Bacterial DNA delays human eosinophil apoptosis

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Oligodeoxynucleotide (ODN) sequences containing unmethylated cytidine phosphate guanosine (CpG) motifs prevalent in bacterial DNA attenuate allergic lung inflammation in experimental models of asthma but failed to inhibit eosinophilia and improve lung function in patients with asthma. Bacterial respiratory tract infections exacerbate asthma in humans. Increased eosinophil survival is a critical factor leading to persistent eosinophilic airway inflammation. Apoptosis is regarded as a key mechanism in the resolution of eosinophilic inflammation. The aim of this study was to investigate the effects of bacterial DNA and CpG ODNs on human eosinophil apoptosis in vitro and to elucidate the signalling pathway. Eosinophils were isolated from healthy peripheral blood by CD16− or CD16+, CD19− and CD304−negative selection. Apoptosis was determined by flow cytometric analysis of relative DNA content, Annexin-V staining and/or morphological analysis. Toll-like receptor 9 (TLR9) expression was studied by using western blotting and intracellular flow cytometry. Bacterial DNA and phosphorothioate-modified CpG ODNs, but not vertebrate DNA, were found to delay spontaneous eosinophil apoptosis. The effect of CpG ODNs was dependent on endosomal acidification and reversed by inhibitory ODN, which suggests involvement of TLR9 pathway. Furthermore, we demonstrated TLR9 expression in eosinophils derived from both atopic and healthy donors. Non-CpG ODNs had occasionally parallel but less profound effect on eosinophil apoptosis, which was not dependent on endosomal acidification. The anti-apoptotic effect of CpG ODNs was dependent on phosphatidylinositol 3-kinase (PI3K) and nuclear factor-κB (NF-κB) but not mitogen-activated protein kinases (MAPKs) as determined by inhibitor studies. Although our results suggest CpG-dependent involvement of TLR9 in the action of phosphorothioate-modified ODNs, we interestingly found that the anti-apoptotic action of native bacterial DNA in eosinophils is not dependent on unmethylated CpG motifs. This suggests that bacterial DNA contains a currently unknown recognition structure lacking from vertebrate DNA. Bacterial DNA-mediated suppression of eosinophil apoptosis is a novel mechanism for exacerbation of eosinophilic lung inflammation associated with bacterial respiratory tract infection.

1. Introduction

Asthma is a chronic inflammatory disease, where eosinophilic granulocytes are numerous in the lungs. Release of eosinophil products such as toxic granule proteins, cysteinyl leukotrienes, pro-inflammatory cytokines and reactive oxygen species leads to epithelial cell damage, mucosal damage, bronchoconstriction and increased mucus secretion and vascular permeability [1]. Additionally eosinophils have recently been demonstrated to have an essential role in airway remodelling and a significant regulatory role in T-helper cells 2 (Th2)-cytokine production [2,3]. Increased eosinophil survival is a critical factor leading to persistent eosinophilic airway inflammation. Apoptotic cell death is considered as an important removal mechanism of eosinophils from the lungs but in patients with asthma eosinophil apoptosis is delayed [4]. Pathogenic components modulate allergic inflammation in several ways. According to the hygiene hypothesis, infections may prevent development of allergic disease. On the other hand, respiratory tract infections seem to exacerbate established asthma and contribute to asthma chronicity [5,6]. These infections, even though originally defined of viral origin, often involve mixed bacterial co-infection and several types of bacterial infections have been associated with acute asthma exacerbations or chronic stable
asthma [6,7]. Eosinophils have been suggested to play an important role in asthma exacerbations [8,9].

Bacterial DNA is characterized by unmethylated cytidine phosphate guanosine (CpG) dinucleotides. In vertebrate DNA, unmethylated CpG dinucleotides are uncommon. DNA containing unmethylated CpG dinucleotides is a pathogen-associated molecular structure recognized by Toll-like receptor 9 (TLR9), a receptor of innate immunity [10]. In mice, bacterial DNA has been previously found to exert both pro-inflammatory and anti-inflammatory effects. Bacterial DNA was reported to induce inflammation in the lower respiratory tract of mice [11]. On the other hand, synthetic CpG oligodeoxynucleotides (ODNs) are currently under intense investigation due to their anti-inflammatory effects in mouse models of asthma [12,13]. Stimulation by CpG ODNs activates TH1-type innate immune response. Synthetic CpG ODNs with different sequences and backbones activate distinct cell types, which has led to their categorization into classes A, B and C. Class A CpG ODNs induce high secretion of type I interferons (IFNs) by plasmacytoid dendritic cells (pDCs), whereas class B CpG ODNs induce interleukin (IL)-6 production and proliferation of B-cells. Class C CpG ODNs were developed to have immunostimulatory activity that is combination of the activities induced by class A and B CpG ODNs [14].

Only scarce information exists of TLR9 function in human eosinophils [15,16]. To our knowledge, no information exists of the effects of native bacterial DNA on eosinophils. Similarly, it is not known whether the effect of CpG DNA on eosinophils involves TLR9 or how the signalling is mediated. To evaluate the role of eosinophils in the modulatory action of CpG DNA in inflammation, we aimed to study the effects of bacterial DNA and synthetic CpG ODNs on human eosinophil apoptosis. Additionally, we aimed to establish the signalling pathway mediating the effect.

2. Materials and methods

2.1. Oligodeoxynucleotides and DNA

ODNs were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. We used ODNs with the following sequences (phosphorothioate bases are shown with small letters and phosphodiester bases with capitals): Class A CpG ODN D19 5'-ggTGCATCCTGAGCA Gggggg-3', non-CpG ODN Dc (control for D19) 5'-ggTG CATCCTGACG CACggggg-3', Class B CpG ODN 1018 5'-tgacgtagtaactttacagtgt-tg-3', non-CpG ODN 1040 (control for 1018) 5'-tgacggctagggctaggt-tg-3', Class B CpG ODN 2006 5'-tgctgtggcttggttctggt-3', Class C CpG ODN C274 5'-tgctgacgtaacgtaggt-3', non-CpG ODN C661 (control for C274) 5'-tgctgg taacgtaggttaacgga-3' and inhibitory ODN 5'-tagggt agggttaggtagg-3'. ODNs were diluted in Tris–EDTA (10 mM Tris, pH 7.5–8.0, 1 mM EDTA) to prevent degradation of the short ODNs known to occur in acidic conditions. Escherichia coli (E. coli) K12 DNA and salmon sperm DNA were purchased from Invivogen, San Diego, CA, USA. They were diluted in nuclease- and endotoxin-free sterile water according to the manufacturer's instructions. E. coli and salmon sperm DNA were made single-stranded before use by heating at 95 °C for 10 min, after which they were rapidly cooled on ice. In each experiment, E. coli DNA and salmon sperm DNA were added in the beginning and once after 16–18 h of culture. For some experiments, E. coli DNA was treated in NE buffer 2 with CpG methyltransferase (2 U/mg DNA) and 160 μM S-adenosylmethionine for 3 h at 37 °C. Methylated and unmethylated DNA (for control) were purified by phenol extraction and ethanol precipitation and dissolved in sterile water. To confirm successful methylation process, we treated DNA with restriction enzyme BstUI followed by agarose gel electrophoresis.

2.2. Other materials

Other reagents were obtained as follows: Anti-TLR9 antibody and its blocking peptide (ProSci Inc., Poway, CA, USA), horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phycoerythrin (PE)-conjugated anti-TLR9 antibody, PE-conjugated rat IgG2a isotype control, fixation buffer and permeabilization buffer (eBioscience, San Diego, CA, USA), CpG methyltransferase M.SssI and BstUI (New England Biolabs, Ipswich, MA, USA), balflomycin A1 from Streptomyces griseus, BMS-345541, pyrrolidine dithiocarbamate (PDTC), SB203580, budesonide, dimethyl sulfoxide (DMSO) and propidium iodide (PI) (Sigma–Aldrich Co., St. Louis, MO, USA), wortmannin, SP600125, negative control for SP600125, PD98059, SB202474 (Merck Biosciences Darmstadt, Germany), anti-CD16, anti-CD19 and anti-CD304 microbeads, fluorescein isothiocyanate (FITC)-conjugated anti-CD303 antibody and the magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-CD122 antibody, FITC-conjugated anti-CD19 antibody, IgG1 isotype control (BD Biosciences Pharmingen, San Jose, CA, USA), PE conjugated anti-TLR9 antibody, IgG2a, FITC-conjugated IgG1 (Chemicon International Inc., Temecula, CA, USA), MG–132 (Tocris Bioscience, Bristol, UK), chloroquine (Invivogen, San Diego, CA, USA). Other reagents were described elsewhere [4,17,18]. Stock solutions of wortmannin, balflomycin A1, mitogen-activated protein kinase (MAPK) inhibitors and their negative controls, BMS-345541 and MG–132 were prepared in DMSO. Final DMSO concentration in the cells was 0.3%. Budesonide stock was prepared in ethanol and the final ethanol concentration in the cells was 0.2%. Similar concentration of the solvent was added to the control cultures.

2.3. Human eosinophil isolation and culture

The blood samples (100 ml) were taken from eosinophilic donors, mostly from patients with asthma and/or allergy. All donors gave written informed consent to a study protocol approved by the Ethical Committee of Tampere University Hospital. Eosinophils were isolated by immunomagnetic CD16-negative selection under sterile conditions as previously described [4,17,19]. Purity of eosinophils after the isolation process was at least 99%. For some experiments, eosinophils were further purified by CD19- and CD304-negative selection to remove possible contaminating B-cells and plasmacytoid dendritic cells. Cells were resuspended at 10⁵/ml and cultured in Dutch modification of RPMI 1640 containing 10% fetal bovine serum, antibiotics and l-glutamine at 37 °C with 5% CO₂ in 96-well plates. If not otherwise stated, eosinophils isolated by CD16-negative selection were used in the experiments.

2.4. Apoptosis assays

Relative DNA fragmentation assay and flow cytometric analysis of PI-stained cells were performed as previously described [4,17,19]. Cells with reduced DNA content were considered as apoptotic. For morphological analysis eosinophils were spun onto cytosin slides (25 g, 5 min), fixed in methanol for 15 min and stained with May–Grünwald–Giemsa. Shrunken cells with nuclear coalescence and chromatin condensation were considered apoptotic. Annexin-V binding assay was performed as previously described [18,19]. The cells displaying positive Annexin-V FITC labelling (FITC+/PI− and FITC+/PI+) were regarded as apoptotic.

2.5. Determination of the amount of contaminating CD19+ and CD123+ CD304+ cells in eosinophil suspensions

The amount of contaminating B-cells (CD19+) and pDC (CD123+ CD304+) after eosinophil isolation was assessed by
immunofluorescence and flow cytometric analysis of 20,000 cells as described by Matsumoto et al. [20]. Briefly, the cells were incubated for 20 min at +4°C in PBS buffer containing 0.5% BSA and 2 mM EDTA. Fc receptor blocking reagent and fluorophore-conjugated monoclonal antibody or corresponding IgG control at concentration recommended by the manufacturer. Cells were washed with PBS buffer and analyzed by flow cytometer.

2.6. TLR9 expression

TLR9 expression was determined by western blotting and intracellular flow cytometry. For western blotting, eosinophils were lysed in ice-cold radioimmuno precipitation assay (RIPA)-buffer, after which protein was mixed in sodium dodecyl sulfate (SDS)-containing loading buffer and loaded onto 8% SDS-polyacrylamide electrophoresis gel. After electrophoresis, proteins were electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, UK, Ltd., Little Chalfont, Buckinghamshire, UK) and blocked for 1 h in Tris-buffered saline with tween (TBST) containing 5% bovine serum albumin (BSA). Membrane was incubated overnight at +4°C in the blocking solution with 1 µg/ml anti-TLR9 or blocked anti-TLR9. Blocking of anti-TLR9 was conducted by incubating the antibody with 1 µg/ml blocking peptide at +37°C for 30 min. For flow cytometric analysis of TLR9 expression, eosinophils were fixed, permeabilized and stained with PE-conjugated TLR9 antibody or IgG control (1 µg/million cells) according to the manufacturer's instructions.

2.7. Statistics

Results are expressed as mean ± standard error of mean (SEM). Apoptosis is expressed as percentage of apoptotic cells (number of apoptotic cells/total number of cells × 100). Statistical significance was calculated by paired t-test or by repeated measures analysis of variance with Dunnett’s post-test. Differences were considered significant when \( p < 0.05 \).

Fig. 1. Effect of bacterial DNA on human eosinophil apoptosis. Eosinophils were incubated in the absence or presence of bacterial or vertebrate DNA for 20 h (A–C, G) or for 40 h (D–F, H–I) after which apoptosis was determined by Annexin-V FITC binding assay (A–C, G), morphological analysis (D–F, H) or relative DNA fragmentation assay (I). In A–C and D–F shown are representative graphs of 6–8 experiments. Percentage of Annexin-V positive cells (FITC+/PI– and FITC+/PI+) is shown in the upper right corners of A–C. Where not stated, DNA concentration was 30 µg/ml. In G–I shown are mean ± SEM of cells isolated from six to eight donors. Asterisk * indicates \( p < 0.05 \) and ** \( p < 0.01 \).
3. Results

3.1. Effect of bacterial DNA on spontaneous eosinophil apoptosis

Human eosinophils undergo spontaneous apoptosis in the culture in the absence of life-supporting cytokines. An early sign of apoptosis, translocation of phosphatidylserine from the inner to the outer leaflet of the lipid bilayer in the cell membrane, was inhibited by bacterial DNA (30 μg/ml) but not by vertebrate DNA (30 μg/ml) as determined by Annexin-V binding assay (Fig. 1A–C, G). Similarly, treatment with bacterial DNA reduced the percentage of eosinophils with apoptotic morphology as compared to treatment with solvent or vertebrate DNA when measured after 40 h of incubation (Fig. 1D–F, H). In addition, treatment with bacterial DNA (30 μg/ml) led to reduction in the number of eosinophils with decreased relative DNA content indicative of reduction in the amount of fragmented DNA (Fig. 1I). In contrast, vertebrate DNA had no effect on DNA fragmentation (Fig. 1).

3.2. Effects of synthetic class A, B and C CpG ODNs on spontaneous eosinophil apoptosis

Bacterial but not vertebrate DNA inhibited human eosinophil apoptosis. To determine whether this is due to the CpG structure more prevalent in bacterial DNA, we tested the effects of class A, B and C CpG ODNs on eosinophil apoptosis. As determined by DNA fragmentation assay, class B CpG ODN 1018 inhibited apoptosis in a concentration-dependent manner, while non-CpG ODN 1040 had no effect (Fig. 2A–C, G, H). Similarly, treatment with 3 μM 1018 resulted in a reduction in the number of eosinophils with apoptotic morphology (Fig. 2D–F, I). Also the number of cells expressing phosphatidylserine on the outer leaflet of the cell (Annexin-V positive) was reduced by 19 ± 3% (p < 0.01, n = 6). Interestingly, however, treatment with non-CpG ODN 1040 reduced the amount of eosinophils with apoptotic morphology (Fig. 2I) and bound Annexin-V (15 ± 2% decrease in apoptosis, p < 0.01, n = 6), although no statistically significant effect occurred in DNA fragmentation assay. Treatment with another class B CpG ODN 2006 or class C CpG...
ODN C274 led to statistically significant reduction in apoptosis (Fig. 2G), whereas non-CpG ODN C661 had no effect. However, class A CpG ODN D19 or the corresponding non-CpG ODN Dc did not affect apoptosis rate of eosinophils (Fig. 2G). The most potent apoptosis-inhibiting CpG ODN 1018, and its corresponding non-CpG ODN 1040 were chosen for further studies.

3.3. Effect of CpG DNA on primary eosinophil necrosis

Inhibition of apoptosis may not only lead to increased cell survival but to induction of other forms of cell death such as cytolysis or primary necrosis of cells. In fact, cytolysis of eosinophils has been described in asthmatic airways [21]. Therefore we tested the effect of CpG ODN 1018 and non-CpG ODN 1040 on primary eosinophil necrosis by PI-staining after 1 h and 2 h incubation. PI can only enter cells with ruptured plasma membrane, which is a typical feature of a necrotic cell. Thereby, necrotic cells are typically positively stained by PI after a short incubation. At 1 h time-point, 1.6 ± 0.1% of untreated, 1.7 ± 0.2% of 1018-treated and 1.7 ± 0.1% of 1040-treated cells showed PI-positive staining (p > 0.05, n = 6). At 2 h timepoint, percentages of PI-positive cells were 1.6 ± 0.1%, 1.9 ± 0.1% and 1.7 ± 0.2% in the absence and presence of 1018 and 1040, respectively (p > 0.05, n = 6). These results suggest that CpG ODN 1018 or non-CpG ODN 1040 do not induce primary necrosis of eosinophils.

3.4. Role of contaminating cells in the anti-apoptotic effect of CpG DNA on eosinophils

There has been a debate over whether the effects of CpG DNA on some cell types are direct or indirect [20,22]. A small amount of contaminating pDC and B-lymphocytes may lead to false interpretations, as they produce high quantities of interferons and/or cytokines in response to CpG DNA. To evaluate this possibility we further purified our eosinophil preparations by using CD16-, CD19- and CD304-negative selection. On average, in anti-CD16 isolated eosinophils we found 0.020 ± 0.010% CD19+ cells and 0.001 ± 0.000% CD123+ CD303+ cells as assessed by flow cytometric analysis (n = 6). After extensive purification flow cytometric analysis revealed complete absence of CD19+ and CD123+ CD303+ cells (n = 6). In these highly purified eosinophil populations, CpG ODN 1018 reduced the proportion of apoptotic cells by 13 ± 3% (p < 0.01, n = 6) as determined by relative DNA fragmentation assay. However, the inhibiting effect of CpG ODN 1018 was somewhat larger in cells simultaneously prepared using CD16-negative selection only (22 ± 5%, p < 0.01, n = 6). Thus, CpG ODNs affect eosinophils directly in the absence of pDC and B-cells. However, in the presence of pDC and B-cells the effect of CpG ODNs may be more pronounced.

3.5. Toll-like receptor 9 expression in human eosinophils

To see whether eosinophils express TLR9 protein and to determine whether the expression is dependent on hypersensitivity, we conducted western blotting and flow cytometric analysis with eosinophils isolated from both healthy and atopic individuals. In western blotting, peripheral blood mononuclear cells served as a positive control. A band corresponding to the size of TLR9 was detected in eosinophil lysates derived from both healthy (data not shown) and atopic donors (Fig. 3A), and the band was not seen if the antibody was first incubated with the specific blocking peptide (Fig. 3A). Expression of TLR9 in eosinophils derived from both healthy and atopic individuals was confirmed by flow cytometric analysis (Fig. 3B and C).

3.6. Effect of CpG methylation on the anti-apoptotic action of bacterial DNA

Methylation of the cytosines in CpG motifs is believed to mask the ability of CpG DNA to activate TLR9. We conducted experiments to see whether the effect of bacterial DNA on eosinophil apoptosis is

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**Fig. 3.** TLR9 expression in human eosinophils. Expression was determined by western blotting (A) and intracellular flow cytometry (B–C) in eosinophils derived from atopic and healthy donors. In A and C shown is one typical experiment of three with similar results. In B, TLR9 expression determined by flow cytometric analysis is shown as mean ± SEM.
abrogated due to CpG methylation, which would support a role for TLR9. Surprisingly, CpG methylation of bacterial DNA did not result in loss of the anti-apoptotic effect (Fig. 4A). Methylated DNA was completely resistant to digestion by restriction endonuclease BstUI (Fig. 4B), indicating successful methylation. These results suggest that the apoptosis-delaying effect of bacterial DNA is not dependent on unmethylated CpG motifs.

3.7. Effect of inhibitors of endosomal acidification and inhibitory ODN on the anti-apoptotic action of CpG ODNs, non-CpG ODNs and bacterial DNA

To explore the role of TLR9 in the effects of CpG ODNs, non-CpG ODNs and bacterial DNA on eosinophils, we employed inhibitors of TLR9-activation pathway. Low endosomal pH is a prerequisite for the activation of intracellularly localized TLR9. Bafilomycin A1 and chloroquine are inhibitors of endosomal acidification with different mechanisms of action. Inhibitory oligodeoxynucleotides are a new, recently identified class of oligodeoxynucleotides, which have been reported to specifically inhibit TLR9 activation induced by CpG DNA [23]. Bafilomycin A1 totally abrogated the effects of 1018 (Fig. 5A) and bacterial DNA (Fig. 5D) on eosinophil apoptosis. Chloroquine also reduced the anti-apoptotic effect of 1018 in a concentration-dependent manner (Fig. 5B) but bacterial DNA induced statistically significant anti-apoptotic effect even in the presence of chloroquine (Fig. 5E). However, preincubation with inhibitory ODN completely abolished the effect of both 1018 (Fig. 5C) and bacterial DNA (Fig. 5F). Bafilomycin (100 nM), chloroquine (10 μM) and inhibitory ODN (10 μM) attenuated spontaneous apoptosis by 23 ± 4%, 10 ± 2% and 17 ± 6%, respectively. Overall, these results indicate that the anti-apoptotic effect of phosphorothioate-modified CpG ODN 1018 is dependent on intracellular TLR9. Evidence was also obtained for TLR9-dependent action of bacterial DNA.

To see, whether the occasionally occurring effects of non-CpG ODN 1040 were dependent on endosomal acidification, we studied the effect of 1040 on eosinophil apoptosis in the presence or absence of bafilomycin A1 and chloroquine. We included only those experiments where 1040 inhibited apoptosis as determined by DNA fragmentation assay. Treatment with 100 nM bafilomycin A1 or 10 μM chloroquine did not affect the anti-apoptotic effect of 1040 (data not shown). The result suggests that the effects of non-CpG on eosinophil apoptosis are not mediated by intracellularly localized TLR9.

3.8. Role of PI3K, NF-κB and MAPKs in the anti-apoptotic effect of CpG ODNs

PI3K, NF-κB and MAPKs have been previously described as mediators of TLR9 signalling pathway [10,24]. To see the involvement of these mediators in CpG DNA-activated pathway leading to delayed eosinophil apoptosis, we conducted experiments with pharmacological inhibitors of PI3K (wortmannin), NF-κB pathway (PDTC, MG-132 and BMS-345541) and MAPKs (SB203580, SP600125 and PD98059). Wortmannin (100 nM), PDTC, BMS-345541 and MG-132 (10 μM) suppressed the effect of CpG ODN 1018 on apoptosis (Fig. 6A–D), even though 1018 retained statistically significant anti-apoptotic effect in the presence of MG-132 (Fig. 6C). Wortmannin (100 nM) slightly enhanced spontaneous apoptosis by 1.2-fold (p < 0.01, n = 6) whereas 1.3–1.5-fold increase in spontaneous apoptosis was found after treatment with 10 μM PDTC, BMS-345541 or MG-132 (in each case p < 0.01, n = 6–13), as previously reported for inhibitors of NF-κB [25]. SB203580, SP600125 or PD98059 (10 μM) did not inhibit the anti-apoptotic effect of CpG ODN 1018 on eosinophils in a statistically significant manner (n = 5–7, data not shown).

3.9. Effect of CpG DNA on GM-CSF-induced eosinophil survival

Granulocyte macrophage-colony stimulating factor (GM-CSF) is an important eosinophil survival-increasing cytokine. Asthmatic patients have elevated levels of GM-CSF in their bronchoalveolar lavage (BAL) fluid [26], which may be a significant cause leading to eosinophilia. We studied next whether CpG ODN 1018 and bacterial DNA increase eosinophil survival in the presence of 10 pM GM-CSF. When apoptosis was measured by DNA fragmentation assay, GM-CSF-treatment decreased eosinophil apoptosis significantly after 40 h of incubation (Table 1). Bacterial DNA or CpG ODN 1018 had no further apoptosis-inhibiting effect in the presence of GM-CSF (Table 1).

Fig. 4. Effect of CpG methylation on the anti-apoptotic action of bacterial DNA. Eosinophils were incubated in the absence or presence of 30 μg/ml unmethylated or methylated bacterial DNA for 20 h after which apoptosis was determined by Annexin-V FITC binding assay (A). Asterisk ** indicates p < 0.01 as compared with the respective solvent control. Agarose gel electrophoresis of unmethylated and methylated bacterial DNA treated with restriction enzyme BstUI for 3 h (B). Lane 1: marker, lane 2: unmethylated DNA with 1 U BstUI/μg DNA, lane 3: unmethylated DNA with 10 U BstUI/μg DNA, lane 4: methylated DNA with 1 U BstUI/μg DNA, lane 5: methylated DNA with 10 U BstUI/μg DNA. Each lane contained 400 ng DNA.
3.10. Effect of CpG DNA on eosinophil apoptosis in the presence of a glucocorticoid

Glucocorticoids reduce the number of eosinophils in the airways of patients with asthma [27]. They enhance spontaneous eosinophil apoptosis [17,18] and partially reverse cytokine-induced eosinophil survival [28]. We used glucocorticoid budesonide to examine whether CpG DNA is able to reverse glucocorticoid-induced apoptosis of eosinophils. As determined by DNA fragmentation assay after 40 h of culture. Experiments were repeated 5–8 times with eosinophils derived from different individuals. Data is shown as mean ± SEM. Asterisk * indicates \( p < 0.05 \) and ** \( p < 0.01 \).

Fig. 5. Effects of inhibitors of endosomal acidification and inhibitory ODN on the anti-apoptotic action of CpG ODN 1018 and bacterial DNA. Eosinophils were pretreated with solvent or indicated concentrations of bafilomycin (A, D) or chloroquine (B, E) for 20 min or with inhibitory ODN (C, F) for 30 min, after which solvent, CpG ODN 1018 (3 \( \mu \)M) or bacterial DNA (30 \( \mu \)g/ml) was added. Apoptosis was measured by DNA fragmentation assay after 40 h of culture. Experiments were repeated 5–8 times with eosinophils derived from different individuals. Data is shown as mean ± SEM. Asterisk * indicates \( p < 0.05 \) and ** \( p < 0.01 \).

Fig. 6. Effects of inhibitors of NF-\( \kappa \)B pathway (A–C) and PI3K (D) on the anti-apoptotic action of CpG ODN 1018. Eosinophils were incubated with solvent or CpG ODN 1018 (3 \( \mu \)M) for 40 h after 1 h pre-treatment with solvent or BMS-345541 (A), PDTC (B) or MG-132 (C) or after 20 min pre-treatment with solvent or wortmannin (D). Apoptosis was determined by relative DNA fragmentation assay. Mean ± SEM of 6–13 experiments with eosinophils from different donors is represented. Asterisk * indicates \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).
a statistically significant manner as previously described (Table 2) [17]. Bacterial DNA and CpG ODN 1018 had no effect in the presence of budesonide (Table 2).

### 4. Discussion

In the present study we showed that bacterial DNA as well as synthetic class B and C CpG ODNs but not vertebrate DNA delay spontaneous apoptosis of human peripheral blood eosinophils in vitro. Additionally, we found TLR9 expression in eosinophils derived from both atopic and healthy individuals. Our results suggest a mechanism dependent on TLR9, PI3K and NF-kB to explain the effects of phosphorothioate-modified CpG ODNs on eosinophil apoptosis. We also showed that unmethylated CpG dinucleotides are not essential for the anti-apoptotic effect of native bacterial DNA in eosinophils suggesting presence of a novel immunostimulatory pattern in bacterial DNA lacking from vertebrate DNA.

Bacterial DNA and CpG ODN were found to reduce spontaneous eosinophil apoptosis maximally by approximately 20%. The size of this effect is smaller than that of the survival-prolonging cytokines IL-3, IL-5 or GM-CSF, but is largely similar to the well characterized effects of β2-agonists and other cyclic AMP elevating agents on human eosinophils [4,29,30].

In the current study, CpG ODN 1018 reduced apoptosis of extensively purified eosinophils, although to a somewhat lesser extent than that of normally purified eosinophils. This result excludes the possibility that the effect is dependent on contaminating B-cells and pDC, which are known to be highly activated by CpG ODNs [20] but suggests that the presence of these contaminating cells may further augment the apoptosis-delaying effect of CpG ODNs on eosinophils. Direct response of eosinophils to CpG ODNs is supported by our finding that eosinophils express TLR9 protein as shown by western blotting and flow cytometric analysis. This is in concordance with a recent report [16]. In addition, human eosinophils have been previously demonstrated to express TLR9 mRNA [15]. Furthermore, treatment with CpG ODNs has been found to resist apoptosis in other TLR9-expressing cells such as pDC [31], B-cells [32] and neutrophils [33]. Interestingly, survival of neutrophils was prolonged with a 10–20-fold lower concentration of bacterial DNA [33] as compared to that found in eosinophils (present study), which may reflect more essential role of neutrophils in the first-line host defence against bacteria as compared to eosinophils.

We found TLR9 expression in eosinophils derived from both healthy and allergic donors indicating that TLR9 expression is not induced by hypersensitivity. It is thought that TLR9 is an intracellular receptor requiring acidic endosomal pH for its activation [34]. Bafilomycin A1, a highly specific inhibitor of vacuolar type H+-ATPase, ablated the effect of CpG ODNs and bacterial DNA on eosinophil apoptosis completely. In contrast chloroquine, which acts as a weak base and accumulates inside endosomes neutralizing their pH [35], reversed most of the effect of synthetic CpG ODNs but not that of bacterial DNA. One possibility is that this inconsistency is due to interaction of DNA with chloroquine [36], which may be influenced by backbone differences. The result may also indicate differences in endosomic processing of bacterial DNA and phosphorothioate-modified CpG ODNs. Inhibitory ODNs have been reported to inhibit TLR9 activation induced by CpG ODNs very recently. Inhibitory ODNs were shown to bind TLR9 with similar affinity to CpG DNA. However, unlike stimulatory CpG DNA, inhibitory ODN did not induce conformational change in TLR9 required for its activation [23]. Preincubation of eosinophils with inhibitory ODNs completely reversed the effect of CpG ODNs and bacterial DNA on apoptosis. Altogether, these results combined with the observation of TLR9 expression in eosinophils suggest that intracellularly localized TLR9 mediates the anti-apoptotic action of synthetic CpG ODNs in human eosinophils. Evidence was obtained also for the involvement of TLR9 in the suppression of eosinophil apoptosis by bacterial DNA but further studies are needed to strengthen this finding.

In this study, treatment with non-CpG ODN 1040 had inhibitory effect on spontaneous apoptosis of eosinophils in some occasions. In contrast to the actions of CpG ODNs, the effect of 1040 was not dependent on endosomal acidification, which is critical for the activation of intracellular TLR9 [34]. Phosphorothioate backbone, where one of the non-bridging oxygens at each phosphodiester linkage is replaced by a sulphur atom, has been previously reported to exert sequence-independent activity on several cellular events [37,38]. Stimulation with phosphorothioate backbone has led to the activation of Jak2 [37], which is a critical component in the survival-promoting effects of IL-5, IL-3 and GM-CSF on eosinophils [39]. It is tempting to speculate that the weak inhibitory effects of non-CpG ODNs are due to TLR9-independent activation of Jak2.

Several previous studies have shown that bacterial DNA stimulates leukocyte populations via a mechanism dependent on unmethylated CpG dinucleotides and TLR9 [33,40,41]. However, recently many groups have demonstrated TLR9 activation independently from unmethylated CpG motifs [42,43]. Also CpG- and TLR9-independent immunostimulatory effects induced by bacterial but not vertebrate DNA have been demonstrated [44]. This suggests that the recognition of bacterial DNA by the host cell may not be as simple as previously thought. Unmethylated CpG motifs may not be the only TLR9-activating pattern in bacterial DNA or also other sensors distinguishing bacterial DNA from host DNA may exist. Our results support the view that immune cells discriminate bacterial DNA from vertebrate DNA also via another molecular pattern than unmethylated CpG motifs. The results with the inhibitors of TLR9-activation pathway suggest involvement of TLR9 in the recognition process of bacterial DNA but we cannot completely rule out other, currently unknown receptors or cytosolic sensors. In previous studies, sequences activating TLR9 in a CpG-independent manner have contained phosphorothioate backbone and 5′-TC dinucleotide in a thymidine-rich background or modified nucleotides with a bicyclic heterobase in the place of C in CpG [42,43,45]. CpG-methylated DNA with phosphodiester backbone has also been

### Table 1

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<tr>
<th>Conditions</th>
<th>Mean ± SEM</th>
<th>Apoptotic cells (%)</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>57 ± 4</td>
<td>9 ± 2***</td>
</tr>
<tr>
<td>GM-CSF 10 pM</td>
<td>9 ± 1</td>
<td></td>
</tr>
<tr>
<td>GM-CSF 10 pM + Bacterial DNA 30 μg/ml</td>
<td>9 ± 1</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>41 ± 5</td>
<td></td>
</tr>
<tr>
<td>GM-CSF 10 pM</td>
<td>9 ± 1**</td>
<td></td>
</tr>
<tr>
<td>GM-CSF 10 pM + CpG ODN 1018 3 μM</td>
<td>10 ± 1</td>
<td></td>
</tr>
<tr>
<td>GM-CSF 10 pM + Non-CpG ODN 1040 3 μM</td>
<td>10 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

N = 5–6, p < 0.01**, p < 0.001*** as compared to untreated cells.

### Table 2

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean ± SEM</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>58 ± 4</td>
<td></td>
</tr>
<tr>
<td>Budesonide 1 μM</td>
<td>70 ± 3*</td>
<td></td>
</tr>
<tr>
<td>Budesonide 1 μM + Bacterial DNA 30 μg/ml</td>
<td>63 ± 2</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>59 ± 7</td>
<td></td>
</tr>
<tr>
<td>Budesonide 1 μM</td>
<td>76 ± 7**</td>
<td></td>
</tr>
<tr>
<td>Budesonide 1 μM + CpG ODN 1018 3 μM</td>
<td>72 ± 9</td>
<td></td>
</tr>
<tr>
<td>Budesonide 1 μM + Non-CpG ODN 1040 3 μM</td>
<td>75 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

N = 5–6, p < 0.05*, p < 0.01** versus untreated eosinophils.
shown to interact with TLR9, however, the interaction was weaker and led to reduced NF-κB activity when compared to unmethylated CpG DNA [34]. Interestingly, Hartmann et al. demonstrated that certain active CpG sequences with proteasome backbone lose their activity when phosphorothioate modified [46]. This suggests that backbone differences lead to different sequence and structure requirements for TLR9 activation. Stiffer phosphorothioate backbone with limited conformations available may have stricter sequence requirements for TLR9 activation as compared to phosphodiester DNA. Altogether, sequence requirements for TLR9 activation seem not to be limited to unmethylated CpG and may be critically influenced by the backbone.

PI3K has been previously described to play an important role in eosi
nophil survival in vivo [47]. In this study, the effect of CpG ODNs on eosi
nophil apoptosis was dependent on member(s) of PI3K family, since it was completely reversed by PI3K inhibitor wort-
mannin. The finding is consistent with the results of a previous study, where regulation of vesicular trafficking concerning TLR9 and CpG DNA was suggested to include PI3K [24]. In dendritic cells, CpG DNA was reported to induce phosphorylation of Akt, which is a known downstream target of PI3K [48].

Activation of transcription factor NF-κB is a critical survival-
prolonging mechanism in eosinophils and many other immune cell types [25]. Stimulation of TLR9 has been described to result in activation of NF-κB [10]. Consistent with this, PDTC and BMS-345541, inhibitors of NF-κB and IkB kinase, respectively, blocked completely the anti-apoptotic effect of CpG ODNs on eosinophils. Also proteasome inhibitor MG-132 which inhibits degradation of IkB, an inhibitory subunit of NF-κB, suppressed the anti-apoptotic effect of CpG ODNs. Taken together, our data suggests a cascade where activation of TLR9 by CpG ODNs leads to activation of PI3K – NF-κB pathway resulting in prolonged survival of human eosinophils. MAPKs seem not to be involved in the action of CpG ODNs in eosinophils. Interestingly, NF-κB and PI3K have been demonstrated to act in concert in several studies. The following mechanisms have been suggested: 1) PI3K-activated Akt enhances degradation of IkB and nuclear translocation of NF-κB by inducing IkB kinase phosphorylation and activation [49], 2) PI3K-Akt axis is not involved in the nuclear translocation of NF-κB but induces Ser536 phosphorylation of the p65 subunit of NF-κB thus increasing its transcriptional activity [50]. Whether these mechanisms are involved in the action of CpG ODNs in eosinophils remains to be determined.

CpG ODNs have exerted anti-inflammatory effects in murine models of asthma and allergy [12,13] but has shown contradictory results in the first clinical trials [51,52]. The present data suggests that treatment with CpG ODNs may lead to exacerbation of eosinophilic inflammation and that particular carefulness should be followed when administrating CpG ODNs to humans.

Pro-inflammatory action of bacterial DNA has been described in mice. Administration of bacterial DNA resulted in significant accumulation of polymorphonuclear leukocytes in the lower respiratory tract suggesting that bacterial DNA may play a patho-
genic role in inflammatory lung diseases [11]. In humans, evidence exists of the role of bacterial infection in the exacerbation of asthma and atopic dermatitis [6,53]. Accumulation of appropriate amounts of bacterial DNA in the infection site is essential for the biological significance of the current results. In the study of Schwartz et al. up to 20 μg/ml bacterial DNA was found from sputum of patients with cystic fibrosis [11]. The anti-apoptotic effect of bacterial DNA on eosinophils occurred between concentrations of 10 μg/ml and 30 μg/ml, suggesting biological relevance. According to the present findings direct contact between bacterial DNA and eosinophils leads to prolonged eosinophil lifespan which presents a novel mechanism of asthma exacerbation during bacterial respiratory tract infection.

In summary, the current results suggest that phosphorothioate-modified CpG ODNs inhibit human eosinophil apoptosis through a pathway involving TLR9, PI3K and NF-κB. We also showed that bacterial but not vertebrate DNA suppresses eosinophil apoptosis but the effect is independent of unmethylated CpG motifs. This indicates existence of a novel, bacterial DNA-specific recognition pattern lacking from vertebrate DNA. The present study revealed an additional pro-inflammatory action of bacterial DNA in vitro, which may have clinical relevance in contributing exacerbation of eosino-
philic lung disease such as asthma.

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