

Neuropeptide S and G protein-coupled receptor 154 modulate macrophage immune responses

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G protein-coupled receptor 154 (*GPR154*) is a recently discovered asthma susceptibility gene upregulated in the airways of asthma patients. We previously observed increased pulmonary mRNA expression of the murine ortholog *Gpr154* in a mouse model of ovalbumin (OVA)-induced inflammation. However, the expression profile of *GPR154* in leukocytes and the cellular functions of the receptor and its endogenous agonist neuropeptide S (NPS) have remained unidentified. Here, we characterized the mRNA expression of NPS and *GPR154* by using real-time RT–PCR in fractionated human blood cells and in peripheral blood mononuclear cells (PBMCs) with monocyte or T cell activation. The expression of *GPR154* in leukocytes was further confirmed by immunoblotting experiments and immunohistochemical staining of human sputum samples. Additionally, we characterized the expression of *GPR154* in the lung tissue samples and in the bronchoalveolar lavage (BAL) fluid of OVA sensitized and challenged BALB/c mice. In human blood and sputum cells, monocyte/macrophages and eosinophils were identified as *GPR154*-positive cells. In PBMCs, monocyte activation with LPS but not T cell activation with anti-CD3/CD28 antibodies resulted in increased NPS and *GPR154* expression. In the lung tissue samples and in the BAL fluid of OVA-challenged mice, *GPR154* expression was upregulated in alveolar macrophages in comparison to controls. In the mouse macrophage RAW 264.7 cell line, NPS-stimulated $G\alpha_s$ - and $G\alpha_q$ -dependent phagocytosis of *Escherichia coli*. The results show that *GPR154* is upregulated in macrophages after antigen challenge and that NPS is capable of inducing phagocytosis of unopsonized bacteria.

INTRODUCTION

We have recently identified a novel susceptibility gene, *GPR154* (G protein-coupled receptor 154 also known as *GPRA*, *PGR14*, *VRR1* and *NPSR*), on human chromosome 7p15–p14 that associates with asthma and high serum total immunoglobulin E (IgE) concentrations (1,2). The findings were recently replicated in large European cohorts in two independent studies, where the associated haplotypes could be allocated into risk and non-risk groups in

agreement with the original results (3,4). There are several options how *GPR154* could have an influence on the disease mechanism of asthma. First, the polymorphisms might affect the signaling, alternative splicing or the allele-specific expression of the two main *GPR154* isoforms, A and B. These mechanisms should result in dysregulation of *GPR154*-mediated signaling in a small fraction of patients. Second, the complex disease mechanisms of asthma might also directly affect to *GPR154* pathway, resulting in abnormal function of the receptor in a large

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proportion of patients. However, evidence concerning the functions of GPR154 in the airway wall is only beginning to accumulate.

A proteomic screening for endogenous ligands of GPR154 resulted in the discovery of a novel linear 20-residue peptide that activated the receptor increasing both intracellular cAMP and Ca²⁺ levels (5). The agonist was named neuropeptide S (NPS). It is highly expressed in the brain and has pharmacological effects related to locomotor activity and anxiolytic-like effects in mice (6). So far, at least, three groups have studied the NPS–GPR154 (NPSR) pathway, and all published evidence (5–8) as well as our unpublished microarray data (J. Vendelin *et al.*, in preparation) support the idea of a specific ligand–receptor pathway. Interestingly, NPS and GPR154 mRNA are co-localized in the bronchial and colon epithelia, revealing the resemblance to the cell profile of the innate immunity and suggesting that GPR154 may be activated by an autocrine or paracrine mechanism (8).

A mouse model of lung inflammation induced by repeated intraperitoneal sensitization with ovalbumin (OVA) has many similarities to human asthma, including elevated total and specific IgE as well as inflammation characterized by infiltration of eosinophils (9). Furthermore, mice sensitized to OVA and then challenged with OVA aerosol exhibit increased airway hyperreactivity (AHR) to inhaled methacholine, a hallmark of asthma. In our recent work, the mRNA levels of the murine ortholog *Gpr154* were significantly upregulated in a mouse model of OVA-induced inflammation (2). Remarkably, public mouse genome databases do not appear to include the sequence for the B isoform. Nevertheless, a GPR154 transcript was detected in human peripheral blood leukocytes (2,6), further suggesting a role for the GPR154 pathway in inflammation. In this study, we characterized the expression of GPR154 in human leukocytes and analyzed the expression of GPR154 in a mouse model of bronchial inflammation at cellular level. Our results implicate a novel role for macrophage-derived GPR154 in the cellular response during antigen challenge.

RESULTS

GPR154 and NPS mRNA expression in human leukocytes

In order to characterize the expression of GPR154 and its agonist in leukocytes, we analyzed GPR154-A, -B and NPS mRNA expression (Fig. 1A–C, respectively) in fractionated cell types and compared the corresponding expression levels with the epithelial and smooth muscle cell lines known previously to express GPR154 (8). GPR154-A mRNA expression in peripheral blood mononuclear cells (PBMCs) was at the same level as in bronchial epithelial (BEAS) and bronchial smooth muscle cells (BSMCs), whereas the expression of GPR154-B mRNA was 3-fold when compared with BEAS cells. In agreement with our previous results (2,8), GPR154-B mRNA expression in BSMCs was very weak. Among fractionated leukocytes, monocytes and eosinophils expressed both GPR154-A and GPR154-B mRNA, whereas CD4⁺ T cells expressed mainly GPR154-B mRNA. NPS mRNA expression in blood cells was strong in comparison to the expression levels in BEAS cells and resembled the

expression profile of GPR154. This supports the idea that NPS may activate GPR154 by an autocrine or paracrine mechanism (Fig. 1C).

Next, we analyzed the effects of LPS-induced monocyte activation on GPR154 and NPS expression and compared the results with those of T cell activation. After a 6 h lag period, the expression of GPR154-A, -B and NPS mRNA was upregulated in PBMCs 16 and 28 h after LPS stimulation. T-cell activation with anti-CD3 and anti-CD28 antibodies decreased the expression of GPR154-A, -B and NPS mRNA 28 h after challenge (Fig. 1D–F, respectively).

GPR154 protein expression in human leukocytes

The changes of GPR154 mRNA levels after LPS stimulation were confirmed at protein level. In western-blotting analysis of PBMC lysates, detection with both anti-GPR154-A and GPR154-B antibodies resulted in one major polypeptide band at ~50 kDa (Fig. 2A and B). This is in agreement with the calculated full-length molecular weights of the GPR154-A and GPR154-B proteins (42.7 and 43.1, respectively). Consistent with the results of the mRNA expression studies, the amount of both GPR154 isoforms was increased in LPS-treated PBMCs 48 h after challenge in comparison to unstimulated samples. Quantification of the band intensities demonstrated that the increases in GPR154-A and -B expression were 1.4-fold and 1.6-fold, respectively (Fig. 2A and B). T cell activation did not modulate GPR154-A or -B expression. The high amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in T cell-activated PBMCs refers to the proliferation of T cells and validates the activation protocol used in the study (Fig. 2C). Blocking experiments with 10 times molar excess of free peptide as a competitor were used to verify the correct polypeptide bands in immunoblotting.

Specificities of the anti-GPR154 antibodies in recombinant GPR154 constructs

For further verification of the antibody specificities, anti-GPR154 antibodies were tested in western-blotting analysis of bacterial lysates with the corresponding polypeptide sequences expressed in *E. coli*. The cytosolic C-terminal tail of the human A isoform was expressed as a dihydrofolate reductase (DHFR) fusion polypeptide (DHFR-GPR154-A), whereas the cytosolic C-terminal tail of the GPR154-B isoform and the third cytoloop and the N-terminus of GPR154 were expressed as glutathione-S-transferase fusion polypeptides [glutathione-S-transferase (GST)–GPR154-B, GST–GPR154-CL3 and GST–GPR154-N, respectively]. Epitope-specific signals were detected with no cross-reactivity between the constructs or with the DHFR or GST construct alone (Fig. 3A–C). Because anti-GPR154-B and anti-GPR154-B-N antibodies detected also shorter GPR154 variants produced as a result of protein disruption, full-length GST-constructs were purified from the lysates with the aid of glutathione affinity chromatography. Immunoblotting of the purified GST-constructs with the corresponding antibodies resulted in detection of a single band as expected (Fig. 3D).

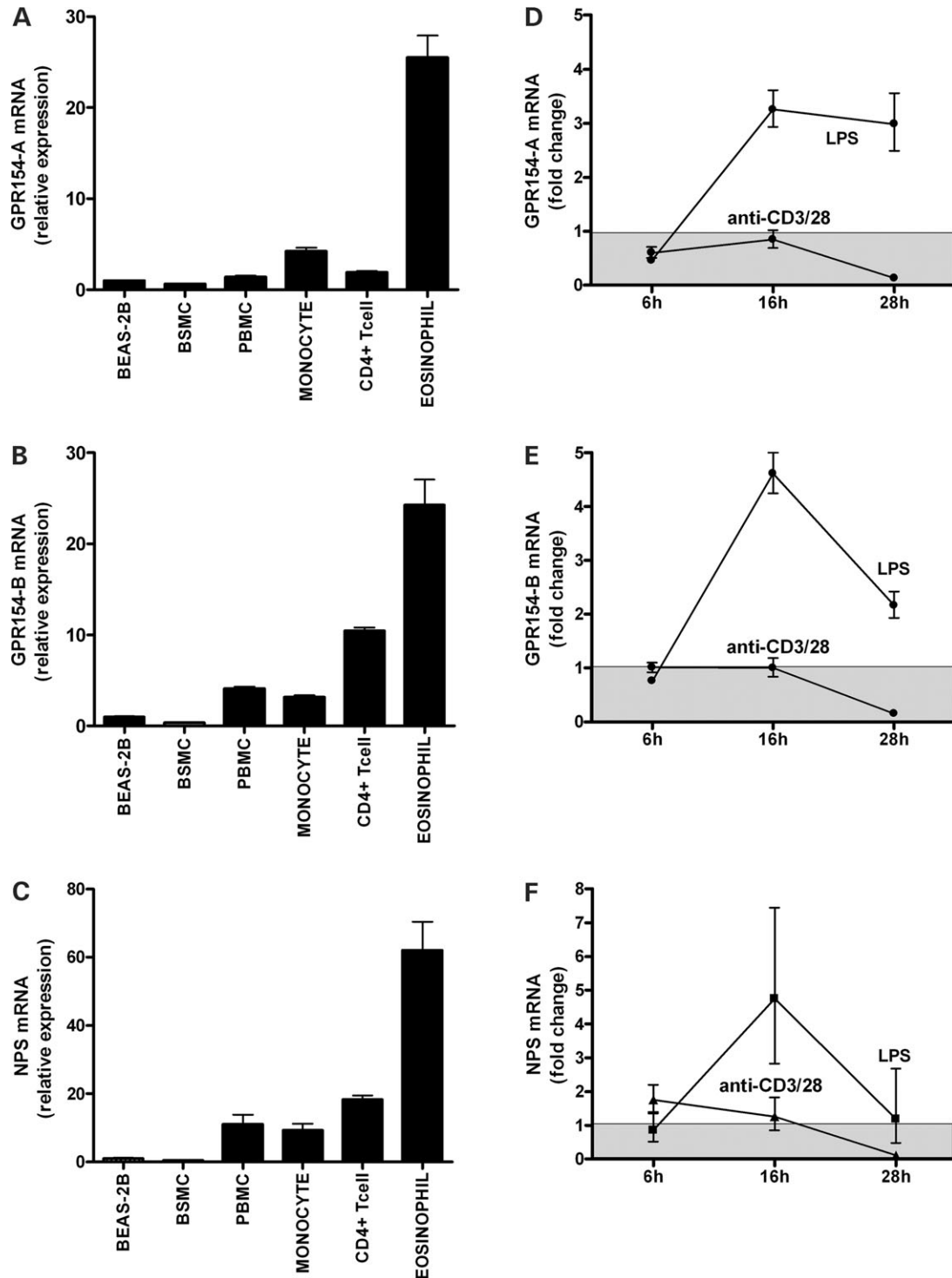


Figure 1. Quantitative real-time RT-PCR analysis of relative GPR154-A (A), GPR154-B (B) and NPS (C) mRNA expression in bronchial epithelial cells (BEAS), BSMCs, PBMCs, monocytes, CD4+ T cells and eosinophils. Relative mRNA expression of GPR154-A (D), GPR154-B (E) and NPS (PBMC) in PBMCs stimulated with LPS and anti-CD3/CD28 antibodies. The results are presented as fold changes in comparison to the relative expression in BEAS cells (A–C) or unstimulated cells indicated with gray area (D–F). Data are expressed as mean $\pm$ 95% confidence intervals.

Expression of GPR154-A and -B in human sputum samples

In order to demonstrate the expression of GPR154 in leukocytes obtained from asthmatic patients, human sputum

samples were studied. In immunoblotting analysis, detection with both anti-GPR154-A and GPR154-B antibodies resulted in one major polypeptide band at ~ 50 kDa with an additional weaker band at ~ 60 kDa (Fig. 2D). The 60 kDa band may

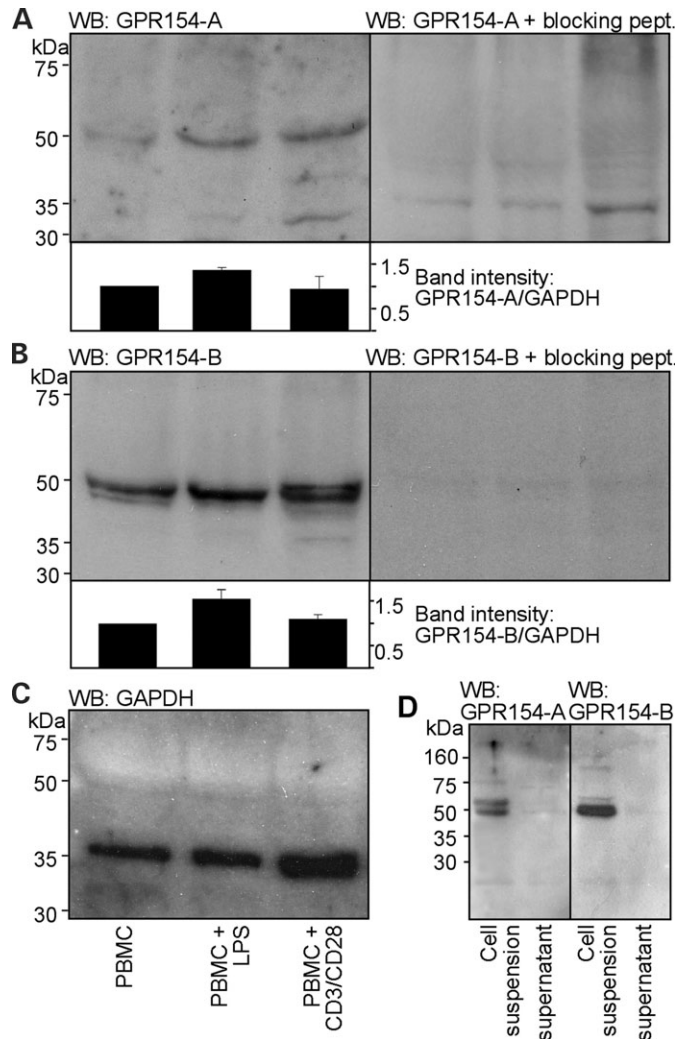


Figure 2. Quantitative analysis of the band intensities after immunoblotting of human PBMC samples with anti-GPR154-A (A) and anti-GPR154-B (B) antibodies. PBMCs were stimulated with LPS and anti-CD3/CD28 antibodies and harvested 48 h after challenge. (C) Immunoblotting with anti-GAPDH antibodies was used for normalization of the protein load in the same samples. The experiment was carried out with PBMCs from two different donors and a representative blot is shown. Data are expressed as mean $\pm$ SD. (D) Immunoblotting of normal human sputum samples with anti-GPR154-A and anti-GPR154-B antibodies.

represent a glycosylated form of the receptor, which is a typical post-translational modification for GPCRs. Omission of the primary antibodies and the negative control experiments with blocking peptides resulted in no signal (data not shown).

In qualitative immunocytochemical analysis of human sputum samples, macrophages and eosinophils expressed GPR154-A and -B (Fig. 4A and B). The anti-GPR154-A antibodies recognize intracellular epitopes of the receptor and thus membrane-associated GPR154 staining in the permeabilized cells could not be properly visualized in the color reaction-based immunohistochemistry setting. However, membrane-associated staining was detectable in macrophages and eosinophils by using non-permeabilized samples stained with the N-terminus-specific antibodies, detecting extracellular epitopes of the receptor (Fig. 4C). The characteristic cell

population profile with high eosinophil count in asthma patients indicates that the cell population in the sputum samples reflects the physiological status of the corresponding study subject (Supplementary Material, Table E1). The proportion of T cells in human sputum was $<1\%$ of total cell count. No staining was observed in T cells and they were excluded from the final analysis. In asthmatic patients, GPR154-A and -B positive eosinophils were detected only in one of the non-atopic subjects, whereas macrophages expressed GPR154-A and -B in the atopic and in four and five of the six non-atopic patients, respectively. In non-asthmatics, GPR154-A and -B positive eosinophils were found in two of the six atopic and two and one of the nine non-atopic subjects, respectively. Macrophages expressed GPR154-A and -B in all of the non-asthmatic subjects except for in one non-atopic subject (Supplementary Material, Table E2). Stainings with the non-immune IgG and omitting the primary antibodies resulted in no signal (2D).

Immunohistochemical analysis of GPR154 in mouse lung tissue samples

To further examine the expression kinetics of GPR154 during inflammation, lung tissue samples of classical OVA-induced mouse model of bronchial inflammation were analyzed by immunohistochemistry. Previously characterized polyclonal anti-GPR154-A antibodies (2,8) raised against the human intracellular sequence CREQRSQDSRMTFRERTER were used to detect the corresponding murine epitope CKMQRSQDSRMTYRERSER (Fig. 5).

In the lung tissue samples of naïve and unsensitized but OVA-challenged mice, GPR154-A was expressed in the epithelial cells of the bronchioles as expected from the previous studies (Fig. 5A and B). In the OVA-challenged mice without sensitization, non-existent to moderate immunostaining was also observed in alveolar macrophages (AMs) (Fig. 5B). In the OVA-sensitized and -challenged mice, GPR154-A expression was decreased in the epithelial cells of the bronchioles, whereas the expression was increased in macrophages when compared with naïve and unsensitized control groups (Fig. 5C and H). The staining of the corresponding sections with the GPR154-A preimmune sera did not result in any immunoreactivity (Fig. 5D).

The sequence of the human and mouse GPR154 differ from their N-terminal parts and thus human antibodies detecting extracellular epitopes of the receptor could not be used in the mouse model. However, the specificity of the immunostaining was further verified with the GPR154-CL3 antibodies, detecting intracellular third cytoloop common to all receptor isoforms. The corresponding murine epitope is identical to the human sequence except for the first amino acid. Staining of the corresponding sections with the GPR154-CL3 antibodies resulted in identical immunoreactivity in overlapping locations to anti-GPR154-A stainings, confirming the results (Fig. 5E–G).

Immunohistochemical analysis of GPR154 in murine BAL samples

In order to confirm the findings obtained from immunohistochemical stainings of the mouse lung tissue samples,

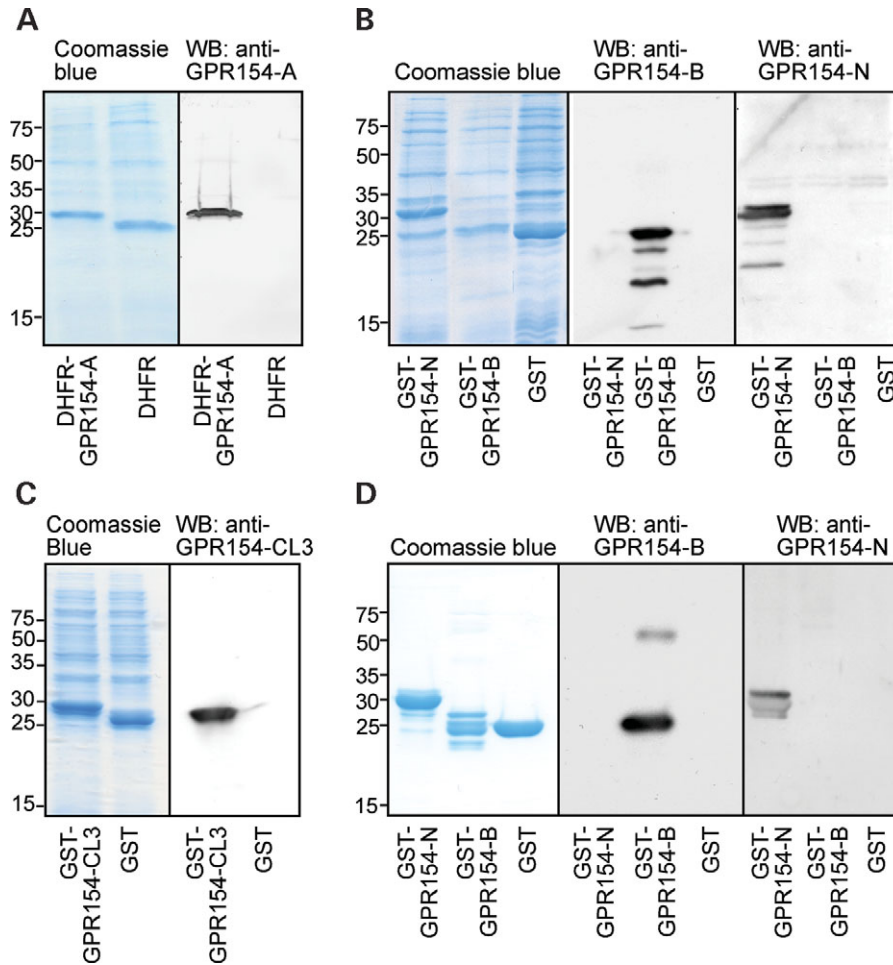


Figure 3. Epitope specificities of the GPR154 antibodies against the recombinant GPR154 constructs produced in *E. coli*. Immunoblotting of bacterial lysates with anti-GPR154-A (A), anti-GPR154-B and anti-GPR154-N (B) as well as anti-GPR154-CL3 (C) antibodies indicates specific detection of the corresponding recombinant polypeptides (DHFR-GPR154-A, GST-GPR154-B, GST-GPR154-N and GST-GPR154-CL3). (D) Detection of purified recombinant proteins with anti-GPR154-B and anti-GPR154-N antibodies is shown for further verification of epitope specificities.

we analyzed the expression of GPR154 in bronchoalveolar lavage (BAL) samples from the corresponding study groups. In the BAL fluid of naïve control mice, GPR154 expression in AMs varied between non-existent to very low (Fig. 5I). On the other hand, moderate GPR154 expression was observed in macrophages of the unsensitized control mice with OVA challenge (Fig. 5J). In the asthma model of OVA-sensitized and -challenged mice, GPR154 staining in macrophages varied from mild to strong (Fig. 5K). Positive GPR154 staining was also detected in eosinophils. These results confirmed the findings indicating upregulated GPR154 expression in tissue infiltrated macrophages upon allergen challenge.

The effect of NPS on macrophage phagocytosis

As the results suggested earlier that inhalation of allergens induces GPR154 expression in macrophages, we studied whether NPS is able to modulate macrophage phagocytosis. Mouse macrophage cell line RAW 264.7 was found to express GPR154 at mRNA and protein levels (data not

shown) as assessed by real-time RT-PCR (Supplementary Material) and immunohistochemistry and was used as a model.

Prestimulation with NPS resulted in a dose-dependent increase in phagocytosis of the fluorescein-labeled *E. coli* (Fig. 6A). Incubation with 1 μ M NPS resulted in up to 10.8-fold increase ($P < 0.001$) in phagocytosis, which was similar to the results obtained with the positive control tuftsin (Fig. 6A). When visualized by fluorescence microscopy, prestimulation with NPS resulted in increased cellular uptake of the fluorescent *E. coli* in the intracellular compartments in comparison to the untreated control cells (Fig. 6C and D). The nuclei of the cells did not emit any fluorescence as expected.

In order to identify the specific signaling pathways involved in the NPS-mediated phagocytosis, specific inhibitors for intracellular Ca^{2+} and PKC involved with the $\text{G}\alpha_q$ pathway and PKA involved with the $\text{G}\alpha_s$ pathway were used. Although NPS has been previously shown to activate both intracellular pathways (5,6), we first verified that NPS increases intracellular Ca^{2+} mobilization and cAMP

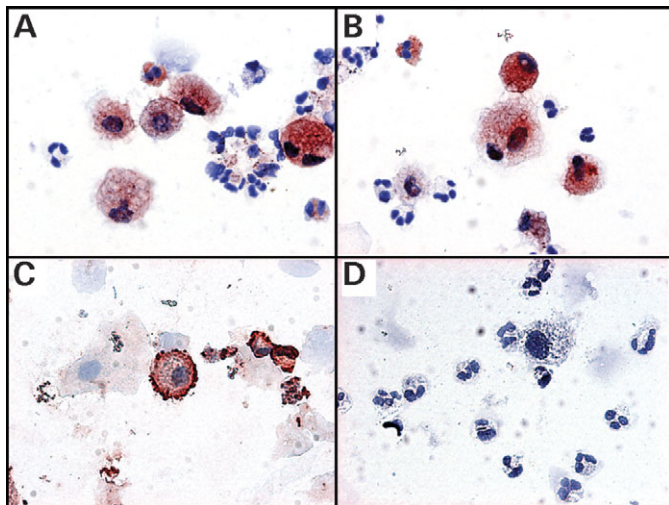


Figure 4. Immunohistochemical analysis of human sputum samples with the GPR154 isoform-specific antibodies detecting intracellular (A and B) or extracellular epitopes (C) of the receptor. Macrophages and eosinophils express GPR154-A (A) and -B (B) in permeabilized human sputum samples. (C) The N-terminal GPR154 antibodies recognizing all receptor isoforms detect membrane-associated GPR154 staining in non-permeabilized samples. (D) Staining with the non-immune rabbit IgG fraction resulted in no signal.

accumulation in our study setting as well. The 293H cell clones stably overexpressing GPR154-A (8) were used as a specific model for the NPS/GPR154 pathway. For measurements of cytoplasmic Ca^{2+} levels, the cells were labeled with Fluo-3 AM as an indicator. NPS ($1 \mu\text{M}$) stimulation increased fluorescence levels 10–15 s after injection, indicating release of Ca^{2+} to the cytoplasm of the GPR154-A overexpressing cells (Fig. 7). No change in Ca^{2+} levels was detected in parental 293H cells (data not shown). In an ELISA-based assay, 20 min stimulation with $1 \mu\text{M}$ NPS resulted in a 36–40% increase in basal cAMP levels (from 48 fmol/10 000 cells to 76–81 f/mol/10 000 cells) in GPR154-A overexpressing cells but not in parental 293H cells (Fig. 7). These findings indicate that NPS is able to activate its cognate receptor GPR154.

In RAW 264.7 cells, incubation with each of the inhibitors for intracellular Ca^{2+} , protein kinase C (PKC) and protein kinase A resulted in a decrease in NPS-mediated phagocytosis when compared with the cells treated with NPS alone (Fig. 6B). However, treatment with none of the inhibitors alone inhibited GPR154-mediated phagocytosis totally to the baseline level of the control cells. The most effective inhibition was obtained with bisindolylmaleimide I (PKC inhibitor), which decreased the NPS-mediated phagocytosis for ~70% ($P < 0.05$). Use of the inhibitors did not decrease phagocytosis in cells not treated with NPS (data not shown). The results indicate that NPS-stimulated phagocytosis is dependent on both $\text{G}\alpha\text{s}$ and $\text{G}\alpha\text{q}$ pathways.

The effect of NPS in macrophage adhesion, cell migration and chemotaxis

In the mouse lung tissue and BAL samples, GPR154 expression correlated with the tissue infiltration of macrophages. Directed

cellular movement is characterized by a dynamic control of both attachment and detachment of the cell surface adhesive receptors with their extracellular matrix ligands. Therefore, we first studied whether NPS modulates cell adhesion in RAW 264.7 cell line. Cells were plated onto fibronectin, collagen type I or poly-L lysine coated wells and incubated in the presence or absence of $1 \mu\text{M}$ NPS. The amount of the cells attached was assessed after 50 min of incubation. NPS slightly but significantly ($P = 0.013$) decreased macrophage adhesion onto immobilized fibronectin, indicating integrin-mediated events in NPS signaling (Fig. 8A). Poly-L-lysine-mediated adhesion resulted in no differences in cell adhesion. For further demonstration of the importance of the immobilized matrix, collagen type I did not support adhesion of RAW 264.7 cells (data not shown).

As detected by a quantitative fluorescence-based cell assay, 2 h stimulation with NPS induced a 2-fold increase ($P = 0.007$) in RAW cell migration up to the same level of the positive control MCP-1 (Fig. 8B). To confirm the results, random migration of the confluent RAW cells was measured with a wound-healing assay. As shown in Figure 8C and D, 24 h stimulation with NPS ($1 \mu\text{M}$) increased cell migration onto the denuded culture dish when compared with the untreated control cells. This further supports the finding that NPS is a chemotactic agent to macrophages *in vitro*.

DISCUSSION

The results of the present study suggest that in addition to the polymorphisms in the GPR154 gene that influence on asthma susceptibility, the GPR154 pathway is likely to play a wider role in the pathogenesis of asthma. The findings in human blood and sputum cells as well as the induced expression of GPR154 in the AMs of the OVA-challenged mice suggest GPR154 to participate in the modulation of macrophage immune response after antigen challenge.

In the current study, several lines of evidence indicated an active role for macrophage-derived GPR154 during inflammation. In blood CD4^+ T cells and especially in eosinophils, the expression of GPR154 and NPS mRNA was relatively strong, but none of the subsequent findings addressed a comprehensible role for GPR154 derived from these cells. Direct T-cell activation with anti-CD3 and anti-CD28 antibodies resulted in reduced GPR154 and NPS mRNA expression in PBMCs as late as 28 h after stimulation. Therefore, the decreased expression may result from induced cytokine production. Accordingly, the expression of GPR154 in PBMCs was not immediately increased upon LPS challenge but was upregulated at later timepoints. Thus, the expression of GPR154 correlates to the maturation of monocytes to macrophages. The mRNA expression profile of NPS was similar to GPR154, suggesting that NPS may activate GPR154 by an autocrine or paracrine mechanisms.

In the mouse model of asthma, the additive effect of OVA sensitization on macrophage GPR154 expression was small when compared with the OVA challenged mice. Although commercial OVA preparations may contain trace amounts of LPS, the reagent used in this study was of high quality grade and has been used similarly in previous studies (2,10).

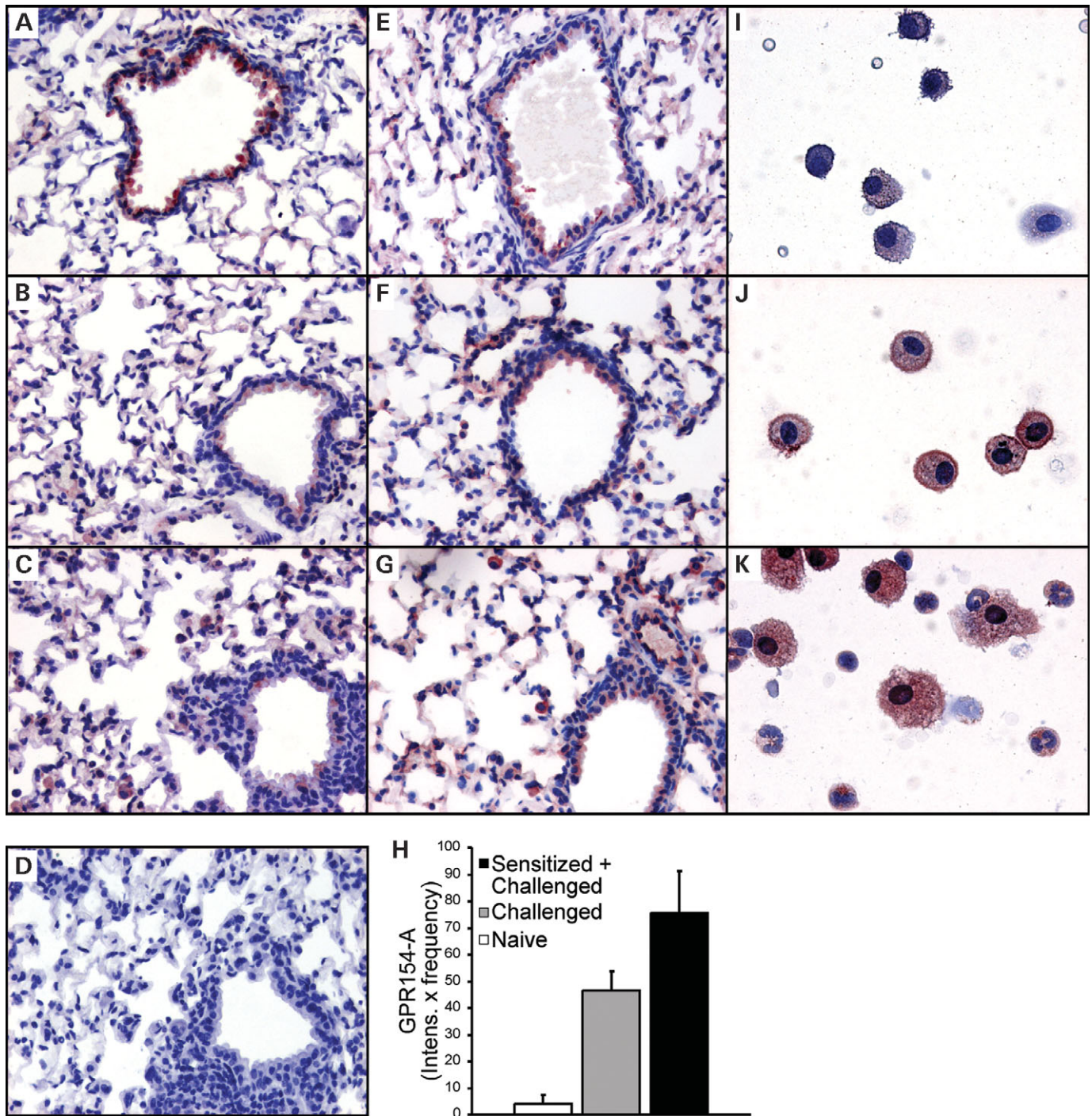


Figure 5. Immunohistochemical analysis of naïve (A, E and I), OVA-challenged (B, F and J) and OVA-sensitized and -challenged (C, G and K) mouse samples with anti-GPR154 antibodies. (A–C and H) The expression of GPR154-A was increased in macrophages after OVA challenge as depicted with photomicrographs and bar diagram. (D) Preimmune serum was used as a negative control. (E–G) Detection with anti-GPR154-CL3 antibodies recognizes intracellular third cytoplasm common to all receptor isoforms and (I–K) GPR154-A expression in the BAL fluid confirms the results.

Hence, the increased levels of GPR154 after OVA challenge may indicate to non-T-cell-mediated events and suggest that inhalation of foreign substance itself may be sufficient to upregulate GPR154 expression.

According to the results of the present study, we propose that autocrine or paracrine secretion of NPS may activate

macrophages to increase migration and phagocytosis resulting in more rapid clearance of tissue invading pathogens. The slightly decreased cell adhesion of RAW 264.7 cells after NPS challenge is more likely to reflect the dynamics of chemotactic events in the cytoskeleton of macrophages upon activation. We suggest that activation of both $G_{\alpha s}$ and

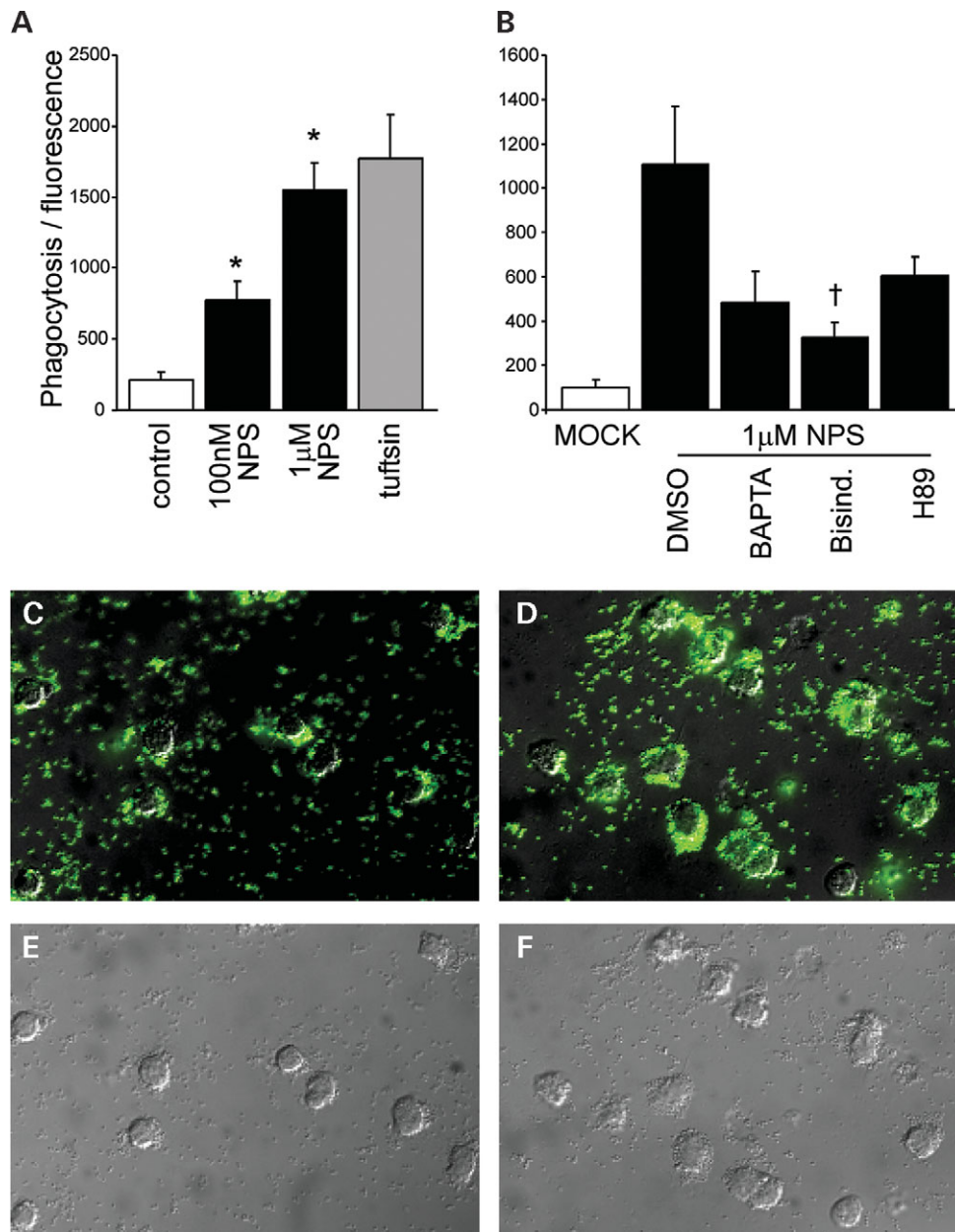


Figure 6. Effect of 1 h NPS prestimulation on phagocytosis of mouse macrophage RAW 264.7 cell line incubated with fluorescein-labeled *E. coli* for 2 h. (A) Quantitative phagocytosis assay was performed in the presence or absence of 100 nM and 1 µM NPS. Extracellular fluorescence was quenched with Trypan blue treatment. Tuftsin at 1 µg/ml was used as a positive control. (B) Treatment with specific inhibitors for intracellular Ca^{2+} (BAPTA), PKC (bisindolylmaleimide I) and protein kinase A (H-89) decreased the NPS-mediated phagocytosis for 70% as a maximum. Use of the inhibitors did not decrease phagocytosis in cells untreated with NPS (data not shown). Photomicrographs of control (C and E) and NPS-treated (D and F) cells visualized by fluorescence microscopy (C and D) and phase contrast (E and F). In opposite to the quantitative analysis mentioned earlier, extracellular fluorescence was not quenched with Trypan blue treatment before image capture. Data are represented as mean $\pm$ SEM. The phagocytosis assay was repeated over 10 times with six parallel wells and representative analyses are shown (* $P < 0.001$; † $P < 0.05$).

Gαq-initiated pathways are linked to the NPS-mediated phagocytosis, as the use of inhibitors for Ca^{2+} , protein kinase A and C decreased the enhancement obtained upon NPS stimulation. Although the use of inhibitors was not observed to decrease phagocytosis in cells not stimulated with NPS, the inhibitors are common to a variety of G protein-coupled receptors (GPCRs) and might decrease the NPS-induced phagocytosis through a GPCR other than GPR154. In our cell line stably

overexpressing GPR154-A, NPS increased both intracellular Ca^{2+} mobilization and cAMP accumulation suggesting that NPS activates GPR154 in a manner typical for GPCRs. Because the specificity of the NPS/NPSR pathway has been confirmed in GPR154 overexpressing cells, future knock-down experiments and the development of GPR154 antagonists will finally help in validating the physiological effects of NPS on endogenous GPR154 expression. In a recent

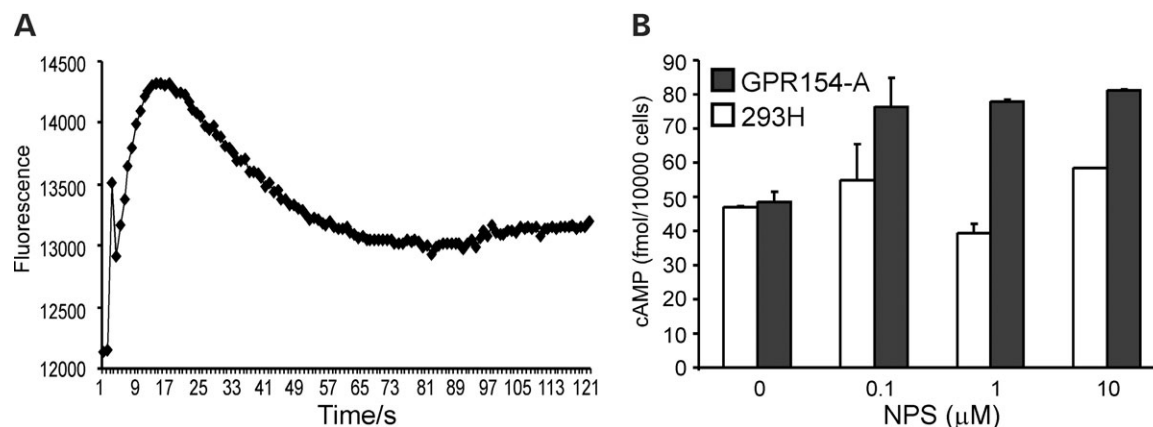


Figure 7. Effects of NPS on intracellular $[Ca^{2+}]$ and cAMP levels in GPR154-A overexpressing 293H cell line. (A) The cell line stably overexpressing GPR154-A was loaded with the Ca^{2+} indicator Fluo-3 AM, and 1μ M NPS was added onto cells prior to measurement of fluorescence (excitation 488 nm and emission max 535 nm). The results represent average of four replicate measurements. (B) cAMP accumulation was measured with an ELISA-based method. The GPR154-A overexpressing (GPR154-A cells) and parental 293H cell lines (293H cells) were cultured for 20 min with NPS (0.1 – 10μ M) stimulation.

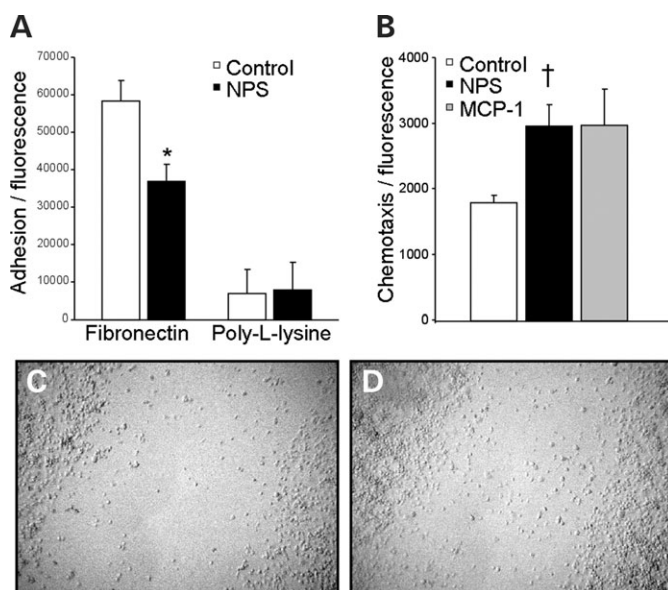


Figure 8. Effects of 1μ M NPS on RAW 264.7 cell adhesion and migration. (A) A 30 min preincubation with NPS decreased fibronectin ($*P = 0.013$), but not poly-L-lysine-mediated cell adhesion 50 min after the cells were seeded onto the wells. (B) NPS incubation increased chemotaxis ($†P = 0.007$) as detected by emission/excitation of the nucleic acid incorporated fluorescence dye 2 h after stimulation ($n = 6$). Random migration was studied using a wound healing assay with (D) or without (C) 24 h stimulation with NPS.

study, NPS induced a dose-dependent proliferation of Colo205 human colon cancer cells endogenously expressing GPR154 and stimulated MAPK phosphorylation in a dose-dependent manner in HEK cells stably expressing GPR154 (7). These findings implicate the potential of NPS to induce functional effects relevant to asthma pathogenesis.

The upregulated GPR154 expression in macrophages upon allergen challenge probably represents a normal response to inhaled allergens. Thus, a defect in GPR154 function might

lead to altered phagocytosis of inhaled particles. This is in good agreement with previous studies, suggesting a link between decreased phagocytic function and asthma (11,12). AMs from human subjects with asthma exhibit impaired Fc γ receptor-mediated phagocytosis associated with reduced surface expression of Fc γ receptor I (12). The clearance of apoptotic cells prevents the dying cells from releasing pro-inflammatory compounds into surroundings. A decrease in LPS-responsiveness of AMs in severe asthma is manifested by defective apoptotic cell uptake and reduced secretion of inflammatory mediators such as prostaglandin E2 and 15-hydroxyeicosatetraenoic acid (13). Furthermore, complement component C3 mediates its effect via a cognate G protein-coupled receptor C3a and plays a major role in phagocytosis and airway inflammation. Interestingly, the gene variants of C3 and its receptor are associated with asthma and related phenotypes (14,15). Furthermore, alleles of the pattern-recognition receptor NOD1 on chromosome 7p14 are associated with asthma and high IgE, suggesting that recognition of bacterial products may contribute to the presence of asthma (16).

In addition to the asthma phenotype, GPR154 is also associated with increased total and specific serum IgE as well as bronchial hyperreactiveness (2–4,17) but not with atopic dermatitis (18). This underlines the different etiopathogenesis of these conditions and the potential effects of allergen inhalation in asthma. Overexpression of GPR154-B in the asthmatic airways points to a potential function in airway hyperresponsiveness (1,8). The expression pattern of GPR154 in macrophages fits well to the hypothetical role of GPR154 in AHR as well. Animals with immunologic memory for an allergen, depleted of their AMs, show highly elevated IgE response and a large infiltration of T cells in the airways, as well as an increase in IgE-secreting B cells in draining lymph nodes following challenge with aerosolized allergen (19–21). In a recent experiment, AMs from Sprague–Dawley rats were able to confer resistance to antigen-induced AHR when transferred into the lungs of otherwise susceptible Brown Norway rats (22). This highlights the importance of AMs in preventing

the development of AHR in sensitized animals. This could result from induction of mediators that suppress pathogenic components contributing to AHR. The activation of such a pathway might require cell–cell interactions such as intake of apoptotic cells. Thus, a defect in phagocytic function of macrophages could lead to increased AHR and other symptoms of asthma. Future studies with high throughput technologies might provide new insights to the interaction of inflammatory signalling and GPR154.

Taken together, our results suggest a link between GPR154 and innate immunity-induced adaptive immunity in the pathogenesis of asthma. Epithelium-derived NPS may activate underlining macrophages to increase migration and phagocytosis to engulf the invading allergens.

MATERIALS AND METHODS

Additional details of the methods are provided online in Supplementary Material.

PBMCs and eosinophils

Peripheral blood cells were isolated from healthy volunteers or buffy coat donors by Ficoll density gradient centrifugations (Biochrom KG, Berlin, Germany). From the granulocyte fraction, erythrocytes were first lysed and eosinophils were further separated by depletion of neutrophils using MACS CD16 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the eosinophil fraction was confirmed by microscopic evaluation of cytopspin samples and was detected to be 99%. Eosinophils were cultured for 24 h with 25 ng/ml GM-CSF in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM non-essential amino acids and vitamins, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Life Technologies, Basel, Switzerland) and 10% fetal bovine serum (Sera-Lab, Sussex, UK). PBMCs were cultured in complete RPMI 1640 overnight and stimulated then for 6, 16, 28 or 48 h either with 10 ng/ml LPS or plate-bound anti-CD3 (1 µg/ml, clone OKT3, ATCC) and anti-CD28 (2 µg/ml, PeliCluster clone 15E8, Sanguin, Amsterdam, The Netherlands). CD4⁺ T cells were isolated from PBMCs using Dynal CD4 Positive Isolation Kit (Dynal, Hamburg, Germany) and rested overnight in serum free AIM-V medium (Life Technologies, Basel, Switzerland). Monocytes were isolated by incubating freshly separated PBMCs in complete RPMI 1640 medium in cell culture bottle for 10–20 min. Unattached cells were washed out and the adherent monocytes were trypsinized, washed and rested overnight in complete RPMI 1640.

Cell lines

Bronchial epithelial cell line BEAS-2B was cultured in DMEM medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and 10% fetal bovine serum (Sera-Lab). BSMC was cultured in SmGM-2 medium (Clonetics, Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA). Mouse macrophage cell line, RAW 264.7 (ATCC, Rockville, MD, USA), was cultured as

described previously (23). All cultures were grown in six-well plates and cells were lysed for mRNA extraction before reaching confluence.

Human sputum samples

Two atopic and six non-atopic patients with asthma diagnosed according to ATS guidelines (24) and six atopic and eight non-atopic, non-asthmatic subjects were recruited for the study approved by the Ethics Committee, Department of Medicine, Helsinki University Hospital. Sputum processing was done as previously described (25). May-Grünwald-Giemsa staining was used for cell population counting.

Experimental animal model

BALB/c female mice, age 6–8 weeks, free of specific pathogens, were obtained from M&B, Ry, Denmark. The mice were housed under pathogen-free conditions and maintained on OVA-free diet. All experiments conducted were approved by the Animal Experimental Committee of the State Provincial Office of Southern Finland. The study groups consisted of six naïve, nine unsensitized but OVA-challenged and nine OVA-sensitized and -challenged BALB/c female mice. Sensitization was performed intraperitoneally on days 1 and 10 with 20 µg grade V OVA (Sigma-Aldrich, St Louis, MO, USA) adsorbed to 2 mg of alum adjuvant (Pierce Biotechnology Inc., Rockford, IL, USA) diluted in saline. Unsensitized groups received 200 µg of saline-diluted alum intraperitoneally. OVA-challenge consisted of a 40-min exposure to 1% (wt/vol) OVA in saline on days 20, 21 and 22. All mice were exposed to aerosol in an exposure chamber connected to the outlet of a six-jet disperser that delivered an aerosol of particles with a mean diameter of 0.3 µm (TSI Inc., St Paul, MN, USA). The paraffin-embedded lung samples and BAL samples were collected and prepared as described previously (10). All experiments were approved by the Animal Experimental Committee of the State Provincial Office of Southern Finland.

Quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kit from 0.1 to 5 × 10⁶ cells lysed and stored frozen in RNeasy lysis buffer, according to the manufacturer's instructions (Qiagen, Hamburg, Germany). cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and random hexamer or oligo dT primers. Quantitative real-time PCR for human samples was performed with ABI PRISM 7500 Sequence Detection System applying SybrGreen chemistry (Applied Biosystems). The primers amplifying specifically the human NPS and GPR154-A and -B isoforms were designed with the Primer Express software version 1.2 (Applied Biosystems). The specificities of the GPR154 primers were tested with cDNA derived from stable cell lines overexpressing GPR154-A and -B (8). The primer sequences were GPR154-A forward 5'-CCTGCAGGGAG CAAAGATCA-3', GPR154-A reverse 5'-AATCTGCATCT CATGCCTCTCA-3', GPR154-B forward 5'-CCTCAACGA GAGAACTGGAAG-3' and GPR154-B reverse 5'-AGA

GCTGTACCTTGGAAGAG-3', NPS forward 5'-CAAAA TGATTAGCTCAGTAAACTCAATC-3' and NPS reverse 5'-GGAAGTGGATAACACCAAAACACA-3'. Elongation factor 1 α (EF-1 α) was amplified as an endogenous reference gene with forward primer 5'-CTGAACCATCCAGGCCAAA T-3' and reverse primer 5'-GCCGTGTGGCAATCCAAT-3'. The relative gene expression differences were calculated with the comparative $\Delta\Delta CT$ method.

For mouse RAW 264.7 samples, real-time quantitative RT-PCR assay was performed according to the manufacturer's (Applied Biosystems) instructions as described previously (23). Mouse GPR154-specific oligonucleotides were 5'-GGC TCATCTCTAAGGCAAAAATCA-3' (forward), 5'-ACGCT CCTTGGTGTCTGGAA-3' (reverse) and 6-FAM-5'-CGTCA TAATCCTTGCTTTCATCTGCTGCTG-3'-TAMRA (probe).

Antibodies

The specific polyclonal goat antibodies against the N-terminus of GPR154 and the rabbit antibodies against the third cytoloop and the two alternative carboxy terminals of GPR154 have been described previously (2,8). Affinity-purified antibodies were further analyzed by immunoblotting of recombinant GPR154 fragments expressed with the pGEX 4T-3 GST fusion expression vector (Amersham Biosciences, Buckinghamshire, UK) as GST-fusion protein in *E. coli* except for the C-terminal tail of the A isoform, which was expressed as a DHFR fusion protein (Qiagen). Glutathione Sepharose was used for protein purification according to the manufacturer's (Amersham Biosciences) instructions.

The goat polyclonal GAPDH antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the horseradish peroxidase-conjugated rabbit anti-goat secondary antibodies from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). The non-immune rabbit IgG fraction was obtained from DAKO (Glostrup, Denmark).

Western blot

For immunoblotting of human sputum samples, 50 μ g of protein from cell pellet or supernatant was lysed into standard sample buffer. For detection of PBMC samples, 1×10^6 cells were lysed into sample buffer. Western-blot analysis with the rabbit GPR154-A and -B antibodies has been described earlier (8). Omitting the primary antibodies was used as a negative control (data not shown). Blocking experiments using 10 times molar excess of free peptide as a competitor were also performed to demonstrate antibody specificity in immunoblotting.

The relative expression of GPR154-A and -B was quantified after normalizing with the relative expression of GAPDH used as an endogenous reference gene. Syngene GeneTools software (Synoptics Ltd, Cambridge, UK) was used for detecting differences between intensities of the corresponding lanes. The unstimulated cells were assigned a relative band intensity value of 1.0.

Immunohistochemistry

Immunohistochemical staining of paraffine-embedded sections has been described previously (2,8). Omission of primary antibody and staining with the preimmune sera did not result in any immunoreactivity. Immunoreactivity was scored from 0–3 (0 = non-existent, 1 = weak, 2 = moderate, 3 = strong) by two different observers. The consensus of the values between the observers was used for final analysis, where the proportion of positive cells and the intensity of the stain in positive cells were multiplied to a final score value.

For immunocytochemical analysis of human sputum and mouse BAL and RAW cells, cytospin samples were fixed with 3.5% paraformaldehyde and stained with the Avidin and Biotinylated horseradish peroxidase macromolecular complex method (Vector Laboratories, Burlingame, CA, USA) with 3-amino-9-ethylcarbazole (Vector Laboratories) as a substrate. For detection of intracellular epitopes, the samples were permeabilized with 0.5% Triton X-100 in PBS for 5 min. The samples were not permeabilized for detection of the extracellular epitopes with the N-terminal GPR154 antibodies. Staining with the non-immune rabbit IgG fraction did not result in any immunoreactivity.

Phagocytosis assay

Phagocytotic activity of RAW 264.7 cells was measured with Vybrant Phagocytosis Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 1×10^5 cells were seeded to the 96 well plates and incubated in RPMI for 1 h in the presence or absence of 100 nM and 1 μ M NPS followed by 2 h incubation with the fluorescein-labeled *Escherichia coli*. The fluorescence from non-internalized *E. coli* was then quenched by the addition of Trypan blue, and the samples were assayed with a Victor2 microplate reader. Tufitsin (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control at a concentration of 1 μ g/ml.

In order to identify the specific signaling pathways involved in the NPS-mediated phagocytosis, the specific inhibitors BAPTA-AM, bisindolylmaleimide I and H-89 (Calbiochem, Madison, WI, USA) for intracellular Ca^{2+} , PKC and protein kinase A, respectively, were incubated with the respective suboptimal concentrations of 50 μ M, 1 μ M and 1 μ M in the presence of 1 μ M NPS. The inhibitors were diluted into DMSO (Sigma-Aldrich).

The results were verified by fluorescence microscopy under a magnification of 6300. Briefly, 4×10^5 cells were seeded onto cover slips and incubated for 1 h in the presence or absence of 1 μ M NPS followed by 2 h incubation with the fluorescein-labeled *E. coli*. In order to avoid cell detachment and bad image quality, the cells were not treated with Trypan blue. After two washes with PBS, the cells were fixed with 3.5% paraformaldehyde in PBS, mounted and visualized by fluorescence microscopy.

Measurements of intracellular Ca^{2+} mobilization and cAMP accumulation

The previously described cell line stably overexpressing GPR154-A (8) was used to study NPS-mediated signaling

events. Cell clones were divided into serum-free SFM II medium at a density of 2.5×10^5 cells/ml. The cells were loaded with 4 μM Fluo-3 AM (Molecular Probes) for 15 min at 37°C followed by a 20 min incubation at RT. After two washes, 50 000 cells were seeded into 96-well plates and Ca^{2+} -dependent fluorescence traces (1 data-point/s, excitation at 488 nm) were measured by Fluostar Optima (BMG Lab-technologies, Offenburg, Germany) microplate reader. NPS (1 μM) was added prior to measurement at 535 nm.

For cyclic adenosine monophosphate (cAMP) assay, the GPR154 overexpressing cell clones and 293H parental cells were divided into cell culture medium at a density of 5×10^4 cells/well and incubated for 3 h at 37°C. Thereafter, increasing concentrations (0.1–10 μM) of NPS were added to the cells for 20 min. The cells were lysed with cAMP detection lysis reagent for 10 min and stored at -80°C . Before storing, viability of the cells was confirmed with Trypan blue exclusion method. Total cellular cAMP was detected with cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences) according to the manufacturer's instructions. The reactions were stopped with 1 M H_2SO_4 and the absorbance values were measured at 450 nm. Duplicate wells were studied and half of the total end volume of 200 μl was used for measurements.

Cell attachment assay

For the cell attachment assay, microtiter wells were coated overnight with 20 ng/ml of fibronectin, 5 ng/ml of collagen or 100 ng/ml of poly-L-lysine at +37°C. The wells were blocked for 60 min with 0.5% BSA in PBS. RAW 264.7 cells were harvested by scraping, washed and resuspended into RPMI medium at a density of 3.5×10^5 cells/ml. The cells were preincubated in suspension in the presence or absence of 1 μM NPS for 30 min on ice. Subsequently, 3.5×10^4 cells were seeded onto the wells, and the plates were incubated for 50 min at 37°C. Cells were washed three times with PBS and the relative number of attached cells was measured using CyQuant reagent (Molecular Probes) and Victor2 plate reader (Perkin-Elmer, Wellesley, MA, USA) using excitation/emission wavelengths of 485/530 nm.

Cell migration assays

Chemotactic migration of the RAW 264.7 cell line was investigated using 24-well transwell culture chambers (Corning, NY, USA) and polystyrene cell culture inserts with 6 μm pore size. Transwell chambers were coated overnight with fibronectin (20 ng/ml) at +4°C. 10^5 cells were seeded onto the cell culture inserts in serum-free RPMI. Lower chambers contained 200 ng/ml MCP-1 (R&D Systems) or 1 μM NPS in serum-free RPMI as a chemoattractant, whereas media alone was added to the control chambers. The cells were allowed to migrate for 2 h at +37°C. The upper side of the membrane was wiped off and the migrated cells in the lower side were lysed into 400 μl of CyQuant reagent (Molecular Probes). Fluorescence was measured with the Victor2 plate reader using excitation/emission wavelengths of 485/530 nm. In 'wound healing assay' demonstrating random cell migration, RAW 264.7 cells were grown to confluency for

14 days on a 24-well plate (Costar, Bethesda, MD, USA). Pipette tips were used to scrape a cell free zone with constant width in the middle of the wells. Cells were washed with PBS and 1 μM NPS in serum-free RPMI was added to the wells to induce cell migration, whereas media alone was added to the control wells. The cells were allowed to migrate for 24 h at +37°C and cell migration was analyzed by light microscopy. The experiment was repeated three times using six parallel wells.

Statistical analysis

All data are expressed as mean $\pm$ SEM unless stated otherwise. Student's *t*-test and analysis of variance were used for parametric comparisons, whereas Mann–Whitney and Kruskal–Wallis tests were used for non-parametric comparisons.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Neuropeptide S and G protein-coupled receptor 154 modulate macrophage immune responses

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Supplementary data

MATERIALS AND METHODS FOR ONLINE SUPPLEMENTARY DATA

Human sputum samples. 2 atopic and 6 non-atopic patients with asthma diagnosed according to ATS guidelines (E1) and 14 non-asthmatic subjects (6 atopic and 8 non-atopic) were recruited for the study (Table E1). Subjects 1, 2, 3, and 7 (Table E1) used inhalation steroid (beclomethasone) 800 µg per day and subject 8 1600 µg per day. The study was approved by the Ethics committee, Department of Medicine, Helsinki University Hospital. Skin prick tests were made using 10 common inhalant allergens (Soluprick SQ, 10 HEP, ALK, Denmark), and positive (histamine dihydrochloride, 10 mg/ml) and negative (solvent) control solutions. A subject was classified as atopic if any allergen induced a red spot of three millimeters or more in diameter and control solutions gave expected results (E2). Standard flow-volume spirometry was carried out at the clinic with a pneumotachograph (Spirotrac III®, Vitalograph, Ltd). Airway responsiveness to histamine, expressed as the histamine dose (mg) resulting in a 20 % decrease in PEF (PD₂₀PEF), was determined at baseline using an automated, inhalation synchronized, dosimetric jet nebulizer (Spira Electro 2, Respiratory Care Center, Hämeenlinna, Finland), as previously described (E3). Bronchial responsiveness was considered to be increased if PD₂₀PEF was less than 1.6 mg.

Sputum was induced by inhalation of nebulized 5 % hypertonic saline via an ultrasonic nebulizer (E4). All subjects were pre-treated with 0.5 mg terbutaline (Bricanyl Turbuhaler®) and PEF was measured before the pre-treatment and after induction.

Sputum processing was done as previously described (E4). Cytospins with a minimum of 300 nonsquamous cells were prepared and stained using the May-Grünwald-Giemsa

(MGG) method or with GPR154 specific antibodies (E5, E6). All analyses were done blind to the clinical characteristics or treatment. The results are expressed as a percentage of individual cells in relation to total nonsquamous cell count.

Quantitative real-time RT-PCR assay for mouse cell line. RAW 264.7 cells cultured in 10-cm dishes were washed three times with PBS. Subsequently total RNAs were isolated with RNeasy Mini kit (Qiagen, Germany) and reverse transcribed into cDNAs with MultiScribe reverse transcriptase (Applied Biosystems) with oligodT primers. cDNA synthesis was performed for 60 min at 48 °C after which the enzyme was inactivated at 95 °C for 5 min.

The real-time quantitative PCR was performed by AbiPrism 7700 Sequence Detector System (Applied Biosystems) according to the manufacturer's instructions. Primers and the probe for the mouse GPR154 and NPS mRNAs were designed by PrimerExpress version 2.0 software (Applied Biosystems, Foster City, CA). Murine GPR154-specific oligonucleotides were 5'-GGCTCATCTCTAAGGCAAAAATCA-3' (forward), 5'-ACGCTCCTTGGTGTCTGGAA-3' (reverse) and 6-FAM-5'-CGTCATAATCCTTGCTTTCATCTGCTGCTG-3'-TAMRA (probe). One to four microliters of the cDNA samples were used in real-time qPCR in a final volume of 25 µl containing 12.5 µl of TaqMan Universal PCR Master Mix and polymerase (Applied Biosystems, Foster City, CA) and 1.25 µl of the mixture of the corresponding primers (300µM) and the probe (200µM). After an initial incubation step at 95 °C for 10 min to activate the DNA polymerase enzyme (AmpliTaqGold), 40 cycles were performed for 15 s at 95 °C and for 1 min at 60 °C. PCR product formation was continuously monitored

during the PCR assay using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA). Accumulation of PCR products was detected directly by monitoring the increase in fluorescence of the FAM reporter dye. The signals were standardized to the endogenous reference $\beta 2$ microglobulin. All runs were performed in triplicate in 96 well optical microtiter plates (Applied Biosystems).

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Table E1. Cell profile of the human sputum samples.

Age		Sex	Cell fractions (%)*		
			Eosinophils	Neutrophils	Macrophages
Asthmatic subjects					
Atopic					
01	31	M	59.1	27.3	11.4
02	42	F	0	30.9	64.1
Non-atopic					
03	52	M	23.3	10.0	58.3
04	59	F	12.3	63.0	22.6
05	53	M	8.8	64.7	23.5
06	42	F	5.5	74.7	19.2
07	82	F	0.5	96.1	3.4
08	47	F	0	39.1	60.9
Healthy persons					
Atopic					
09	19	M	0.57	93.75	5.68
10	20	M	0.68	86.49	11.49
11	18	M	0.34	96.56	3.09
12	18	M	0.00	62.96	37.04
13	20	M	0.00	66.36	33.64
14	17	M	0.00	59.80	34.31
Non-atopic					
15	19	M	0.00	94.17	4.85
16	20	M	0.56	91.62	7.26
17	20	M	2.34	89.63	6.35
18	18	M	0.51	77.16	22.34
19	17	M	0.00	58.38	40.72
20	16	M	0.00	67.82	31.61
21	16	M	0.00	77.96	21.63
22	17	M	0.00	96.93	3.07

* Of leukocytes, only eosinophils, neutrophils and macrophages were taken into account.

Table E2. GPR154-A and -B expression in the cells in the human sputum samples.

		GPR154 A			GPR154 B		
		Eosinophils	Neutrophils	Macrophages	Eosinophils	Neutrophils	Macrophages
Asthmatic subjects							
Atopic							
01	0/-	-		+	-	-	+
02	0/-	-		+	-	-	+
Non-atopic							
03	0/-	-		+	-	-	+
04	4/3	-		+	+	-	+
05	0/-	-		+	-	-	-
06	0/-	-		+	-	-	+
07	0/-	-		-	-	-	+
08	0/-	-		-	-	-	+
Healthy persons							
Atopic							
09	-	-		+	-	-	+
10	+	+		+	+	-	+
11	+	+		+	+	-	+
12	-	-		+	-	-	+
13	-	-		+	-	+	+
14	-	-		+	-	-	+
Non-atopic							
15	-	+		+	-	-	+
16	-	+		+	-	-	+
17	+	+		+	+	-	+
18	+	+		+	-	-	-
19	-	-		+	-	+	+
20	-	-		+	-	-	+
21	-	+		+	-	+	+
22	-	+		+	-	+	+