Nociceptin/OrphaninFQ (N/OFQ) modulates immunopathology and airway hyperresponsiveness representing a novel target for the treatment of asthma.

Shailendra R Singh, PhD1,2, Nikol Sullo, PhD1,3, Maria Matteis, PhD3, Giuseppe Spaziano, PhD3, John McDonald, PhD1, Ruth Saunders, PhD2, Lucy Woodman, PhD2, Konrad Urbanek, MD, PhD3, Antonella De Angelis, PhD3, Raffaele De Palma, MD3, Rachid Berair, MRCP2, Mitesh Pancholi, BSc2, Vijay Mistry, BSc2, Francesco Rossi, MD3, Remo Guerrini, PhD5, Girolamo Calò, PhD4, Bruno D’Agostino, MD3, Christopher E Brightling, MD2*, David G Lambert, PhD1*

1Department of Cardiovascular Sciences, University of Leicester, Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, Leicester, LE2 7LX. UK.
2Institute for Lung Health, Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK.
3Department of Experimental Medicine, Section of Pharmacology L Donatelli, Second University of Naples, Naples, Italy.
4Department of Medical Sciences, Section of Pharmacology, University of Ferrara, Italy.
5Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Italy.

*DGL and CEB are joint senior authors and contributed equally. BD’A co-ordinated animal experiments in Naples.

Corresponding author:
Professor David G Lambert,
Department of Cardiovascular Sciences, University of Leicester,
Division of Anaesthesia, Critical Care and Pain Management,
Leicester Royal Infirmary, Leicester, UK.
Tele: (+44) 0116 252 3161
E.mail: dgl3@le.ac.uk

Short running title: N/OFQ-NOP system modulates airway immune response

Author Contributions:
SRS: involved in the planning and design of the study, data collection and interpretation; performed and analysed quantitative PCR, migration experiments, in vitro ELISA measurements, collagen gel contraction assays, RIA, cAMP and 3H-Thymidine incorporation assays; coordinated recruitment of healthy and asthmatic volunteers for sputum and eosinophil collection; analysed relevant clinical data, IL-8 measurements in HMC-1 cells, wound healing assays, IHC on airway tissues and contributed to writing of the manuscript.
NS: Performed quantitative PCR, migration experiments, in vitro ELISA measurements, collagen gel contraction assays; involved in co-ordinating animal experiments in Naples and contributed to writing of the manuscript.
JM performed IL-8 measurements in HMC-1 cells. RS and LW performed HASM and HBEC wound healing experiments respectively. KU and ADA performed histochemistry and immunofluorescence analysis and contributed to writing of the manuscript. RDP: performed cytokine assays. RB: Coordinated recruitment of healthy and asthmatic volunteers for sputum and eosinophil collection and collected all relevant clinical patient data. MP: performed
Sputum processing and sputum cell counts. VM: performed immunohistochemistry on human airway tissues. FR: made substantial contributions to the final version. GC and RG: provided N/OFQ peptide and made an intellectual contribution to the pharmacology of the manuscript. BD: Coordinated animal experiments in Naples, made substantial contributions to the conception and design of the animal study and to drafting of the manuscript and supervised NS. CEB: Involved in the planning and design of the study, data collection and interpretation; coordinated recruitment of healthy and asthmatic volunteers for sputum and eosinophil collection; analysed IHC on airway tissues; contributed to writing of the manuscript and supervised SRS, NS, RS, LW, RB, MP and VM. DGL: Involved in the planning and design of the study, data collection and interpretation; established collaborative links with RG, GC, BD; performed and analyzed cAMP and 3H-Thymidine incorporation assays; contributed to design of animal study and drafting of the manuscript and supervised SRS, NS ad JM. All authors approved the final draft of the manuscript.

Keywords: nociceptin/orphanin FQ, asthma, inflammation, chemotaxis, bronchoconstriction, immunomodulation, wound healing, mast cells, eosinophils, ova-sensitization
Abstract:

**Background and purpose:** There is evidence supporting a role for Nociceptin/OrphaninFQ (N/OFQ) receptor (NOP) and its endogenous ligand N/OFQ in the modulation of neurogenic inflammation, airway tone and calibre. We hypothesised that NOP activation has beneficial effects upon asthma immunopathology and airway hyper-responsiveness. Therefore, the expression and function of N/OFQ-NOP was examined in healthy and asthmatic human airway tissues. The concept was further addressed in an animal model of allergic asthma.

**Experimental approach:** NOP expression was investigated by qRT-PCR. Sputum N/OFQ was determined by RIA. N/OFQ function was tested using several assays including proliferation, migration, collagen gel contraction and wound healing. The effects of N/OFQ administration *in vivo* were studied in ovalbumin(OVA)-sensitised and challenged mice.

**Key Results:** NOP is expressed on a wide range of human and mouse immune and airway cells. Eosinophils express N/OFQ-precursor mRNA and their number correlates with N/OFQ concentration. N/OFQ is found in human sputum and increases in asthma. Additionally, elevated N/OFQ immunoreactivity is seen in asthmatic human lung. NOP activation inhibits migration of immunocyte and increases wound healing in airway structural cells. In addition, N/OFQ relaxes spasmogen-stimulated gel contraction. Remarkably, these findings were mirrored in OVA-mice where N/OFQ treatment before or during sensitisation substantially reduced airway constriction and immunocyte trafficking to the lung; in particular eosinophils. N/OFQ also reduced inflammatory mediators and mucin production.

**Conclusions and Implications:** We have demonstrated a novel dual airway immunomodulator/bronchodilator role for N/OFQ and suggest targeting this system as an innovative treatment for asthma.

**Abbreviations list:** N/OFQ: nociception orphanin FQ; NOP: nociception orphanin FQ peptide receptor; ppN/OFQ: prepronociceptin; HASM: human airway smooth muscle; HLMC: human lung mast cells; HMC-1: human mastocytoma cell line; HBEC: human bronchial epithelial cells; EOL-1: eosinophil-like cell line; EFS: electrical field stimulation; OVA: ovalbumin; SCF: stem cell factor; GINA: Global initiative for asthma; CHOhNOP: CHO cells transfected with recombinant human NOP; ECM: epithelial conditioned media; TFA: trifluoroacetic acid; AUC: area under curve; PBEs: peripheral blood eosinophils; AHR: airway hyperresponsiveness
Introduction

Nociceptin/Orphanin FQ (N/OFQ) is the endogenous peptide activator of the N/OFQ receptor (NOP), classified by IUPHAR as a non-opioid (or non-classical) member of the opioid family. Since the discovery of NOP and its deorphanisation, N/OFQ-NOP system has revealed some intriguing pharmacology. Not least a dual action in pain processing with anti-opioid actions supraspinally and antinociceptive actions in the spinal cord [Halford et al., 1999; Lambert, 2008]. The vast range of additional peripheral actions for NOP activation have reinvigorated interest also regarding its immunomodulatory actions and effects on isolated airway tissues.

The increasing prevalence of asthma is a major health problem affecting 235 million worldwide with an annual mortality of ~0.25million[WHO, 2013]. Asthma is a complex heterogeneous and devastating disease characterised by variable degree of airflow obstruction, airway hyper-responsiveness, chronic airway inflammation and airway remodelling [Brightling et al., 2012]. These changes are the result of a crosstalk between resident structural airway smooth muscle (ASM) and epithelial cells, progenitors including fibrocytes, infiltrating airway submucosal inflammatory cells (eosinophils and T cells) [Brightling et al., 2002], localised mast cells within ASM bundles [Brightling et al., 2002] and Th2 cells (and their cytokines) [Brightling et al., 2002].

With respect to airways, NOP activation abolishes capsaicin- and electrical field stimulation (EFS)-induced contraction in guinea pig airways [Corboz et al., 2000; Shah et al., 1998] and EFS-induced contractions of an ex vivo human bronchial ring preparation [Basso et al., 2005]. These effects have been attributed to inhibition of airway acetylcholine and sensory neuropeptide release [Corboz et al., 2000; Patel et al., 1997]. In ovalbumin (OVA)-sensitized mice, capsaicin induces increased airway hyperresponsiveness (AHR) that may be partly mediated by reduced endogenous N/OFQ. More significantly, N/OFQ inhibits capsaicin-induced bronchoconstriction in both naïve and (OVA)-sensitized mice [D’Agostino et al., 2012]. In addition, NOP agonists are antitussive in preclinical models [McLeod et al., 2001].

In a clinical setting, combined anti-inflammatory/bronchodilator therapy is effective in controlling asthma, however ~10% of asthmatics display variably steroid resistant patterns of inflammation [Bousquet et al., 2009]. Development of new therapies combining both
bronchodilator and steroid free immuno-suppressor profiles offer several advantages over the current treatments not least a simplified dosing regimen.

We hypothesized that N/OFQ-NOP system plays a critical role in the pathogenesis of airway inflammation, airflow obstruction and hyperresponsiveness; hallmarks of asthma. There are currently no data on N/OFQ-NOP expression in cells from human airways and as such its potential role in human asthma is unknown. We have addressed this hypothesis by investigating NOP, ppN/OFQ mRNA and N/OFQ peptide expression and function within airway tissue. We have used ex vivo human tissue from phenotyped asthmatic and non-asthmatic patients and volunteers and compared data with an established in vivo ovalbumin(OVA)-sensitised mouse model of asthma. We show that N/OFQ is a candidate dual immunomodulator and bronchodilator.

Methods

Detailed methods are available in the Online Supplement.

Subjects

Asthmatic subjects and healthy controls were recruited in Leicester, UK and clinical characteristic are reported in Table 1 with the approval of the Leicestershire Ethics Committees. All patients gave written informed consent. Asthmatic subjects had a consistent history and objective evidence of asthma. Asthma severity was defined by Global Initiative for Asthma treatment steps (mild-moderate GINA 1-3, severe GINA 4-5). Subjects underwent extensive clinical characterization including sputum induction and video assisted fibreoptic bronchoscopic examination.

Cell isolation and culture

Pure human airway smooth muscle (HASM) bundles [Brightling et al., 2005], primary human bronchial epithelial cells (HBEC) [Martin et al., 2011] and human lung mast cells (HLMC) [Sanmugalingam et al., 2000] were isolated and cultured as described. HMC-1(human mastocytoma cell line) and EOL-1(human eosinophil-like) cell lines were cultured, as described [Butterfield et al., 1990]. Peripheral blood eosinophils(PBEs) were isolated from heparinized peripheral venous blood from healthy control subjects and asthmatic volunteers using LS columns (Miltenyi Biotech-United Kingdom).
Membrane preparation and [125I] radioisotope dilution assay

Membranes were prepared from freshly harvested cells, HASM (asthmatic and non-asthmatic) and HMC-1 cells at confluence. Cells were suspended in homogenising buffer of Tris-HCl (50mM), MgSO4 (5mM) pH7.4 with KOH and were homogenised followed by centrifugation at 13,500rpm, for 10min at 4°C. Membrane protein (20μg of CHO_NOP, 200μg of HASM and HMC-1) was incubated in 0.5 ml of homogenisation buffer containing 0.5% BSA, 10μM peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon-Sigma-Aldrich, Poole, UK) and various concentrations of 125I-N/OFQ (~10pM – 1nM, Perkin Elmer, UK) for 1h at room temperature. Nonspecific binding (NSB) was defined in the presence of 1μM unlabelled N/OFQ. Bound and free radioactivities were separated by vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters. Filter-bound radioactivity was assessed by a gamma counter and receptor density was calculated from dilution isotherms.

Quantitative real time PCR (qRT PCR)

All airway and immune cells from volunteers and patients or cultured cells were prepared immediately or stored in RINAlater® (Ambion, Warrington, UK) before RNA extraction. Total RNA was extracted and final RNA pellets (patient or cultured cells) were resuspended in PCR grade water. The mass of RNA was determined using an Eppendorf Biophotometer and RNA purity crudely assessed from the 260/280 nm ratio which was in the range of 1.9 to 2.1 for all samples using a nanodrop (Thermo Scientific, UK). Total RNA extracted was processed using Turbo DNA-free® kit and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Quantitative RT-PCR assessed mRNA quantity using commercially available TaqMan® gene expression assays from Applied Biosystems for the human NOP receptor (Hs00173471_m1), human prepronociceptin (ppN/OFQ or ppNOC; Hs00173823_m1), human CCL11 (eotaxin-1; Hs00237013_m1), human CCL26 (eotaxin-3; Hs00171146_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β2 microglobulin (β2M). TaqMan probes for the genes under investigation and GAPDH contained different dyes and so were used in a duplex assay format. The thermal profile for quantitative real-time PCR (Q-PCR) reactions in the StepOne instrument (Applied Biosystems) was 2min at 50°C, 10 min at 95°C, 40 or 50 cycles of 15 s at 95°C, and 1 min at 60°C. Non-template controls were included for all samples. Results were expressed as ΔCt: the difference in cycle threshold (Ct) of the gene of interest
and the housekeeper gene, GAPDH or β2 microglobulin. Typical methodology is further described in [Leonard et al., 2009].

**Immunohistochemistry**
Sequential 2μm sections were cut from glycomethacrylate embedded asthmatic and healthy bronchial biopsies and stained using a polyclonal anti-human nociceptin/orphanin FQ antibody (1 in 200 dilution, Phoenix Europe GmbH, Germany), and appropriate isotype control rabbit IgG (Phoenix Europe GmbH, Germany). N/OFQ staining in airway smooth muscle was assessed using a semi-quantitative score of no staining=0, very low=1, low=2, moderate=3, high=4, and very high=5.

**Cell Migration**
24-well or 96-well Transwell migration assay was used to measure the migration of HMC-1 cells, HLMCs, EOL-1 cells and peripheral blood eosinophils in response to chemoattractants. To investigate mast cell migration, 450μL of SCF (10ng/ml, R & D Systems, Abingdon, UK), CXCL10 (10ng/ml, R & D Systems, Abingdon, UK) and human airway smooth muscle supernatants stimulated with recombinant TNF-α for 24hours (10ng/ml, R & D Systems, Abingdon, UK) were used as chemoattractants. We used epithelial conditioned media (ECM) and sputum from severe asthmatic volunteers (with very high levels of N/OFQ) as chemoattractants to study peripheral blood eosinophil and EOL-1 cell migration. Chemoattractants were added to the bottom compartment of each well, with the exception of the negative controls. Cells were then added to the top chamber of each well (2.5x10^5 HMC-1 cells; 1x10^5 HLMCs; 2.5x10^4 eosinophils and EOL-1 cells). N/OFQ was added to the top compartment. To allow migration, mast cells (HMC-1 and HLMCs) were incubated for 4 hours at 37°C and EOL-1 cells, eosinophils were incubated for 90minutes at 37°C. Following this, cells present in the lower well were recovered and then counted on a flow cytometer or resuspended in trypan blue (0.4%) stain for counting with a haemocytometer by a blinded observer [Kaur et al., 2006].

**Measurement of CCL11 and CCL26 by ELISA**
HASM cells or undifferentiated HBECs (both 1x10^5 cells/well) were grown to confluence in a 6-well culture plate. Cells were then serum starved for 24hours and preincubated with 300nM of N/OFQ for 60minutes at 37°C in the presence of peptidase inhibitors. Next HASM
cells were stimulated in the presence or absence of TNF-α (10ng/ml, R & D Systems, Abingdon, UK) and HBEC in the presence or absence of IL13 (10ng/ml, R & D Systems, Abingdon, UK) for 24hours at 37°C. After 24h, supernatants were collected, spun down to remove debris and stored at -80°C till further analysis. Colorimetric human CCL11 and CCL26 ELISAs (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's protocol.

**Measurement of IL8 release from HMC-1 cells**

HMC-1 cells were grown in 6-well culture plates and then pre-incubated in the presence or absence of N/OFQ (300nM) for 60minutes at 37°C in the presence of peptidase inhibitors. Next cells were stimulated in the presence or absence of SCF (10ng/ml, R & D Systems, Abingdon, UK for 24hours at 37ºC. After 24h, supernatants were collected, centrifuged at 1500rpm for 5minutes to remove debris and stored at -80ºC till further analysis. IL-8 concentrations were determined using a DuoSet ELISA Development kit from R&D Systems.

**Wound healing**

Non-asthmatic and asthmatic HBEC cells were seeded onto 8-well culture plates, serum deprived for 24h, and then wounded using a sterile 200-μl pipette tip in a predetermined grid pattern. After wounding cells were washed and treated in the presence or absence of N/OFQ (3nM and 300nM). Non-asthmatic and asthmatic HASM cells were seeded onto 8-well fibronectin-coated plates, serum deprived for 24h in ITS medium, and then wounded using a sterile 200-μl pipette tip in a predetermined grid pattern. After wounding HASM cells were washed and treated in the presence or absence of N/OFQ (3nM, 30nM and 300nM). Wounds were then photographed at baseline, after 6 h and 24 h. Wound areas were analyzed by a blinded observer using Image J software and the extent of repair calculated and expressed as a percentage of wound healed area.

**Sputum N/OFQ measurement by RIA**

Acidified sputum samples were extracted using Strata C18-E solid-phase extraction cartridges with eluate. N/OFQ measured by RIA as described [Williams et al., 2008].
Assessment of airway smooth muscle contraction by Collagen Gel Analysis

Contractile properties of airway smooth muscle cells were assessed using collagen gel contraction assay. Collagen gels were impregnated with HASM cells (2.5x10^5 cells) resuspended in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100μg/mL), amphotericin (0.25μg/mL), non-essential amino-acids (100μM) (Invitrogen), sodium pyruvate (1mM) and insulin-transferrin-selenium (1%) (Sigma). Next 450μL of gel mixture was added to each well of a PBS 2% BSA pre-coated 24-well plate and allowed to polymerize at 37°C for 90min. After polymerization, 500μL DMEM with Glutamax-1 (supplemented as above) was added to each well and the gel was detached from the plastic surface to allow free contraction. Collagen gels were then incubated in the presence or absence of N/OFQ (3nM and 300nM) for 24hours at 37°C. Carbachol (100µM) or bradykinin (10nM) were then added to each well in an equal volume of the above media. The collagen gels were photographed at specified time points over a 3h period by a blinded observer and the gel size as a percentage of the well area was calculated at specific time points using ImageJ software (National Institutes of Health, USA). All gel conditions were performed in duplicate.

ANIMAL STUDIES

All experimental procedures were in accordance with Italian DLgs 26/2014, application of the EU Directive 2010/63/EU.

Experimental protocol

Female BALB/c mice were used in this study. Animals were sensitized to ovalbumin (OVA) by subcutaneous (s.c.) injection with 0.4 ml of 10μg OVA, absorbed to 3.3mg of aluminium hydroxide gel in sterile saline at day 0 and 7. From day 21 to 23, all OVA sensitized mice were aerosol-challenged (7-minutes long daily sessions) with 1% ovalbumin in phosphate-buffered saline using an ultrasonic nebulizer (De Vilbiss Health Care, UK Ltd., Heston, Middlesex, UK). We used untreated animals (naïve mice) and OVA sensitized mice, treated with 100μl of saline solution (vehicle) or 100μl of N/OFQ (15 microg/Kg). Two different experimental protocols were used for N/OFQ treatment. In the first protocol (postOVA-sensitization) vehicle or N/OFQ were administered intraperitoneally from day 21 to 23, 30 min before each OVA aerosol-challenge (Figure 2a), while in the second protocol (preOVA-sensitization) vehicle or N/OFQ were administered intraperitoneally, at day 0 and 7, 30 min
before each allergen injection (Figure 2b). 24 hours after the last aerosol-challenge animals were sacrificed and bronchopulmonary function, pulmonary tissue and BAL fluid collection were performed.

**Airway hyperresponsiveness**

AHR to acetylcholine was assessed in an isolated and perfused mouse lung model as described in detail in [Roviezzo et al., 2007]. After 60 min, mean tidal volume was 0.21±0.02ml (n=61), mean airway resistance 0.23±0.08 cmH\(_2\)O ml\(^{-1}\), and mean pulmonary artery pressure 2.9±1.4cmH\(_2\)O. The measured airway resistance was corrected for the resistance of the pneumotachograph and the tracheal cannula of 0.6cmH\(_2\)O ml\(^{-1}\).

**Bronchoalveolar lavage**

Mouse BAL fluid was collected as follows: 1.5 ml of saline was instilled and withdrawn from the lungs via an intratracheal cannula; this lavage was performed three times, and different samples were collected. Bronchoalveolar lavage fluid was centrifuged at 1000 g for 10 min at 4 °C. The supernatant was transferred into tubes and stored at −70 °C before use to analyze the cytokine production. Cell pellets were resuspended in phosphate-buffered saline to a final volume of 0.5 ml for total and differential cell counting.

**Total and differential cell count**

Total cell count was performed using the Countess automated cell counter (Invitrogen), which evaluates cell number and viability using trypan blue stain according to the manufacturer’s instructions. Differential counting was performed on Diff-Quik (Reagena, Gentaur Italy) stained cytospins. At least 200 cells were counted on each cytospin according to standard morphologic criteria under light microscopy.

**Cytokine assays**

Measurement of cytokines in bronchoalveolar lavage fluid were performed taking advantage of the well-established Luminex xMAP technology, that allows measurement of a panel of analytes in a small sample volume (100µl) simultaneously. The assays were performed using a Milliplex Cytokine Panel plate (Millipore-Merck, Vimodrone-Milan, Italy) according to the manufacturer’s instructions on an automated immunoassay analyzer (Luminex® 200™ System, Invitrogen, Milan, Italy) as detailed previously [Vignali, 2000]. All samples were run in
duplicate. Data were analyzed using Xponent software (1.9 version, Luminex® 200™ System, Invitrogen, Milan, Italy).

**Histochemistry and immunofluorescence**

Lungs were perfused and fixed in 10% phosphate-buffered formalin. Tissue was embedded in paraffin and cut in 5 µm sections for histological analysis. For immunofluorescence, after deparaffinization and rehydration, tissue sections were treated with 10% normal donkey serum for 30min. at room temperature and then incubated with the primary antibodies diluted in PBS. After being washed several times with PBS, the sections were incubated with the fluorescein isothiocyanate (FITC)-conjugated and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were stained with DAPI. For the assessment of inflammation, sections were stained with hematoxylin–eosin (HE). To facilitate the recognition of eosinophils, a modified HE protocol was used [Meyerholz et al., 2009]. The number of eosinophils per mm² of the peribronchial tissue was measured. The number of mast cells per mm² of the lung tissue, was measured after staining with toluidine blue. Mucin production was assessed by immunolabelling with anti-mucin 5AC antibody (Abcam). Mucin-positive cells were quantified in the epithelial layer of the bronchi by counting labelled cells per total number of cells within the airway epithelium. N/OFQ expression within the mouse airways was investigated by immunolabelling with anti-N/OFQ antibody (Novus Biologicals, Italy). All samples were analyzed with a Leica fluorescence microscope and a Zeiss LSM 700 confocal microscope. The values of Corrected Total Fluorescence of N/OFQ per unit area of a peribronchial tissue from control (n=3) and OVA mice (n=3) were obtained using ImageJ software (imagej.nih.gov), (adapted from Burgess A et al., 2010).

**Measurement of OVA-specific IgE and IgG levels in BAL fluid by ELISA**

Briefly, BAL fluid samples were mixed with an equal volume of trifluoroacetic acid (TFA: 1% v:v). Acidified samples were then loaded onto Strata C18-E solid-phase extraction cartridges and washed twice with 0.1% TFA. Samples were eluted with 0.1 % TFA 3ml 60% acetonitrile, lyophilised using a centrifugal evaporator and then freeze dried. Before assay, the sample was reconstituted in assay buffer. OVA-specific IgE (Cambridge Bioscience, UK; assay range 20.7 pg/ml - 20ng/ml) and OVA-specific IgG (2B Scientific, Oxford, UK, assay range 1.56 U/ml – 100 U/ml) ELISA was performed according to the manufacturer’s protocol.
**Statistical Analysis**

Analysis between groups was performed (PRISM Version 6 (GraphPad, CA) by paired or unpaired t-tests and across groups by one-way / two-way ANOVA with appropriate post hoc comparisons. Post hoc tests were only performed if F was significant. Due to the limitations in the availability of tissue material, some animal and human studies were performed with n<5. However no statistical analysis was performed for sample sizes less than 5. For sputum N/OFQ measurements, subjects were categorized into three groups: healthy, mild/moderate asthmatics (GINA1-3) and severe asthmatics (GINA4-5). Between group differences were analysed by unpaired t-tests or Fisher’s exact test. Correlations were assessed by Spearman rank (rs) coefficients. Values of p<0.05 was considered significant.

**Results**

**N/OFQ expression in asthmatic human airways and blood eosinophils.**

Severe asthmatics (GINA4-5) had significantly higher levels of N/OFQ in sputum relative to healthy volunteers and GINA1-3 subjects (Figure 1a). There was no statistical difference between levels of N/OFQ in the sputum from healthy and asthmatic (mild-mod). Although N/OFQ and FEV₁/FVC did not correlate (Figure E1 online supplement), we observed a weak correlation between the increased number of eosinophils in asthmatic patients (mild-moderate and severe) and sputum N/OFQ (Figure 1b, Table 1). In a search for the source of N/OFQ peptide we assessed its expression in human airway tissue using IHC. N/OFQ staining appeared to be increased in asthma biopsies with a preferential sub-epithelial and extracellular matrix location and a weak staining in HASM bundles (Figure 1c, d). These observations are consistent with sputum measurements and indicate that the eosinophils potentially release N/OFQ within the airways. In fact, ppN/OFQ mRNA transcripts were found in peripheral blood eosinophils (PBE), but not in human airway structural cells, from healthy (expression observed in 5/9 independent samples;ΔCt = 21.73±1.57) and asthmatic (expression observed in 5/7 independent samples;ΔCt = 18.31±2.66) patients with no statistically significant differences (p<0.05; Figure 1e).

**NOP receptor expression on HLMCs, HMC-1 and airway structural cells**

Interestingly, HLMCs and HMC-1 cells expressed higher levels of NOP mRNA transcript than HASM cells and HBECs. NOP mRNA transcript was also detected in EOL-1
(eosinophil like) cells and native eosinophils isolated from the peripheral blood of healthy and asthmatic volunteers. In contrast to N/OFQ, no differences were observed between healthy and asthmatic subjects documenting no disease signal (Figure 1f). Additionally, in a series of $^{125}$I N/OFQ radioligand isotope dilution assays, cell membrane NOP receptor density was quantified on HASM ($B_{\text{max}}; 7.3 \pm 1.1$ fmol/mg protein, n=9) and HMC-1 cells ($B_{\text{max}}; 17 \pm 5.9$ fmol/mg protein, n=3). NOP receptor density was significantly increased in HMC-1 cells compared to HASM ($p<0.05$; unpaired t-test). A CHO cell line expressing recombinant human NOP (positive control) expressed $1321 \pm 60$ fmol/mg protein (n=4) of NOP. Moreover, N/OFQ treatment of activated HMC-1 cells induced a small inhibition of the signaling messenger cAMP (19.3% at 300nM N/OFQ; Figure E2 online supplement).

Overall, these data demonstrate that N/OFQ-NOP system is not only present in human airways but may also have a role in asthma as suggested by increased N/OFQN/OFQ-NOP system may be positively involved in airway pathophysiology, we therefore considered whether the concentration was insufficiently increased to completely ameliorate inflammation in vivo and whether an additional exogenous N/OFQ supplement is required to induce beneficial effects.

**Exogenous N/OFQ improves functional parameters and reduces inflammation in experimental allergic asthma.**

To investigate the impact of exogenous N/OFQ on allergic asthma, N/OFQ was administered in mice prior to allergen sensitization or during the challenge period to examine effects on established airway inflammation (Figure 2a,b). Airways of OVA challenged mice showed increased N/OFQ expression (Figure 2c), remarkably similar to that seen in human airways. N/OFQ treatment, either pre or post OVA sensitisation, reduced Ach-induced bronchoconstriction. Importantly, N/OFQ given during OVA aerosol-challenge (post OVA-sensitization) appeared to induce a greater degree of inhibition in Ach-induced AHR when compared with N/OFQ treatment during OVA sensitization (pre OVA sensitization) (62.5% vs. 51%; Figure 2d,e).

BAL analysis showed that N/OFQ administration significantly reduced total cell counts in OVA mice. Of note OVA-induced increase in eosinophils was markedly reduced by N/OFQ in both protocols, while the effect on lymphocytes was apparent only when N/OFQ was administered during OVA challenge (Figure 3a,b). There was no significant effect on
neutrophils (Figure 3a,b) or alveolar macrophages (data not shown). Histological evaluation revealed an increased peribronchial inflammatory infiltrates in the lungs of animals sensitized and challenged with OVA and this was markedly reduced by N/OFQ (Figure 3c,d). Additionally, N/OFQ treatments appeared to reduce peribronchial eosinophil infiltration in OVA-challenged mice (Figure 3e,f). Although bronchial and peribronchial mast cell accumulation was not observed, the total number of mast cells within the lung tissue of OVA-sensitized and challenged mice increased. This was lower in N/OFQ treated groups (Figure 3g,h).

Because Th1 and Th2 cytokines play an important role in allergic inflammation, to determine if N/OFQ affects inflammatory mediators in vivo, cytokine profile was analyzed in BAL. When N/OFQ was administered during OVA sensitisation and during OVA challenge phase, the treatment significantly reduced allergen-induced increases in IL-4, IL-5, IL-12 and IL-13 (Figure E3 a-d; Figure 4a-d). Further administration of N/OFQ during OVA challenge significantly potentiated OVA-stimulated increase in BAL IL-10 levels and reduced OVA-induced IL-17 (Figure 4e-f). No effect was observed on IFN-γ levels (not shown). However it had no effect on IL-10 and IL-17 levels following administration during the OVA sensitisation phase (Figure E3 e-f).

Finally, OVA-specific IgE in BAL fluid and plasma samples were significantly reduced by N/OFQ (Figure 4g,h). N/OFQ treatment had no effect on OVA-specific IgG levels in either BAL fluid or plasma (Figure 4i,j). These observations suggest a Th2-selective immunomodulatory effect of N/OFQ in vivo.

**N/OFQ inhibits agonist-induced human airway smooth muscle cell contraction**

Next we determined whether the exposure to exogenous N/OFQ affects the functional properties of human airway structural cells. As an index of in vitro airway smooth muscle contractility, carbachol induced a significant time-dependent collagen gel contraction initiating at 30 minutes post-stimulation with a maximum effect after 180 minutes. There was a significant concentration and time-dependent inhibition of carbachol-induced contractility following 24h pre-treatment with 3nM and 300nM N/OFQ (p<0.05 following repeated measures by 2-way ANOVA). These inhibitory responses were significant from 60 to 180 minutes post-agonist treatment (Figure 5a,b,c). We observed a similar effect of N/OFQ on
bradykinin-induced gel contraction (Figures E4 online supplement). There was no significant
difference between the effect of N/OFQ on agonist-induced contraction of healthy and
asthmatic HASMs.

**N/OFQ inhibits migration of human mast cells and eosinophils.**
Sputum from severe asthmatics containing 15-35 pg/ml (8-19pM) N/OFQ induced a
significant increase in EOL-1 and PBE migration. Addition of 30 or 300nM exogenous
N/OFQ significantly inhibited these effects (Figure 6a,b; Figure E5a and E5b online
supplement).

Chemoattractants including stem cell factor (SCF), CXCL10 and supernatants from HASM
stimulated with 10ng/ml of TNF-α (HASM sn) induced an increase in HMC-1 migration
(SCF:2.5 fold over control; CXCL10:1.98 fold over control; HASM sn:2.67 fold over
control). SCF-induced HLMC migration (2.34 fold over control) was inhibited by N/OFQ
(Figure 6c; Figure E5c online supplement). Epithelial conditioned media (ECM) stimulated
eosinophil migration (2.33 fold over control) that was also attenuated by 300nM N/OFQ
(Figure 6c; Figure E5d online supplement). Further, N/OFQ (300nM) significantly inhibited
SCF, CXCL10 and HASM sn-stimulated HMC-1 migration (Figure 6c; Figure E5e and E5f
online supplement).

**N/OFQ inhibits the release of chemoattractants that mediate human mast cell and
eosinophil migration**
IL-8 and TNF-α play vital roles in the recruitment of mast cells to sites of inflammation
[Nilsson et al., 1999; Olsson et al., 2004]. SCF-stimulated IL-8 release from HMC-1 cells
was significantly inhibited by N/OFQ (Figure 6d). However there was no effect on TNF-α
release (Figure E6 online supplement).

CCL11 and CCL26 are involved in the activation and recruitment of PBEs [Garcia-Zepeda et
al., 1996; White et al., 1997]. N/OFQ treatment demonstrated a trend towards reduced
(increased ΔCt). TNF-α stimulated CCL11 mRNA expression in HASM (Figure 6e) and
TNF-α stimulated CCL11 release appear to be inhibited by N/OFQ (Figure 6f). N/OFQ
treatment appeared to reduce IL-13 stimulated CCL26 mRNA transcript and protein
expression in HBEC (Figure 6g and 6h).
**N/OFQ promotes wound repair of human airway structural cells but has no effect on cell proliferation.**

Features of airway remodelling include epithelial cell damage and mucus hypersecretion. Airway epithelial repair is regulated through proliferation, migration and differentiation of cells adjoining the damaged area [Tam et al., 2011].

To test the effects of N/OFQ on repair capacity of bronchial epithelium, confluent monolayers of undifferentiated healthy and asthmatic HBEC were scratch-wounded in the presence or absence of the peptide. Although a complete wound closure (a combination of proliferation and migration or chemotaxis) was not achieved, N/OFQ appeared to promote wound repair of healthy HBECs and significantly induced wound closure of asthmatic HBECs (Figure 7a,b); the magnitude of effect was larger in asthmatic HBECs. Similarly, N/OFQ also promoted wound closing in HASM cultures. Specifically, wound closure of non-asthmatic HASM was promoted only by 300nM N/OFQ, while asthmatic HASM wound closure was markedly increased with 3nM, 30nM and 300nM N/OFQ (Figure 7c,d). There was a significant time (p<0.05) and dose dependent closure of HASM wound (p<0.05; repeated measures by 2-way ANOVA).

Additionally, mitogen-induced proliferation of HASMs, HMC-1 cells alone (Figure E7; online supplement) or when co-cultured with HASMs was not influenced by N/OFQ (data not shown).

Finally, as the *in vivo* counterpart, the response of airway epithelium to the exogenous N/OFQ was evaluated. In animals sensitized and challenged with OVA, administration of N/OFQ reduced by ~50% the extent of epithelial damage (not shown) and decreased the fraction of mucin-labelled epithelial cells (Figure 7e,f).

**Discussion**

Using a combination of complementary *in vitro* human and *in vivo* mouse studies, we showed that AHR, eosinophil and mast cell migration and inflammatory mediator release in the lungs were dramatically inhibited by N/OFQ. This is the first study to report a critical role for this system in asthma and describes a novel agent with combined anti-hyperresponsivness and immunomodulatory properties.
There is emerging evidence suggesting a generalised immunomodulatory role for the N/OFQ-NOP system [Miller and Fulford, 2007] and our data showed that asthmatic sputum had significantly elevated levels of N/OFQ. This may come from the increased eosinophil counts as there was a correlation between increased eosinophils and elevated N/OFQ. N/OFQ inhibited eosinophil and mast cell migration and attenuated the release of inflammatory mediators that play key roles in their recruitment. Interestingly, N/OFQ expression was found to be up regulated in the lung biopsies from asthmatic patients. In particular, N/OFQ was increased in sub-epithelial layer and extracellular matrix. We also demonstrated significant NOP expression in human airway structural and inflammatory cells with ppN/OFQ expression only in eosinophils. However, we did not detect any significant increase in NOP expression in asthma implying no disease signal. Functional in vitro studies showed that N/OFQ significantly inhibited agonist-induced HASM-embedded gel contraction and we hypothesise that this could be an additional effect to its anti-inflammatory role; a response that requires further investigation.

Asthmatic sputum contains several cytokines and chemokines that regulate eosinophil migration including IL-5, IL-8, RANTES, IgA and complexes of IL-8-IgA [Louis et al., 1997]. The increase in the levels of endogenous N/OFQ in sputum (~8-19 pM; 15-35pg/ml) of asthmatic patients was several orders of magnitude lower than that required to exert beneficial effects \textit{in vitro}. We therefore suggest that additional exogenous N/OFQ administration in the airways might present a new therapeutic strategy for asthma. This hypothesis is supported by our observation that spiking sputum from severe asthmatics (with reported high levels of endogenous N/OFQ) with additional N/OFQ (30 nM and 300 nM), which is over 2000-fold higher than measured endogenously, significantly attenuated migration of eosinophils towards asthmatic sputum.

N/OFQ is a naturally occurring peptide and does not cross the blood brain barrier [Lambert et al., 2008] and no significant adverse effects were reported in clinical trial evaluating the urodynamic effects of intravesical administration of 1 \( \mu \)M N/OFQ in patients with neurogenic detrusor activity [Lazzeri et al., 2003]. Therefore, any systemic/local administration of N/OFQ is unlikely to induce any unwanted central effects.
As a proof of concept, our in vivo experiments confirmed the hypothesis that exogenous N/OFQ can ameliorate the course of asthma. OVA sensitization followed by challenge has been widely used as a model of airway inflammation although this may not entirely reflect human asthma pathology. It does, however retain many features of human allergic asthma including Th2 cytokine production, goblet cell hyperplasia, mast cell degranulation, IgE production, AHR and airway remodelling [Gelfand, 2002; Kumar and Foster, 2002; Kumar et al., 2008; Han et al., 2013]. We found a significant reduction of allergen-increased levels of IL-4, IL-5, IL-12 and IL-13; Th2 cytokines linked to inflammation [Brightling et al., 2002]. These observations were consistent with previous reports demonstrating inhibition of IL-2 release and T cell proliferation by N/OFQ [Miller and Fulford, 2007; Easten et al., 2009]. Surprisingly, we did not observe any significant effect of N/OFQ on IFN-γ production, that is the principal effector of Th1-mediated inflammation and has a protective effect against Th2 driven immune responses [Teixeira et al., 2005]. Additionally, N/OFQ was able to inhibit levels of OVA-specific IgE in BAL fluid. However, it failed to modulate OVA-specific IgG levels. These observations suggest a Th2 selective immunomodulatory effect of N/OFQ in vivo.

Of note, we have administered N/OFQ either prior to or concurrent with OVA; this has important consequences for potential treatment paradigms. The observation that N/OFQ has efficacy in both models indicates that use in a clinical setting could involve both prophylaxis and control of acute symptoms. These data confirm and extend our recent study with the highly selective non-native NOP agonist UFP-112 [Sullo et al., 2013].

One of the characteristic features of airway remodeling is epithelial cell damage. Abnormal epithelial shedding in asthmatic patients with a variable degree of epithelial damage has been observed [Liu et al., 2013]. Repair of airway epithelium is regulated through proliferation, migration and differentiation of cells adjoining the damaged area [Tam et al., 2011]. Glucocorticoids, one of principal drug classes used in asthma, play a key immunomodulatory role in airway inflammation and provide a sustained repair potential for mechanically injured human airway epithelial cells [Barnes, 2002; Wadsworth et al., 2006]. However, studies have also shown that steroids can adversely affect the repair process by suppressing early-stage migration and proliferation of airway epithelial cells [Liu et al., 2013; Wadsworth et al., 2006]. Therefore, to identify a role for N/OFQ in repair, we explored the effect of this
peptide on HBEC and HASM cell wound healing. We observed a significant up regulation in wound repair that relates more to lung cell homeostasis than immunomodulation. Importantly, this effect was predominant in cells from asthmatic airways. Additionally, N/OFQ reduced mucus production in vivo.

An important issue in chronic treatments is GPCR signalling desensitisation due to persistent ligand binding at high doses. NOP receptor signalling is regulated by the process of homologous desensitization [Donica et al., 2013]. Several factors are known to regulate this process including NOP receptor density, dose and duration of exposure, peptide and non-peptide agonists. Acute exposure to its agonist N/OFQ, does not induce NOP receptor down regulation [Dautzenberg et al., 2001]. However, long-term exposure to N/OFQ differentially induces down-regulation in a time-dependent manner [Hashimoto et al., 2002]. Our in vitro assays demonstrate beneficial effects of N/OFQ at 3nM, 30 nM and 300 nM following administration of a single-dose for either 4 hours or 24 hours. It would therefore be interesting to investigate whether this N/OFQ administration regimen would result in NOP desensitisation. Another interesting observation would be to determine whether this would activate any compensatory mechanism(s) to maintain NOP receptor expression.

The main limitations of our study relate to lack of antagonist data and mechanistic information. Use of the antagonist UFP-101 in several other studies is questionable as it may have partial agonist activity [Mahmoud et al., 2010] making interpretation problematic. Mechanistic details would be useful in particular the role of downstream signalling events in the N/OFQ-NOP system including calcium channel modulation, activation of protein kinase C, MAP kinase, extracellular signal regulated kinase 1/2 and Rho kinases; this would need further investigation [Baiula et al., 2013]. Despite these limitations, key strengths of our findings include consistency of the observations between human data and an animal model and the magnitude of the effects observed. The anti-inflammatory effects upon eosinophilic inflammation in the in vivo model are larger than typically observed with corticosteroids [Lee et al., 2008] and are similar to IL5 neutralisation [Leckie et al., 2000]. The magnitude of the effect upon cell migration was also larger than observed in response to corticosteroids or any anti-inflammatory therapy we have investigated [Wardlaw et al., 2000]. The effect upon airway hyper-responsiveness in vivo was similar to those described for anti-IL13 and anti-IL17 [Yang et al., 2004; Kinyanjui et al., 2013], but the effect upon inhibiting in vitro agonist-induced airway smooth muscle contraction was the largest.
Clinical translation of this data set is a critical future development but clearly there are ‘non therapeutic’ issues with N/OFQ as it is a natural product already in the public domain. Supraspinal NOP activation has the potential to produce hyperalgesic/antiopioid effects [Schröder et al., 2014] but our observations in human and mouse suggests the main effects on airways are purely peripheral. A simple clinical trial of nebulised N/OFQ both as prophylaxis and during an exacerbation is clearly warranted. The use of a nebulised formulation would reduce total body dosing, negate the likelihood of central spread and offer the advantage of a single entity combining anti-hyper responsiveness and immuomodulatory actions.

Our data suggest that endogenous N/OFQ is elevated in asthma but its concentrations are too low to substantially modulate the immune system and airway hyperresponsiveness indicating that the supplementation with exogenous N/OFQ is needed.

In conclusion, we have identified an important and an innovative role for N/OFQ in counteracting non-neurogenic airway inflammatory responses and airway hyperresponsiveness. This combination of beneficial effects is rarely observed and supports our assertion that this opens a completely new potential target/strategy in the treatment of asthma.

Acknowledgments: We would like to thank Prof Peter Bradding Institute for Lung Health, Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK for providing HMC-1 and human lung mast cells.
References


http://www.who.int/respiratory/asthma/en/

## Table I. Clinical Characteristics of Patients

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=29)</th>
<th>GINA 1-3 (n=30)</th>
<th>GINA 4-5 (n=55)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age in years</strong> #</td>
<td>50 ± 3</td>
<td>55 ± 2</td>
<td>56 ± 2</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>Male, n (%)</strong></td>
<td>19 (66)</td>
<td>20 (67)</td>
<td>39 (58)</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Smoking, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>9 (31)</td>
<td>12 (40)</td>
<td>16 (29)</td>
<td>0.99</td>
</tr>
<tr>
<td>Ex</td>
<td>7 (22)</td>
<td>2 (17)</td>
<td>3 (19)</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking (pack years)</strong> #</td>
<td>3.9 ± 1.5</td>
<td>6.3 ± 1.9</td>
<td>4.5 ± 1.2</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>FEV₁% predicted</strong> #</td>
<td>105.3 ± 3.6</td>
<td>84.2 ± 4.1</td>
<td>69.3 ± 3.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>FEV₁/FVC%</strong> #</td>
<td>78.5 ± 1.2</td>
<td>71.3 ± 2.1</td>
<td>34.5 ± 4.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Sputum neutrophils, %</strong> *</td>
<td>50 (31-61.5)</td>
<td>62.4 (55-78.5)</td>
<td>56.3 (49-69)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Sputum macrophages, %</strong> *</td>
<td>39.3(32.5-60.8)</td>
<td>23.8(14.3-36.5)</td>
<td>19.9 (14-28)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Sputum eosinophils, %</strong> *</td>
<td>1.8 ± 0.6 (0-5)</td>
<td>0.8 (0.5-1.8)</td>
<td>2.9 (1.8-6.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Sputum epithelial cells, %</strong> *</td>
<td>2.0 (1.0-3.3)</td>
<td>1.0 (0.8-2.3)</td>
<td>2.5 (1.8-4.0)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Sputum lymphocytes, %</strong> *</td>
<td>0.3 (0-0.3)</td>
<td>0.0 (0.0-0.3)</td>
<td>0.3 (0.0-0.5)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Table 1:* Clinical characteristics of healthy and asthmatic volunteers recruited for sputum analysis. #Mean ± SEM; * Median (interquartile range). P value represents comparisons between healthy subjects and GINA 4-5 patients.
Figure Legends:

Figure 1. Endogenous N/OFQ expression is increased in human asthmatic airways; NOP is expressed in human airways.
(a) Sputum N/OFQ levels from healthy (n=29 subjects; n=5 below detection limit), mild to moderate asthmatic (GINA1-3; n=30 patients; n=7 below detection limit) and severe asthmatic (GINA 4-5; n=55 patients; n=2 below detection limit) donors, (b) Correlation between sputum N/OFQ (pg/ml) and sputum eosinophils (%) in asthmatics (mild-moderate and severe), (c) IHC staining of human airway tissues for N/OFQ (representative image, n=3 healthy donors and n=6 asthmatic donors), Scale bars 100 µm, (d) Semi-quantitative IHC staining score (n<5 for healthy human airway tissue), (e) Quantitative RT-PCR demonstrating ppN/OFQ mRNA expression on PBEs (n=5 healthy and n=5 asthmatic donors ), (f) Quantitative RT-PCR demonstrating NOP mRNA expression in human airway structural and immune cells(n numbers represent cells from individual donors for HASM, HBEC, HLMC and peripheral blood eosinophils and independent experiments for HMC-1 and EOL-1 cell lines) . Data expressed as mean±SEM. Comparisons made by unpaired t-test or one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05.

Figure 2. N/OFQ inhibits bronchial hyperreponsiveness
(a) In vivo experimental protocol: N/OFQ administered 30 mins prior to OVA-challenge (N/OFQ post-OVA sensitization), (b) In vivo experimental protocol: N/OFQ administered 30 mins prior to OVA-sensitization (N/OFQ pre-OVA sensitization), (c) Representative image of N/OFQ (green) qualitative and quantitative expression in vivo by immunofluorescence, Scale bars 20 µm, (d) Measurement of Ach-induced lung resistance in an in vivo model (N/OFQ post-OVA sensitization) with area under curve (AUC) (inset, n=6 mice), (e) Measurement of acetylcholine (Ach)-induced lung resistance in an in vivo model (N/OFQ pre-OVA sensitization) with area under curve (AUC) (inset), n=4 mice. Data expressed as mean±SEM. Statistical comparisons by one-way or two-way ANOVA followed by appropriate post hoc tests where relevant *p<0.05. N/OFQ administered at 1µM is equivalent to 15microg/Kg.

Figure 3. N/OFQ inhibits inflammatory cell infiltration in vivo and recruitment of inflammatory cells within mouse airway tissues.
(a) Total and differential cell count in mouse BAL fluid in an in vivo model (N/OFQ post-OVA sensitization, n=6 mice), (b) Total and differential cell count in mouse BAL fluid in an in vivo model (N/OFQ pre-OVA sensitization, n=3 mice), (c) Representative image of Haematoxylin/Eosin (HE) staining in a N/OFQ post-OVA sensitized mouse airways (n=6 mice). Scale bars, 50 µm, (d) Representative image of Haematoxylin/Eosin (HE) staining in a N/OFQ pre-OVA sensitization mouse airways (n=3 mice). Scale bars, 50 µm, (e) Modified HE staining showing the kinetics of eosinophilia in a N/OFQ post-OVA sensitization model (representative image, n=7 mice) within peribronchial tissue. Scale bars, 10µm, (f) Modified HE staining showing the kinetics of eosinophilia in a N/OFQ pre-OVA sensitization model (representative image, n=4 mice) within peribronchial tissue, Scale bars, 10µm, (g) Representative image of mast cells detected by toluidine blue staining, n=7 mice. Scale bars, 20 µm, (h) Quantitative estimation of mast cells/mm² of mice airway tissue in a N/OFQ pre-OVA sensitization mouse airways (n=4 mice), Data expressed as mean±SEM and analysed by one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05.

Figure 4. Administration of N/OFQ following sensitisation and challenge with OVA regulates release of inflammatory mediators in vivo.
(a) IL-4, (b) IL-5, (c) IL-12, (d) IL-13, (e) IL-10 and (f) IL-17 cytokine levels in mouse BAL fluid obtained from different treatment groups, n=5-9 mice. Data expressed as pg/ml (mean±SEM), (g) Measurement of OVA-specific IgE in BAL fluid (n=5 mice) and (h) in plasma (n=10-12 mice). Data expressed as ng/ml (mean±SEM), (i) Measurement of OVA-specific IgG levels in mouse BAL fluid and (j) in mouse plasma. Data expressed as U/ml (mean±SEM). Statistical comparisons by one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05.

Figure 5. N/OFQ-NOP activation modulates agonist-induced HASM contraction.
(a) Carbachol-induced time-dependent gel contraction [n=7 (HASM cells from 7 independent donors); *statistical comparisons between carbachol and control; #statistical comparisons between N/OFQ and carbachol), (b) AUC-carbachol response, (c) AUC-carbachol response normalized to control (expressed as 100%). Data expressed as mean ± SEM. Comparisons by one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05; #p<0.05.
Figure 6. N/OFQ inhibits inflammatory cell migration by blocking mediator release in vitro.
(a) Migration of EOL-1 cells (n=8 replicates) and (b) PBEs towards asthmatic sputum (n=PBEs from 7 independent donors), (c) Migration of mast cells and eosinophils through an 8-μm pore-size transwell membrane (n=5-7 independent donors). [*statistical comparisons with control, #statistical comparisons with relevant chemotactic stimuli], (d) SCF-induced IL8 release from HMC-1 cells (n=7 replicates), (e) HASM CCL11 mRNA (n=HASM cells from 4 independent donors) expression, (f) CCL11 levels in HASM supernatants (n= HASM cells from 6 independent donors), (g) HBEC CCL26 mRNA (n=HBECs from 3 independent donors) expression, (h) CCL26 levels in HBEC supernatants (n=HBECs from 3 independent donors). Data expressed as mean ± SEM. Comparisons by one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05; #p<0.05.

Figure 7. N/OFQ-NOP activation promotes wound repair of human airway structural cells and inhibits recruitment of mucin-labelled cells in vivo.
(a) Wound repair of undifferentiated healthy (n= HBECs from 3 independent donors) and (b) asthmatic HBEC (n= HBECs from 5 independent donors ), (c) Wound repair of non-asthmatic (n=HASM cells from 5 independent donors) and (d) asthmatic HASMs (n=HASM cells from 5 independent donors), (e) Mucin-labelled cells (green) in airway epithelium following administration of N/OFQ post-OVA sensitization (n= airway tissue from 7 mice), Scale bars 20 µm, (f) Mucin-labelled cells (green) in airway epithelium following administration of N/OFQ prior to OVA sensitization (n=airway tissue from 4 mice). Data expressed as mean ± SEM. Comparisons by one-way ANOVA followed by appropriate post hoc tests where relevant. *p<0.05; #p<0.05. In panels (c and d) there was a significant time (p<0.05) and dose dependent closure of HASM wound (p<0.05; repeated measures by 2-way ANOVA).
Figure 3

(a) Total Cell Counts: Eosinophils, Lymphocytes, Neutrophils

(b) Total Cell Counts: Eosinophils, Lymphocytes, Neutrophils

(c) N/OFQ, post OVA-sensitisation

d) N/OFQ, pre OVA-sensitisation

(e) N/OFQ, post OVA-sensitisation

(f) N/OFQ, pre OVA-sensitisation

(g) Naive, OVA, OVA + OFQ (post sensitisation)

(h) N/OFQ, pre OVA-sensitisation
Figure 6

(a) [Graph showing EDA migration (fold change).]
(b) [Graph showing EDA proliferation (fold change).]
(c) [Graph showing Migration (fold change).]
(d) [Graph showing IL-α conc. (pg/ml).]

(e) [Graph showing CCL17 ACC values.]
(f) [Graph showing CCL23 conc. (pg/ml).]
(g) [Graph showing CCL26 conc. (pg/ml).]
(h) [Graph showing CCL13 conc. (pg/ml).]
Figure 7

(a) Healthy HBEC  
(b) Asthmatic HBEC  
(c) Non-Asthmatic HASM  
(d) Asthmatic HASM

(e) N/OFG post OVA-sensitisation  
(f) N/OFG pre OVA-sensitisation
Online Supplementary Material

Nociceptin/OrphaninFQ (N/OFQ) exerts dual immunomodulatory and bronchodilatory roles and represents a novel target for the treatment of asthma.

Shailendra R Singh, PhD, Nikol Sullo, PhD, Maria Matteis, PhD, Giuseppe Spaziano, PhD, John McDonald, PhD, Ruth Saunders, PhD, Lucy Woodman, PhD, Konrad Urbanek, MD, Antonella De Angelis, PhD, Raffaele De Palma, MD, Rachid Beraïr, MRCP, Mitesh Pancholi, BSc, Vijay Mistry, BSc, Francesco Rossi, MD, Remo Guerrini, PhD, Girolamo Calò, PhD, Bruno D’Agostino, MD, Christopher E Brightling, MD*, David G Lambert, PhD*

*DGL and CEB are joint senior authors and contributed equally. BD’Aco-ordinated animal experiments in Napoli.

Corresponding author:
Professor David G Lambert,
Department of Cardiovascular Sciences, University of Leicester,
Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, Leicester, UK.
Tele: (+44) 0116 252 3161
E.mail: dgl3@le.ac.uk
Materials and Methods (for experiments in supplement)

**Cell isolation and culture**

Pure human airway smooth muscle (HASM) bundles were isolated from biopsies obtained at bronchoscopy and from lung resection from well-characterised asthmatics and healthy volunteers. HASM cells were cultured in DMEM with Glutamax-1 supplemented with 10% FBS, 100U/mL penicillin, 100nicillin, 100mycin, 0.25 and healthy volunteers. HASM cells were cultured in DMEM with Gum pyruvate. HASM cell characteristics were determined by immunofluorescence and light microscopy with α-smooth muscle actin-FITC direct conjugate (Sigma, Gillingham, Dorset, UK).

Primary human bronchial epithelial cells (HBEC) were isolated from bronchial brushes, and grown to confluence on 1% PureCol-coated surfaces (Inamed Biomaterials, Nutacon, The Netherlands) as submerged cultures using bronchial epithelial growth medium (BEGM, Lonza Verviers, Belgium) supplemented with 0.3% Fungizone, Belgium) su (Gibco, Invitrogen, Paisley, UK) and 1% antibiotic-Antimycotic (AA) (Gibco).

Human lung mast cells (HLMC) were dispersed from macroscopically normal lung obtained within 1h of surgical resection for lung cancer using immunoaffinity magnetic selection as described previously. Mast cell purity and viability were tested and typically >95% were observed to be pure and viable. HLMCs were then cultured in DMEM, 10% FBS, antibiotic/antimycotic solution, SCF (100ng/ml), IL-6 (50ng/ml) and IL-10 (10ng/ml). The HMC-1 cell lines were cultured in Iscove's medium containing 10% iron-supplemented fetal calf serum and 1.2mM-thioglycerol. Cells were split 1:10 every 3 days and resuspended in fresh medium.

EOL-1 (eosinophil-like) cells were cultured in RPMI media supplemented with 10% FBS and antibiotic/antimycotic solution.

**Isolation of eosinophils from peripheral blood**

Eosinophils were isolated from heparinized peripheral venous blood from healthy control subjects and asthmatic volunteers. Briefly, 30-40mL of peripheral blood was collected and layered on top of an equal volume of Polymorph prep solution (Axis-Shield Point-of-Care Division, Oslo, Norway). This was then centrifuged at 600g for 45minutes at 20°C. The polymorphonuclear cell layer was isolated, mixed with an equal volume of PBS and centrifuged at 600g for 10minutes at 20°C. Supernatant was then discarded and red blood cells in the cell pellet were lysed with cell lysis buffer (BD Biosciences, UK) by incubating for 15minutes at room temperature. Red blood cells were discarded following centrifugation at 600g for 10minutes at 20°C. Next eosinophils were negatively selected by using neutrophil-specific anti-CD16c anti-CD16emicrobeads and LS columns (Miltenyi Biotech, Surrey, United Kingdom). Finally, purity was assessed with Kimura stain. Eosinophil purity was consistently >95% and the viability of freshly isolated eosinophils was >99% as evaluated by Trypan blue dye exclusion.

**Proliferation Assays**

HMC-1 cells were grown at a density of 1e e5 cells per well in 6-well culture plates in their normal growth medium until they achieved 70% confluency. Later cells were serum starved for 24 hours and then incubated with SCF (10ng/ml) or 10% FBS supplemented DMEM media in the presence or absence of N/OFQ (300nM) for either 24 or 48hours. Cells were then harvested and counted with a haemocytometer by a blinded observer. In addition, MTS proliferation assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, according to the manufacturer's instructions (Promega, Southampton, UK). HMC-1 cells were cultured at a
density of 1ty^4 cells per well in 96-well culture plates until the cells achieved 70% confluency. Next cells were serum starved for 24 hours and then incubated with SCF (10ng/ml) or 10% FBS supplemented DMEM media in the presence or absence of N/OFQ (300nM) for either 24 or 48 hours. Next, 20μL of CellTiter 96 Aqueous One Solution Reagent (Promega, Southampton, United Kingdom) was added to each well. After 4 hours in culture, the cell viability was determined by measuring the absorbance at 490nm with a Multiskan* Ascent Microplate Photometer (ThermoFisher Scientific, UK). Results were expressed as fold change over the control (mean±SEM).

**HASM/HMC-1 co culture proliferation assay**

HASM (non-asthmatic and asthmatic) were seeded onto 6-well plates at a density of 16x10^4 and grown in DMEM media containing 10% FBS, 1% antibiotic/antimycotic, 1% non-essential amino acids, and 1% sodium pyruvate till the cells achieved complete confluency. Cells were then serum starved for 24 hours in DMEM media containing ITS (insulin, transferrin and selenium), 1% antibiotic/antimycotic, 1% nonessential amino acids, and 1% sodium pyruvate. Because analysis of bronchial biopsies reveal a mean mast cell density in asthmatic ASM bundles of ~4 HASM cell:1 mast cells, 4ds^3 HMC-1 cells were then seeded onto confluent HASM cells in the presence or absence of N/OFQ (300nM) for 48 hours. Cell numbers during the culture period was assessed with a haemocytometer by a blinded observer using Kimura staining, which readily differentiates red metachromatic mast cells from unlabeled ASM cells. Results were expressed as fold change over the control (mean±SEM).

**[^3]H| thymidine incorporation in intact cells**

Human ASM cells were cultured in 24-well plates in duplicate at a seeding density of 2.5X10^4 cells/well at 37°C in DMEM medium supplemented with 10% FBS, 1% antibiotic/antimycotic, 1% nonessential amino acids, and 1% sodium pyruvate. Subconfluent cultures (60-70%) were washed and then incubated in DMEM containing 0.1% FBS, 1% antibiotic/antimycotic, 1% nonessential amino acids, and 1% sodium pyruvate for 30h to growth arrest the cells. Platelet derived growth factor (PDGF-AB, Sigma-Aldrich, Poole, UK) at a concentration of 20ng/ml was then added for 16h in the presence or absence of N/OFQ (300nM) along with 1μM of peptidase inhibitors (amastatin / bestatin / phosphorhamidon / captoril).[^3]H|thymidine (0.5μdine (0.55/ captoril). [ orill 24h of the incubation. At the end of this period, the supernatant was aspirated, and the cells were washed with PBS and lysed with 500μl of 0.4M percholic acid. Four hundred microliters of the supernatant were transferred to a scintillation vial along with 4.5ml of scintillation fluid and counted using liquid scintillation spectroscopy, the results being expressed as fold change over the control value. Proliferation rates were calculated as fold change over control and expressed as mean±SEM.

**Measurement of cAMP formation**

Confluent HMC-1 or HASM cell cultures (grown in T125 flasks) were incubated in 0.3ml Krebs buffer containing 0.5% BSA, 1mM isobutylmethylxanthine (IBMX) and forskolin (1μMol (1forskolin (1IBMX) and forskolin (1n T125 flasks) were incubated with 10M HCl and neutralised with 10M NaOH/1 mM Tris, pH7.4. The concentration of extracted cAMP was measured using a protein-binding assay against known cAMP standards. Results were calculated as fold change over basal and expressed as mean±SEM.
ANIMAL STUDIES

Measurement of airway hyperresponsiveness

Lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1 ml/min resulting in a pulmonary artery pressure of 2-3 cm H$_2$O. The perfusion medium used was RPMI 1640 lacking phenol red (37°C circulating fashion through the pulmonary artery at a constant flow of 0.2 O$_2$/min and a tidal volume of about 200ml circulating fashion through the pulmonary artery at a constant flow of 0.2 O$_2$/min). Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45-24) and airflow velocity with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45-15). The lungs respired humidified air. The arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne) which was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analysed with the Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). The data were analysed through the following formula: $P = Vt - 1 + R_L \cdot R_{tou} - 1$, where $P$ is chamber pressure, $C$ pulmonary compliance, $V$ tidal volume, $R_L$ airway resistance. The airway resistance value registered was corrected for the resistance of the pneumotachometer and the tracheal cannula of 0.6 cm H$_2$O s ml$^{-1}$. Lungs were perfused and ventilated for 45 min without any treatment in order to obtain a baseline state. Subsequently, lungs were challenged with acetylcholine (10$^{-8}$-10$^{-3}$M). Repetitive dose response curve of acetylcholine was administered as 50s response curve of or 45 min without any treatment in order to obtain a baseline state. Subse

Figure legends

Figure E1. N/OFQ levels in asthmatic sputum do not correlate with FEV1/FVC ratio. Spearman correlation between sputum N/OFQ and FEV$_1$/FVC ratio.

Figure E2. NOP receptors on HMC-1 cells are coupled to inhibition of cAMP production. Measurement of cAMP formation in HMC-1 cells (n=7 independent experiments) following forskolin stimulation (1 µM) significantly inhibited (p<0.05) forskolin-stimulated increase in cAMP formation. Data expressed as fold change over basal and was analysed by one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05.

Figure E3. Administration of N/OFQ during OVA-sensitisation regulates release of inflammatory mediators in vivo. (a) IL-4, (b) IL-5, (c) IL-12, (d) IL-13, (e) IL-10 and (f) IL-17 cytokine levels in mouse BAL fluid obtained from different treatment groups (N/OFQ pre and post OVA-sensitization), n=3. Data expressed as pg/ml (mean±SEM)

Figure E4. N/OFQ-NOP activation modulates agonist-induced HASM contraction. (a) Bradykinin-induced time-dependant gel contraction (n=HASM cells from 7 independent donors) and (b) AUC-bradykinin response. Data expressed as mean±SEM. Comparisons made by two-way ANOVA. Contraction data are a combination of cells harvested from healthy and asthmatic patients.

* represents comparisons between bradykinin and control; # represents comparisons between N/OFQ and bradykinin. *p<0.05; #p<0.05.
**Figure E5. N/OFQ inhibits inflammatory cell migration in vitro.**
(a) Migration of EOL-1 cells (n=8 replicates) and (b) PBEs towards asthmatic sputum (n=PBEs from 7 independent donors), (c) Migration of HLMC towards SCF (n=7 independent donors), (d) Migration of PBEs (n=PBEs from 6 independent donors) to epithelial conditioned media, (e) Migration of HMC-1 to SCF and CXCL10 (n=5 replicates), (f) Migration of HMC-1 to ASM supernatants (n=8 replicates). Data expressed as mean ± SEM. Comparisons by one-way ANOVA. *p<0.05.

**Figure E6. N/OFQ had no effect on TNF-α release from HMC-1 cells.**
SCF-induced TNF-α release from HMC-1 cells (n=6 independent experiments) was not modulated by N/OFQ pre-treatment. Data expressed as pg/ml (mean ± SEM) and analysed by paired t-test.

**Figure E7. N/OFQ does not modulate mitogen-induced proliferation of HASMs and HMC-1 cells.**
(a) [³H] thymidine incorporation was measured in HASM cultures stimulated with platelet-derived growth factor (PDGF-AB, 20 ng/ml) for 24 h in the presence or absence of N/OFQ expressed as fold change, (b) MTS colorimetric assay was performed to detect HMC-1 cell viability and proliferation following stimulation with stem cell factor (SCF, 10 ng/ml) for 24 h in the presence or absence of N/OFQ expressed as fold change, (c) [³H] thymidine incorporation was measured in HASM cultures stimulated with platelet-derived growth factor (PDGF-AB, 20 ng/ml) for 24 h in the presence or absence of N/OFQ expressed as actual counts, (d) MTS colorimetric assay was performed to detect HMC-1 cell viability and proliferation following stimulation with stem cell factor (SCF, 10 ng/ml) for 24 h in the presence or absence of N/OFQ expressed as actual counts. All data represent mean ± SEM (n=6 independent experiments). Comparisons made by one-way ANOVA. *p<0.05.