with changes in cell survival (15, 16), we studied the impact of CpG-DNA on PMN viability. Consistent with our previous study (7), after 4 h in vitro, CpG-DNA slightly increased the percentage of viable PMN from 88 ± 2 to 93 ± 2% (n = 5; p < 0.05), whereas thymus DNA or met-CpG-DNA was without effect (PMN viability, 89 ± 2 and 90 ± 2%, respectively; n = 5; both p > 0.1 vs untreated).

**Discussion**

The present study provides evidence that a novel mechanism by which CpG-DNA and nonmethylated CpG motifs, in particular, may affect PMN responses, and the inflammatory process is inducing ONOO− formation. This is associated with increased nuclear accumulation of NF-κB and subsequent induction of IL-8 gene expression and production. Because recent studies have increasingly pointed to the pivotal role of IL-8 in directing leukocyte traffic into inflamed areas, our observations bear directly on the mechanism for the proinflammatory actions of bacterial DNA.

**CpG-DNA induces IL-8 mRNA expression and its inhibition by L-NAME.** PMN were incubated for 20 min with L-NAME (1 mM) and challenged with CpG-DNA for 4 h at 37°C. A, Representative RNase protection assay using probes for IL-8 and GAPDH. B, Densitometric analysis of autoradiographs of the samples probed for IL-8 and GAPDH. The IL-8 results are expressed as a percentage of the control (unstimulated) value after normalization with the GAPDH values. The results represent the mean ± SEM of blots from four experiments with different blood donors. *p < 0.05 (vs untreated).

**CpG-DNA induces the formation of superoxide, NO, and ONOO− in PMN.** PMN were challenged with CpG-DNA at 37°C for 4 h. Intracellular formation of superoxide and NO was monitored simultaneously using dihydroethidine (5 μM) and DAF (5 μM), respectively, and is expressed as RFU. ONOO− formation was assessed as L-NAME (1 mM)-inhibitable oxidation of DHR 123 to rhodamine and is expressed as RFU. Values are the mean ± SEM (n = 5–8), *p < 0.05; **p < 0.01 (vs unstimulated).

**Effect of CpG-DNA on the expression of NOS isoforms.** Freshly isolated PMN (0 h) or PMN incubated without or with CpG-DNA for 2 and 4 h were harvested for RNA preparation. RT-PCR was performed with specific primers for human nNOS, iNOS, eNOS, and β-actin. The PCR products were resolved on a 1.2% agarose gel by electrophoresis and stained with ethidium bromide. RNA isolated and amplified from human chondrocytes stimulated with IL-1β for 4 h served as a positive control for iNOS. The results are representative of four independent experiments using PMN from different donors.
Among the cytokines measured from CpG-DNA-stimulated PMN, IL-8 is the most abundant. In comparison, CpG-DNA induced a modest secretion of IL-6 and a very low production of TNF-α. In whole blood, CpG-DNA evoked the release of IL-8 and, to a lesser degree, IL-6 and TNF-α, indicating that leukocytes other than PMN also respond to CpG-DNA to release cytokines. A controversy exists on the ability of PMN to produce IL-6. We found that high purity (≥99%) PMN released considerably less, although still detectable, amounts of IL-6 than standard PMN preparations. IL-8 release was only slightly affected by depletion of mononuclear cells, confirming the ability of PMN to produce IL-8. Furthermore, L-NAME suppression of IL-8, but not IL-6, release suggests the existence of different signaling mechanisms for cytokine production. Our results demonstrate that CpG-DNA is a potent activator of key responses of leukocytes in amplifying the inflammatory response in whole blood and in a culture milieu that is biologically relevant. Thus, our results enhance previous findings with intestinal epithelial cells (33) and PMN (5) and confirm the ability of CpG-DNA to regulate the production of IL-8 and other proinflammatory cytokines in human PMN. By contrast, human PMN were reported to synthesize IL-8 in response to a class A CpG oligonucleotide (GTTGCATCGATGCAGGGGGG) only if pretreated with GM-CSF (5). Although the biological actions of this oligonucleotide have not been fully characterized, short CpG oligonucleotides are generally less potent immunostimulators than E. coli DNA (2, 34).

Our results indicate that differences in methylation patterns would enable PMN to selectively recognize CpG-DNA. Methylation of cytosines in CpG dinucleotides in CpG-DNA completely abolished its actions, and calf thymus DNA did not reproduce the

**FIGURE 7.** CpG-DNA stimulates nuclear accumulation of NF-κB and c-Fos. PMN were incubated without or with CpG-DNA (1.6 μg/ml) for the indicated times or with various concentrations of CpG-DNA for 30 min in the absence or the presence of L-NAME (1 mM). PMN nuclei or nuclear extracts were prepared for immunostaining and NF-κB DNA binding assay, respectively, as described in Materials and Methods. A, Representative fluorescence histograms of singlet nuclei of PMN after staining for NF-κB/p65 (left panel) or c-Fos (right panel). Also shown is the staining of nuclei prepared from PMN incubated with vehicle as controls (curves labeled C) and staining with normal rabbit IgG, followed by FITC-labeled anti-rabbit IgG Ab (curves labeled IgG). As a negative control, Abs were preincubated for 2 h with the appropriate blocking peptide (curves labeled BP) before addition to nuclei. B, L-NAME inhibits nuclear accumulation of NF-κB/p65 and c-Fos. Time- and concentration-dependent actions of CpG-DNA on nuclear accumulation of NF-κB/p65 (C) or binding to DNA (D). DNA binding of NF-κB was detected by ELISA using an immobilized κB consensus sequence and is expressed as the OD. E, Effects of CpG-DNA on nuclear accumulation of c-Fos. The results are the mean ± SEM of four or five experiments with different blood donors. *, *p < 0.05; **, p < 0.01 (vs unstimulated).
FIGURE 8. PDTC and gliotoxin suppress CpG-DNA-induced nuclear accumulation of NF-κB/p65 and IL-8 release. PMN were incubated with PDTC (25 or 100 μM) or gliotoxin (150 or 600 nM), then challenged with CpG-DNA (1.6 μg/ml; A) for 30 min to assess nuclear accumulation of NF-κB/p65 by flow cytometry or for 4 h to measure IL-8 release by enzyme immunoassay (B). Values are the mean ± SEM (n = 5). **, p < 0.01 (vs untreated). ††, p < 0.01 (vs CpG-DNA).

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effects of CpG-DNA. The effects of CpG-DNA were not due to endotoxin contamination, because culture of PMN with 0.02 ng/ml LPS (the highest level of LPS detected in our DNA preparations) did not result in detectable effects. Endosomal acidification of bacterial DNA is a critical step to initiate intracellular signaling through binding to TLR-9 (30, 35). Human PMN do express TLR-9 mRNA (5, 6) and stain positively with anti-TLR-9 Abs (Refs. 5 and 7 and the present study). Bafilomycin A, which inhibits vacuolar H⁺-ATPase (29), and chloroquine, which accumulates in and increases the pH in endosomes (30), rendered PMN unresponsive to CpG-DNA, consistent with signaling through TLR-9.

It is unclear why CpG-DNA selectively promotes IL-8 synthesis in PMN, although it is known that PMN have a limited protein synthesis profile (36). One possibility is that CpG-DNA preferentially activates the intracellular signaling pathways required for IL-8 synthesis. Indeed, CpG-DNA-induced IL-8 gene and protein expressions in PMN were markedly inhibited by L-NAME, indicating an NO-dependent mechanism. Albeit NO formation is a prerequisite for the induction of IL-8 release, NO by itself evokes a very modest IL-8 release from human leukocytes (18). By contrast, ONOO⁻ formed in the reaction of NO with superoxide is a potent inducer of IL-8 expression, and it mediates LPS-, IL-1β-, and TNF-α-stimulated IL-8 gene expression (18–20). Our present results demonstrate that CpG-DNA also activates this pathway. Indeed, CpG-DNA evoked simultaneous formation of O₂⁻ and NO, as monitored by dihydroethidium and diaminofluorescein fluorescence, respectively. We detected dihydroethidium fluorescence in unstimulated PMN. However, this fluorescence was not due to O₂⁻, because dihydroethidium undergoes significant oxidation in resting leukocytes, possibly through the uncoupling of mitochondrial oxidative phosphorylation (37). Resting PMN do not release detectable amounts of superoxide, as measured by the ferricytochrome c reduction assay (24). Our RT-PCR analysis points toward eNOS as the source of CpG-DNA-stimulated NO production, because only eNOS-specific products were amplified in unchallenged and CpG-DNA-treated PMN. Augmented NO formation was probably due to enhanced eNOS activity, because CpG-DNA did not induce detectable increases in eNOS mRNA in our RT-PCR assay. These observations are consistent with previous findings (38), although others failed to detect eNOS mRNA in human PMN (39). The reasons for this apparent discrepancy are not known, but it might be attributed to various amounts of contaminating cells or may reflect differences in the assays used to detect eNOS. We could not detect iNOS mRNA in PMN challenged with CpG-DNA for up to 4 h. Although iNOS-positive PMN have been shown in tissue samples and exudates (25), at least 16-h exposure of human PMN to LPS or cytokines in vitro is required for iNOS expression (40). Whether CpG-DNA is capable of inducing iNOS expression in PMN after prolonged incubation periods remains to be investigated.

Parallel with increases in O₂⁻ and NO production, CpG-DNA also enhanced ONOO⁻ formation. We monitored intracellular ONOO⁻ formation as NO-dependent oxidation of DHR 123 to rhodamine and measured nitration of protein tyrosine residues; we obtained consistent results with these two assays. A significant portion of rhodamine fluorescence in CpG-DNA-stimulated leukocytes can be attributed to ONOO⁻, because it depends on NO-related species for it can be inhibited by L-NAME, whereas NO per se does not oxidize DHR 123 (26). Furthermore, the NO donor, spermine NONOate, restored DHR 123 oxidation in L-NAME-treated PMN, indicating that the effect of L-NAME in the DHR 123 assay was not due to inhibition of assembly of NADPH oxidase. The nitration reactions have been attributed to the interaction of NO with O₂⁻ at a rate constant of 6.7 × 10⁹ M⁻¹s⁻¹ (pH 7.4) to form ONOO⁻ (15). There is evidence that the nitration yield may be increased via formation of the nitrosoperoxycarbonian anion from physiologic concentrations of CO₂ and bicarbonate, thereby enhancing the reactivity of ONOO⁻ (41). Although nitrotyrosine formation is considered a specific fingerprint of ONOO⁻ (15), myeloperoxidase-dependent tyrosine nitration has also been reported (42), indicating that nitrotyrosine may, instead, serve as an indicator of reactive nitrogen species. Intriguingly, in human PMN, ONOO⁻ appears to be the predominant mechanism for tyrosine nitration (43). Although a controversy exists about whether ONOO⁻ is capable of tyrosine nitration at physiologic pH (reviewed in Ref. 44), our previous results with exogenous ONOO⁻ clearly indicate the existence of such a reaction in human PMN (24). These reactions represent important pathways by which CpG-DNA may modulate the oxidative chemistry of NO, O₂⁻, and ONOO⁻.

The IL-8 gene contains cis-regulatory elements for NF-κB, AP-1, and NF-IL-6 (45). Of these transcription factors, NF-κB plays a key role in the induced expression of IL-8 (46). Accordingly, we found that CpG-DNA stimulates nuclear accumulation of NF-κB and DNA binding by NF-κB/p65 and induces IL-8 gene transcription. In human PMN, the dominant form of NF-κB induced by CpG-DNA appears to be a p50/p65 heterodimer, consistent with findings in murine macrophages, but not B cells, where CpG-DNA activates the p50/c-Rel heterodimer (2). The low amounts of NF-κB/p65 DNA-binding activity detected in the nuclei of unstimulated PMN (Refs. 24 and 47 and the present study)
probably contribute to constitutive expression of transcripts encoding κB-dependent genes. PDTC or giotoxin effectively blocked nuclear accumulation of NF-κBp65 and inhibited ~75% of IL-8 release. We also observed CpG-DNA induction of nuclear accumulation of c-Fos, a finding consistent with the role of AP-1 in the enhancement of IL-8 gene expression (45). However, human PMN express c-Jun at a very low level, and many PMN agonists failed to evoke c-Jun expression (48). It remains to be determined whether CpG-DNA could induce c-Jun expression, or whether CpG-DNA-induced c-Fos and low levels of c-Jun could form AP-1 in quantities sufficient to regulate IL-8 gene transcription. Consistent with the suppression of ONOO⁻ formation and IL-8 mRNA and protein expression, t-NAME effectively inhibited the nuclear accumulation of NF-κB and c-Fos. The mechanism by which ONOO⁻ increases the nuclear binding of NF-κB is not fully understood. The positive correlation of phosphorylation and nitration of tyrosine residues in IkB-α with NF-κB translocation (20) would suggest that CpG-DNA, through increasing ONOO⁻ formation, might have induced nitration of IkB-α. Recently, CpG-DNA was reported to evoke epithelial IL-8 production independently of the NF-κB pathway (33). Thus, the mechanism of CpG-DNA-induced IL-8 expression may also be cell specific.

The present findings may have relevance to chronic inflammatory diseases. If CpG-DNA is proinflammatory, as indicated by the present and previous results (8), then simply killing bacteria may be insufficient to fully suppress the inflammatory response. Indeed, DNA from dead bacteria would be still present even when bacteria cannot be detected by standard microbiological techniques and could trigger PMN responses. This possibility is supported by the detection of CpG-DNA under pathological conditions that are also associated with nitrosative stress and PMN activation and/or accumulation (8–12). Thus, strategies would have to be developed to degrade bacterial DNA to block its receptors and/or stimulatory effects.

In summary, the current study provides evidence for CpG-DNA activation of ONOO⁻ signaling in human PMN, which underlies IL-8 gene expression and IL-8 production. Increased ONOO⁻ formation is also consistent with increased cytotoxicity. These are important additions to the bioactivity profile of CpG-DNA. Combined with previous observations of CpG-DNA up-regulation of β2 integrin expression on PMN (6) and suppression of PMN apoptosis (7), bacterial DNA and unmethylated CpG motifs, in particular, emerge as potent regulators of PMN functions and thereby may contribute to aggravation and maintenance of the inflammatory response.

Disclosures
The authors have no financial conflict of interest.

References