

# Characterization of bacterial DNA binding to human neutrophil surface

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Bacterial DNA activates neutrophils through a CpG- and TLR9-independent mechanism. Neutrophil activation does not require DNA internalization, suggesting that it results from the interaction of bacterial DNA with a neutrophil surface receptor. The aim of this study was to characterize the interaction of bacterial DNA with the neutrophil surface. Bacterial DNA binding showed saturation and was inhibited by unlabeled DNA but not by other polyanions like yeast tRNA and poly-A. Resembling the conditions under which bacterial DNA triggers neutrophil activation, binding was not modified in the presence or absence of calcium, magnesium or serum. Treatment of neutrophils with proteases not only dramatically reduced bacterial DNA binding but also inhibited neutrophil activation induced by bacterial DNA. Experiments performed with DNA samples of different lengths obtained after digestion of bacterial DNA with DNase showed that only DNA fragments greater than  $\approx 170$ –180 nucleotides competed bacterial DNA binding and retained the ability to trigger cell activation. Treatment of neutrophils with chemoattractants or conventional agonists significantly increased bacterial DNA binding. Moreover, neutrophils that underwent transmigration through human endothelial cell monolayers even in the absence of chemoattractants, exhibited higher binding levels of bacterial DNA. Together, our findings provide evidence that binding of bacterial DNA to neutrophils is a receptor-mediated process that conditions the ability of DNA to trigger cell activation. We speculate that neutrophil recognition of bacterial DNA might be modulated by the balance of agonists present at inflammatory foci. This effect might be relevant in bacterial infections with a biofilm etiology, in which extracellular DNA could function as a potent neutrophil agonist.

*Laboratory Investigation* (2008) 88, 926–937; doi:10.1038/labinvest.2008.59; published online 14 July 2008

**KEYWORDS:** bacterial DNA binding; biofilm; CpG; inflammation; neutrophils; TLR9

Neutrophils are crucial players in the first line of defense against invading microbial pathogens. They mediate the phagocytosis and destruction of these microorganisms through oxygen-dependent and -independent mechanisms. They also inform and shape immune responses and contribute to the repair of tissues as well as their breakdown.<sup>1,2</sup>

It is well established that unmethylated-CpG motifs in bacterial DNA trigger immune cell activation by interacting with Toll-like receptor 9 (TLR9).<sup>3–5</sup> However, we have previously demonstrated that neutrophils are activated by bacterial DNA through a CpG- and TLR9-independent mechanism.<sup>6,7</sup> Additional studies provided evidence for the existence of pathways unrelated to the presence of CpG motifs through which DNA may be able to trigger cell activation by TLR9-dependent or -independent mechanisms that involve endosomal translocation.<sup>8–12</sup> Moreover, *in vivo* studies conducted in transgenic mice lacking TLR9 revealed a CpG-independent mechanism that contributes to the toxic effects associated with systemic delivery of cationic lipid-plasmid DNA complexes in nonviral gene therapy protocols.<sup>13</sup> On the other hand, more recent studies defined a mechanism by which bacterial DNA can activate TLR9-independent and IRF-3-dependent innate immune responses when it gains access to the cytosol.<sup>14</sup> In contrast to the mentioned reports, our previous results indicated that neutrophil activation by bacterial DNA is mediated by a mechanism that does not require DNA internalization and which can be triggered by immobilized DNA, suggesting that

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Received 02 December 2007; revised 14 May 2008; accepted 18 May 2008

a surface molecule is involved in DNA recognition and neutrophil activation.<sup>6</sup> Of note, many different works have suggested that bacterial DNA and synthetic oligodeoxynucleotides (ODNs) are taken up by cells through a sequence-independent receptor-mediated mechanism, even though the identity of the receptor involved remains undefined.<sup>4,15</sup>

Several studies have demonstrated the presence of extracellular DNA in bacterial cultures.<sup>16–19</sup> Naturally transformable bacterial species release chromosomal DNA into culture media.<sup>19–23</sup> Extracellular bacterial DNA has also been proposed to play a role as a nutrient during starvation. In fact, previous studies have shown that *Escherichia coli* strains that consume DNA are more fit than those mutants unable to metabolize DNA.<sup>19</sup> On the other hand, several independent observations have adjudicated an important role for DNA in the formation and composition of biofilms, which are structured communities of cells enclosed in self-produced hydrated polymeric matrix adherent to an inert or living surface.<sup>24,25</sup> Thus, we hypothesized that extracellular DNA in bacterial microenvironments might be recognized by a neutrophil surface molecule able to trigger neutrophil activation. Accordingly, here we have characterized the interaction between bacterial DNA and neutrophils.

## MATERIALS AND METHODS

### Reagents

RPMI 1640 medium and PBS were purchased from HyClone Laboratories Inc. (Logan, UT, USA). Formyl-methionyl-leucyl-phenylalanine (FMLP), polyadenylic acid (poly-A), yeast transfer RNA (tRNA), phorbol myristate acetate (PMA) and EDTA were acquired from Sigma-Aldrich (St Louis, MO, USA). Ficoll and Dextran T-500 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Hypaque from Winthrop Products (Buenos Aires, Argentina); proteinase K (prot K) from Qiagen (GmbH, Germany); penicillin/streptomycin and trypsin from Life Technologies (Grand Island, NY, USA); human serum albumin (HSA) from Laboratorio de Hemoderivados (Córdoba, Argentina); agarose and weight markers were obtained from Promega (Madison, WI, USA). Interleukin-8 (IL-8) was purchased from Peprotech (Mexico). DNA from *E. coli* was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). DNA was purified by extraction with phenol:chloroform:isoamyl alcohol and ethanol precipitation and was further passed through a polymyxin-agarose column (Sigma-Aldrich) to eliminate endotoxins. Plasmid DNA pBluescript II-KS+ (pBS; Stratagen, La Jolla, CA, USA) was purified with a commercial kit (Qiagen) and was further passed through a polymyxin-agarose column to eliminate endotoxins. Human placenta DNA was from Sigma-Aldrich. Phosphodiester ODNs (P-ODNs) or phosphorothioate ODNs (S-ODNs) were purchased from Sigma Genosys (The Woodlands, TX, USA) and Invitrogen Corporation (Rockville, MD, USA). The sequence of the CpG-containing ODN 2006

was TCGTCGTTTTGTCGTTTTGTCGTT. The sequence of the ODN with CpG inverted dinucleotides 2006K was TGCTGCTTTTGTGCTTTTGTGCTT. All materials employed were sterile and endotoxin free.

### Neutrophil Isolation

Blood samples were obtained from healthy donors by venipuncture of the forearm vein. Neutrophils were isolated from heparinized human blood by Ficoll-Hypaque gradient centrifugation and dextran sedimentation as previously described.<sup>6</sup> Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (>96% of neutrophils on May-Grunwald-Giemsa-stained cytopreps) were resuspended in RPMI 1640 supplemented with 5 mg/ml endotoxin-free human albumin, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium) or in PBS as indicated. Human albumin was employed to protein supplement medium instead of fetal calf serum in order to avoid possible effects produced by contaminating LPS of the DNA samples, because as we and others have demonstrated, under these conditions, LPS fails to activate neutrophils.<sup>6,26,27</sup>

### Endothelial Cell Culture and Transendothelial Migration Assay

Human umbilical vein endothelial cells (HUVEC) were obtained according to the method of Jaffe *et al.*<sup>28</sup> Cells were seeded on 1% gelatin-coated 25-cm<sup>2</sup> tissue culture flasks and identified by morphology and von Willebrand factor antibody (Immunotech, Marseille, France) binding. Cells were grown in RPMI 1640 medium supplemented with fetal bovine serum (FBS; 10%; Gibco, Rockville, MD, USA), heparin (100 µg/ml), endothelial cell growth factor supplement (50 µg/ml), sodium pyruvate (2 mM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO<sub>2</sub> incubator. Having reached confluence, cells were detached by mild treatment with trypsin/EDTA (0.05% and 0.1 mM, respectively), washed with 10% FBS-supplemented PBS (Ca<sup>2+</sup>- and Mg<sup>2+</sup> free; PBS/FBS) and seeded over filters bearing 3-µm pore in Transwell culture plate inserts (Costar, Cambridge, MA, USA), pretreated with 0.01% gelatin 24 h before seeding and 60 µg/ml fibronectin 2 h before being used as previously described.<sup>29</sup> After 5 days of culture, HUVEC monolayers were activated with 10 ng/ml TNF-α for 24 h at 37°C. Then, neutrophils (5 × 10<sup>5</sup> in 100 µl) were seeded onto the upper chamber of the Transwell and allowed to migrate for 3 h at 37°C. Neutrophils that reached the lower compartment were collected and evaluated for DNA binding.

### *E. coli* DNA Biotinylation

*E. coli* DNA and pBS were biotinylated with Photoprobe (long arm) biotin (Vector Laboratories, Burlingame, CA, USA) by thermal coupling or photocoupling according to manufacturer's instructions. A 4:1 DNA:biotin ratio (w:w) was employed.

### Cell Surface DNA Binding

Neutrophils ( $5 \times 10^6$  per ml) suspended in PBS with 5 mg/ml HSA (assay buffer) were incubated with the indicated concentrations of biotinylated-*E. coli* single stranded DNA (biot-DNA) at 4°C for 30 min. Nonspecific binding was determined by preincubating cells with  $10 \times E. coli$  single stranded DNA (ssDNA). After a 30-min incubation period, cells were washed with cold PBS and incubated for 10 min at 4°C in the dark with avidin DCS-FITC (10 µg/ml; Vector Laboratories) in assay buffer. After washing with cold PBS, cell surface DNA binding was analyzed by FACScan and confocal laser scanning microscopy (CLSM).

### Confocal Laser Scanning Microscopy

A confocal laser scanning microscope (Carl Zeiss LSM510-Axiovert 100M) was used to visualize the interaction between neutrophils and bacterial DNA. Neutrophils and biot-DNA were incubated and revealed with avidin-FITC as described above. The DNA binding to the cell surface was observed in chambered coverglass slides containing a borosilicate glass base of 170 µm thickness (Lab-Tek Nunc no. 155411). Confocal images were reconstructed using the Zeiss LSM Image Browser Version 3.2.0. The images were acquired by sequentially scanning with settings optimal for FITC (488 nm excitation with argon laser line and detection of emitted light between 505 and 530 nm; pseudocoloured green). A C-Apochromat  $\times 63/1.2$  water immersion objective was used.

### *E. coli* DNA Digestion

*E. coli* DNA was partially digested with 2 U of DNase RQ1 (Promega) per mg of DNA by incubation at 37°C for 0, 2, 5, 20, 80 and 1000 (ON) min. Then, DNase was inactivated by heating at 95°C for 10 min and the reaction products were dialyzed against NaCl 0.15 M, EDTA 1 mM at room temperature and then quantified by determining absorbance at 260 nm. Fragments were visualized on 0.8% denaturing agarose gel electrophoresis containing 50 mM NaOH and on 5% denaturing polyacrylamide gel containing urea 7 M in TAE buffer. The size of the DNA fragments obtained was estimated by comparison with a set of DNA size markers in the 0.8% denaturing agarose gel (for fragments obtained after 0, 2 or 5 min of digestion) or in 5% polyacrylamide gel (for fragments obtained after 20 min of degradation) by analyzing the line profile plots of each lane (ImageJ, NIH). The size ranges of fragments obtained in each digestion were calculated by excluding 10% of the sample mass at the edges of each line profile plot.

### CpG Methylation of *E. coli* DNA

For some experiments, *E. coli* DNA was methylated with CpG methylase SssI according to the manufacturer's instructions (New England BioLabs Inc., Beverly, MA, USA). Briefly, DNA was treated with 1 U CpG methylase SssI per µg DNA for 4 h at 37°C in NE Buffer 2 supplemented with 160 µM S-adenosylmethionine, and replenishment of S-adenosylmethionine

after 2 h. Methylation status was verified by digestion with *HpaII* and electrophoresis on 1% agarose gels, as described.<sup>30,31</sup>

### Measurement of IL-8 Production

Neutrophils suspended in complete medium ( $5 \times 10^6$  per ml) were seeded into round-bottom 96-well plates and were stimulated with the indicated concentrations of *E. coli* DNA, digested DNA, ds pBS or medium (control), and cultured for 3 h at 37°C. Culture supernatants were collected and IL-8 concentration was measured by ELISA (R&D, Minneapolis, MN, USA). IL-8 released to culture supernatants was neo-synthesized and did not represent a cytokine released from a preformed pool. It was suggested by the fact that stimulation of neutrophils with *E. coli* DNA in the presence of Actinomycin D abrogated IL-8 release to culture supernatants, indicating that it required *de novo* mRNA synthesis (data not shown).

### Neutrophil Treatment with Proteolytic Enzymes

Neutrophils ( $5 \times 10^6$  per ml) suspended in PBS were treated with 100 µg/ml trypsin or prot K for 30 min at room temperature. After three washes with PBS supplemented with 10 mg/ml HSA, *E. coli* DNA binding or IL-8 production in response to DNA was determined as previously described. The degree of cell surface proteolysis was monitored by evaluating the expression of CD16, a marker susceptible to the action of proteases, by immunostaining and flow cytometry.

### Statistical Analysis

Statistical significance was determined using the nonparametric Wilcoxon or Friedman test for multiple comparisons with Dunn's post-test. Statistical significance was defined as  $P < 0.05$ .

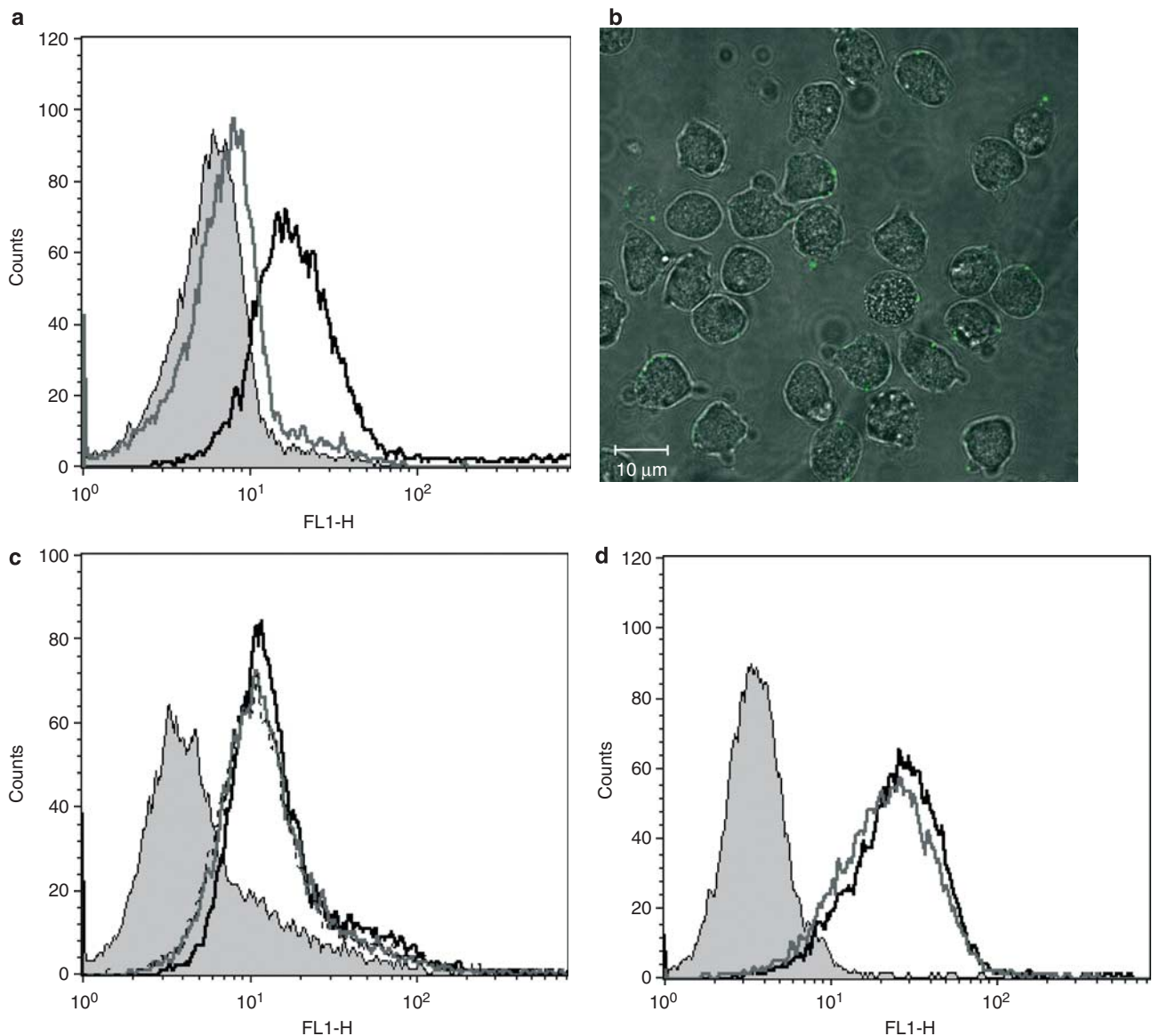
## RESULTS

### DNA Binding to Human Neutrophil Surface

We have previously determined that pretreatment of neutrophils with chloroquine and wortmannin, compounds that inhibit endosome acidification/maturation, does not affect the ability of bacterial DNA to induce neutrophil activation. In agreement with these findings, we also found that bacterial DNA immobilization does not modify its neutrophil stimulating capacity, suggesting that a cell surface molecule might be involved in the activation process.<sup>6</sup> Thus, to further analyze the mechanism involved in neutrophil activation by bacterial DNA, we first evaluated the ability of bacterial DNA to interact with neutrophil surfaces by determining the binding of biot-DNA. Preliminary experiments indicated that biot-DNA effectively triggered IL-8 production by human neutrophils, suggesting that biotinylation did not impair DNA ability to induce neutrophil activation (data not shown). Thus, cells were incubated for 30 min at 4°C with 60 µg/ml biot-DNA, a concentration that has been previously

shown to clearly induce neutrophil activation.<sup>6</sup> After the addition of FITC-avidin, binding was revealed by flow cytometry (Figure 1a) or laser confocal microscopy analysis (Figure 1b). Results showed that neutrophils effectively bind bacterial DNA. As these assays were performed at 4°C, the detected fluorescence represented biot-DNA binding to neutrophil surfaces. This fact was confirmed by the ability of trypan blue to completely quench the fluorescence emitted by neutrophils, which had bound biot-DNA (Figure 1a). Binding of DNA was independent of the presence of Ca<sup>2+</sup> and

Mg<sup>2+</sup> because no differences were detected when binding was achieved in PBS supplemented with both cations or in PBS plus EDTA 1.5 mM (Figure 1c). Furthermore, binding was not modified when it was evaluated in PBS supplemented with FBS or with HSA (Figure 1d). Of note, our previous results indicated that bacterial DNA was equally efficient to activate neutrophils suspended in culture medium, PBS supplemented with Ca<sup>2+</sup> and Mg<sup>2+</sup>, PBS with EDTA and in the presence or absence of FBS or HSA, suggesting that conditions under which bacterial DNA binding takes



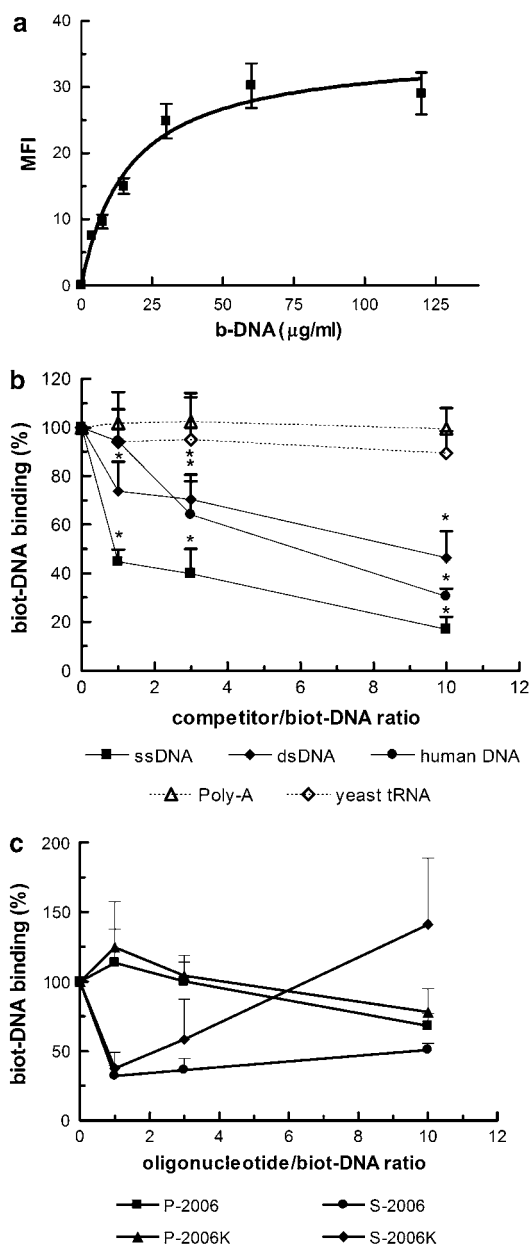
**Figure 1** Binding of bacterial DNA to human neutrophils. (a) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence (black line) or absence (shaded histogram) of single stranded biotinylated-*E. coli* DNA (biot-DNA;  $60 \mu\text{g/ml}$ ) for 30 min at 4°C. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Gray line represents the fluorescence after the addition of trypan blue. (b) Neutrophils treated as in (a) were evaluated by CLSM. Magnification  $\times 630$ . (c) Binding of biot-DNA in PBS (black line), PBS supplemented with 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (gray line) or in PBS with EDTA 1.5 mM (dashed line). (d) Binding of biot-DNA in PBS supplemented with HSA (black line) or in PBS supplemented with FBS (gray line). Shaded histograms represent fluorescence in the absence of biot-DNA. Histograms are representative of at least four independent experiments.

place match with those required to trigger cell activation.<sup>6</sup> Based on the above described results, the rest of the experiments were performed in PBS supplemented with HSA.

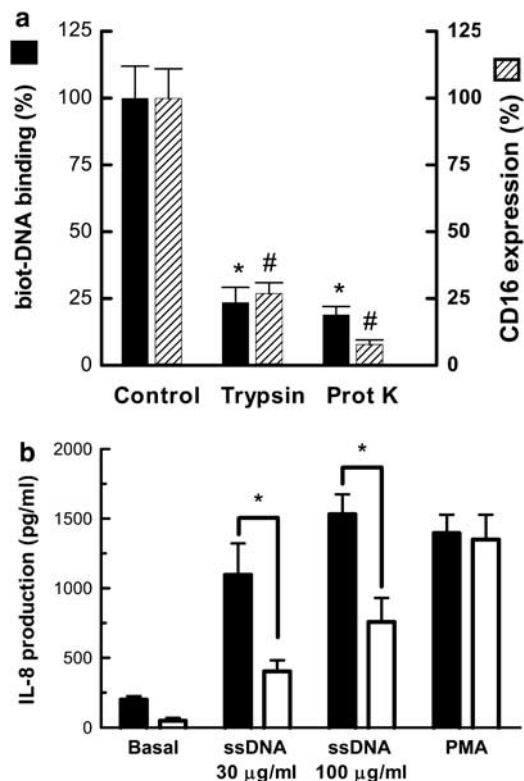
To determine whether bacterial DNA binding is saturable, we then incubated neutrophils with different concentrations of biot-DNA ranging from 3.5–120  $\mu\text{g/ml}$ . As shown in Figure 2a, the binding curve showed saturation of bacterial DNA binding at  $\approx 60 \mu\text{g/ml}$ . The amount of DNA required to attain 50% of maximal biot-DNA binding was  $17 \pm 4 \mu\text{g/ml}$ . To test the specificity of the binding, cells were preincubated with different concentrations of unlabeled competitors (ssDNA, double stranded DNA (dsDNA), poly-A, yeast tRNA or human DNA) and then incubated with 60  $\mu\text{g/ml}$  of biot-DNA. A 10-fold excess of unlabeled ssDNA reduced biot-DNA binding by  $\sim 85\%$  whereas the same excess of unlabeled dsDNA inhibited biot-DNA binding but less efficiently than ssDNA (Figure 2b). By contrast, poly-A and yeast tRNA were unable to reduce biot-DNA binding (Figure 2b). Interestingly, human DNA also displaced bacterial ssDNA binding similarly to what was observed with bacterial dsDNA, even though human DNA does not activate human neutrophils.<sup>6</sup> Although our findings do not rule out the possibility that human DNA is competing with biot-DNA for binding to the same putative receptor, considering that human DNA is unable to induce neutrophil activation, it is also possible that it exerts a steric hindrance in bacterial DNA binding to neutrophils by interacting nonspecifically with the cell surface. Taken together, findings depicted in Figure 2a and b suggest that bacterial DNA binding is a receptor-mediated event.

Considering that our previous studies showed that P-ODNs either containing or lacking CpG motifs do not induce neutrophil activation whereas S-ODNs activate neutrophils independently of the presence of CpG motifs, we determined their ability to compete biot-DNA binding to cell surface. Results in Figure 2c show that P-ODNs did not significantly inhibit biot-DNA binding even employed at 10-fold higher concentrations than biot-DNA, indicating that P-ODNs have a lower avidity than large DNA molecules to bind to neutrophil surface. Regarding S-ODNs, we found that S-ODN 2006 inhibited biot-DNA binding to neutrophil surface at all concentrations tested whereas S-ODN 2006K exerted variable effects depending on the concentration used (Figure 2c).

To characterize the nature of the putative DNA receptor, we then evaluated biot-DNA binding on neutrophils previously treated with trypsin or prot K. Figure 3a shows that cell treatment with proteases markedly inhibited biot-DNA binding, suggesting a role for a surface protein in DNA binding. The effectiveness of protease treatment was corroborated by evaluating the expression of CD16, a surface molecule which undergoes cleavage by the action of proteases.<sup>32</sup> We also observed a direct relationship between the inhibition of bacterial DNA binding to the surface of protease-treated neutrophils and the susceptibility of these cells to be activated by bacterial DNA. As Figure 3b shows, IL-8



**Figure 2** DNA-binding curve of bacterial DNA to neutrophil surface. (a) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence of 0, 3.5, 7, 15, 30, 60 or 120  $\mu\text{g/ml}$  of biot-DNA for 30 min at  $4^\circ\text{C}$ . After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Data represent the mean  $\pm$  s.e.m. of five experiments. (b) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence of 60 (1  $\times$ ), 180 (3  $\times$ ) or 600  $\mu\text{g/ml}$  (10  $\times$ ) of cold ssDNA, cold dsDNA, sonicated cold human placenta DNA, poly-A or yeast tRNA during 20 min at  $4^\circ\text{C}$  or alternative (c) they were incubated in the presence of 60 (1  $\times$ ), 180 (3  $\times$ ) or 600  $\mu\text{g/ml}$  (10  $\times$ ) of cold ODNs P-2006, P-2006K, S-2006 or S-2006K for 20 min at  $4^\circ\text{C}$ . Then, neutrophils were incubated for additional 30 min in the presence of 60  $\mu\text{g/ml}$  of biot-DNA. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Results are expressed as percentage of the maximal biot-DNA binding observed in the absence of competitors considered as 100%. Data represent the mean  $\pm$  s.e.m. of 4–8 experiments. \* $P < 0.05$  vs biot-DNA binding in the absence of competitors.



**Figure 3** Neutrophil DNA binding is protease sensitive. (a) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence or absence of  $100 \mu\text{g/ml}$  proteinase K (prot K) or trypsin during 30 min at room temperature. Then, they were washed three times with PBS supplemented with  $10 \text{ mg/ml}$  HSA, and binding of biot-DNA and the expression of CD16 was evaluated. Results are expressed as percentage of biot-DNA binding or percentage of CD16 expression observed in cells not treated with proteases, considered as 100%. Data represent the mean  $\pm$  s.e.m. of five experiments. \* $P < 0.01$  vs binding in cells not treated with the corresponding protease, # $P < 0.01$  vs CD16 expression in cells not treated with the corresponding protease. (b) Neutrophils were left untreated (black bars) or treated with prot K (white bars) as described in (a) and after were incubated in the presence or absence of ssDNA or PMA ( $10 \text{ ng/ml}$ ) for 3 h at  $37^\circ\text{C}$ . Then, the IL-8 released to supernatants was evaluated by ELISA. Data represent the mean  $\pm$  s.e.m. of five experiments. \* $P < 0.05$  vs IL-8 production in cells not treated with prot K (control cells).

production triggered by bacterial DNA was markedly inhibited in neutrophils treated with prot K as compared to that observed in cells not treated with this protease. As expected, protease treatment did not affect IL-8 production induced by PMA, because this compound triggers cell activation without interacting with cell surface receptors (Figure 3b).

### Analysis of Bacterial DNA Properties Enabling its Recognition by Neutrophils

To determine whether there is a relationship between the length of DNA and its binding avidity to neutrophil surface, we partially digested *E. coli* DNA with DNase I for different periods of time. Then we dialyzed and quantified the products, and evaluated the size of the obtained fragments by comparison to a set of DNA size markers in 0.8% denaturing

agarose gel electrophoresis (for fragments obtained after 0, 2 or 5 min of digestion) or in 5% denaturing polyacrylamide gel electrophoresis (for fragments obtained after more than 20 min of degradation). For simplicity, the pattern of DNA degradation is only shown in a representative agarose gel (Figure 4a). We employed these DNA fragments of differing lengths in competition assays, evaluating their ability to prevent the binding of undigested biot-DNA to neutrophil surface. As Figure 4b shows, samples containing fragments between 30 and 170 nucleotides even employed at a 10-fold excess than biot-DNA were unable to inhibit its binding to neutrophil surface. By contrast, fragments longer than 290 bases, used at 10-fold excess reduced biot-DNA binding by  $\sim 60\%$ . Surprisingly, DNA samples containing fragments ranging from 50 to 390 bases enhanced, although not significantly, biot-DNA binding, whereas those containing fragments of  $\approx 90$ – $930$  exhibited a marked variability in their capacity to modify biot-DNA binding.

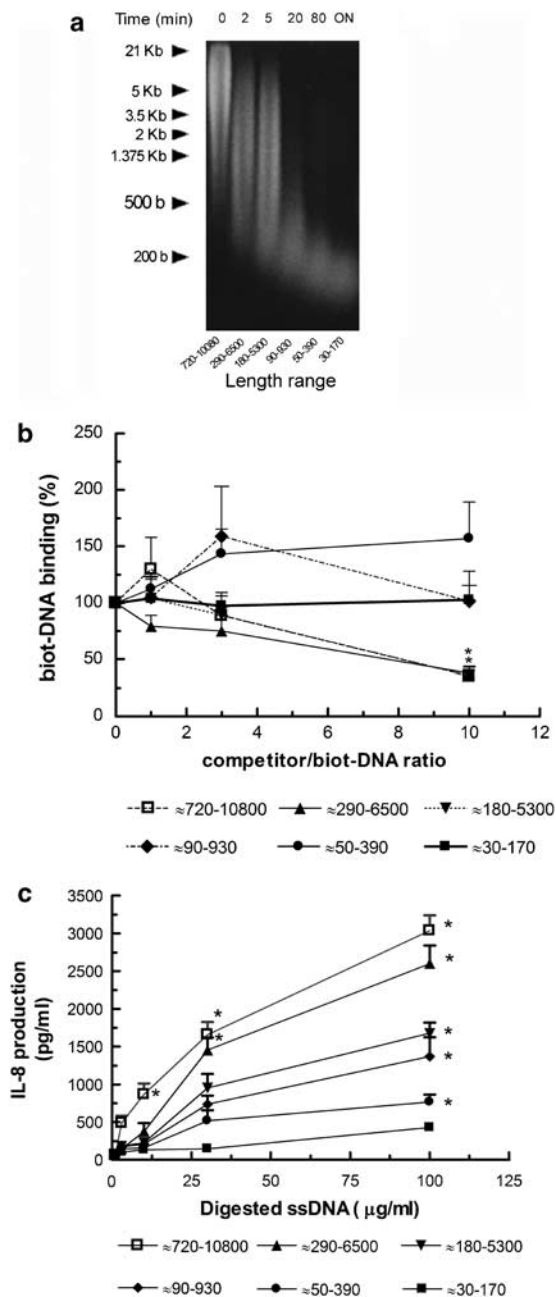
We then evaluated whether the length of bacterial DNA affected its ability to trigger cell activation by analyzing IL-8 production induced by DNA samples of differing lengths. Results depicted in Figure 4c show both that only the DNA samples containing fragments longer than 170 bases induced IL-8 production, and that the samples enriched in fragments longer than 290 bases showed the highest ability to trigger IL-8 production.

As plasmid DNA is currently used in DNA immunization and gene therapy protocols, we wondered whether it might be able to bind and trigger neutrophil activation. Thus, we performed additional experiments using biotinylated double stranded plasmid DNA (ds pBS). Plasmid DNA both efficiently bound to neutrophil surface (Figure 5a) and triggered IL-8 production (Figure 5b).

Considering that CpG methylation does not affect DNA ability to induce neutrophil activation,<sup>6</sup> we speculated that it would not modify its ability to bind to neutrophil surface. To test this premise, we treated *E. coli* DNA with SssI methylase and then evaluated its ability to compete the binding of biot-DNA. Results in Figure 5c show that methylated ssDNA was equally efficient as unmethylated ssDNA (ssDNA) to inhibit biot-DNA binding to neutrophil surface.

### Modulation of Bacterial DNA Binding to Neutrophil Surface by Stimulation with Conventional Agonists

We then evaluated whether stimulation of neutrophils with conventional agonists affected their ability to bind bacterial DNA. To this end, human neutrophils were stimulated with either of two chemoattractants, FMLP ( $5 \times 10^{-9} \text{ M}$ ) or IL-8 ( $5 \text{ nM}$ ), or with PMA ( $20 \text{ ng/ml}$ ), for 5, 15 and 30 min, and then their ability to bind bacterial DNA was analyzed by employing  $120 \mu\text{g/ml}$  of biot-DNA. Results from flow cytometric assays depicted in Figure 6a–c show that neutrophil stimulation with these agonists markedly increased cell surface biot-DNA binding, a fact that was confirmed by CLSM analysis (Figure 6d–e). Furthermore, neutrophils pretreated



**Figure 4** Identification of the minimal length of the oligomer able to bind to neutrophil surface. (a) Electrophoresis in 0.8% denaturing agarose gels of *E. coli* DNA treated with DNase RQ1 for the indicated times. (b) Neutrophils ( $5 \times 10^6$  per ml) were incubated during 30 min at 4°C in the presence or absence of 60 (ratio 1), 180 (ratio 3) or 600 μg/ml (ratio 10) of unlabeled fragments obtained after DNase digestion of bacterial DNA, for 20 min at 4°C. Then, neutrophils were incubated for additional 30 min in the presence of 60 μg/ml of biot-DNA. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Results are expressed as percentage of the maximal biot-DNA binding observed in the absence of competitors considered as 100%. Data represent the mean  $\pm$  s.e.m. of six experiments. (c) Neutrophils ( $5 \times 10^6$  per ml) were incubated during 3 h at 37°C in the presence or absence of the indicated concentrations of fragments obtained after DNase digestion of bacterial DNA. Then, IL-8 release to culture supernatant was evaluated by ELISA. Data represent the mean  $\pm$  s.e.m. of six experiments evaluated in triplicate. \* $P < 0.05$  vs basal IL-8 production.

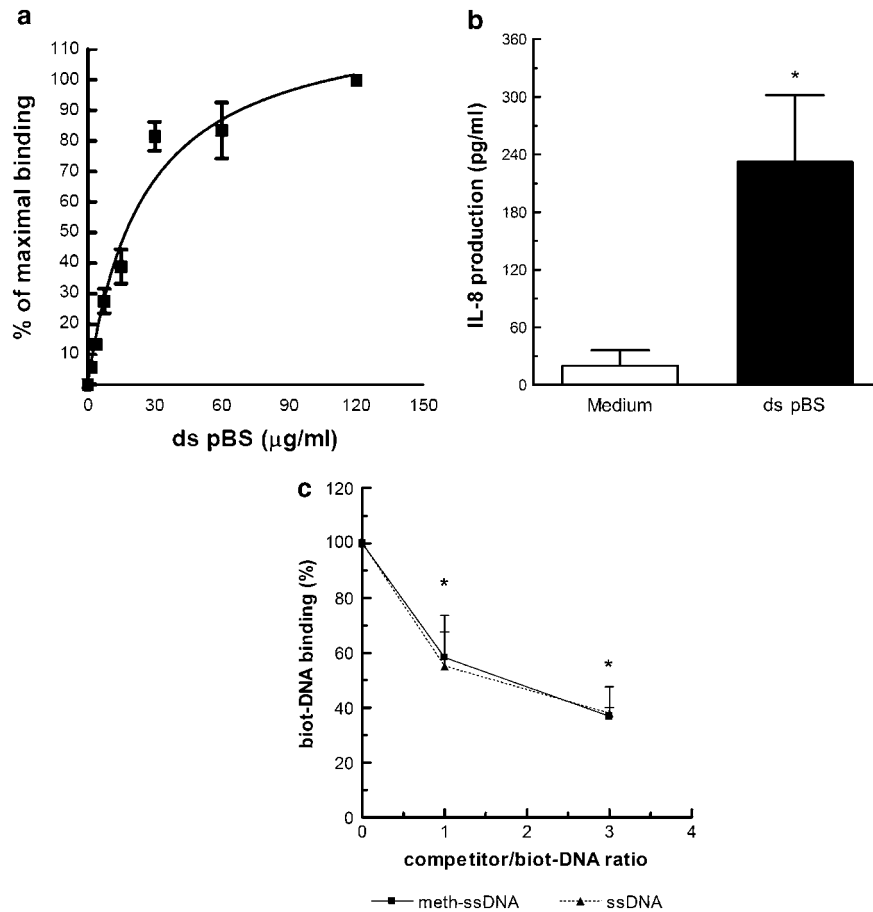
with FMLP exhibited an enhanced capacity to produce IL-8 in response to bacterial DNA (Figure 6f), showing a direct relationship between the intensity of DNA binding and the level of cellular activation.

As expected, unlabeled ssDNA, but not poly-A, effectively inhibited the binding of biot-DNA to stimulated neutrophils (Figure 6g–n), supporting that neutrophil activation results in an increased ability to specifically recognize bacterial DNA which is not related to an enhanced capacity to bind poly-anions in a nonspecific manner.

Results from Figure 6a–c show that the increase in bacterial DNA binding upon neutrophil activation was noticeable as early as 5 min post stimulation (the first time-point analyzed), indicating that it did not depend on *de novo* synthesis of a putative receptor. Thus, it could be accounted for by an increase in the expression of DNA receptors from a preexisting pool present in the membrane of granules that become part of plasma membrane due to granule exocytosis in response to stimulation with the above-described agonists. To test this hypothesis, neutrophils were treated with pentoxifylline (PXF, 5 mM), a methylxanthine derivative able to inhibit neutrophil degranulation,<sup>33</sup> and then were stimulated with FMLP for 15 min at 37°C. Afterwards, both the binding of biot-DNA and the expression of CD11b were evaluated. The expression of CD11b was determined to address the degree of inhibition of degranulation mediated by PXF treatment, because it is expressed in the membrane of specific and gelatinase granules as well as in secretory vesicles.<sup>34</sup> Results from Figure 7 show that PXF inhibited in a similar fashion both the enhancement of biot-DNA binding (Figure 7, left y axis) and the upregulation of CD11b expression in FMLP-treated neutrophils (Figure 7, right y axis). Similar results were obtained when exocytosis of all four granule types was inhibited by incubation of neutrophils under hypertonic conditions (data not shown).

### Effect of Cell Migration on Bacterial DNA Binding to Neutrophil Surface

It is well established that during extravasation to inflammatory foci, neutrophils undergo degranulation, an event that increases the expression of molecules involved in the adhesion cascade and diapedesis. Thus, we questioned whether transendothelial migration might modulate the DNA-binding capacity of human neutrophils. To address this issue, neutrophils were seeded on confluent monolayers of HUVECs cultured on Transwell filters, which had been previously activated by TNF- $\alpha$  treatment. Later, neutrophils were allowed to migrate across endothelial cells for 3 h, and their ability to bind biot-DNA was determined. Transmigrated neutrophils showed a markedly increased capacity to bind biot-DNA as compared to nonmigrated neutrophils (Figure 8). Of note, we did not evaluate the DNA-binding capacity of neutrophils that transmigrate in response to chemoattractants because, as we had previously determined, these stimuli



**Figure 5** Plasmid DNA binding to neutrophil surface. **(a)** Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence of 1.9, 3.8, 7.5, 15, 30, 60, or 120  $\mu\text{g/ml}$  of biotinylated double stranded plasmid DNA (ds pBS) for 30 min at 4°C. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. **(b)** Neutrophils ( $5 \times 10^6$  per ml) were incubated for 3 h at 37°C in the presence or absence of ds pBS (30  $\mu\text{g/ml}$ ). Then, IL-8 release to culture supernatant was evaluated by ELISA. Data represent the mean  $\pm$  s.e.m. of three experiments. \* $P < 0.05$  vs absence of ds pBS. **(c)** Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence of 60 ( $1 \times$ ) or 180 ( $3 \times$ )  $\mu\text{g/ml}$  of cold unmethylated-ssDNA or methylated-ssDNA for 20 min at 4°C. Then, neutrophils were incubated for additional 30 min in the presence of 60  $\mu\text{g/ml}$  of biot-DNA. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Results are expressed as percentage of the maximal biot-DNA binding observed in the absence of competitors considered as 100%. Data represent the mean  $\pm$  s.e.m. of 4–8 experiments.

significantly increase *per se* the neutrophil ability to bind DNA.

## DISCUSSION

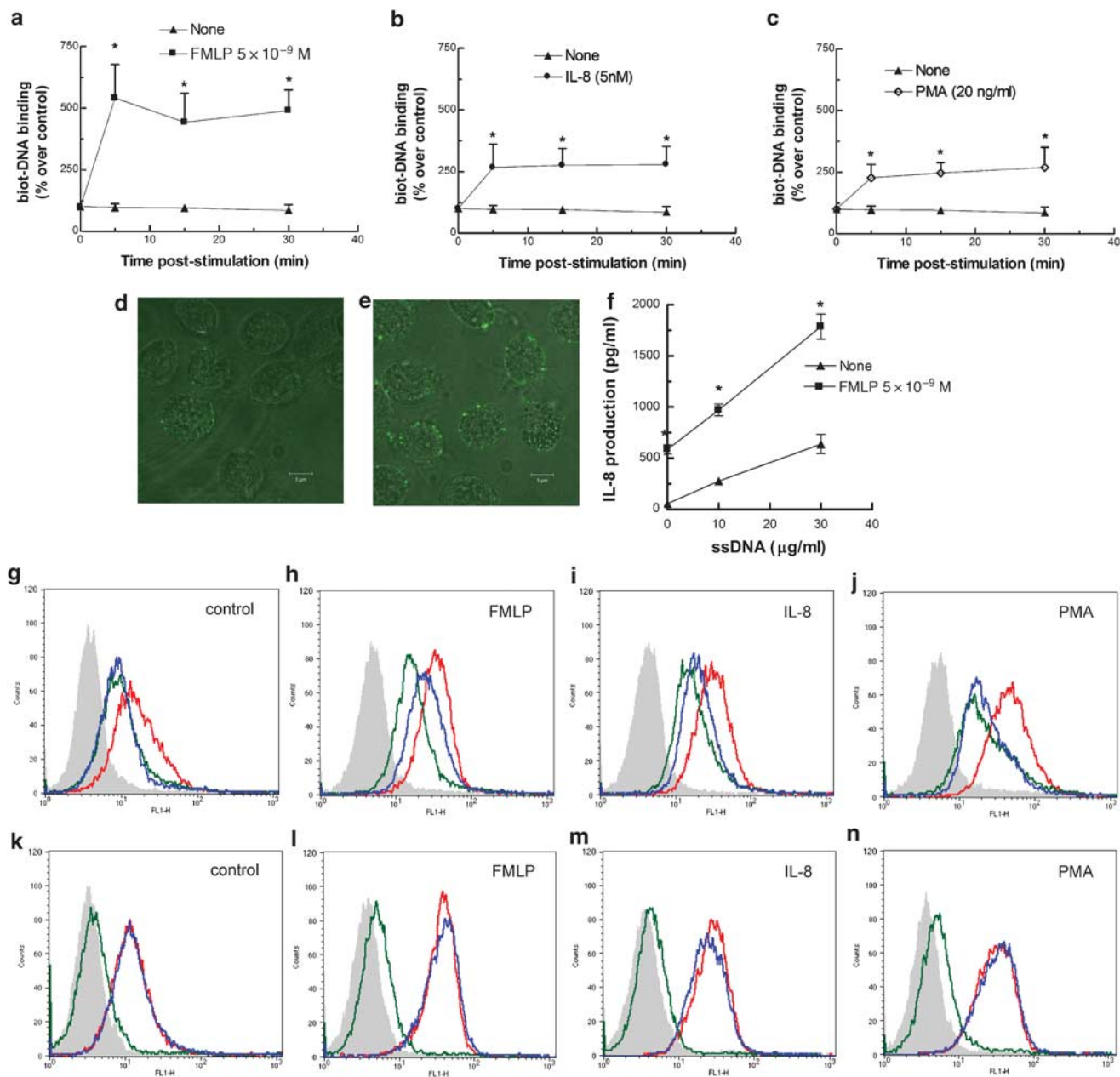
In this study we determined that bacterial DNA binds to human neutrophil surface. Binding was saturable and was dose-dependently inhibited by competition with an excess of unlabeled DNA but not by other polyanions like poly-A or yeast tRNA, suggesting that it is a receptor-mediated process. Binding was prevented by previous treatment of cells with trypsin or prot K, and this effect was associated to an incapacity of neutrophils to produce IL-8 in response to bacterial DNA, strongly suggesting that DNA binds to a protease-sensitive specific surface receptor.

Previous studies performed by Bennett *et al*<sup>35</sup> supported the presence of a DNA-binding receptor on neutrophil plasma membrane which would be responsible for the endocytosis and degradation of exogenous DNA. Contrasting with

our findings, they found that binding of  $\lambda$ -phage DNA to neutrophils was dependent upon the presence of calcium and magnesium as well as sulfates. The reasons for these discrepancies could be due to differences in the origin of DNA assessed or to distinct experimental settings employed. In fact, Bennett *et al* used [<sup>3</sup>H]-DNA and evaluated binding to neutrophils pretreated for an hour with DNase, a procedure that could have activated cells by integrin-mediated interactions and modified the DNA-binding properties of neutrophils.

Other works have also determined the presence of DNA binding proteins in leukocytes, fibroblasts, keratinocytes and Kupffer cells.<sup>36–40</sup> It has been reported that an 80 kDa protein mediates the transport of ODNs in HL-60 cells.<sup>36</sup> The authors reported that ODNs binding was sequence independent and could be inhibited by 60–80% by twofold excess (25  $\mu\text{M}$ ) of unlabeled ODNs, as well as by desoxyribonucleotides, ribonucleotides, plasmid DNA and yeast tRNA. However, the

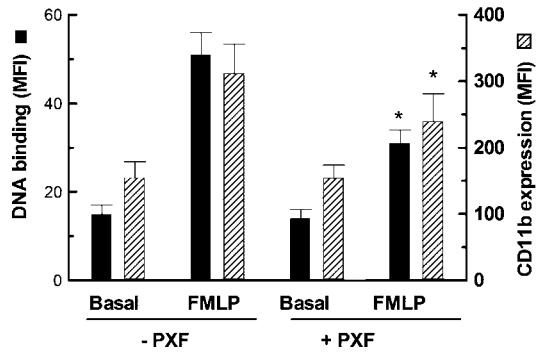




**Figure 6** Neutrophil activation increases binding of bacterial DNA. (a–c) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence or absence of  $5 \times 10^{-9}$  M FMLP, 5 nM IL-8 or 20 ng/ml PMA during the indicated periods of time, and then, binding was analyzed by employing 120  $\mu$ g/ml biot-DNA. (d, e) Confocal laser scanning microscopy (CLSM) images of neutrophils incubated in the absence (d) or presence of  $5 \times 10^{-9}$  M FMLP (e) during 15 min at 37°C and then for 30 min at 4°C with 120  $\mu$ g/ml biot-DNA followed by FITC-avidin; magnification  $\times 630$ . (f) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence or absence of  $5 \times 10^{-9}$  M FMLP for 15 min at 37°C and then stimulated with the indicated ssDNA concentrations during additional 3 h at 37°C. Then, IL-8 released to supernatants was evaluated by ELISA. Data represent the mean  $\pm$  s.e.m. of five experiments; \* $P < 0.05$  vs IL-8 production in cells not stimulated with FMLP. (g–n) Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence or absence of the indicated agonists for 15 min at 37°C. Then, they were incubated in the presence or absence of a fivefold excess of cold ssDNA (g–j) or 1.2 mg/ml poly-A (k–n), during 20 min at 4°C. Then, without being washed, neutrophils were incubated for additional 30 min in the presence of 120  $\mu$ g/ml of biot-DNA. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Histograms are representative of at least four experiments. Shaded histograms represent nonspecific binding of avidin-FITC, nonspecific avidin-FITC binding to unlabeled DNA or poly-A (green line), biot-DNA binding of neutrophils incubated in the absence (control) or presence the indicated agonists (red line) and biot-DNA binding of neutrophils preincubated in the absence (control) or presence of the indicated agonists and then incubated with fivefold excess of unlabeled DNA (g–j) or poly-A (k–n) (blue line).

binding of DNA and the activation of neutrophil described here do not appear to be mediated by this protein, because our results shown that (1) DNAs smaller than 170 nucleo-

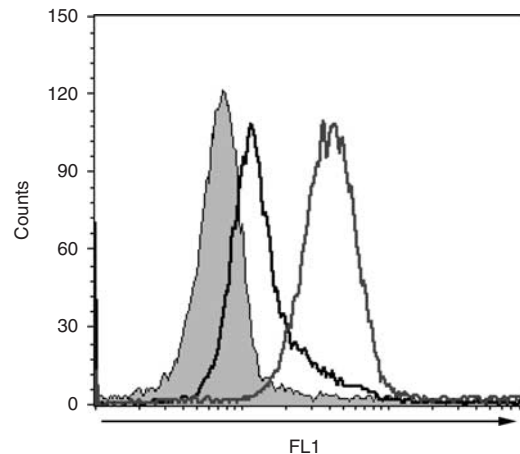
tides neither significantly competed binding of undigested biot-DNA to neutrophils nor were able to induce their activation and (2) yeast tRNA and poly-A were completely



**Figure 7** Inhibition of degranulation significantly reduces the increase of bacterial DNA binding observed after neutrophil stimulation. Neutrophils ( $5 \times 10^6$  per ml) were incubated in the presence or absence of 5 mM PXF, then were stimulated or not with  $5 \times 10^{-9}$  M FMLP during 15 min at 37°C. After, binding of biot-DNA (left axis) and expression of CD11b (right axis) was evaluated by FACScan. Data represent the mean  $\pm$  s.e.m. of five experiments. \* $P < 0.05$  vs FMLP-stimulated cells incubated in the absence of PXF.

unable to inhibit bacterial DNA binding to neutrophils. Other two DNA-binding proteins (79 and 90 kDa) have been detected in L929 mouse fibroblasts.<sup>37</sup> In human keratinocytes, two plasma membrane-actin linkers, ezrin and moesin, were identified as DNA-binding proteins.<sup>38</sup> Using southwestern analysis and gel shift and supershift assays, the authors found that these proteins were able to bind plasmid DNA,  $\lambda$ -phage DNA, ODNs irrespective of their sequences and calf thymus DNA, but not RNA. On the other hand, Benimetskaya *et al*<sup>39</sup> demonstrated the ability of Mac-1 to bind ODNs. We have previously demonstrated, however, that this protein is not involved in bacterial DNA recognition by human neutrophils<sup>6</sup> and recently confirmed these findings by evaluating bacterial DNA binding and its activating capacity in neutrophils of a patient suffering from leukocyte adhesion deficiency-1 (JI Fuxman Bass, unpublished results). The scavenger receptor A (SRA) has also been proposed to function as a DNA-binding molecule, although when cytokine production and DNA uptake by cells lacking the SRA were analyzed, similar levels to those of cells that express this receptor were detected, indicating that SRA, whereas able to bind DNA, is not essential for immune stimulation by DNA.<sup>41</sup> Early studies in Kupffer cells have reported binding of calf thymus DNA fragments (250–350 bases) to a trypsin-sensitive receptor.<sup>40</sup> In agreement with our study, they found that DNA binding was independent of serum factors. More recent work showed that RAW264.7 macrophages not only bind DNA but also their exposure for 18 h to DNA or LPS increases cell surface DNA binding and internalization.<sup>42</sup> In contrast to our findings, these authors observed that PMA treatment of RAW264.7 macrophages did not increase DNA binding.

Our observations indicated that samples enriched in fragments shorter than 170 nucleotides did not affect undigested biot-DNA binding. These results, however, should be interpreted with caution, because they do not rule out that fragments shorter than 170 nucleotides might actually bind to neutrophils. Our smaller DNA samples contain distinct fragments ranging from 30 to 170 nucleotides, thus it is



**Figure 8** Neutrophil transmigration increases binding of bacterial DNA.

Confluent monolayers of HUVECs cultured on Transwell filters were activated during 24 h with TNF- $\alpha$  (10 ng/ml). Then, monolayers were washed to eliminate residual TNF- $\alpha$ , and neutrophils ( $5 \times 10^5$  per well) were seeded on HUVECs and allowed to migrate for 3 h at 37°C. Transmigrated cells were recovered and DNA binding was evaluated by employing 120  $\mu$ g/ml of biot-DNA as previously described, and compared to biot-DNA binding in cells incubated during the 3 h in the lower chamber of the transmigration system. Binding of biot-DNA on transmigrated neutrophils (gray line), on nonmigrated neutrophils (black line) and fluorescence in absence of biot-DNA (shaded histogram). Histograms are representative of three independent experiments.

conceivable that a proportion of these fragments, perhaps those longest among the sample, can interact with the putative receptor although the binding of these fragments cannot be enough to be detected in competition assays. On the other hand, samples containing DNAs longer than 290 nucleotides triggered the highest levels of IL-8 production and were able to efficiently preclude binding of undigested biot-DNA, suggesting that this DNA length is critical for both efficient binding and activation, a fact that might reflect an increase in the affinity/avidity of the putative receptor if DNA has a length that allows it to interact simultaneously with two or more receptors.

Remarkably, DNAs ranging from 50 to 390 nucleotides enhanced biot-DNA binding whereas those ranging from 90 to 930-mers showed a variable behavior, suggesting that some oligomers might facilitate DNA binding to neutrophil surface. Our data do not provide an answer as to how this binding-promoting effect might be brought about; however, similar findings were previously reported to occur in DNA binding to TLR9. Kindrachuk *et al*<sup>43</sup> demonstrated that sequences that are unable to mediate direct activation of TLR9 influence TLR9 function by sensitizing the receptor for *in vitro* ligand binding as well as *in vivo* activation. Their studies indicated that ODNs promote plasmid binding to TLR9 without being incorporated into the complex TLR9-plasmid. They also found that both CpG- and non-CpG-phosphodiester ODNs were equally effective in promoting plasmid binding, but the phosphorothioate ODNs were far less effective in this capacity. We have not been able to detect

facilitation of biot-DNA binding by small ODNs ( $\approx 24$ -mer), however, further studies employing ODNs with different sequences are required to generalize this observation.

We also found that not only unlabeled ssDNA but also dsDNA and ss-meth-DNA inhibited binding of biot-DNA to neutrophil surface. These findings evidenced a direct relationship between DNA binding and activation, because we have previously shown that both dsDNA and ss-meth-DNA activate human neutrophils.<sup>6</sup> Moreover, dsDNA was less efficient than ssDNA to competitively inhibit binding of biot-DNA, in accordance with our previous findings indicating that dsDNA activates human neutrophils less effectively than ssDNA.<sup>6</sup> Additionally, we showed here that plasmid DNA not only binds but also activate human neutrophils. These findings might reflect similarities in DNA-binding properties between the neutrophil surface putative DNA receptor and the canonical DNA receptor, TLR9, for which both binding of ssDNA as well as plasmid DNA, preferentially in a supercoiled state, has been demonstrated.<sup>43,44</sup>

Our results also indicated that human DNA is able to inhibit bacterial biot-DNA binding to cell surface. Although these findings do not rule out the possibility that human DNA is competing with biot-DNA for binding to the same putative receptor, considering that human DNA is unable to induce neutrophil activation, it is also possible that long human DNA molecules exert a steric hindrance in bacterial DNA binding to neutrophils by interacting nonspecifically with the cell surface. Another possibility could be that human DNA effectively binds to the same receptor as bacterial DNA does, but being unable to trigger cell activation. Supporting this possibility, previous studies on TLR9 function have shown that TLR9 binds to stimulatory and inhibitory DNA; however, only stimulatory DNA led to substantial conformational changes in the TLR9 ectodomain that results in the activation of transductional pathways.<sup>45</sup> Irrespective of the mechanisms through which human DNA interacts with neutrophil surface, the ability of human DNA to inhibit bacterial DNA binding might have consequences *in vivo* in reducing the proinflammatory capacity of bacterial DNA. In fact, recent studies have demonstrated the release of genomic DNA by neutrophils undergoing death by 'NETosis'.<sup>46</sup> During 'NETosis', neutrophils release extracellular traps (NETs), composed of chromatin and specific proteins from the neutrophilic granules.<sup>47</sup> Considering that bacteria are potent stimulators of NETs formation, it is possible to speculate that human DNA contained in NETs might compete with bacterial DNA for binding to viable neutrophils, thus reducing its capacity to trigger inflammatory responses. Supporting this possibility, we have observed that a 10-fold excess of human DNA significantly inhibited the production of IL-8 triggered by bacterial DNA (JI Fuxman Bass, unpublished results).

We also found that binding of bacterial DNA is markedly enhanced upon treatment of neutrophils with both chemottractants and PMA. The increased capacity of neu-

trophils to bind DNA was evidenced 5 min after stimulation, suggesting that it did not involve *de novo* synthesis of a putative receptor. By contrast, it probably accounted for an increased exposition of a DNA-binding protein from a preexisting pool present in the membrane of granules that become part of plasma membrane due to granule exocytosis in response to stimulation with the above-described agonists. This presumption was supported by findings indicating that the inhibitor of degranulation PXF, inhibited in a similar fashion both the upregulation of CD11b and the enhancement of DNA binding triggered by FMLP. However, our results do not rule out that the DNA-binding protein whose expression is increased upon neutrophil activation is not a DNA receptor responsible for activation but a protein able to bind DNA and consequently cooperate in DNA binding to a transducing receptor. In fact, previous studies demonstrated that lactoferrin, a cationic protein contained in specific granules, is mobilized to plasma membrane upon neutrophil activation<sup>48</sup> and exhibits DNA-binding properties.<sup>49</sup> Moreover, we have also determined that myeloperoxidase and cathepsin G, two cationic proteins contained in azurophilic granules, are able to bind DNA (JI Fuxman Bass, unpublished results). However, our results showing that the increase in bacterial DNA binding due to neutrophil stimulation with conventional agonists could be inhibited by competence with unlabeled DNA but not by another polyanion like poly-A, favor the view that neutrophil activation results in the expression of additional DNA-specific receptors.

Finally, we found that transmigration across confluent HUVECs monolayers markedly increases neutrophil DNA-binding capacity. It is possible to speculate that in a physiological setting, after extravasation, neutrophils are better prepared to recognize and be activated by bacterial DNA.

Extracellular DNA has been detected in virtually all environments inhabited by bacteria,<sup>16,18,19,50-52</sup> and has also been implicated as a major structural component of bacterial biofilms.<sup>24,25,53</sup> Thus, it is possible that bacterial DNA recognition by a surface receptor has evolved as a signal to alert the immune system to the presence of bacterial invasion, mainly in infections which course with bacteria growing as biofilms. Further studies are underway in our laboratory to identify the receptor responsible for DNA recognition.

#### ACKNOWLEDGEMENTS

We thank Yanina Powazniak, Evelia López, Mabel Horvat and Beatriz Loria for their valuable assistance. We also thank Dr Tomás Santa Coloma for making confocal microscope available. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT20433), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP6133), the Third World Academy of Sciences (RG 04-0389) and Fundación Alberto Roemmers (Argentina).

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