

Received Date : 21-Aug-2015

Revised Date : 11-Sep-2015

Accepted Date : 21-Sep-2015

Article type : Original Article: Experimental Allergy and Immunology

Editor : Hans-Uwe Simon

Osteopontin binds and modulate functions of eosinophil-recruiting chemokines

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Short title: Osteopontin modulates chemokine functions

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12771

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ABSTRACT

Background: Allergic asthma is characterized by eosinophilic inflammation and airway obstruction. There is also an increased risk of pulmonary infection caused by *Streptococcus pneumoniae*, in particular during severe asthma where high levels of the glycoprotein, osteopontin (OPN) are present in the airways. Eosinophils can be recruited by chemokines activating the receptor CCR3 including eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, RANTES/CCL5, and MEC/CCL28. In addition to inducing chemotaxis, several of these molecules have defensin-like antibacterial properties. This study set out to elucidate the functional consequences of OPN-binding to eosinophil-recruiting chemokines.

Methods: Antibacterial activities of the chemokines were investigated using viable count assays and electron microscopy. Binding studies were performed by means of surface plasmon resonance. The potential interference of OPN with antibacterial, receptor-activating, and lipopolysaccharide-neutralizing abilities of these chemokines was investigated.

Results: We found that OPN bound all eosinophil-recruiting chemokines with high affinity except for CCL5. The eosinophil-recruiting chemokines all displayed bactericidal activity against *S. pneumoniae*, but only CCL26 and CCL28 retained high antibacterial activity in the presence of sodium chloride at physiologic concentrations. Preincubation of the chemokines with OPN strongly inhibited their antibacterial activity against *S. pneumoniae* but did not affect their ability to activate CCR3. All chemokines investigated showed LPS-neutralizing activity that was impaired by OPN only in the case of CCL24.

Conclusions: The data suggest that OPN may impair host defense activities of the chemokines without affecting their eosinophil-recruiting properties. This could be one mechanism explaining the increased vulnerability to acquire pneumococcal infection in parallel with sustained allergic inflammation in asthma.

Keywords: allergy; asthma; chemokines; eosinophils; osteopontin

Introduction

Asthma is a chronic inflammatory disease of the airways, affecting and disabling a large number of individuals worldwide. The disease is mediated by the up-regulation of multiple pro-inflammatory proteins, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and cell surface receptors (1). In allergic asthma, the inflammation is characterized by production of Th2 cytokines including TSLP, IL-4, IL-5, and IL-13 (2). Subsequently, several chemokines are produced that bind and activate the CCR3 receptor on eosinophils promoting influx of these cells to the airways, in particular eotaxin-1/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26 but also MEC/CCL28 and RANTES/CCL5 (3, 4). Several chemokines have been described to possess antimicrobial properties (5). Similarly, several of the eosinophil-recruiting chemokines have defensin-like antibacterial properties and may therefore contribute to airway host defense during allergic inflammation (6-8).

Osteopontin (OPN) is an anionic, phosphorylated glycoprotein first described as a protein of the bone matrix (9). In recent years, high levels of OPN have been detected in the airways of patients suffering from asthma, increasing with disease severity (10, 11). OPN can activate neutrophils and in an animal model, instillation of OPN to the airways resulted in recruitment of neutrophils (12-14). OPN may also play important roles during allergic inflammation. In a mouse model of allergic asthma using

ovalbumin as allergen, OPN exerted pro-inflammatory activity during sensitization and had anti-inflammatory effects during antigenic challenge (15).

Several recent studies have shown an association between asthma and susceptibility to respiratory infections, particularly those caused by *Streptococcus pneumoniae* (16). The risk of invasive pneumococcal disease (IPD) in patients suffering from allergic asthma is increased as much as 5-10 fold compared with healthy individuals (16, 17). The underlying cause is unknown but the dysregulated inflammatory response seen in asthma is likely to contribute to the vulnerability. Interestingly, it was shown *in vivo* that mice deficient in OPN had improved resistance to pneumococcal infection (18). It was suggested that the mechanism could be that OPN bind and stabilize the bacterial cell wall and membrane, thus promoting bacterial survival.

In this study, we investigated whether OPN affects the antibacterial and receptor-activating properties of eosinophil-recruiting chemokines, possibly impairing host defense and increasing the vulnerability to acquire pneumococcal infection.

Materials and methods

Special reagents

Recombinant human RANTES/CCL5, eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, MEC/CCL28, and OPN were purchased from Peprotech, London, UK. TTK25 and GSD35 were synthesized using fmoc-chemistry by Schafer-N, Copenhagen, Denmark.

Surface plasmon resonance spectrometry

Human OPN was immobilized via amine coupling in flow cells of a CM5 sensorchip (GE Healthcare, Uppsala, Sweden) at approximately 400 resonance units. A flow cell subjected to the coupling

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reaction without added protein was used as a control for bulk refraction index changes. The analyte was injected in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.005% surfactant P20, and 3.4 mM EDTA) at different concentrations over the coated surface. Following peptide injection, the coated surfaces were regenerated by injection of 0.5 M NaCl followed by a wash procedure. After X and Y normalization of data, the blank curves from the control flow cell of each injected concentration were subtracted. The association (K_A) and dissociation (K_D) rate constants were determined simultaneously using the equation for 1:1 Langmuir binding in the BIA Evaluation 3.1 software (GE Healthcare). The binding curves were fitted locally, and the equilibrium dissociation constants (K_D) were obtained from mean values of the rate constants.

Bacteria and growth condition

The *Streptococcus pneumoniae* strain TIGR4 is a clinical encapsulated isolate of serotype 4 sequenced by The Institute for Genomic Research TIGR (ATCC BAA-334; Rockville, MD; <http://www.tigr.org>). The strain was routinely grown in Todd-Hewitt (TH; Difco/Becton & Dickinson, Franklin Lakes, NJ) broth supplemented with 0.5% yeast extract (THY) liquid medium or on blood-agar plates in 5% CO₂ and at 37°C.

Bacterial killing Assay

All bacteria were cultivated to mid-logarithmic phase (optical density at 620 nm was 0.4) in broth, washed, and diluted (1:1,000) in incubation buffer (10 mM Tris-HCl, containing 5 mM glucose, pH 7.4). 50 µl of bacteria (106 colony forming units (cfu)/ml) were incubated in the absence or presence of chemokines at various concentrations, (0.01-1 µM) for one hour at 37°C. To quantify antibacterial activity, serial dilutions of the incubation mixtures were plated on blood agar plates, incubated overnight at 37°C, and the number of cfu determined. In order to investigate the antibacterial activity of the chemokines in the presence of OPN, the same procedure was followed with preincubations of

chemokine and OPN at different ratios performed for 20 minutes at room temperature prior to their addition to bacteria.

Scanning electron microscopy

For scanning electron microscopy (SEM), bacteria were incubated at 37°C for 1 hour in buffer alone, with OPN (1 μ M), with CCL5, CCL11, CCL24, CCL26, and CCL28 (all at 1 μ M), or with the chemokines separately preincubated with OPN at equimolar concentrations for 20 minutes. To verify bacterial killing, part of the sample was used for the viable count assay described above. The samples were fixed in buffered glutaraldehyde (0.5%), processed for SEM and examined in an XL 30 FEG scanning electron microscope (Philips, Eindhoven, The Netherlands).

Negative staining

To visualize the molecular interaction of OPN with chemokines in the bacterial context, negative staining and transmission electron microscopy were used. In short, CCL26 and OPN were conjugated with 5 nm and 20 nm colloidal gold particles, respectively. The conjugated peptides were incubated with bacteria for 60 min at 37°C and negatively stained with 0.75% uranyl formate. Samples were examined in a Philips/FEI CM100 BioTwin transmission electron microscope (Philips).

Endotoxin-neutralizing effect

THP-1 cells stably transfected with a reporter plasmid expressing a secreted embryonic alkaline phosphatase gene (Invivogen, San Diego, CA) were cultured in RPMI supplemented with 10% FBS, L-glutamine, and Pen-Strep (50 U/ml/50 μ g/ml). For selection, the cells were cultured with the addition of zeocin (200 μ g/ml) and G418 (1 mg/ml). 20 μ l of peptide sample (50 μ M) that was pre-incubated with LPS (100 ng/ml) at room temperature for 30 min was added per well, including LPS

and PBS as positive and negative controls, respectively. 180 μ l of cells were suspended in pre-warmed growth medium, loaded at 200,000 cells/well of a flat bottom 96-well cell culture plate (Nunc, Glostrup, Denmark) and incubated at 37°C in a CO₂ incubator for 18-24 hours. Following overnight incubation, 20 μ l of THP-1 cells supernatant was mixed with 180 μ l QUANTI-Blue substrate (InvivoGen) per well of the 96-well plate and incubated at 37°C followed by measurement in a spectrophotometer at a wavelength of 640 nm.

Calcium mobilization assay

The assay was performed essentially as previously described (8).

Measurement of eosinophil chemotaxis

Eosinophils were purified from peripheral blood via negative selection as per the manufacturer's instructions (MACSxpress® human Eosinophil Isolation Kit; MiltenyiBiotec, Auburn, CA). After hypotonic lysis of erythrocytes, eosinophils were washed in PBS supplemented with 2% FBS and 1mM EDTA and resuspended in RPMI supplemented with 25mM HEPES and 0.1% BSA (running buffer) at a concentration of 2.5×10^6 cells/ml. Chemotaxis was carried out using a modified Boyden chamber. Briefly, the lower chamber of a CHEMOTx® plate (#101-5, Neuroprobe Inc, MD) was blocked with RPMI supplemented with 25 mM HEPES and 1% BSA for 30 minutes at room temperature (blocking buffer). After aspirating the blocking buffer, CCL26 and/or OPN was added to the wells in duplicate, using a range of dilutions prepared in the running buffer. The filter membrane (5 μ m pores) was placed on top and 20 μ l of the eosinophil suspension (50,000 cells/well) was placed on top of the filter membrane. The entire chamber was then incubated for 2 hours at 37°C, 5% CO₂ in a humidified box. Cells that migrated through the membrane were spun into a 96 well opaque white plate by centrifugation at 300g for 5 minutes with the use of a funnel plate (Neuroprobe). The chemotactic signals were determined by the addition of an equal volume of the ATP-sensitive dye

CellTiter-Glo® (Promega, Madison, WI, USA) followed by luminescence measurements on a TopCount (Packard Bioscience, CT). Chemotactic indices (CI) were obtained by dividing the migratory signal induced by chemokine with the basal migratory signal obtained with media alone. The means of duplicates were calculated for data points, with the experiment repeated four times using eosinophils from four different donors.

Molecular modeling

Structural models of Th2 chemokines were prepared using the package PyMOL version 1.7.4 (<http://www.pymol.org>). The positively charged arginines and lysines are highlighted in blue and orange, respectively.

Statistics

Statistical significances were calculated by multiple *t*-test using GraphPad Prism 6 (GraphPad Software, San Diego, CA). All values are displayed as means $\pm$ SD and P-values < 0.05 were considered to be significant.

Results

OPN binding to eosinophil-recruiting chemokines

To investigate if OPN binds eosinophil-recruiting chemokines (i.e. CCL11, CCL24, CCL26, CCL5, and CCL28), OPN was immobilized to a biosensor chip by means of amino coupling. Varying concentrations of the chemokines were injected over the biosensor surface and association and

dissociation kinetics were measured (Figure 1). Analysis of the sensograms revealed a dose-dependent binding affinity for eosinophil-recruiting chemokines, but insignificant binding to CCL5.

Bactericidal activity of eosinophil-recruiting chemokines and modulation by OPN

The antibacterial activity of the eosinophil-recruiting chemokines (i.e. CCL5, CCL11, CCL24, CCL26, and CCL28) against *S. pneumoniae* (TIGR4 strain) was investigated using a viable counts assay. Bacteria grown to mid-logarithmic phase were incubated with the chemokines at concentrations ranging from 0.01-1 μ M for 60 minutes followed by plating on agar over night. The number of cfu obtained after incubation with chemokines were compared with those obtained after incubation in buffer alone and expressed as percent killing. The eotaxins (CCL11, CCL24, and CCL26), as well as CCL28 showed significant bactericidal activity while CCL5 was less active. The salt concentration in the periciliary liquid covering the surface of bronchial epithelial cells has been a matter of debate and a low salt concentration was previously suggested (19). Recently, a salt concentration closer to that of plasma (150 mM) was recently demonstrated (20). Therefore, the antibacterial activity was also investigated in the presence of sodium chloride at a concentration of 150 mM conditions under which only CCL26 and CCL28 retained high antibacterial activity (Figure 2).

To investigate if OPN interferes with the bactericidal activity of the chemokines investigated, the viable counts assay against *S. pneumoniae* was used as described above. Chemokines alone (1 μ M) or preincubated with OPN at ratios of 1:0.1 or 1:1 respectively, were incubated with bacteria for one hour followed by viable counts. OPN caused a dose-dependent inhibition of the bactericidal activity in the case of all peptides investigated. However, in the presence of sodium chloride (150 mM), low or no antibacterial activity was seen for CCL11, CCL24, and CCL5 while a dose-dependent inhibition from addition of OPN remained for CCL26 and CCL28 respectively (Figure 3A).

Using scanning electron microscopy, the morphology of bacteria was investigated (Figure 3B). *S. pneumoniae* incubated with chemokines showed protrusions and lost integrity which was reflected as

killing in parallel viable counts experiments. Preincubation of chemokines with OPN at equimolar ratios protected the bacteria that remained intact and had an appearance similar to that of bacteria incubated in buffer alone.

To investigate interactions between a chemokine and OPN at the bacterial surface, CCL26 and OPN were labeled with colloidal gold of different sizes, incubated with *S. pneumoniae*, followed by negative staining and transmission electron microscopy (Figure 3C). In experiments where CCL26 alone was incubated with bacteria, the chemokine was found associated with the bacterial cell wall and membrane. Upon pre-incubation, CCL26 and OPN co-localized at the bacterial surface and the bacteria remained intact.

OPN binds a COOH-terminal but not NH₂-terminal fragment of CCL26

Mast cells are effector cells during allergic inflammation and release serine proteases chymase and tryptase. Chymase cleaves CCL26, resulting in a 25 amino acid fragment spanning the COOH-terminal region (TTK25), while tryptase cleaves the molecule resulting in a fragment spanning the NH₂-terminal region (GSD35) (Figure 4) (8). TTK25 has bactericidal properties whereas GSD35 has LPS-neutralizing properties. Using surface plasmon resonance, we observed that TTK25 but not GSD35 bound OPN with high affinity (Figure 4).

Effect from OPN on LPS-neutralizing activity of the chemokines

LPS-neutralizing activity has been demonstrated for CCL11, CCL24, and CCL26 (8). To ascertain if this is also the case for CCL5 and CCL28, a monocytic cell line transfected with an NFκ-B responsive reporter gene was used. CCL28 showed strong LPS-neutralizing activity while CCL5 showed no activity in this respect (Figure 5). Preincubation of the chemokines with equimolar amounts of OPN only showed significant inhibition of the LPS-neutralizing activity in the case of CCL24.

The eosinophil-recruiting chemokines are known to signal through CCR3, leading to cellular activation, for example, chemotaxis of eosinophils (3). Intracellular signaling of CCR3 includes mobilization of calcium, which can be measured by means of flow cytometry using calcium sensitive probes. A murine cell-line stably expressing CCR3 was loaded with calcium-sensitive Fluo-4 and Fura red followed by stimulation with CCL5, CCL11, CCL24, CCL26 and CCL28 (all at 1, 10 and 100 nM). All induced calcium mobilization at these concentrations with the exception of CCL28 (Suppl. Figure 1). In a previous publication, 0.3 μ M of CCL28 induced chemotaxis in CCR3 transfectants but a dose-plateau was not reached (21). Thus, we investigated increasing concentrations of CCL28 reaching 1 μ M but without detecting any calcium mobilization. To investigate if OPN interferes with CCR3-activation, the active chemokines were preincubated with OPN (at ratios of 1:1 and 1:10 respectively) followed by stimulation of the cells (Suppl. Figure 2). There was no significant reduction in calcium flux following preincubation with OPN, indicating that this does not interfere with the ability of the ligands to induce CCR3 activation. Furthermore, chemotactic experiments with primary eosinophils, CCL26 induced chemotaxis whereas OPN alone lacked chemotactic activity (Suppl. Figure 2). Nor did co-incubation of OPN interfere with chemotaxis induced by CCL26 in eosinophils (not shown).

Discussion

In this study, we show that OPN binds eosinophil-recruiting chemokines, impairing their defensin-like antibacterial properties against *Streptococcus pneumoniae* without interfering with their receptor-activating properties. This interaction may, at least in part, explain the increased vulnerability to acquire pneumococcal infection during asthma, a risk that increases in parallel with increased OPN expression in the airways.

The different physiologic roles of OPN are intriguing since this molecule acts as a matrix protein of bone, is present at high concentrations in breast milk, and has cytokine functions (22). The biophysical characteristics of OPN are striking, being highly anionic with a pI close to 4 on an amino acid level (Suppl. Table 1). In addition, it is subject to extensive post-translational modifications, including addition of sialic acid containing carbohydrates and phosphorylation, altogether further adding to its negative charge (16). Most chemokines are highly cationic and it is likely that the interaction between chemokines and OPN is electrostatic in nature, which is supported by the findings of the present study using surface plasmon resonance.

Several cell surface-expressed receptors for OPN have been identified including CD44, $\alpha 9\beta 4$, $\alpha 4\beta 1$, and $\alpha 9\beta 1$, the latter, when expressed on neutrophils, resulting in chemotaxis (13). In a recent study, we found no binding of OPN to the neutrophil-recruiting CXC-chemokines GRO- β /CXCL2, GCP-2/CXCL6, or IL-8/CXCL8 nor did it reduce the receptor-activating properties of these chemokines (23). Furthermore, influx of neutrophils after OPN-instillation was seen in non-inflamed airways of mice and in ozone-induced lung injury, BALF neutrophils were significantly reduced in OPN-deficient compared with wild-type mice (12, 14). The roles of OPN during allergic inflammation are complex, being proinflammatory during sensitization and anti-inflammatory during allergen challenge respectively through the regulation of different subsets of dendritic cells (15). Preformed OPN is stored in the cytoplasmic granules of eosinophils and may be released from these cells at sites of inflammation (24). In addition, OPN has been reported to induce chemotaxis in eosinophils (25, 26). This is in contrast to our finding where we could not detect any chemotactic response in primary eosinophils by OPN alone. OPN can bind and cross-link $\alpha 4\beta 1$ integrins on eosinophils through an RGD-motif (8). One explanation for the lack of activity from OPN alone in this study could be that the molecule can undergo polymerization, thereby cross-linking integrins (Nishimichi, 2011). Thus, OPN from different sources may have different propensity to polymerize.

Both constitutive and induced innate antibiotics (AMPs) contribute to airway host defense. The expression of most induced AMPs is promoted by Th1-cytokines (27). Structurally, the eosinophil-recruiting chemokines have defensin-like properties including three-stranded β -sheet and

an overlying α -helix and two adjacent cysteine residues close to the N-terminus, and may serve as defensin-like AMPs during a Th2-profiled inflammation in the airways (Figure 4 and Suppl. Table 1) (28). Recently, we found that CCL26 has potent bactericidal activity against a broad range of airway pathogens (8). An important study, supporting the findings of the current study, showed that OPN impaired host defense against pneumococcal infection of the airways, comparing wild-type and OPN knockout mice (18).

Why does OPN inhibit the antibacterial activity of the chemokines investigated but not the receptor-activation and only weakly impair the LPS-neutralizing properties? Most of the cationic charge of the chemokines is localized to the amphipathic and α -helical COOH-terminal, which is most likely to associate with anionic regions of OPN (Figure 4). The antibacterial activity of CCL26 has been mapped to the COOH-terminal region while the NH₂-terminal harbors the LPS-neutralizing properties (8). In addition, the NH₂-terminal domain of CCL26 is important for the activation of CCR3 (29). This distribution of the functions to the NH₂- and COOH-terminal domains may also be the case for the other chemokines investigated and can explain why binding to OPN mainly impairs their antibacterial properties. Recently, a soluble poxvirus-encoded protein, named viral CC chemokine inhibitor, was shown to bind through electrostatic interactions with CCL11. However, the functional consequences thereof were not investigated (30).

The findings presented in this study may also have bearing in other diseases, such as cancer. In colorectal carcinoma, patients with a high number of tumor-infiltrating eosinophils have a better prognosis (31). On the other hand, high expression of OPN is seen in many neoplastic diseases and possible interactions between eosinophil-recruiting chemokines and OPN may occur, thus affecting disease progression (32).

Is OPN a potential pharmaceutical target in asthma? Corticosteroids are commonly used to treat asthma. In an animal model of allergic asthma, corticosteroids inhibited OPN production but future targets should be more specific, circumventing the side effects of corticosteroids (33).

Taken together, this study shows that OPN binds and modulate the functions of eosinophil-recruiting chemokines, resulting in an altered inflammatory response and impaired activity of these molecules as innate antibiotics.

Acknowledgments

We thank Pia Andersson and Maria Baumgarten for excellent technical assistance.

This work was supported by the Swedish Research Council, the Swedish Heart and Lung Foundation, the Medical Faculty of Lund University, Swedish Government Funds for Clinical Research (ALF), the Swedish Foundation for Strategic Research, Fysiografen Sällskapet, and the foundations of Th. C. Bergh, and Alfred Österlund.

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Figure legends

Fig. 1. OPN-binding to eosinophil-recruiting chemokines. In order to investigate OPN binding to eosinophil-recruiting chemokines, surface plasmon resonance was used. OPN was immobilized via amine coupling to a sensorchip and the analyte (chemokine) was injected at concentrations ranging from 31.25 - 500 nM over the coated surface. The binding curves were fitted locally to obtain the equilibrium dissociation constants (KD).

Fig. 2. Bactericidal properties of the eosinophil-recruiting chemokines. The antibacterial activity of the eosinophil-recruiting chemokines (i.e. CCL5, CCL11, CCL24, CCL26, and CCL28) against *Streptococcus pneumoniae* was investigated using a viable counts assay in the absence and presence of sodium chloride (NaCl at 150 mM). The number of bacterial colonies (cfu) obtained after incubation with chemokines were compared with those obtained after incubation in buffer alone and expressed as percent killing. The eotaxins (CCL11, CCL24, and CCL26) as well as CCL28 showed high bactericidal activity while CCL5 was less active. In the presence of sodium chloride, only CCL26 and CCL28 retained high antibacterial activity. The data represent mean and SD from three separate experiments. ***P<0.001.

Fig. 3. OPN impairs the bactericidal activity of eosinophil-recruiting chemokines.

A. To investigate if OPN interferes with the bactericidal activity of the chemokines investigated, a viable counts assay against *S. pneumoniae* was used as described above. Chemokines alone (1 μ M) or preincubated with OPN at ratios of 1:0.1 or 1:1 were incubated with bacteria for one hour followed by viable counts. OPN caused a dose-dependent inhibition of the bactericidal activity in the case of all peptides investigated. However, at physiologic salt conditions (NaCl at 150 mM), only weak antibacterial activity was seen for CCL11, CCL24, and CCL5 while a dose-dependent inhibition after

addition of OPN remained for CCL26 and CCL28 respectively. The data represent mean and SD from three separate experiments. * $P < 0.05$ and *** $P < 0.001$.

B. To investigate the morphology of bacteria, scanning electron microscopy was used. *S. pneumoniae* was incubated in buffer alone, in the presence of chemokines (1 μM), or with chemokines that had been preincubated with equimolar amounts of OPN for 20 minutes. To verify bacterial killing, part of the sample was used for the viable count assay. Bacteria incubated with chemokines show protrusions and lost integrity that was reflected as killing in the viable count assay while bacteria incubated with a combination of chemokine and OPN, or in a buffer alone remain intact and viable. Bar scale is 5 μm .

C. To investigate the interaction at an ultrastructural level at the bacterial surface, CCL26 and OPN were conjugated with 5 nm and 20 nm colloidal gold particles, respectively followed by negative staining and transmission electron microscopy. Bacteria were incubated in buffer, with CCL26 alone (1 μM) or CCL26 preincubated with OPN at a ratio of 1:1. In buffer, the bacteria are intact and the cell wall is visible (I and II). In the presence of CCL26, bacteria are disrupted and CCL26 accumulate in the area of the cell wall (5 nm gold particles) (III and IV). CCL26 preincubated with OPN appear colocalized at the cell wall and bacteria remain intact and viable (V and VI). Bar scale for V and VI is 1 μm and 100 nm, respectively.

Fig. 4. OPN binding to the CCL26-derived peptides. TTK25 and GSD35 fragments were injected at increasing concentration over immobilized OPN. The COOH-terminal derived TTK25 showed dose dependent binding to OPN, whereas the NH₂-terminal GSD35 showed no binding.

Fig. 5. Endotoxin-neutralizing activity of eosinophil-recruiting chemokines. The LPS-neutralizing activity of the eosinophil-recruiting chemokines was investigated using a THP-1 monocytic cell-line transfected with a NF-kappaB responsive element where activation results in production of alkaline phosphatase (ALP). LPS of *P. aeruginosa* (final concentration 10 ng/ml) was preincubated for 20

minutes in medium alone, chemokine or chemokines that had been preincubated with OPN at equimolar ratios for 20 min (all at a final concentration of 5 μ M) followed by incubation with the cell-line for 24 hours. The ALP-activity was used as readout and the response to LPS alone at 10 ng/ml was set to 100%. All chemokines, with the exception of CCL5, showed LPS-neutralizing activity as reflected by reduced NF-kappaB activation. However, preincubation with OPN displayed a significant reduction of the LPS-neutralizing activity only in the case of CCL24. The data represent mean and SD from three separate experiments. **P<0.01.

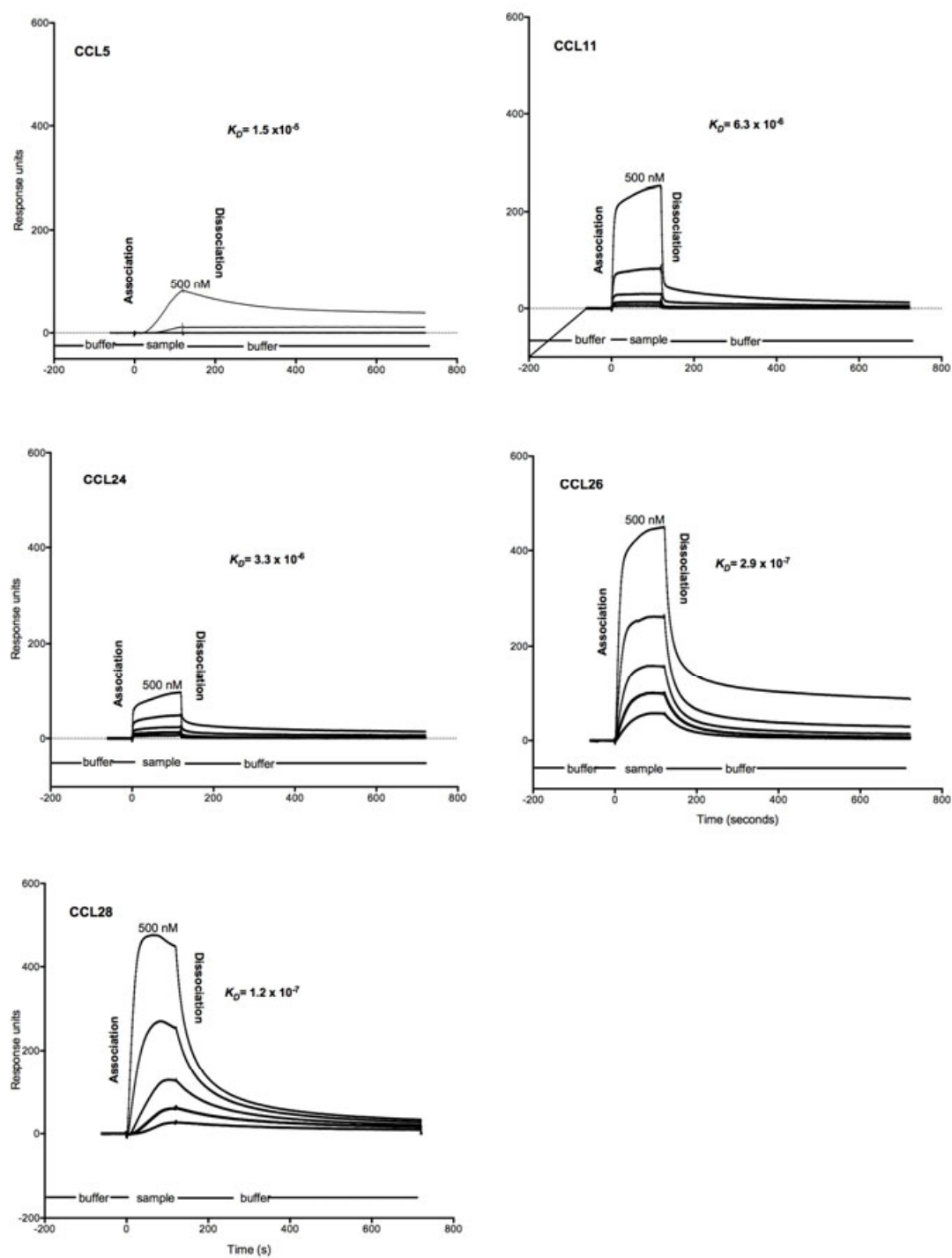
Fig. 1: Gela *et al.*

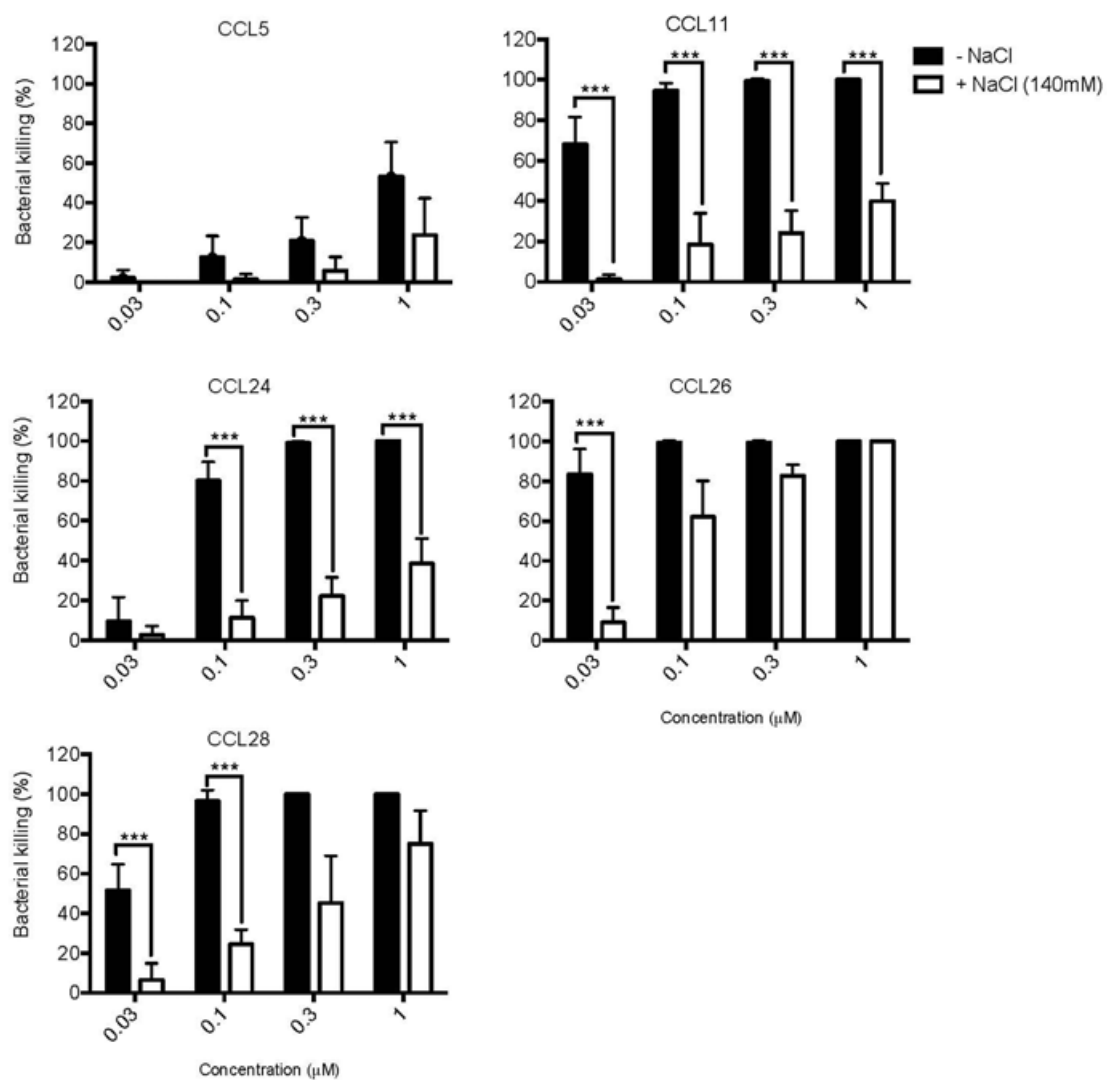
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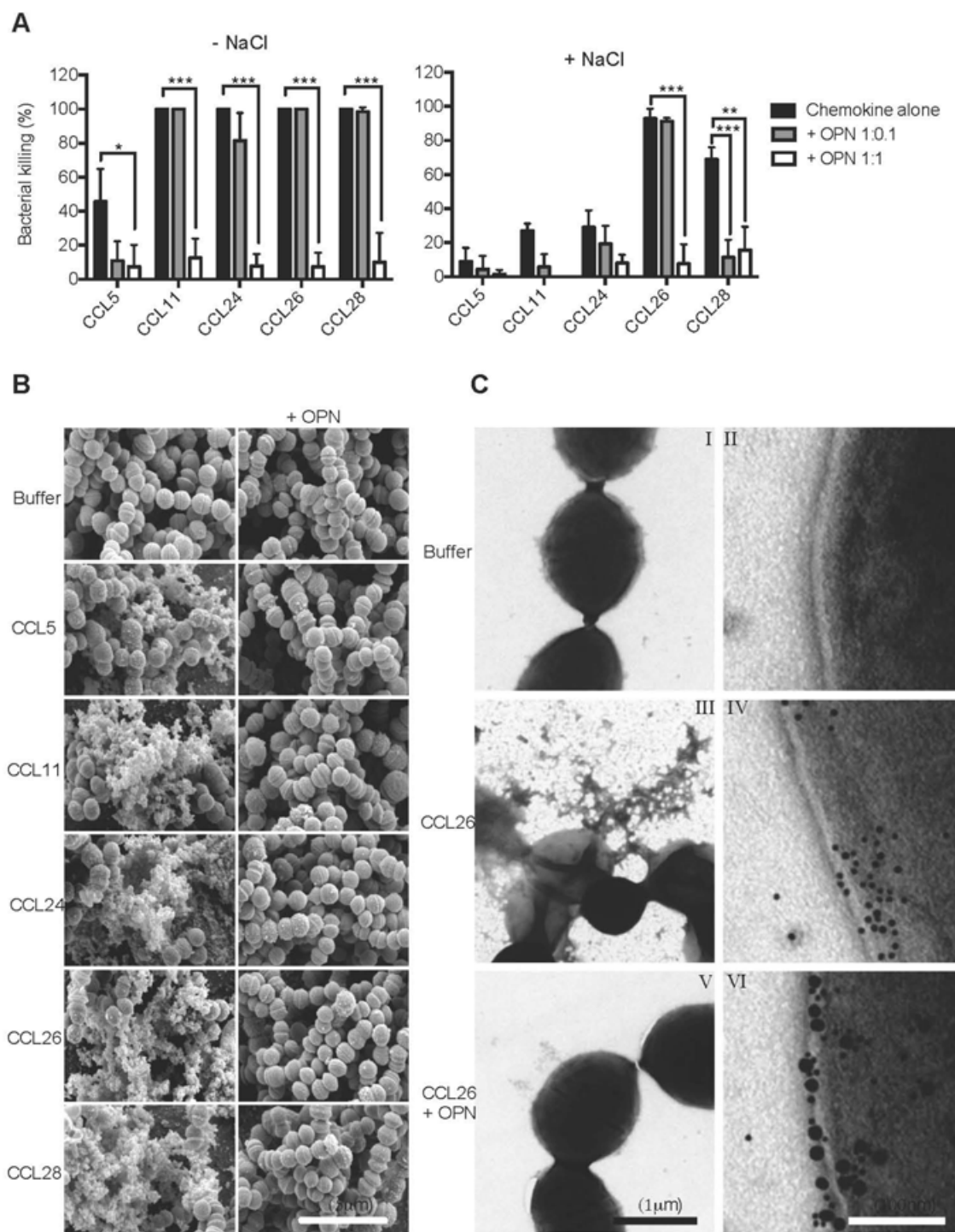
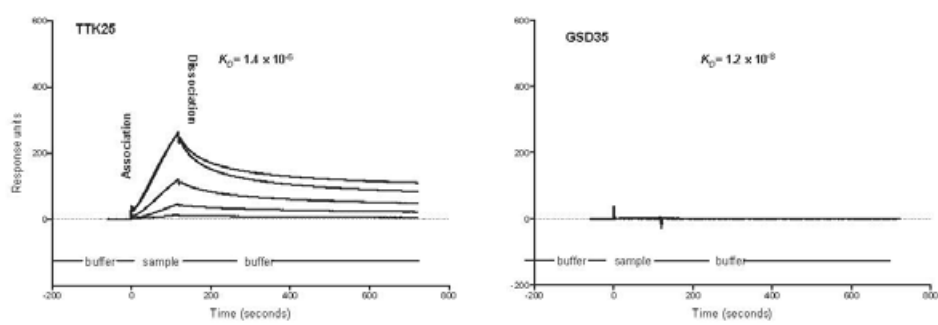
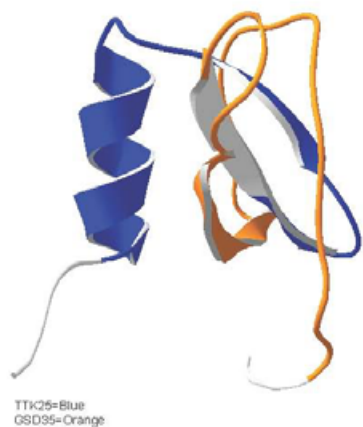
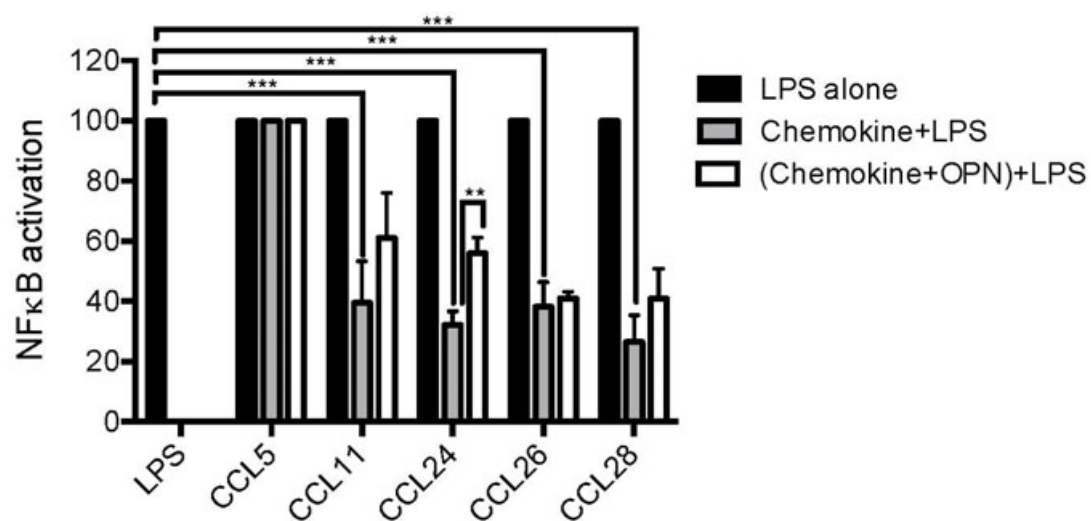
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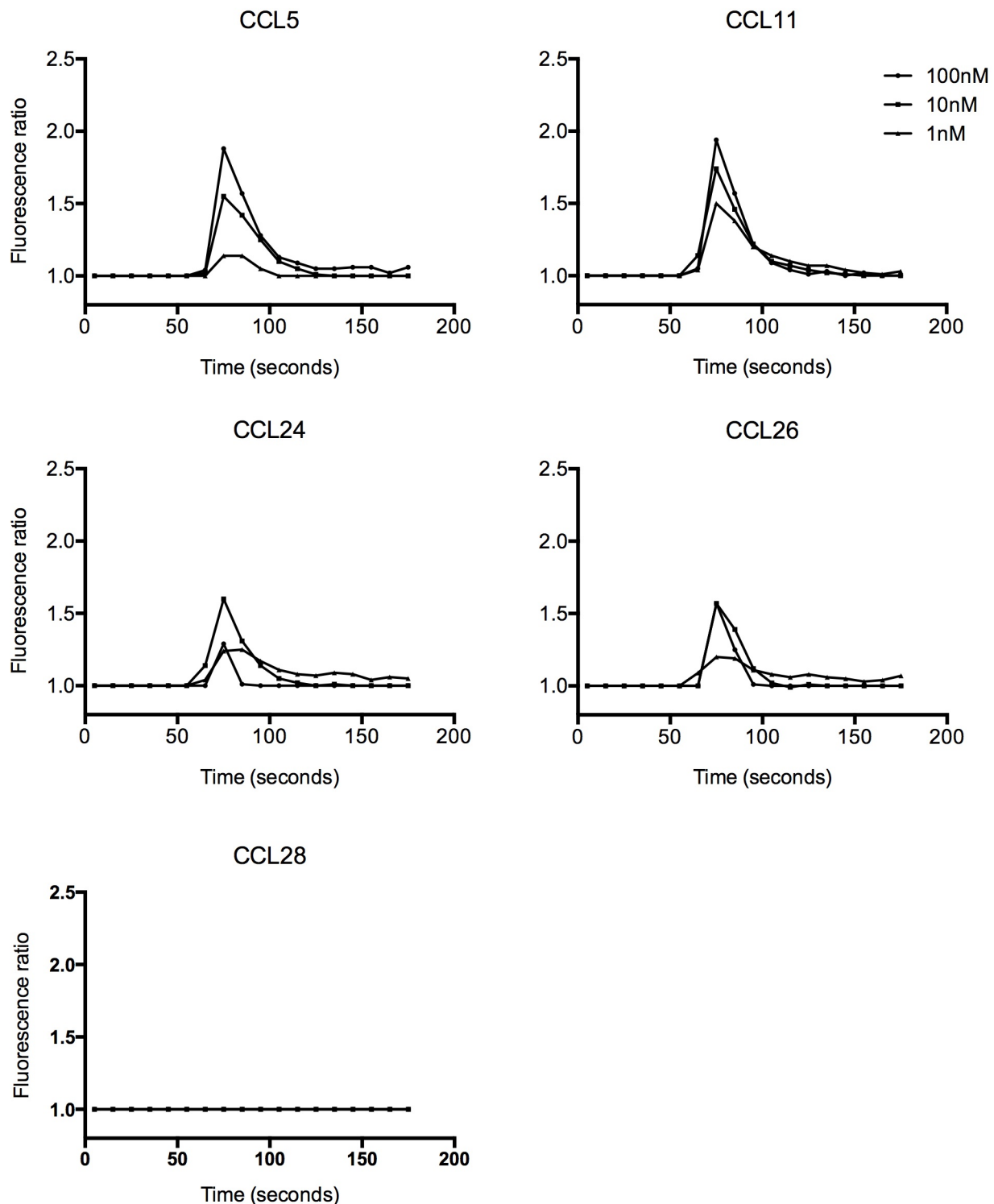
Fig. 4: Gela *et al.*

CCL26(TRGSDISKTCFCFQYSHKPLPWTWVRSYEFTSNSCSQRVAVIFITKRGKKVCITPRKKWVQKYISLLKTPKQL)



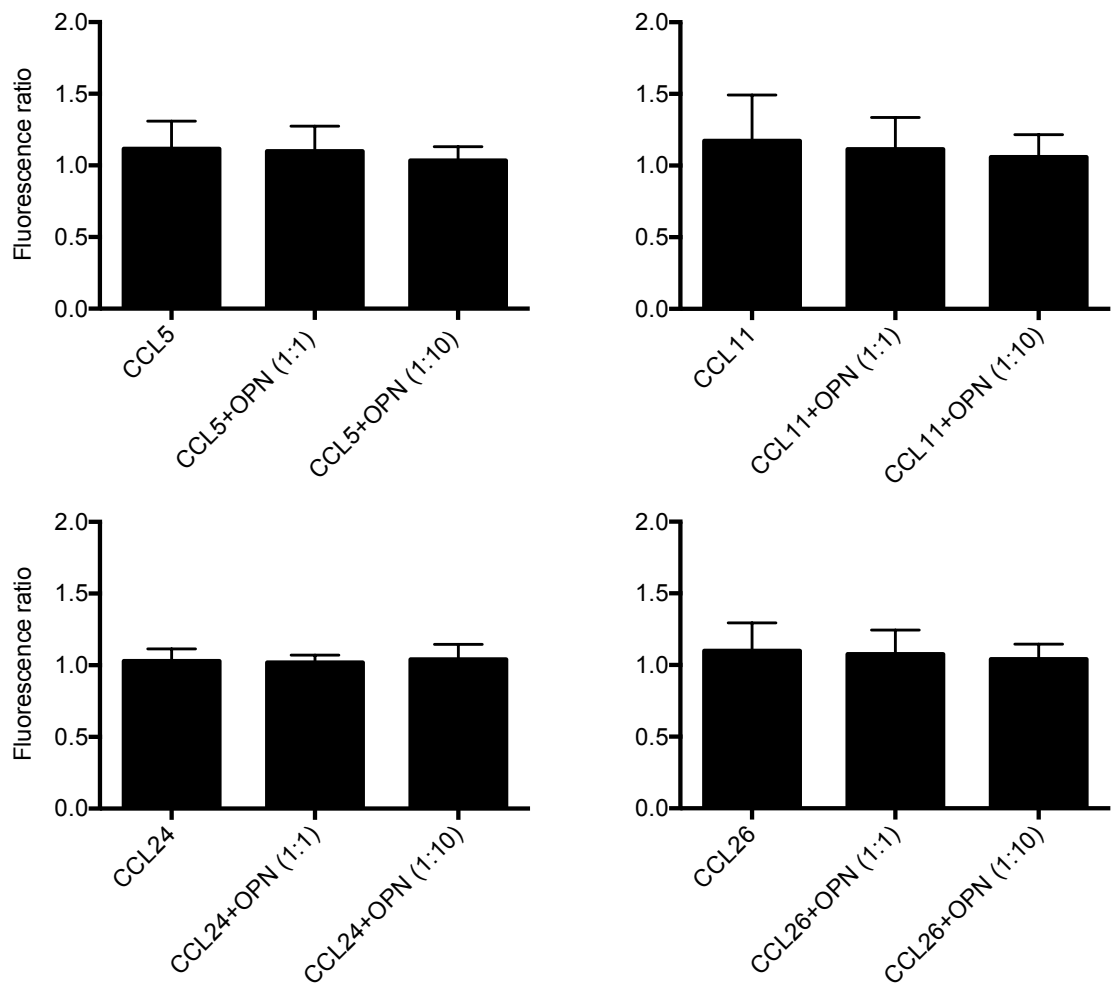
	No of amino acids	Net charge	Isoelectric point	Molecular weight (kDa)
TTK25	25	+9	10.8	3.0
GSD35	35	+2	8.6	4.1

Fig. 5: Gela *et al.*

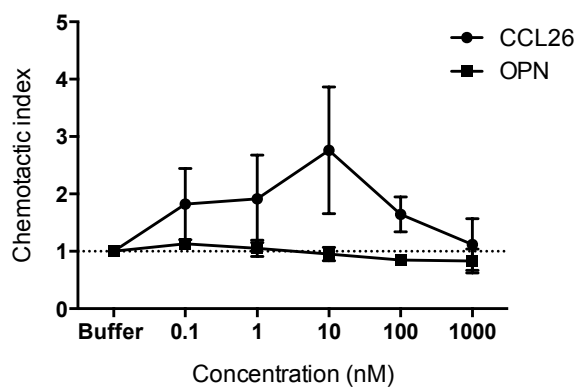


Suppl. Figure 1: A murine cell line stably expressing human CCR3 were loaded with the calcium-sensitive probe Fluo-4 and Fura-red, thereafter stimulated with eosinophil-recruiting chemokines (all at 1, 10, and 100 nM). Each chemokine elicited a similar mobilization of intracellular calcium in a dose dependent manner, with the exception of CCL28. Compiled data from three separate representative is shown.

A

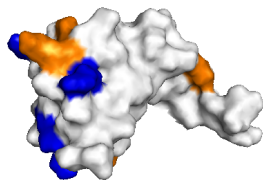
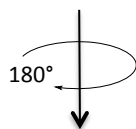
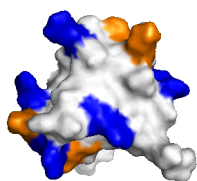


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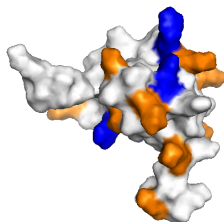
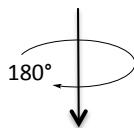
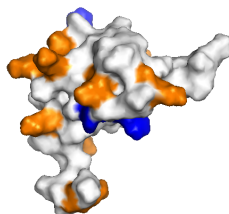


Suppl. Fig. 2. OPN interaction with CCR3-activation of eosinophil-recruiting chemokines. A. To investigate if OPN interferes with receptor activation, the chemokines were preincubated with OPN (1:1 and 1:10) followed by stimulation of cells (a murine pre B cell leukemia cell line transfected with the human CCR3 receptor). Intracellular mobilization of calcium was measured using a calcium-sensitive fluorescent probe and flow cytometry. Compiled data from the three separate experiments show no significant difference with respect to chemokine-induced receptor-activation in the absence and presence of OPN. OPN alone did not mobilize calcium (not shown). (B) Eosinophils, purified by negative magnetic selection, were allowed to migrate in response to increasing concentrations of CCL26 and OPN. The chemotactic response is expressed as a ratio of the migration observed in response to buffer alone (chemotactic index). The data represent mean and SD from four separate experiments.

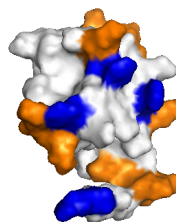
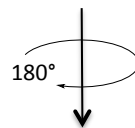
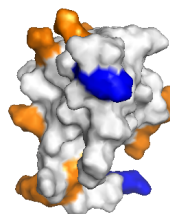
CCL5



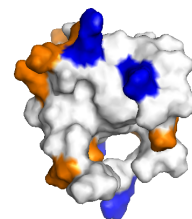
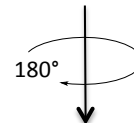
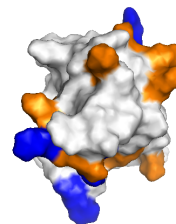
CCL11



CCL24



CCL26



ARG=BLUE
LYS=ORANGE

Suppl. Figure 3. Molecular models of eosinophil-recruiting chemokines. The structure of CCL28 has not been determined.

Suppl. Table 1

Biophysical properties of Th2 chemokines in comparison to osteopontin

	No of amino acids	Net charge	Isoelectric point	Molecular weight (kDa)
CCL5	68	+5	9.2	7.8
CCL11	74	+11	9.9	8.4
CCL24	78	+11	10.1	8.8
CCL26	71	+13	10.2	8.4
CCL28	108	+15	10.2	12.4
OPN	298	-47	4.3	33.7