Bacterial DNA activates human neutrophils by a CpG-independent pathway

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Bacterial DNA stimulates macrophages, monocytes, B lymphocytes, NK cells, and dendritic cells in a CpG-dependent manner. In this work we demonstrate that bacterial DNA, but not mammalian DNA, induces human neutrophil activation as assessed by L-selectin shedding, CD11b upregulation, and stimulation of cellular shape change, IL-8 secretion, and cell migration. Induction of these responses is not dependent on the presence of unmethylated CpG motifs, as neutrophil stimulatory properties were neither modified by CpG-methylation of bacterial DNA nor reproduced by oligonucleotides bearing CpG motifs. We found that human neutrophils express Toll-like receptor (TLR) 9 mRNA. However, as expected for a CpG-independent mechanism, activation does not involve a TLR9-dependent signaling pathway; neutrophil stimulation was not prevented by immobilization of bacterial DNA or by wortmannin or chloroquine, two agents that inhibit TLR9 signaling. Of note, both single-stranded and double-stranded DNA were able to induce activation, suggesting that neutrophils might be activated by bacterial DNA at inflammatory foci even in the absence of conditions required to induce DNA denaturation. Our findings provide the first evidence that neutrophils might be alerted to the presence of invading bacteria through recognition of its DNA via a novel mechanism not involving CpG motifs.

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1 Introduction

The innate immune cells of vertebrates detect the presence of infection through the recognition of pathogen-associated molecular patterns (PAMP) [1]. Bacterial DNA behaves as a PAMP, exerting stimulatory effects on human and murine immune cells, both *in vitro* and *in vivo* [2, 3]. These effects include triggering of B cell proliferation and immunoglobulin secretion [4–8], activation of monocytes, macrophages, and dendritic cells to secrete Th1-like cytokines and induce Th1-like responses [8–12], and activation of NK cells [13]. These properties were reported to be dependent on the presence of unmethylated CpG dinucleotides (CpG motifs) in certain base contexts [2, 3, 5–13]. CpG motifs are at least 20-fold more common in bacterial DNA than in vertebrate DNA; in eukaryotic DNA the dinucleotide CpG is suppressed and mostly methylated [2, 14]. It has been proposed that the immune system of vertebrates recognizes the molecular pattern characteristic of bacterial DNA as a "danger signal" and activates appropriate immune responses to protect the body against the potentially harmful effects of microorganisms [15]. Indeed, it was demonstrated that CpG DNA is taken up by cells and interacts with TLR9 in endosomal vesicles, triggering a signaling cascade that results in cellular activation [2].

Given the essential role of neutrophils in host defense against bacterial infections, it is surprising that to date, the effect of bacterial DNA on neutrophil physiology has not been addressed. Our results demonstrate for the first time that bacterial DNA induces activation of human neutrophils that is not related to the presence of unmethylated CpG motifs and does not involve a TLR9-dependent signaling pathway.

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Abbreviations: CpG motif: Unmethylated CpG dinucleotide **TLR:** Toll-like receptor **ODN:** Oligodeoxynucleotide **CpG ODN:** ODN containing CpG motif **S-ODN:** Phosphorothioate ODN **P-ODN:** Phosphodiester ODN **Meth-*Ecoli* DNA:** CpG-methylated *E. coli* DNA **ds:** Double-stranded **ss:** Single-stranded

2 Results

2.1 Bacterial DNA but not mammalian DNA induces neutrophil activation

To determine whether bacterial DNA is able to induce human neutrophil activation, we first evaluated its capacity to induce shedding of the adhesion molecule L-selectin (CD62L), which occurs rapidly upon leukocyte activation [16, 17]. Neutrophils cultured with DNA from both gram-negative bacteria (*Escherichia coli* or *Acynetobacter baumannii*) and gram-positive bacteria (*Clostridium perfringens* or *Mycrococcus lysodeikticus*) experienced a significant reduction in L-selectin expression (Fig. 1A) that was, with the exception of *C. perfringens* DNA, comparable in magnitude to that observed with FMLP. In contrast, mammalian DNA obtained from human placenta and calf thymus did not modify L-selectin expression (Fig. 1A). Responses induced by bacterial DNA could not be attributed to the presence of contaminating LPS, as *E. coli* LPS at concentrations higher than those usually present in DNA preparations [6, 7, 13] did not modify L-selectin expression (Fig. 1A). These results are in agreement with previous reports demonstrating that under serum-free conditions (those employed throughout our studies), LPS fails to activate neutrophils [18, 19]. Furthermore, *E. coli* DNA digested with DNase I for 2 h at 37°C did not stimulate neutrophil activation (not shown). All bacterial DNA also induced significant shape change (Fig. 1B). In contrast, mammalian DNA induced only negligible modifications in forward light scatter, while *E. coli* LPS did not stimulate neutrophil shape change (Fig. 1B). Similarly, bacterial DNA, but not calf thymus DNA, induced production of IL-8 significantly higher than constitutive release (Fig. 1C).

2.2 Neutrophil migration induced by bacterial DNA

Additional experiments were conducted to evaluate whether bacterial DNA was also able to induce neutrophil migration. All bacterial DNA induced neutrophil migration (Fig. 2A), while mammalian DNA was unable to support neutrophil locomotion. Again, *E. coli* LPS failed to exert any stimulatory effect. To determine whether neutrophil movement in response to bacterial DNA was directed (chemotactic) or random (chemokinetic) in

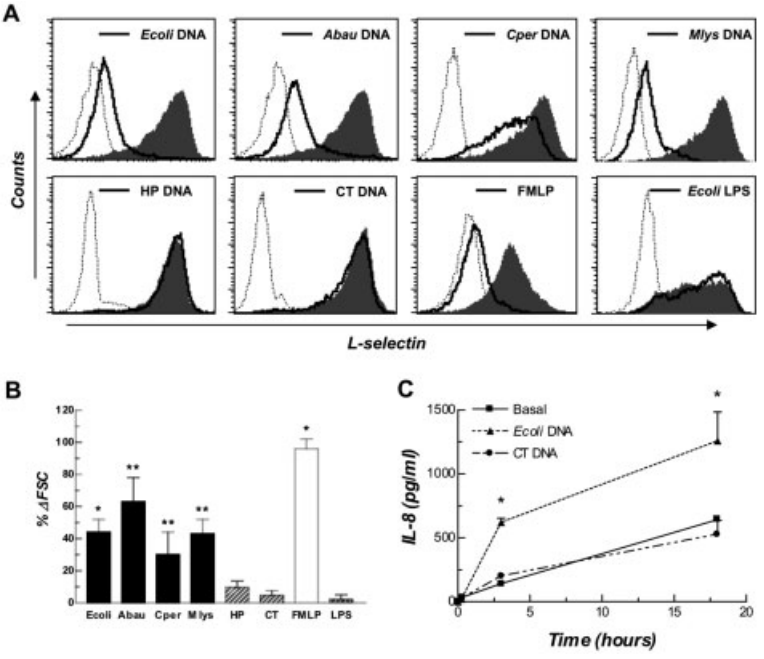

Fig. 1. Bacterial DNA induces L-selectin shedding, shape change, and IL-8 production in human neutrophils. Neutrophils were incubated in the presence or absence of 100 μg/ml DNA from *E. coli*, *A. baumannii* (*Abau*), *C. perfringens* (*Cper*), *M. lysodeikticus* (*Mlys*), human placenta (HP) or calf thymus (CT), FMLP (10^{-7} M), or *E. coli* LPS (100 ng/ml) at 37°C, and L-selectin expression (A), shape change (B), and IL-8 production (C) were assessed after different periods of time. (A) Histograms are representative of seven experiments (dotted line, isotype control; solid line, stimulated neutrophils; gray shaded histograms, unstimulated neutrophils). (B) Shape change is expressed as the percent increase in forward scatter (FSC) compared to unstimulated cells. (C) IL-8 release (pg/ml) per 10^6 neutrophils is shown as the mean ± SEM of seven to ten experiments (*$p<0.01$ and **$p<0.05$ compared to unstimulated cells).

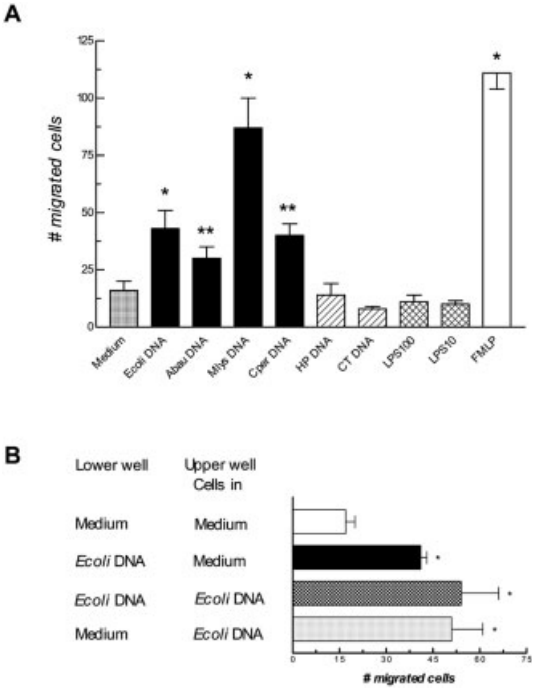

Fig. 2. Neutrophil migration and chemokinesis induced by bacterial DNA. (A) Migration of neutrophils in response to the indicated DNA (100 μg/ml), *E. coli* LPS (10 and 100 ng/ml), or FMLP (10^{-7} M) was assessed. (B) Checkerboard assay was assessed using *E.coli* DNA (100 μg/ml) as described [58]. Results represent the mean ± SEM of eight to ten donors evaluated in duplicate (*$p<0.01$ and **$p<0.05$ compared to control values).

nature, checkerboard analysis was performed. Locomotion of neutrophils in response to *E. coli* DNA placed in the lower well of the chamber (positive gradient) was compared with the migration of neutrophils suspended in *E. coli* DNA and exposed to either *E. coli* DNA (gradient excluded) or culture medium (inverted gradient) placed in the lower well. Neutrophil migration was not dependent on the presence of a positive gradient of *E.coli* DNA (Fig. 2B), indicating that cell movement must be attributed to a chemokinetic mechanism. Similar results were obtained with DNA from *A. baumannii*, *C. perfringens*, and *M. lysodeikticus* (not shown).

2.3 Effect of ODN containing CpG motifs on neutrophil function

Previous work has shown that the stimulatory properties of bacterial DNA on immune cells can be reproduced by synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG motifs (CpG-ODN) [2, 3, 5–13]. We analyzed whether CpG-ODN are able to induce neutrophil activation, employing five nuclease-resistant ODN with phosphorothioate backbones (S-ODN), two containing CpG motifs (2006 and DSP30), their controls with inverted CpG dinucleotides (2006 K and DSP30 K), and another ODN without CpG motif (H05). All the tested S-ODN stimulated neutrophil shape change and L-selectin shedding to a comparable degree (Fig. 3A, B). Moreover, all S-ODN with the exception of DSP30 and its control DSP30 K were able to support neutrophil locomotion (Fig. 3C). Taken together, these results suggest that S-ODN stimulate neutrophils in a CpG-independent manner. This observation is further strengthened by the finding that CpG methylation of S-ODN 2006 (SM-2006) did not affect its ability to induce neutrophil activation (Fig. 3A–C).

We then evaluated whether ODN with sequences identical to those previously employed, but with unmodified phosphodiester backbones (P-ODN) instead of phosphorothioate backbones, were able to induce neutrophil activation. Neither these P-ODN nor another CpG P-ODN (P2080) with recognized immuno-modulatory properties on human cells [20] induced neutrophil activation or supported neutrophil migration (Fig. 3D–F).

2.4 Effect of CpG methylation on the ability of DNA to induce neutrophil activation

Because CpG methylation of bacterial DNA abrogates its immunostimulatory effects exerted on different cell types, we next evaluated whether this procedure could also impair the ability of *E.coli* DNA to trigger neutrophil activation. *E.coli* DNA was methylated with *Sss*I methylase, and the extent of methylation was tested by analyzing resistance to *Hpa*II digestion (Fig. 4A). Methylated *E.coli* DNA induced neutrophil activation in a fashion similar to unmethylated *E.coli* DNA (Fig. 4B, C, D).

2.5 Bacterial DNA obtained from an *E. coli* Dam$^-$ Dcm$^-$ strain retains the ability to induce neutrophil activation

Prokaryotes contain DNA with N^6-methyladenine, 5-methylcytosine, and N^4-methylcytosine because of the presence of adenine- and cytosine-DNA methyltransferases [21]. In contrast, adenine-methyltransferases have not been described in higher eukaryotes, in which only cytosine-C5 methylation has been found. Moreover, cytosine methylation in mammals mainly occurs at CG sequences [21]. To determine whether content of adenine methylation or cytosine methylation other than CG methylation in bacterial DNA may account for its stimulatory capacity, we used bacterial DNA isolated from an *E.*

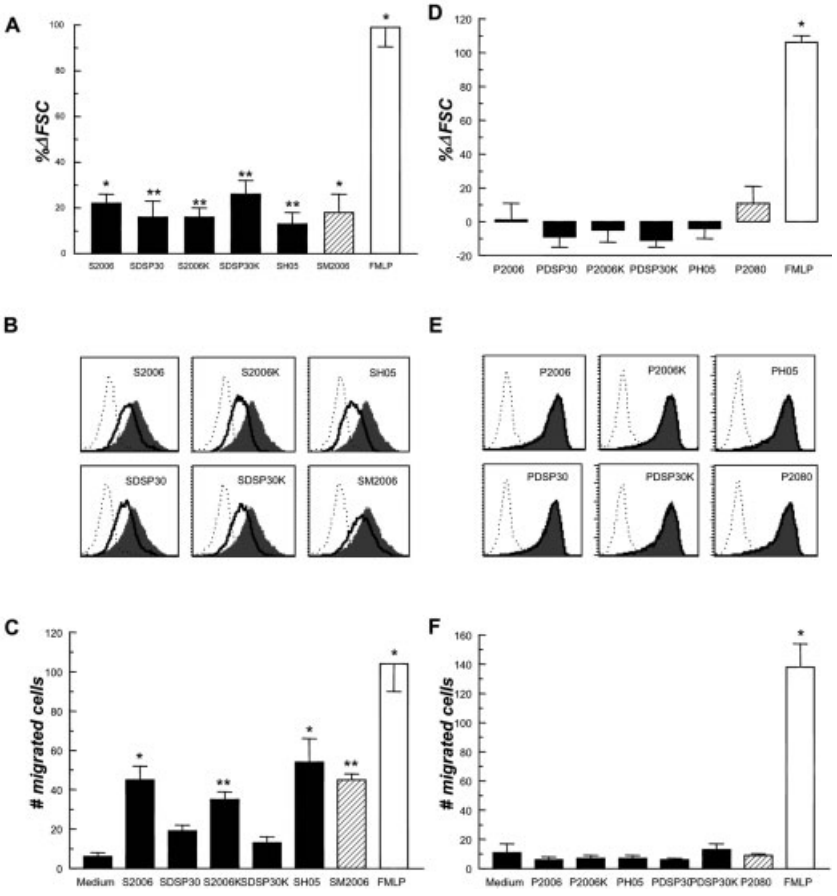

Fig. 3. CpG-independent stimulation of human neutrophils by S-ODN. Shape change (A and D), L-selectin down-regulation (B and E), and migration (C and F) of neutrophils stimulated by 2 μM S-ODN (A, B, and C), 30 μM P-ODN (D, E, and F), or 10^{-7} M FMLP were assessed as described in Sect. 4. Data represent the mean ± SEM of eight to nine experiments (*$p<0.01$ and **$p<0.05$ compared to control). Histograms show L-selectin expression by unstimulated neutrophils (gray shaded), isotype control (dotted line), or neutrophils stimulated with the indicated ODN (solid line) and are representative of eight experiments.

coli strain deficient in both adenine and cytosine methyltransferases (*E. coli* SCS110 strain Dam^- Dcm^-). DNA from the *E. coli* control and strain SCS110 were equally capable of stimulating neutrophil activation (Fig. 5A, B).

2.6 Neutrophil activation induced by bacterial DNA does not involve a TLR9-dependent signaling pathway

Previous studies established that the immunostimulatory activity of CpG ODN is eliminated by ODN immobilization on a solid support and enhanced by ODN lipofection [2, 22, 23]. These findings suggest that although ODN cellular uptake may involve binding to cell surface proteins, its stimulatory effects most likely requires binding to an intracellular receptor. Studies performed with TLR9 knockout mice and TLR9-transfected fibroblasts strongly suggest that TLR9 is the intracellular receptor involved in CpG-induced signaling [24–28]. Recent studies have also indicated that immune stimulation by CpG DNA is wortmannin sensitive; wortmannin inhibits the uptake and colocalization of CpG DNA with TLR9 in endocytic vesicles, presumably by inhibiting members of the PI3-kinase family different from DNA-dependent protein kinase [24]. Moreover, CpG-induced signaling is also inhibited by chloroquine, indicating that endosomal acidification is required for CpG-mediated activation to proceed [25, 29].

Our findings support the activation of neutrophils by bacterial DNA via a CpG-independent pathway, suggesting that TLR9 might not be involved in the activation mechanism. To confirm this presumption, we first examined the expression of TLR9 mRNA in human neutrophils and then evaluated its involvement in neutrophil activa-

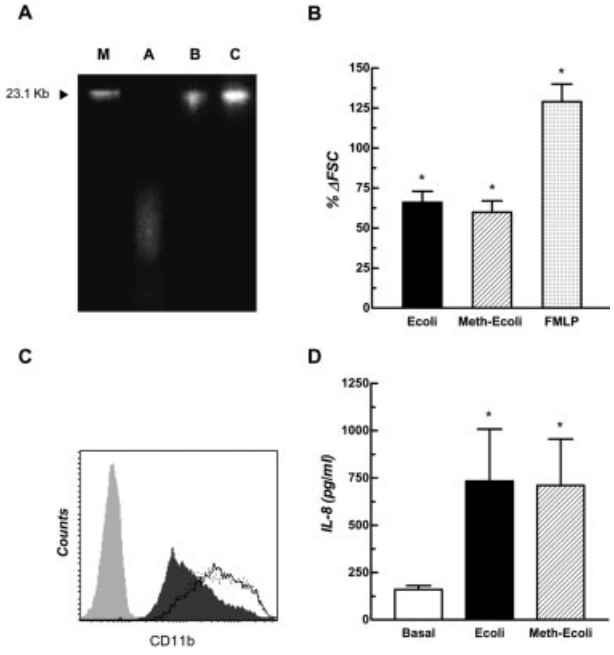

Fig. 4. CpG methylation of *E. coli* DNA does not affect its ability to induce neutrophil activation. (A) Resistance of *Sss*I-methylated *E. coli* DNA to *Hpa*II digestion. *E. coli* DNA (2 μg) pretreated with buffer only (lane A) or *Sss*I methylase (lane B) was digested with *Hpa*II as described and resolved on a 1% agarose gel together with a non-methylated, non-*Hpa*II-treated *E. coli* DNA preparation (lane C) and a molecular weight marker (lane M). (B) Shape change induced by CpG-methylated *E. coli* DNA (Meth-Ecoli; 100 μg/ml), unmethylated *E.coli* DNA (Ecoli; 100 μg/ml), or FMLP (10^{-7} M). Data represent the mean ± SEM of six experiments (*$p<0.01$ compared to unstimulated neutrophils). (C) Histograms show CD11b expression on unstimulated neutrophils (dark gray shaded), isotype control (light gray shaded), and neutrophils stimulated with 100 μg/ml CpG-methylated *E. coli* DNA (dotted line) or unmethylated *E. coli* DNA (thin black line) for 30 min and are representative of five experiments. (D) IL-8 release from neutrophils incubated 3 h in the absence (basal) or presence of 100 μg/ml CpG-methylated *E. coli* DNA or unmethylated *E. coli* DNA. Data represent the mean ± SEM of five experiments (*$p<0.05$ compared to basal values).

tion. In accordance with Zarember & Godowski [30], we clearly detected the presence of TLR9 transcripts in neutrophils (Fig. 6A). We observed that immobilized *E. coli* DNA and soluble DNA triggered neutrophil activation to similar degrees (Fig. 6B, C), and neither wortmannin (Fig. 6D, E) nor chloroquine (Fig. 6F, G) prevented neutrophil activation by *E. coli* DNA, supporting TLR9-independent neutrophil activation by bacterial DNA.

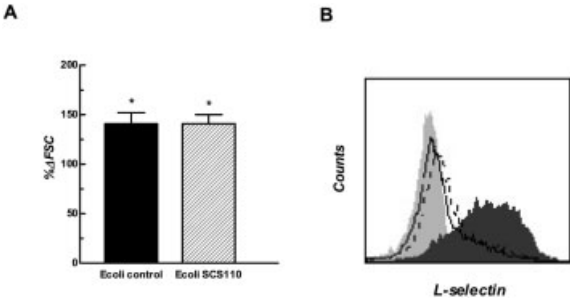

Fig. 5. DNA from *E. coli* Dam-Dcm- (SCS110) induces neutrophil activation. Shape change (A) and L-selectin shedding (B) induced by 100 μg/ml DNA from *E. coli* SCS110 or the *E. coli* control are shown. (A) Shape change (forward scatter) data represent the mean ± SEM of six experiments (*$p<0.01$ compared to values from unstimulated cells). (B) Histograms show L-selectin expression (isotype control, light gray shaded; untreated cells, dark gray shaded; control *E. coli* DNA-treated, plain line; SCS110 DNA-treated, dashed line) and are representative of five experiments.

2.7 Mac-1 is not involved in neutrophil activation induced by bacterial DNA

Benimetskaya et al. showed that Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein [31]. We analyzed whether Mac-1 plays a role in neutrophil activation mediated by bacterial DNA. Incubation with soluble fibrinogen, a Mac-1 ligand previously shown to inhibit ODN binding to Mac-1 [31], did not affect *E. coli* DNA-induced L-selectin shedding (Fig. 7A) or IL-8 release (fibrinogen pre-treated, 627±87 pg/ml *vs.* untreated cells, 707±160 pg/ml; n=3). Moreover, saturating amounts of an Fab fragment directed to the αM subunit (CD18) of Mac-1 did not inhibit neutrophil shape change (% ΔFSC 51±9 *vs.* 60±10 for neutrophils cultured in the absence or presence of anti-CD18 mAb, respectively; n=4) or IL-8 production (not shown) induced by *E. coli* DNA. Finally, as Mac-1 ligand recognition is dependent on the presence of extracellular Ca^{2+} and Mg^{2+}, experiments were also performed in medium and PBS without Ca^{2+} and Mg^{2+} in the presence or absence of EDTA. Neutrophil activation was also observed under Ca^{2+}- and Mg^{2+}- free conditions (Fig. 7B), confirming that Mac-1 plays no role in mediating neutrophil activation by bacterial DNA.

2.8 ds bacterial DNA stimulates neutrophil activation

Finally, we evaluated whether ds *E. coli* DNA is able to trigger neutrophil activation. As shown in Fig. 8A and B, ds *E. coli* DNA efficiently induced L-selectin shedding

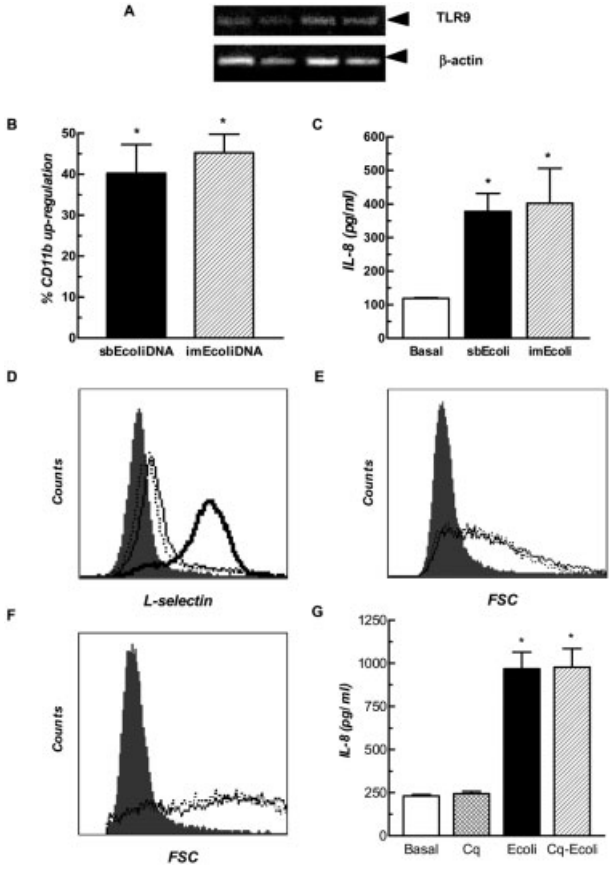

Fig. 6. TLR9 is not involved in neutrophil activation induced by bacterial DNA. (A) Expression of TLR9 mRNA in neutrophils from four donors. (B) CD11b upregulation in response to 100 μg/ml immobilized *E. coli* DNA (ImEcoliDNA) or soluble *E. coli* DNA (sbEcoliDNA). (C) IL-8 production by neutrophils incubated with 100 μg/ml immobilized *E. coli* DNA or soluble *E. coli* assessed after 3 h culture. Pretreatment of neutrophils with 1 μM wortmannin (D and E) or 5 μg/ml chloroquine (Cq, F and G) for 15 min at 37°C did not prevent L-selectin shedding (D), shape change (E, F), or release of IL-8 (G) induced by *E. coli* DNA (100 μg/ml); histograms are representatives of six experiments. (D) Histogram shows L-selection expression (isotype control, gray shaded; untreated cells, thick black line; *E. coli* DNA-treated cells, plain thin line; and wortmannin pre-treated cells incubated with *E. coli* DNA, dotted line). (E, F) Histograms show shape change (forward scatter) of untreated cells (gray shaded), *E. coli* DNA-treated cells (thin black line), and wortmannin (E) or chloroquine (F) pre-treated cells incubated with *E. coli* DNA (dotted line). (G) IL-8 release (pg/ml) as the mean ± SEM of four experiments (*$p<0.05$ compared to unstimulated cells).

and IL-8 secretion. In contrast, ds calf thymus DNA did not exert stimulatory effects (not shown). Similar results were observed when the ability of ds *E. coli* DNA to trigger the shape change response was evaluated (% ΔFSC

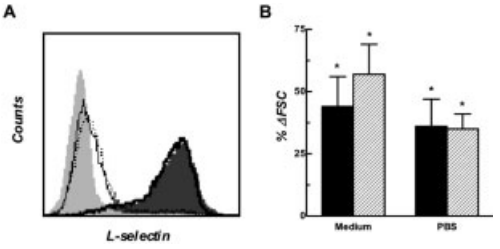

Fig. 7. Mac-1 plays no role in neutrophil activation induced by bacterial DNA. (A) Pretreatment of neutrophils with soluble fibrinogen (6 μM) did not inhibit L-selectin shedding induced by *E. coli* DNA (100 μg/ml). Histograms show isotype control (light gray shaded), untreated cells (dark gray shaded), fibrinogen-treated cells (plain thick line), cells stimulated with *E. coli* DNA (plain thin line), and cells treated with fibrinogen and stimulated with *E. coli* DNA (dotted line) and are representative of six experiments. (B) Shape change responses induced by *E. coli* DNA (100 μg/ml) assessed in complete medium or PBS in the absence (black bars) or presence (dashed bars) of 2 mM EDTA. Data represent the mean ± SEM of six experiments (*$p<0.05$ compared to unstimulated cells).

=47±8 *vs.* 52±9 for neutrophils cultured in the presence of 100 μg/ml ss *E. coli* DNA or ds *E. coli* DNA, respectively; $n = 4$).

3 Discussion

Previous studies demonstrated that bacterial DNA activates macrophages, monocytes, B lymphocytes, NK cells, and dendritic cells in a CpG-dependent manner [2–13, 32, 33]. This conclusion was supported by findings showing that: *(1)* selective CpG methylation abolishes the stimulatory activity of bacterial DNA, and *(2)* synthetic ODN bearing CpG motifs, but not ODN devoid

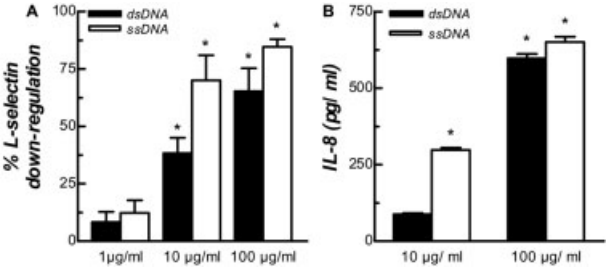

Fig. 8. Activation of human neutrophils by ds bacterial DNA. L-selectin shedding (A) and IL-8 production (B) induced by ds *E. coli* DNA and ss *E. coli* DNA. Data represent the mean ± SEM of five experiments (basal IL-8 release, 45±7 pg/ml, *$p<0.05$ compared to unstimulated cells).

of CpG motifs, reproduce the effects of bacterial DNA [2–13, 20, 33–35].

In the current study, we demonstrate for the first time that bacterial DNA, but not mammalian DNA, induces human neutrophil activation. The exposure of neutrophils to DNA from four different bacterial species stimulated L-selectin shedding, shape change, CD11b upregulation, and IL-8 secretion, responses considered characteristic of neutrophil activation. Moreover, bacterial DNA supported neutrophil migration by a mechanism of chemokinetic nature.

In contrast to the observations made in other leukocyte populations, stimulation of neutrophils by bacterial DNA appears to be mediated by a pathway unrelated to the presence of unmethylated CpG motifs. Different findings support this conclusion: *(1)* CpG methylation of *E. coli* DNA did not suppress its ability to trigger neutrophil activation; *(2)* P-ODN containing CpG motifs in their sequences did not stimulate neutrophil functions; *(3)* the stimulatory activity of nuclease-resistant S-ODN was not related to the presence of CpG motifs; and *(4)* CpG methylation of an active CpG S-ODN did not reduce its ability to induce neutrophil activation.

In agreement with Bylund et al. [36], we found that P-ODN were unable to stimulate neutrophils regardless of CpG content. In contrast, S-ODN used at concentrations 15-fold lower than those for P-ODN efficiently triggered neutrophil activation. The presence of unmethylated CpG motifs, however, appears unnecessary for stimulation of neutrophil functions, as a similar degree of activation was observed using S-ODN containing or lacking these motifs. Although the half-life of P-ODN in culture is estimated at 4 h, while that of S-ODN is 14 h [37, 38], it seems unlikely that differences in the stimulatory potential of the two classes of ODN could be attributed to a higher rate of P-ODN degradation; we assessed responses within a 30 min incubation period. Our observations that S-ODN stimulates neutrophil functions are also in agreement with Bylund et al. [36], who found that S-ODN induce neutrophil NADPH-oxidase activation, leading to the release of reactive oxygen species, degranulation, and L-selectin shedding in a CpG-independent but phosphorothioate-dependent manner. Similarly, Hyuck Baek et al. [39] reported that S-ODN induce macrophage chemotaxis in the absence of CpG motif, while McCluskie et al. [40] showed that non-CpG S-ODN exert adjuvant effects when administered at mucosal sites. These data suggest that the phosphorothioate backbone itself might have immunostimulatory properties.

Previous studies have indicated that bacterial DNA binds to an undefined, sequence-independent membrane receptor that mediates its endocytosis, allowing CpG DNA to interact with TLR9 in endosomal vesicles and stimulate robust innate immune responses [24, 26–28]. Our results suggest that bacterial DNA activates neutrophils via a TLR9-independent pathway. This proposal is supported not only by our finding that activation is independent of CpG motifs, but also by experiments performed with immobilized bacterial DNA which indicated that endocytosis of DNA is not required for neutrophil activation. It was further strengthened by the fact that neither wortmannin nor chloroquine, compounds with the recognized ability to prevent CpG DNA-triggered cellular activation in different cell systems [7, 24, 25, 29], were unable to inhibit neutrophil activation by bacterial DNA.

Our findings suggest that a cell surface receptor is involved in neutrophil activation by bacterial DNA. Several membrane proteins have been shown to play a role in binding and uptake of natural and synthetic DNA, but with the exception of Mac-1 and Scavenger Receptor A (SRA), putative oligonucleotide binding proteins have not been characterized either structurally or functionally [36, 41–46]. Benimetskaya et al. [31] demonstrated that Mac-1 can bind oligonucleotides, but its role as a protein involved in binding of high molecular weight DNA has not yet been addressed. Our observations suggest that Mac-1 is not involved in bacterial DNA recognition, since compounds shown to block oligonucleotide binding to human neutrophils [31] were unable to inhibit neutrophil activation triggered by bacterial DNA. This conclusion is supported by the finding that neutrophil stimulation by bacterial DNA also occurs under Ca^{2+}- and Mg^{2+}-free conditions. We performed a number of experiments designed to analyze involvement of SRA in neutrophil activation by bacterial DNA; agents such as polyinosinic acid, dextran sulfate, and fucoidan, which block DNA binding to SRA [43–46], exhibited intrinsic neutrophil stimulatory activity (not shown), perhaps reflecting their capacity to interact with molecules other than SRA, such as CD18 and L-selectin [47, 48]. More importantly, all the agents additively potentiated neutrophil activation induced by bacterial DNA, suggesting that SRA is not involved in this process (Trevani, unpublished results).

The physiological relevance of bacterial DNA as an innate immune stimulator has not yet been established [49]. Because unmethylated ss CpG ODN have well-recognized therapeutic properties, much information about the *in vivo* role of bacterial DNA has been inferred from studies employing ss ODN or ss (heat-denatured) bacterial DNA [4–7, 12, 20]. However, because ds DNA remains intact for long periods in the presence of activated neutrophils, even when localized within the endosomes [50], it is conceivable that bacterial DNA interacts

predominantly with neutrophils as dsDNA during the course of anti-bacterial acute inflammatory responses. While our results show that ds bacterial DNA efficiently triggers neutrophil activation, further studies are required to establish its effectiveness in other leukocyte populations.

The motifs that enable recognition of bacterial DNA by neutrophils remain to be elucidated. Our findings suggest that the distinct ability of bacterial DNA and mammalian DNA to induce neutrophil activation cannot be attributed to differences in methylation patterns. Possible causes for the dissimilar behavior of bacterial and mammalian DNA may be (1) differences in the content of pyrimidine clusters [51], (2) differences in coding sequences, and (3) the existence of inhibitory sequences in mammalian DNA capable of masking DNA stimulatory motifs, as reported for CpG-triggered immune responses [52, 53]. Although the mechanism enabling activation of neutrophils by bacterial DNA is unclear, the results presented here suggest the presence of a novel pathway through which neutrophils can be alerted to the presence of invading bacteria.

4 Materials and methods

4.1 Reagents

RPMI 1640 medium and PBS were purchased from HyClone Laboratories, Inc. (Logan, UT). Human albumin, FMLP, LPS (from *E. coli* 0111:B4), glutaraldehyde, chloroquine, wortmannin, *E.coli* DNA, *C. perfringens* DNA, *M. lysodeikticus* DNA, human placenta DNA, and calf thymus DNA were obtained from Sigma (St Louis, MO). Ficoll and Dextran T-500 came from Pharmacia Fine Chemicals (Uppsala, Sweden). Penicillin/Streptomycin were purchased from Life Technologies (Grand Island, NY). Anti-CD62L mAb was purchased from BD PharMingen (San Diego, CA), and anti-mouse IgG, anti-CD11b, and anti-CD66b were ordered from Immunotech (Marseille, France). Methylase *Sss*I and *Hpa*II were purchased from New England BioLabs, Inc. (Beverly, MA). The ELISA for IL-8 detection came from R&D (Minneapolis, MN).

4.2 Genomic DNA and ODN preparations

DNA was purified by extraction with phenol:chloroform:isoamyl alcohol and ethanol precipitation [54]. *A. baumannii* DNA was obtained from strains isolated from intensive care unit patients and prepared as described previously [55]. *E. coli* DNA with unmethylated adenines and cytosines, was obtained from SCS110 strain (*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lac-proAB)* [F' *traD36 proAB lacI^{q}ZDM15*]). All DNA was suspended in DNase-free, LPS-free distillated water. The final A_{260}/A_{280} ratio for all DNA preparations was greater than 1.8. Unless otherwise stated, DNA preparations were made ss before use by heating at 95°C for 10 min, followed by rapid cooling on ice. For some experiments, *E. coli* DNA was methylated with CpG methylase *Sss*I according to the manufacturer's instructions. The efficiency of methylation was determined by measuring resistance to cleavage with *Hpa*II, followed by electrophoresis on 1% agarose, as described [5, 6]. When indicated, DNA digestion was carried out in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 units DNaseI/μg DNA. The reactants were incubated 2 h at 37°C, and DNase was inactivated by heating 10 min at 95°C. In some experiments, *E. coli* DNA was immobilized in 98-well U-shaped microplates by overnight incubation in immobilization buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA) followed by five washes with saline to remove unbound DNA [56].

P-ODN and S-ODN were purchased from Sigma Genosys (The Woodlands, TX) and Invitrogen Corporation (Rockville, MD). The sequences of the CpG-containing oligonucleotides were: 2006, T<u>CG</u>T<u>CG</u>TTTTGT<u>CG</u>TTTTGT<u>CG</u>TT [8, 20; DSP-30, T<u>CG</u>T<u>CG</u>CTGTCTC<u>CG</u>CTTCTTCTTGCC [32]; and 2080, T<u>CG</u>T<u>CG</u>TTCCCCCCCCCCCC [20]. The sequences of the oligonucleotides with CpG inverted dinucleotides were: 2006 K, T*GC*T*GC*TTTTGT*GC*TTTTGT*GC*TT [8] and DSP-30 K, T*GC*T*GC*CTGTCTC*CC*CTTCTTCTTGCC [8]. The oligonucleotide without CpG motif was: H05, GAGACCCTGAACAGTTGATC [33]. The sequence corresponding to SM-2006 oligonucleotide with methyl-cytosines was: TZGTZGTTTTGTZGTTTTGTZGTT [20]. All ODN were used ss and HPLC-purified and were chosen on the basis of their abilities to induce strong activation of human immune cells [8, 20, 32, 33].

4.3 Cell preparation

Neutrophils were obtained and purified as previously described [57]. Cells were suspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 5 mg/ml human albumin and penicillin/streptomycin (complete medium).

4.4 Flow cytometric measurements

Assays were performed using a FACScan (Becton Dickinson, San Jose, CA). Expression of L-selectin was determined by indirect immunofluorescence using IgG anti-CD62L mAb and FITC-conjugated goat anti-mouse IgG. Neutrophil shape change was measured following fixation of DNA-treated neutrophils with an equal volume of 1% glutaraldehyde in PBS; shape change was determined by shift in the forward light scatter.

4.5 Chemotactic and chemokinetic assays

Cell migration was quantified using by 48-well microchemotaxis as previously described [58]. DNA preparations in com-

plete medium were deposited in the lower wells of the chambers. Neutrophils (2×10^6 cells/ml in complete medium) were placed in the upper wells of the chambers, and migration was scored by counting the number of cells that reached the lower surface of the filter after a 30 min incubation period. Five high-power (×1000) microscopic fields were scored per well. Chemokinesis was evaluated by checkerboard assay performed as previously described [58].

4.6 Detection of TLR9 mRNA expression in human neutrophils

Neutrophils were purified by conventional techniques and subjected to positive-selection using anti-CD66b and anti-mouse magnetic beads. Magnetic bead-associated neutrophils (5×10^6 cells) were lysed in TRIzol (Gibco BRL, Gaithersburg, MD), and total RNA was obtained by chloroform extraction and isopropanol precipitation. RNA (2 μg) was used for RT-PCR. Reverse transcription of mRNA was done using oligoDT primers, and 1/25 of the cDNA was used in one amplification by PCR. Message for β-actin was amplified using specific primers. The sequences of primer sets used to detect TLR9 were: sense, 5'-GGACCTCTGGTACTGCTTCCA-3'; and antisense, 5'-GTACACCCAGTCTGCCACTGC-3'.

4.7 Statistical analysis

Statistical significance was determined using the nonparametric Friedman test for multiple comparisons with Dunns post-test. Statistical significance was defined as $p<0.05$.

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