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GM-CSF enhances a CpG-independent pathway of neutrophil activation triggered by bacterial DNA

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ABSTRACT

We have previously demonstrated that bacterial DNA induces neutrophil activation through a CpG- and TLR9-independent but MyD88-dependent-pathway. In this study we determined that GM-CSF enhances the activation of neutrophils by bacterial DNA. Granulocyte-macrophage colony-stimulating factor increased IL-8 and IL-1β secretion, and CD11b-upregulation induced by single-stranded bacterial DNA. It also enhanced neutrophil IL-8 production induced by double-stranded bacterial DNA, methylated single-stranded DNA, plasmid DNA, and phosphorothioated-CpG and non-CpG-oligodeoxynucleotides. Together these observations indicated that GM-CSF enhances neutrophil responses triggered by bacterial DNA in a CpG-independent fashion. We also found that GM-CSF enhanced the activation of the MAPKs p38 and ERK1/2 induced by bacterial DNA. Moreover, the pharmacological inhibition of these pathways significantly diminished GM-CSF ability to increase neutrophil activation by bacterial DNA. Finally, we observed that GM-CSF was unable to increase the activation of MyD88$^{-/-}$ neutrophils by bacterial DNA. Our findings suggest that GM-CSF modulates the CpG-independent, MyD88-dependent neutrophil response to bacterial DNA, by increasing the activation of the MAPKs p38 and ERK1/2.

1. Introduction

Neutrophils play a crucial role in the innate immune response to bacterial infections by mediating the phagocytosis and destruction of these microorganisms through oxygen-dependent and -independent mechanisms. They also produce cytokines, chemokines and growth factors that contribute to shape the immune response as well as to repair the damaged tissues (Nathan, 2006; Witko-Sarsat et al., 2000).

It is widely accepted that unmethylated-CpG motifs in bacterial DNA trigger immune cell activation by interacting with the endosomal Toll-like receptor 9 (TLR9) (Akira et al., 2006; Ishii and Akira, 2006; Kawai and Akira, 2006). However, our previous studies in neutrophils demonstrated that bacterial DNA induces cell activation through a CpG- and TLR9-independent mechanism that does not require DNA internalization (Alvarez et al., 2006; Trevani et al., 2003). Similarly, studies conducted by other researchers have also suggested the existence of pathways unrelated to the presence of CpG motifs by which DNA may be able to trigger cell activation, through either TLR9-dependent or -independent mechanisms (Elias et al., 2003; Stetson and Medzhitov, 2006; Wang and Krieg, 2003; Yasuda et al., 2006; Yasuda et al., 2005; Yoshinaga et al., 2002; Zhao et al., 2004).

The presence of extracellular DNA in bacterial cultures has been extensively documented (Allesen-Holm et al., 2006; Lorenz et al., 1991; Lorenz and Wackernagel, 1994; Palchevskiy and Finkel, 2006). Naturally transformable bacterial species release chromosomal DNA into culture media (Moscoso and Claverys, 2004; Palchevskiy and Finkel, 2006; Steinberger and Holden, 2005; Steinmoen et al., 2002). Moreover, bacterial DNA plays an important role in the formation and composition of biofilms (Petersen et al., 2005; Whitchurch et al., 2002), a mode of bacterial growth that is relevant in the pathogenesis of different human diseases

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such as oral infections, chronic infections like in cystic fibrosis (CF), bacterial endocarditis, infectious kidney stones, and osteomyelitis (Hall-Stoodley et al., 2004; Parsek and Singh, 2003).

Bacterial infectious diseases involve the development of inflammatory responses in which neutrophil recruitment and activation are crucial. Taking into account both, the presence of extracellular DNA in bacterial microenvironments and our previous studies showing that extracellular DNA represents a powerful stimulus for neutrophils, it is reasonable to speculate that the interaction of neutrophil with extracellular bacterial DNA plays an important role in the host immune response to bacterial infections, particularly in those having a biofilm etiology.

There are no previous studies directed to identify factors able to modulate the activation of neutrophils by bacterial DNA. In the present study, we have analyzed whether GM-CSF, a cytokine which is critical in regulating neutrophil survival and function, is able to modulate the response to bacterial DNA and/or the mechanisms through which DNA triggers the activation of neutrophils.

2. Materials and methods

The studies performed in this work have been reviewed and approved by the institutional review board and local ethical committee.

2.1. Antibodies and reagents

Endotoxin-free reagents and plastics were used in all experiments. RPMI 1640 and PBS were purchased from HyClone Laboratories Inc. (Logan, UT) and human albumin (HSA) from UNC Hemoderivados (Cordoba, Argentina). Clinical grade recombinant human GM-CSF (rhGM-CSF) was from Laboratorios Gautier (Buenos Aires, Argentina) and recombinant mouse GM-CSF (rmGM-CSF) was purchased from R&D (Minneapolis, MN). PE-conjugated anti-human CD11b (cat. No. IM2581) was purchased from Immunotech (Marseille, France). Antibodies (Abs) against Thr180/Tyr182-phospho-p38 MAPK (cat. No. 9211), Thr202/Tyr204-phospho-ERK1/ERK2 (cat. No. 9106), Ser536-phospho-NF-κB p65 (cat. No. 3033) and HRP-conjugated goat anti-mouse Ab (cat. No. 7076) were obtained from Cell Signaling Technology Inc. (Beverly, MA). Anti-phospho Thr186/Tyr185-JNK (cat. No. sc-6254) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). PE-anti-mouse CD11b (cat. No. 12-0112) and FITC-anti mouse Ly-6G (Gr 1) (cat. No. 11-5931) were purchased from ebioscience (San Diego, CA). Anti-IκBα rabbit polyclonal Ab (cat. No. 554135) and HRP-conjugated goat anti-rabbit IgG Ab (cat. No. 554021) were purchased from BD Pharmingen (San Diego, CA). PD98059, SB203580, SP600125, ALLN, MG-132 and sulfasalazine (SSZ) were obtained from Calbiochem (EMD Biosciences Inc., La Jolla, CA). *E. coli* LPS (*E. coli* O111:B4), polymyxin-agarose and PMA were purchased from Sigma–Aldrich (St. Louis, MO). DNA from *E. coli* was purchased from Worthington Biochemical Corporation (Lakewood, NJ). *E. coli* DNA, purified as previously described (Trevani et al., 2003), and plasmid DNA (pBluescript II-KS+, Stratagen, La Jolla, CA), were passed through a polymyxin-agarose column to eliminate endotoxins. Unless otherwise stated, *E. coli* DNA preparations were made single-stranded before use by heating at 95 °C for 10 min, followed by rapid cooling on ice (ssDNA). For some experiments, *E. coli* DNA was methylated with CpG methylase *Sss*I (New England BioLabs, Beverly, MA) as described (Trevani et al., 2003).

2.2. Human neutrophil isolation

Neutrophils were isolated from heparinized human blood from healthy donors by Ficoll–Hypaque gradient centrifugation (Ficoll, GE Healthcare, Uppsala, Sweden; Hypaque, Winthrop Products, Argentina) and dextran sedimentation. Contaminating erythrocytes were removed by hypotonic lysis. After washing with saline, the cells were suspended in RPMI 1640 supplemented with 5 mg/ml HSA, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). We used HSA for protein supplement medium instead of FCS to avoid possible effects produced by minimal levels of contaminating LPS of the DNA samples, since, under these conditions, LPS fails to activate neutrophils (Hailman et al., 1994; Soler-Rodriguez et al., 2000; Trevani et al., 1999).

All neutrophil preparations were FACs-analyzed after purification to guarantee that their FSC/SSC parameters were compatible with non-activated cells. To minimize neutrophil spontaneous activation, cells were used immediately after isolation.

Only samples with <0.3% of monocyte contamination, as judged by their FSC/SSC distribution, were employed for IL-8 and IL-1 determinations.

2.3. Measurement of cytokines production

Neutrophils (5×10^6 ml^{-1}) were incubated in the presence or absence of rhGM-CSF for the indicated periods of time. Then, they were seeded into round-bottom 96-well plates and stimulated with DNA or medium (mock) and cultured for 3 h (for IL-8) or 18 h (for IL-1β) at 37 °C. Finally, culture supernatants were collected and IL-8 or IL-1β concentrations were measured by ELISA (R&D, Minneapolis, MN).

2.4. Western blotting

Neutrophils (4×10^6) were incubated in the presence or absence of rhGM-CSF (15 ng/ml) for 30 min at 37 °C and then stimulated with ssDNA (100 μg/ml) for 0–60 min at 37 °C. The stimulation was terminated by the addition of 1 ml of ice-cold saline with 1 mM PMSF and rapid centrifugation. The pellets were immediately frozen in dry ice after aspiration of the supernatants, then were dissolved in sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol and trace amounts of bromophenol blue dye in 62.5 mM Tris–HCl, pH 6.8), heated for 5 min at 95 °C and stored at −70 °C until subjected to gel electrophoresis. After SDS-PAGE, proteins were electro-transferred from the gel to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) for 2 h and then blocked in PBS containing 0.05% Tween 20 and 5% non-fat milk for 1 h at 25 °C. The membranes were immunoblotted overnight with the indicated antibodies at 4 °C. After washing, bound antibodies were visualized with HRP-conjugated antibodies against rabbit or mouse immunoglobulin G (IgG) by using the ECL Western Blotting System (Amersham Biosciences, Germany).

2.5. Mice

C57BL/6 (H-2b) mice were purchased from Division of Animal Production, Animal Core Facility, Faculty of Veterinary Sciences, La Plata University, Buenos Aires, Argentina. Male mice (7–8 weeks age) were γ-irradiated (900 rad) and reconstituted with 5×10^6 MyD88-deficient or control bone marrow cells, to generate MyD88$^{-/-}$ or wild-type chimeras. MyD88$^{-/-}$ bone marrow-derived cells were obtained from the Centre de Développement des Techniques Avancées pour l'Expérimentation Animale, Orleans, France. At 6–8 weeks post-reconstitution, BM cells were aspirated and layered over a discontinuous density gradient composed by Histopaque 1077 and 1119 (Sigma–Aldrich, St. Louis, MO) as previously described (Tumpey et al., 2002). Two bands, one enriched with neutrophils and another in mononuclear cells were isolated

by centrifugation. Mononuclear cells were cultured for 24 h in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 0.055 mM mercaptoethanol. Then, non-adherent cells were removed and the ability of adherent cells to produce IL-6 and TNF-α in response to a CpG-ODN, LPS or PMA was tested by intracellular flow cytometry. As expected, MyD88$^{-/-}$ adherent cells produced neither IL-6 nor TNF-α when stimulated by the TLR-agonists CpG-ODN or LPS, but produced these cytokines when stimulated by PMA. Wild-type chimeric-derived macrophages produced cytokines in response to all agonists (not shown).

The neutrophil-enriched band obtained from the Histopaque gradient contained 70–75% of Gr-1^{high} cells. Neutrophils were incubated in the presence or absence of rmGM-CSF (2000 U/ml) for 30 min at 37 °C and then stimulated with ssDNA (100 μg/ml), double-stranded plasmid DNA (30 μg/ml), Pam3CSK4 (1 μg/ml), or PMA (10 ng/ml) for 1 h at 37 °C. Then, cells were stained with FITC-anti-Gr 1 and PE-anti-CD11b mAbs for 20 min at 4 °C and fluorescence was measured with a FACScan argon laser flow cytometer.

2.6. Statistical analysis

Statistical significance was determined using the nonparametric Friedman test for multiple comparisons with Dunns post-test or Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. GM-CSF enhances neutrophil responses induced by bacterial DNA

First, we cultured neutrophils with different concentrations of rhGM-CSF (5–100 ng/ml) for 90 min at 37 °C, and evaluated the production of IL-8 induced by single-stranded *E. coli* DNA (ssDNA) (30 μg/ml). Fig. 1A shows that rhGM-CSF enhanced IL-8 production induced by ssDNA, at all concentrations tested, being the most pronounced effect already achieved at 15 ng/ml rhGM-CSF. Then we cultured neutrophils for 30, 90 or 180 min with rhGM-CSF and evaluated IL-8 secretion induced by ssDNA (10–100 μg/ml). The rhGM-CSF significantly enhanced IL-8 production induced by all concentrations of ssDNA tested, being the effect more marked at 100 μg/ml of bacterial DNA (Fig. 1B). As similar levels of IL-8 production were observed regardless the duration of the pretreatment with GM-CSF, in ahead, their effects were evaluated after a 30-min pretreatment period.

Recombinant human GM-CSF also enhanced IL-8 secretion when double-stranded *E. coli* DNA (dsDNA) (Fig. 2A), double-stranded plasmid DNA (ds pBS) (Fig. 2B) and unmethylated or SssI-methylated ssDNA (meth-ssDNA) (Fig. 3A) were used as stimuli. Our previous studies indicated that human neutrophils are activated by phosphorothioated-oligonucleotides (S-ODNs) in a CpG-independent mode (Trevani et al., 2003). Here, we evaluated the effect of rhGM-CSF on IL-8 production induced by both a CpG-B ODN (S2006) and a GpC ODN (S2006K). rhGM-CSF strikingly increased IL-8 secretion induced by both S-ODNs (Fig. 3B) to similar levels. Together, these data support that GM-CSF modulates IL-8 production induced by bacterial DNA through a CpG-independent mechanism.

As shown in Fig. 4A, rhGM-CSF also enhanced the upregulation of the expression of CD11b and IL-1β secretion induced by ssDNA (Fig. 4B). Since experiments were performed by employing neutrophil preparations that contained <0.3% of monocyte contamination, IL-1β production was adjudicated to neutrophil population, a conclusion that was strengthened by the fact that GM-

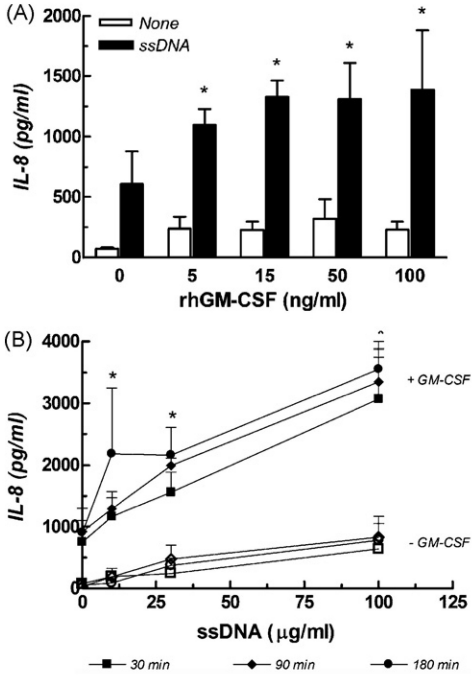

Fig. 1. GM-CSF enhances IL-8 production induced by single-stranded bacterial DNA (ssDNA). (A) Neutrophils (5×10^6 ml^{-1}) were preincubated with the indicated concentrations of rhGM-CSF for 90 min at 37 °C and further incubated in the presence (black bars) or absence (open bars) of 30 μg/ml of ssDNA for 3 h at 37 °C. (B) Neutrophils (5×10^6 ml^{-1}) were incubated in the presence (filled symbols) or absence (open symbols) of 15 ng/ml of rhGM-CSF for 30, 90 or 180 min at 37 °C. Then, they were stimulated with 10, 30 or 100 μg/ml of ssDNA and incubated for three additional hours at 37 °C. After incubation, IL-8 released to culture supernatants was assessed by ELISA. Data are expressed as the mean ± S.E.M. of five experiments. $^*p < 0.05$ compared to ssDNA-stimulated cells incubated in the absence of GM-CSF.

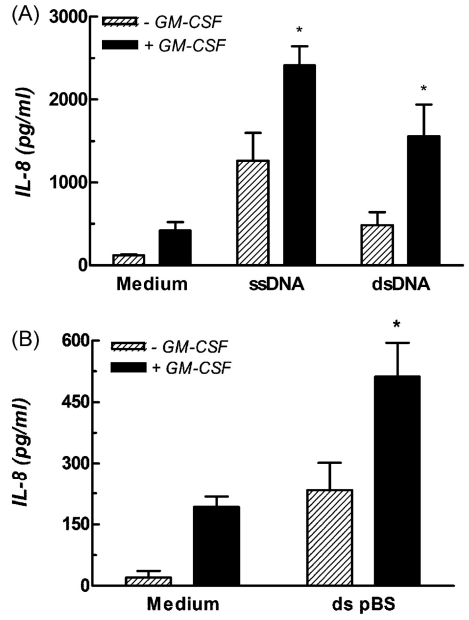

Fig. 2. GM-CSF enhances IL-8 production induced by double-stranded bacterial DNA (dsDNA) and plasmid DNA (ds pBS). Neutrophils (5×10^6 ml^{-1}) were incubated in the presence or absence of 15 ng/ml rhGM-CSF for 30 min at 37 °C. Then, they were stimulated with either ssDNA or dsDNA (30 μg/ml) (A) or with ds pBS (20 μg/ml) (B), and incubated for three additional hours at 37 °C. The IL-8 released to culture supernatants was assessed by ELISA. Data are expressed as the mean ± S.E.M. of four experiments. $^*p < 0.05$ compared to DNA-stimulated cells not pre-treated with GM-CSF.

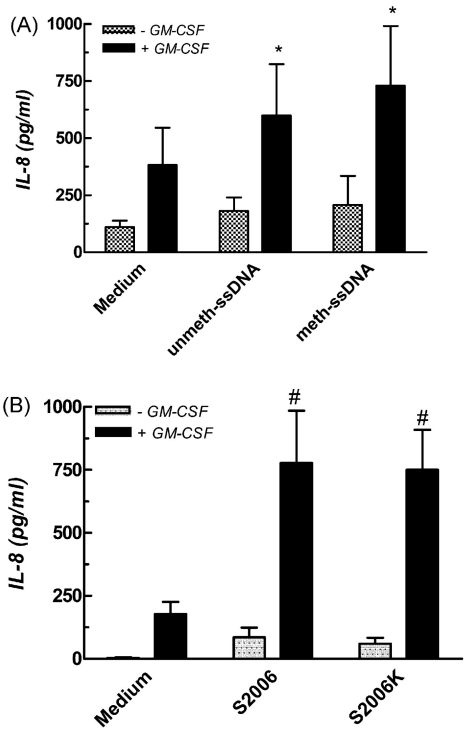

Fig. 3. GM-CSF enhances IL-8 production induced by CpG-methylated DNA. Neutrophils (5×10^6 ml^{-1}) were incubated in the presence or absence of 15 ng/ml rhGM-CSF for 30 min at 37 °C. Then, they were stimulated with CpG-methylated-ssDNA (meth-ssDNA) or unmethylated-ss DNA (unmeth-ssDNA) (10 μg/ml) (A) or with SODNs 2006 and 2006K (2 μM) (B). The IL-8 released to culture supernatants was assessed by ELISA and is expressed as the mean ± S.E.M. of 4–6 experiments. $^{*}p < 0.05$ compared to DNA-stimulated cells not pre-treated with GM-CSF; and $^{\#}p < 0.001$ compared to S-ODN-stimulated cells not pre-treated with GM-CSF.

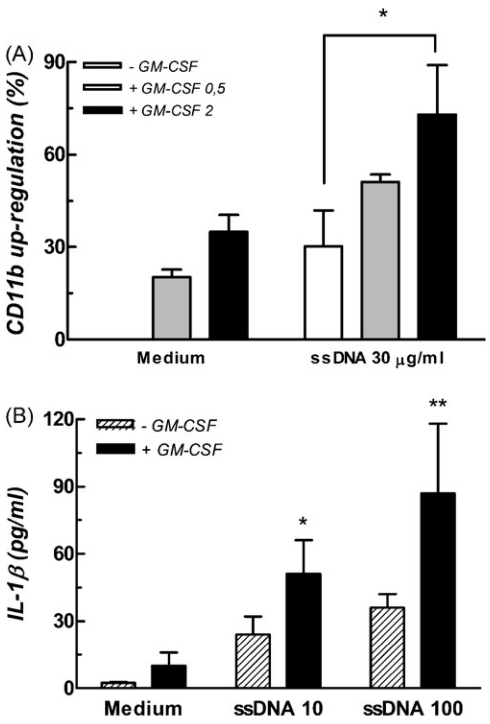

Fig. 4. GM-CSF increases CD11b expression (A) and IL-1β production (B) induced by bacterial DNA. (A) Neutrophils (5×10^6 ml^{-1}) were incubated in the presence or absence of 0.5 or 2 ng/ml rhGM-CSF for 15 min at 37 °C. Then, they were stimulated with ssDNA (30 μg/ml) for 60 min at 37 °C and CD11b expression was evaluated by FACs. Results are expressed as percentage of upregulation of CD11b expression. (B) Neutrophils (5×10^6 ml^{-1}) were incubated in the presence or absence of 15 ng/ml of rhGM-CSF for 30 min at 37 °C. Then, were stimulated with ssDNA (10–100 μg/ml) for 18 h at 37 °C and the IL-1β released was evaluated in culture supernatants by ELISA. Data represent the mean ± S.E.M. of four experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to DNA-stimulated cells not pre-treated with GM-CSF.

CSF was unable to increase IL-1 production induced by bacterial DNA in mononuclear cells (data not shown).

3.2. GM-CSF enhances bacterial DNA-triggered neutrophil responses by increasing p38 and ERK1/2 MAPK activation

We also evaluated the signaling intermediates activated in human neutrophils pretreated with rhGM-CSF (15 ng/ml) and then stimulated with ssDNA (100 μg/ml). The phosphorylation of p38 MAPK induced by ssDNA was markedly enhanced by pretreatment of neutrophils with rhGM-CSF (Fig. 5A). This effect was detected 10 min after stimulation with bacterial DNA, but maximal enhancement was observed at 30 min, followed by a gradual decrease by 60 min post-stimulation. rhGM-CSF *per se* induced a slight activation of p38 (Fig. 5A). By contrast, rhGM-CSF pretreatment itself induced a fast and strong ERK1/2 phosphorylation (Fig. 5A), however it was even more marked in neutrophils pretreated with rhGM-CSF and stimulated with bacterial DNA as compared to those pre-incubated with medium and stimulated with ssDNA or those only pretreated with rhGM-CSF (Fig. 5A). A marked phosphorylation of ERK1/2 in rhGM-CSF-pretreated and DNA-stimulated neutrophils was even detected by 30 min post-stimulation, a time point at which only a weak ERK phosphorylation was observed in cells pre-incubated with medium and stimulated with ssDNA or those only pretreated with rhGM-CSF. Low levels of p38 and ERK1/2 activation were detected in unstimulated neutrophils which had undergone a 30-min preincubation period (Fig. 5A, first lane). Similar findings were previously reported in other studies carried out with human neutrophils (Derouet et al., 2004; Jozsef et al., 2002; Nakamae-Akahori et al., 2006; Suzuki et al., 2001).

We then evaluated JNK phosphorylation and detected similar levels of phosphorylation of a predominant species of 46-kDA, compatible with JNK-1, at all time intervals investigated under all the tested conditions (Fig. 5B). These results suggest that JNK is not involved in the GM-CSF-mediated potentiation of IL-8 secretion induced by bacterial DNA. The reasons why JNK is also phosphorylated under control conditions, could rely on activation of JNK due to integrin-mediated interactions that could have been established during the GM-CSF pretreatment period (30 min), as it has been previously observed in TNF-α- or LPS-stimulated neutrophils (Arndt et al., 2004).

We also examined the effect of the specific pharmacological inhibitors of MAPKs on IL-8 production. Both SB203580 (a p38 inhibitor) and PD98059 (an inhibitor of MEK, an ERK upstream kinase) partially reduced IL-8 secretion induced by ssDNA in both rhGM-CSF- or mock pretreated-neutrophils as well as that induced by rhGM-CSF alone (Fig. 5C). However, the inhibitory effect exerted by SB203580 was stronger in those neutrophils stimulated by ssDNA either pretreated or not with GM-CSF than in those neutrophils only pre-treated with GM-CSF. Acting together, both inhibitors nearly abrogated IL-8 production induced either by bacterial DNA or by rhGM-CSF, while markedly reduced the production of IL-8 by neutrophils pretreated with rhGM-CSF and subsequently stimulated by ssDNA (inhibition: 71%; Fig. 5C).

As expected, the JNK inhibitor SP600125 did not significantly affect IL-8 production under all the conditions tested (Fig. 5D).

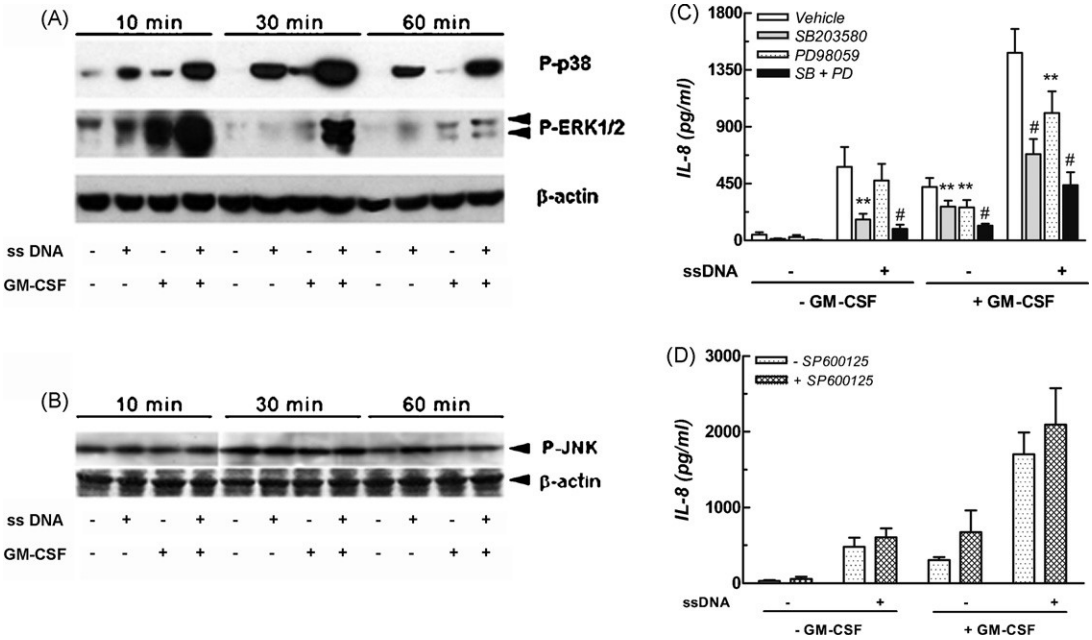

Fig. 5. GM-CSF modulates neutrophil MAPKs activation induced by bacterial DNA. Neutrophils (4×10^6 ml^{-1}) were incubated in the presence or absence of 15 ng/ml of rhGM-CSF for 30 at 37 °C. Then, they were stimulated by ssDNA (100 μg/ml) for the indicated times at 37 °C. Phosphorylation of the MAPKs p38 and ERK1/2 (A) and JNK (B) was analyzed by immunoblotting using specific antibodies against the phosphorylated forms of each protein. Immunoblots are representative of at least three independent experiments. (C and D) Effect of pharmacological inhibitors of MAPKs on neutrophil IL-8 production by GM-CSF-pretreated and ssDNA-stimulated neutrophils. Cells were pre-treated with SB203580 (5 μM) or PD98059 (5 μM) (C), or with SP600125 (10 μM) (D) for 15 min at 37 °C, followed by incubation in the presence or absence of rhGM-CSF (15 ng/ml) for 30 min at 37 °C. Then, were stimulated with ssDNA (30 μg/ml) for 3 h at 37 °C and the production of IL-8 was evaluated by ELISA. Data represent the mean ± S.E.M. of six experiments. $^{**}p < 0.01$ and $^{\#}p < 0.001$ compared to cells incubated in the absence of inhibitors.

3.3. GM-CSF enhances NF-κB activation triggered by bacterial DNA

We then evaluated whether rhGM-CSF modulates NF-κB activation induced by ssDNA by analyzing the IκBα levels by Western blotting. As shown in Fig. 6A, GM-CSF itself did not induce IκBα degradation beyond that observed in non-stimulated control neutrophils, however, it accelerated the kinetic of IκBα degradation induced by bacterial DNA. In fact, a marked IκBα degradation was observed in GM-CSF-pretreated neutrophils stimulated for 10 min with ssDNA, while ssDNA alone required 60 min to induce similar levels of IκBα degradation (Fig. 6A). Thus, we evaluated the effect of NF-κB inhibitors to determine its involvement in the GM-CSF-enhancement of responses triggered by ssDNA. We observed that ALLN and MG-132, two proteasome inhibitors, significantly inhibited IL-8 production induced by bacterial DNA in neutrophils either pretreated or not with GM-CSF, but not that induced by GM-CSF alone (Fig. 6B). However, when the more specific NF-κB inhibitor sulfasalazine (SSZ) was employed only a significant reduction in IL-8 production induced by bacterial DNA was observed.

It is well established that NF-κB transcriptional activity is modulated through phosphorylation of the p65 subunit (Vermeulen et al., 2003; Yang et al., 2003). Our previous studies indicated that the NF-κB complex found in nuclear extracts of neutrophils stimulated by bacterial DNA mainly consists of both p65/p50 and p50/p50 dimers (Alvarez et al., 2006). Thus, we evaluated p65 phosphorylation in response to bacterial DNA stimulation in rhGM-CSF-pretreated and non-pretreated neutrophils. GM-CSF *per se* induced a hardly detectable phosphorylation of p65. By contrast, ssDNA induced p65 phosphorylation, which was markedly enhanced by rhGM-CSF pretreatment (Fig. 6C).

3.4. GM-CSF enhances MyD88-dependent neutrophil responses triggered by bacterial DNA

We also found by employing neutrophils from MyD88$^{-/-}$ bone marrow chimeric mice that, in accordance with our previous findings, MyD88$^{-/-}$ neutrophils did not upregulate CD11b expression in response to bacterial genomic or plasmid DNA. GM-CSF itself increased CD11b expression both in the wild type and MyD88$^{-/-}$ neutrophils, but it was completely unable to upregulate CD11b expression in response to bacterial genomic or plasmid DNA in MyD88$^{-/-}$ neutrophils (Fig. 7). Responses induced by both Pam3CSK4, a TLR2 agonist which transduces through a MyD88-pathway and PMA, a TLR-independent agonist, are shown as controls (Fig. 7). The value of adding up of individual effects mediated by GM-CSF and ssDNA or pBS in WT-BMC neutrophils were significantly different from the CD11b levels observed when WT-BMC neutrophils were pre-treated with GM-CSF and treated with ssDNA or pBS respectively, suggesting that the enhancing effect mediated by GM-CSF on neutrophil responses triggered by bacterial DNA requires a functional MyD88 pathway.

4. Discussion

In this study, we demonstrated that rhGM-CSF increases neutrophil IL-8 and IL-1 production as well as CD11b upregulation induced by bacterial DNA.

Previous studies showed that human neutrophils were not activated by a CpG-containing type A ODN, but become responsive by pretreatment with GM-CSF (Hayashi et al., 2003). The authors concluded that GM-CSF enables CpG-DNA to trigger neutrophil activation through a TLR-9-dependent pathway. By contrast, we found that the response to type-B ODNs was CpG-independent and was markedly increased by GM-CSF pretreatment. Moreover, we

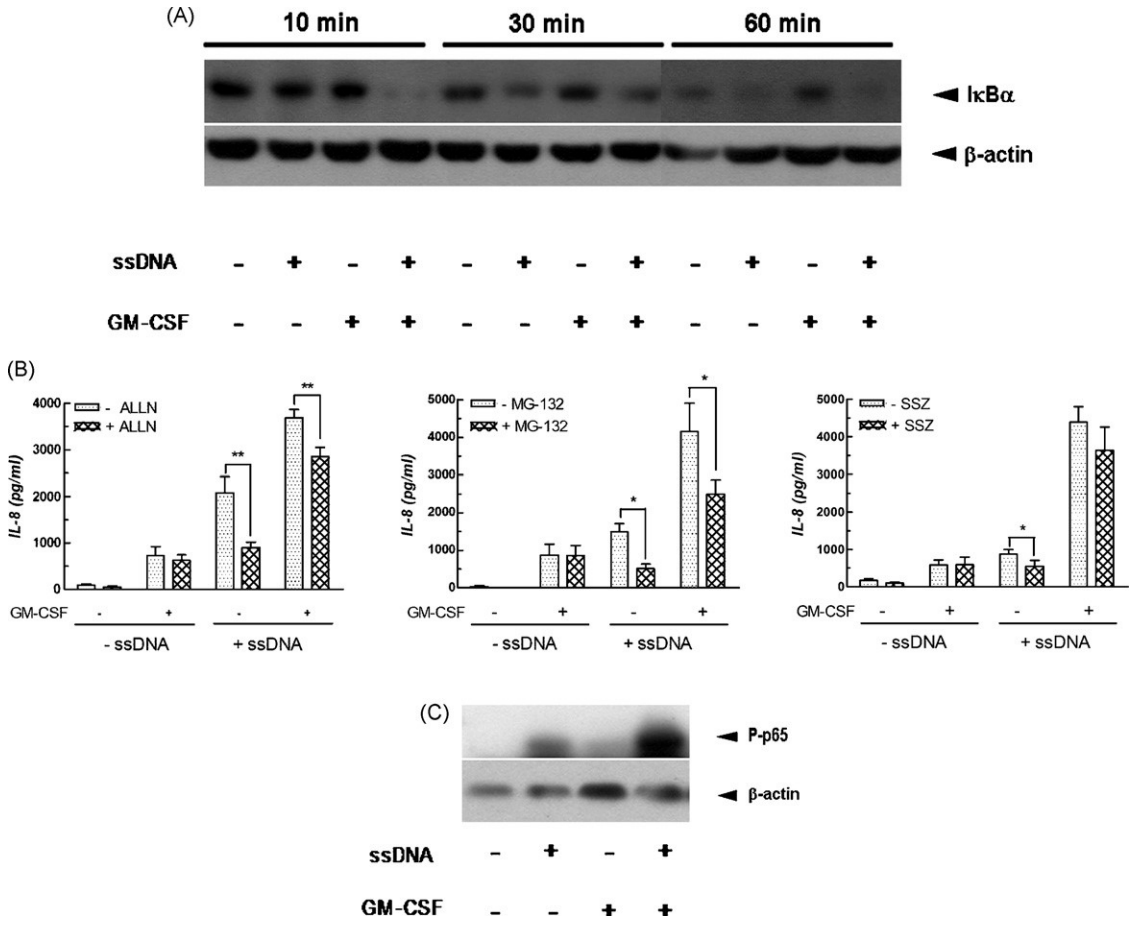

Fig. 6. GM-CSF modulates NF-κB activation induced by bacterial DNA. (A) Neutrophils (4×10^6) were incubated in the presence or absence of 15 ng/ml of rhGM-CSF for 30 min at 37 °C. Then, were stimulated by ssDNA (100 μg/ml) for the indicated times at 37 °C. Then, IκBα was analyzed by immunoblotting using a rabbit polyclonal specific Abs. Immunoblots are representative of four independent experiments. (B) Effect of ALLN (10 μM), MG-132 (30 μM) and sulfasalazine (SSZ; 100 μM) on neutrophil IL-8 production by GM-CSF-pre-treated and ssDNA stimulated neutrophils evaluated as in Fig. 5D. Data represent the mean ± S.E.M. of 5–7 experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$. Each graph depicts data obtained from different sets of experiments. (C) Neutrophils (4×10^6) were incubated in the presence or absence of 15 ng/ml of rhGM-CSF for 30 min at 37 °C. After, cells were stimulated with ssDNA (100 μg/ml) for 30 min at 37 °C. Finally, phospho-p65 expression was analyzed by immunoblotting using a rabbit polyclonal specific Ab. Immunoblots are representative of four independent experiments.

found that GM-CSF increased to similar levels neutrophil response to unmethylated and CpG-methylated DNA and to double-stranded genomic bacterial DNA or plasmid DNA, suggesting that GM-CSF increases neutrophil activation by bacterial DNA through a CpG-independent pathway.

We have previously demonstrated that MyD88$^{-/-}$ neutrophils were non-responsive to bacterial DNA. Here, we showed that although MyD88$^{-/-}$ BMC-derived neutrophils increased CD11b expression by GM-CSF pretreatment, this cytokine did not increase CD11b expression after stimulation with bacterial genomic or plasmid DNA in MyD88$^{-/-}$ neutrophils. Since the enhancing effect exerted by GM-CSF in DNA-stimulated WT-BMC neutrophils is synergic, our findings suggest that GM-CSF requires a functional MyD88 pathway to enhance DNA-triggered responses.

Noteworthy, previous work showed that GM-CSF pretreatment increased neutrophil responses to TLR2, TLR4, TLR5, TLR7, TLR8 and TLR9 agonists (Hayashi et al., 2003). Although the authors found an enhanced expression of TLR2 and TLR9 after GM-CSF pretreatment, receptor expression alone could not explain the enhanced response to TLR stimulation, because GM-CSF pretreatment also resulted in the enhancement of responses through TLRs whose expression was not affected by GM-CSF. The authors hypothesized that an altered activation of signaling molecules could also play a role in TLR/GM-CSF-receptor synergy. Thus, it is possible to speculate that the enhancement of neutrophil responses to bacterial DNA mediated by GM-CSF, is part of a broader phenomenon by which GM-CSF upregulates neutrophil responses to agonists that activate the MyD88-signaling pathway.

It is well established that the IL-8 promoter contains NF-κB, AP-1 and C/EBP binding sites (Holtmann et al., 1999; Mukaida et al., 1994), and previous studies performed with human KB epithelial cells and HEK293 have indicated that maximal IL-8 synthesis requires the coordinate action of at least three different signal transduction pathways which cooperate to induce mRNA synthesis and suppress mRNA degradation (Holtmann et al., 1999). These pathways included NF-κB, p38 and JNK MAPKs. Results from Fig. 5 suggest that the enhancing effects mediated by GM-CSF in DNA-stimulated neutrophils are caused, at least in part, by an increase in MAPK phosphorylation. Furthermore, results showing that GM-CSF pretreatment markedly accelerated IκBα degradation induced by bacterial DNA (Fig. 6A) are indicative of an enhanced NF-κB nuclear translocation under these conditions. However, whether this increased NF-κB nuclear translocation is involved in the enhancing effects mediated by GM-CSF on IL-8 production is controversial, since contrasting findings were made by employing different NF-κB inhibitors. In fact, the proteasome inhibitors ALLN and MG-132 but not SSZ, an IκB kinase inhibitor, significantly inhibited IL-8 production in GM-CSF-pretreated and

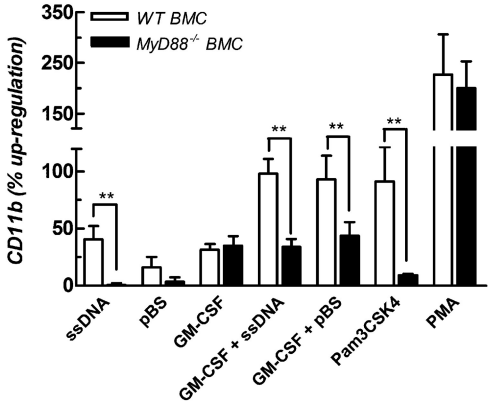

Fig. 7. GM-CSF enhances MyD88-dependent neutrophil responses triggered by bacterial DNA. Neutrophils from wild type and MyD88$^{-/-}$ BMC mice were incubated in the presence or absence of rmGM-CSF (2000 U/ml) for 30 min at 37 °C. Then were stimulated with either ssDNA (100 μg/ml), plasmid DNA (pBS; 20 μg/ml), Pam3CSK4 (1 μg/ml), PMA (20 ng/ml) or medium for 1 h at 37 °C. Then, were stained with FITC-conjugated anti-Gr 1 and PE-conjugated anti-CD11b mAbs or isotype controls mAbs for 20 min at 4 °C. Finally, the expression of CD11b was evaluated on the high Gr 1-expressing cells by flow cytometry. Data represent the mean ± S.E.M. of five experiments. $^{**}p < 0.01$; $p < 0.05$ comparison between adding up of the values obtained in GM-CSF-treated WT-BMC neutrophils and ssDNA or pBS, and those in WT-BMC neutrophils pre-treated with GM-CSF and treated with ssDNA or pBS, respectively.

DNA stimulated-neutrophils. On the other hand, the increased level of p65 phosphorylation in Ser536 detected in GM-CSF-pretreated and DNA-stimulated neutrophils also suggests that p65 phosphorylation might represent another step in the regulation of IL-8 production under these conditions, since p65 phosphorylation is an event that has been shown to modulate NF-κB transcriptional activity (Yang et al., 2003; Vermeulen et al., 2003). Interestingly, a previous report demonstrated the constitutive expression of a Ser536-phosphorylated form of p65 in the cytoplasm of T cells, which undergoes nuclear translocation not regulated by IκBκ upon cell activation (Sasaki et al., 2005). Of note, this phosphorylated-p65 was recruited to the IL-8 promoter and regulated IL-8 transcription upon cell activation. Thus, this noncanonical NF-κB pathway, which is unaffected by proteasome inhibition and by IκB kinase inhibiton, could also contribute to the increased expression of IL-8 in GM-CSF pre-treated and DNA-stimulated neutrophils. On the other hand, our previous findings indicating that p38 and ERK1/2 regulate neutrophil activation by bacterial DNA (Alvarez et al., 2006), and results from this work, showing that together, SB203580 and PD98059, markedly inhibited IL-8 production induced by bacterial DNA in GM-CSF pretreated neutrophils, suggest that p38 and ERK1/2 are also involved in the enhancement of IL-8 production mediated by GM-CSF pretreatment. As mentioned, the core IL-8 promoter contains AP-1-C/EBP-binding sites, which unlike NF-κB sites, are dispensable for transcriptional activation in some cells, but contribute to maximal gene expression in others (Hoffmann et al., 2002). Thus, p38 AND ERK1/2 might contribute to enhance IL-8 expression by GM-CSF in DNA-stimulated neutrophils by activating AP-1 (Hoffmann et al., 2005). In fact, we have previously demonstrated that bacterial DNA induces AP-1 activation in neutrophils. Alternatively, p38 may also be involved in increasing IL-8 production by GM-CSF in bacterial DNA-stimulated neutrophils, by stabilizing IL-8 mRNA. In fact, IL-8 mRNA harbors AU-rich elements (ARE) in the 3′-untranslated region which mediate IL-8 post-transcriptional regulation (Holtmann et al., 1999). Other studies have demonstrated that activation of p38 MAPK pathway stabilizes the IL-8 mRNA through an ARE-targeted mechanism (Hoffmann et al., 2002). Moreover, CpG-DNA has been shown to increase the half-life of the IL-8 mRNA in human bronchial epithelial cells in response to IL-1 stimulation, through a mechanism which might involve p38 activation (Parilla et al., 2006).

In conclusion, our studies show that neutrophil responses to bacterial DNA can be enhanced by GM-CSF. This effect might be relevant in bacterial infections with a biofilm etiology, in which extracellular DNA could function as a potent neutrophil agonist.

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