

## RESEARCH PAPER

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

**Correspondence** Professor David G Lambert, Department of Cardiovascular Sciences, University of Leicester, Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, Leicester, UK. E-mail: dgl3@le.ac.uk

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Shailendra R Singh<sup>1,2</sup>, Nikol Sullo<sup>1,3</sup>, Maria Matteis<sup>3</sup>, Giuseppe Spaziano<sup>3</sup>, John McDonald<sup>1</sup>, Ruth Saunders<sup>2</sup>, Lucy Woodman<sup>2</sup>, Konrad Urbanek<sup>3</sup>, Antonella De Angelis<sup>3</sup>, Raffaele De Palma<sup>6</sup>, Rachid Berair<sup>2</sup>, Mitesh Pancholi<sup>2</sup>, Vijay Mistry<sup>2</sup>, Francesco Rossi<sup>3</sup>, Remo Guerrini<sup>5</sup>, Girolamo Calò<sup>4</sup>, Bruno D'Agostino<sup>3</sup>, Christopher E Brightling<sup>2\*</sup> and David G Lambert<sup>1,\*</sup>

<sup>1</sup>Department of Cardiovascular Sciences, University of Leicester, Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, Leicester LE2 7LX, UK, <sup>2</sup>Institute for Lung Health, Department of Infection, Immunity and Inflammation and Health Sciences, University of Leicester, Leicester, UK, <sup>3</sup>Department of Experimental Medicine, Section of Pharmacology L Donatelli, Second University of Naples, Naples, Italy, <sup>4</sup>Department of Medical Sciences, Section of Pharmacology, University of Ferrara, Ferrara, Italy, <sup>5</sup>Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy, and <sup>6</sup>Department of Clinical and Experimental Medicine, Division of Immunology, Second University of Naples, Naples, Italy

\*D. G. L. and C. E. B. are joint senior authors and contributed equally. B. D'A co-ordinated animal experiments in Naples.

### BACKGROUND AND PURPOSE

There is evidence supporting a role for the nociceptin/orphanin FQ (N/OFQ; NOP) receptor and its endogenous ligand N/OFQ in the modulation of neurogenic inflammation, airway tone and calibre. We hypothesized that NOP receptor activation has beneficial effects upon asthma immunopathology and airway hyperresponsiveness. Therefore, the expression and function of N/OFQ and the NOP receptor were examined in healthy and asthmatic human airway tissues. The concept was further addressed in an animal model of allergic asthma.

### EXPERIMENTAL APPROACH

NOP receptor expression was investigated by quantitative real-time 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)-sensitized and challenged mice.

### KEY RESULTS

NOP receptors were expressed on a wide range of human and mouse immune and airway cells. Eosinophils expressed N/OFQ-precursor mRNA and their number correlated with N/OFQ concentration. N/OFQ was found in human sputum and increased in asthma. Additionally, in asthmatic human lungs N/OFQ immunoreactivity was elevated. NOP receptor activation inhibited migration of immunocytes and increased wound healing in airway structural cells. Furthermore, N/OFQ relaxed spasmogen-stimulated gel contraction. Remarkably, these findings were mirrored in OVA-mice where N/OFQ treatment before or during sensitization 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 demonstrated a novel dual airway immunomodulator/bronchodilator role for N/OFQ and suggest targeting this system as an innovative treatment for asthma.

## Abbreviations

AHR, airway hyperresponsiveness; ECM, epithelial conditioned media; EFS, electrical field stimulation; EOL-1, eosinophil-like cell line; GINA, Global Initiative for Asthma; HASM, human airway smooth muscle; HBEC, human bronchial epithelial cells; HLMC, human lung mast cells; HMC-1, human mastocytoma cell line; NOP receptor, N/OFQ peptide receptor; N/OFQ, nociceptin/orphanin FQ; OVA, ovalbumin; PBES, peripheral blood eosinophils; ppN/OFQ, prepronociceptin; SCF, stem cell factor; TFA, trifluoroacetic acid

## Tables of Links

TARGETS
NOP receptor

LIGANDS				
ACh	CCL26	IL-5	IL-13	TNF- $\alpha$
Bradykinin	CXCL10	IL-8 (CXCL8)	IL-17	UFP-101
Carbachol	IFN- $\gamma$	IL-10	N/OFQ	
CCL11	IL-4	IL-12	Stem cell factor	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

## Introduction

Nociceptin/orphanin FQ (N/OFQ) is the endogenous peptide activator of the N/OFQ receptor (NOP receptor), classified by The International Union of Basic and Clinical Pharmacology (IUPHAR) as a non-opioid (or non-classical) member of the opioid family. Since the discovery of the NOP receptor and its deorphanization, the N/OFQ-NOP receptor 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.*, 1995; Lambert, 2008). The vast range of additional peripheral actions for NOP receptor activation have also reinvigorated interest 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 ~0.25 million (WHO, 2013). Asthma is a complex heterogeneous and devastating disease characterized by variable degree of airflow obstruction, airway hyperresponsiveness, chronic airway inflammation and airway remodelling (Brightling *et al.*, 2012). These changes are the result of a crosstalk between resident structural airway smooth muscle and epithelial cells, progenitors including fibrocytes, infiltrating airway submucosal inflammatory cells (eosinophils and T-cells) (Brightling *et al.*, 2002a), localized mast cells within airway smooth muscle bundles (Brightling *et al.*, 2002a) and Th<sub>2</sub> cells (and their cytokines) (Brightling *et al.*, 2002b).

With respect to airways, NOP receptor activation abolishes capsaicin- and electrical field stimulation (EFS)-induced contraction in guinea pig airways (Shah *et al.*, 1998; Corboz *et al.*, 2000) 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 ACh and sensory neuropeptide release (Patel *et al.*, 1997; Corboz *et al.*, 2000). 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.*, 2010). In addition, NOP receptor 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 variable steroid-resistant patterns of inflammation (Bousquet *et al.*, 2009). The development of new therapies *combining* both bronchodilator and steroid-free immunosuppressive profiles offer several advantages over the current treatments not least a simplified dosing regimen.

We hypothesized that the N/OFQ-NOP receptor system plays a critical role in the pathogenesis of airway inflammation, airflow obstruction and hyperresponsiveness, the hallmarks of asthma. There are currently no data on N/OFQ-NOP receptor 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 receptor, prepronociceptin (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* OVA-sensitized mouse model of asthma. We showed that N/OFQ is a candidate dual immunomodulator and bronchodilator.

## Methods

Detailed methods are available in the Supporting Information.

## Subjects

Asthmatic subjects and healthy controls were recruited in Leicester, UK, and their 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 (GINA) treatment steps (mild-moderate

**Table 1**

Clinical characteristics of healthy and asthmatic volunteers recruited for sputum analysis.

	Healthy (n = 29)	GINA 1–3 (n = 30)	GINA 4–5 (n = 55)	P value
Age in years <sup>#</sup>	50 ± 3	55 ± 2	56 ± 2	0.48
Male, n (%)	19 (66)	20 (67)	39 (58)	0.85
Smoking, n (%)	9 (31)	12 (40)	16 (29)	0.99
Current	2 (22)	2 (17)	3 (19)	
Ex	7 (78)	10 (83)	13 (81)	
Smoking (pack years) <sup>#</sup>	3.9 ± 1.5	6.3 ± 1.9	4.5 ± 1.2	0.60
FEV <sub>1</sub> % predicted <sup>#</sup>	105.3 ± 3.6	84.2 ± 4.1	69.3 ± 3.3	<0.001
FEV <sub>1</sub> /FVC% <sup>#</sup>	78.5 ± 1.2	71.3 ± 2.1	34.5 ± 4.5	<0.001
Sputum neutrophils, % <sup>*</sup>	50 (31–61.5)	62.4 (55–78.5)	56.3 (49–69)	0.03
Sputum macrophages, % <sup>*</sup>	39.3(32.5–60.8)	23.8(14.3–36.5)	19.9 (14–28)	<0.001
Sputum eosinophils, % <sup>*</sup>	1.8 ± 0.6 (0–5)	0.8 (0.5–1.8)	2.9 (1.8–6.9)	<0.001
Sputum epithelial cells, % <sup>*</sup>	2.0 (1.0–3.3)	1.0 (0.8–2.3)	2.5 (1.8–4.0)	0.03
Sputum lymphocytes, % <sup>*</sup>	0.3 (0–0.3)	0.0 (0.0–0.3)	0.3 (0.0–0.5)	0.25

P value represents comparisons between healthy subjects and GINA 4–5 patients.

<sup>#</sup>Mean ± SEM;

<sup>\*</sup>Median (interquartile range).

GINA 1–3 and severe GINA 4–5). Subjects underwent extensive clinical characterization including sputum induction and video-assisted fiberoptic 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. Human mastocytoma (HMC-1) and human eosinophil-like (EOL-1) cell lines were cultured, as described previously (Butterfield *et al.*, 1990). Peripheral blood eosinophils (PBEs) were isolated from heparin-treated peripheral venous blood from healthy control subjects and asthmatic volunteers using LS columns (Miltenyi Biotec, UK).

### Membrane preparation and <sup>125</sup>I 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 homogenizing buffer of Tris–HCl (50 mM), MgSO<sub>4</sub> (5 mM) pH 7.4 with KOH and were homogenized followed by centrifugation at 4890 g for 10 min at 4°C. Membrane protein (20 µg of CHO<sub>hNOP</sub>, 200 µg of HASM and HMC-1) was incubated in 0.5 mL of homogenization buffer containing 0.5% BSA, 10 µM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon, Sigma-Aldrich, Poole, UK) and various concentrations of [<sup>125</sup>I]-N/OFQ (~10 pM–1 nM, Perkin Elmer, UK) for 1 h at room temperature. Non-specific binding was defined in the presence of 1 µM unlabelled N/OFQ. Bound and free radioactivities were separated by vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters (Fisher Scientific, Loughborough, UK). Filter-bound radioactivity was assessed by a γ counter, and receptor density was calculated from dilution isotherms.

### Quantitative real-time PCR

All airway and immune cells from volunteers and patients or cultured cells were prepared immediately or stored in RNAlater® (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 (Fisher Scientific, Loughborough, UK) 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 ppN/OFQ or ppNOC (Hs00173823\_m1), human CCL11 (eotaxin-1; Hs00237013\_m1), human CCL26 (eotaxin-3; Hs00171146\_m1) and GAPDH or β2 microglobulin. 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 reactions in the StepOne instrument (Applied Biosystems) was 2 min 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 are expressed as ΔCt: the difference in cycle threshold 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 2 µm sections were cut from glycomethacrylate-embedded asthmatic and healthy bronchial biopsies and stained using a polyclonal anti-human N/OFQ antibody (1 in 200 dilution, Phoenix Europe GmbH, Germany) and appropriate isotype

control rabbit IgG (Phoenix Europe GmbH). 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

The 24-well or 96-well Transwell migration assay was used to measure the migration of HMC-1 cells, HLMCs, EOL-1 cells and PBEs in response to chemoattractants. To investigate mast cell migration, 450  $\mu\text{L}$  of stem cell factor (SCF) ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , R&D Systems, Abingdon, UK), CXCL10 ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , R&D Systems, Abingdon, UK) and HASM supernatants stimulated with recombinant TNF- $\alpha$  for 24 h ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , 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 PBE 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 ( $2.5 \times 10^5$  HMC-1 cells;  $1 \times 10^5$  HLMCs;  $2.5 \times 10^4$  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 h at  $37^\circ\text{C}$  and EOL-1 cells, eosinophils were incubated for 90 min at  $37^\circ\text{C}$ . 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  $1 \times 10^5$  cells per well) were grown to confluency in a six-well culture plate. Cells were then serum starved for 24 h and pre-incubated with 300 nM of N/OFQ for 60 min at  $37^\circ\text{C}$  in the presence of peptidase inhibitors. Next, HASM cells were stimulated in the presence or absence of TNF- $\alpha$  ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , R&D Systems, Abingdon, UK) and HBEC in the presence or absence of IL-13 ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , R&D Systems, Abingdon, UK) for 24 h at  $37^\circ\text{C}$ . After 24 h, supernatants were collected, spun down to remove debris and stored at  $-80^\circ\text{C}$  till further analysis. Colorimetric human CCL11 and CCL26 ELISAs (R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's protocol.

### Measurement of IL-8 release from HMC-1 cells

HMC-1 cells were grown in six-well culture plates and then pre-incubated in the presence or absence of N/OFQ (300 nM) for 60 min at  $37^\circ\text{C}$  in the presence of peptidase inhibitors. Next, cells were stimulated in the presence or absence of SCF ( $10 \text{ ng}\cdot\text{mL}^{-1}$ , R&D Systems, Abingdon, UK) for 24 h at  $37^\circ\text{C}$ . After 24 h, supernatants were collected, centrifuged at 2000 g for 5 min to remove debris and stored at  $-80^\circ\text{C}$  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 eight-well culture plates, serum deprived for 24 h and then wounded using a sterile 200  $\mu\text{L}$  pipette tip in a predetermined

grid pattern. After being wounded, cells were washed and treated in the presence or absence of N/OFQ (3 and 300 nM). Non-asthmatic and asthmatic HASM cells were seeded onto eight-well fibronectin-coated plates, serum deprived for 24 h in ITS medium, and then wounded using a sterile 200  $\mu\text{L}$  pipette tip in a predetermined grid pattern. After being wounded, HASM cells were washed and treated in the presence or absence of N/OFQ (3, 30 and 300 nM). Wounds were then photographed at baseline, after 6 and 24 h. Wound areas were analysed by a blinded observer using IMAGEJ software, and the extent of repair was 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 was measured by RIA as described previously (Williams *et al.*, 2008).

### Assessment of airway smooth muscle contraction by collagen gel analysis

Contractile properties of airway smooth muscle cells were assessed using a collagen gel contraction assay. Collagen gels were impregnated with HASM cells ( $2.5 \times 10^5$  cells) resuspended in DMEM with GlutaMAX-1 supplemented with penicillin ( $100 \text{ U}\cdot\text{mL}^{-1}$ ), streptomycin ( $100 \mu\text{g}\cdot\text{mL}^{-1}$ ), amphotericin ( $0.25 \mu\text{g}\cdot\text{mL}^{-1}$ ), non-essential amino acids ( $100 \mu\text{M}$ ) (Invitrogen, Paisley, United Kingdom), sodium pyruvate (1 mM) and insulin-transferrin-selenium (1%) (Sigma-Aldrich). Next, 450  $\mu\text{L}$  of gel mixture was added to each well of a PBS 2% BSA pre-coated 24-well plate and allowed to polymerize at  $37^\circ\text{C}$  for 90 min. After polymerization, 500  $\mu\text{L}$  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 (3 and 300 nM) for 24 h at  $37^\circ\text{C}$ . Carbachol ( $100 \mu\text{M}$ ) or bradykinin (10 nM) 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 3 h period by a blinded observer, and the gel size was calculated at specific time points, as a percentage of the well area, using IMAGEJ software (National Institutes of Health, USA). All gel conditions were performed in duplicate.

### Animal studies

Animal studies reported are in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). The investigation was approved by the Veterinary Animal Care and Use Committee of the Second University of Naples (1966/7.17.2012) and conforms to the National Ethical Guidelines of the Italian Ministry of Health and the Guide for the Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA, revised 1996). All experimental procedures were in accordance with Italian DLgs 26/2014, application of the EU Directive 2010/63/EU. BALB/c mice were obtained from Harlan Laboratory (Udine, Italy). Mice were housed in the animal facility of the Second University of Naples in standard conditions. Food and water were supplied *ad libitum*. Room temperature was  $22^\circ\text{C}$ – $24^\circ\text{C}$ , relative humidity was 40%–50%, and the day/night cycle was set at 12 h/12 h. Mice were acclimatized for 1 week before starting any procedures in 5 mice per cage.



## Experimental protocol

Female BALB/c mice were used in this study. Animals were sensitized to OVA by s.c. injection with 0.4 mL of 10 µg OVA, adsorbed to 3.3 mg of aluminium hydroxide gel in sterile saline at days 0 and 7. From day 21 to 23, all OVA-sensitized mice were aerosol challenged (7-min-long daily sessions) with 1% OVA in PBS 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 100 µL of saline solution (vehicle) or 100 µL of N/OFQ (15 µg·kg<sup>-1</sup>). Two different experimental protocols were used for N/OFQ treatment. In the first protocol (post-OVA sensitization), vehicle or N/OFQ were administered i.p. from day 21 to 23, 30 min before each OVA aerosol challenge (Figure 2A), while in the second protocol (pre-OVA sensitization), vehicle or N/OFQ were administered i.p., at day 0 and 7, 30 min before each allergen injection (Figure 2B). Twenty-four hours after the last aerosol challenge, animals were killed, and bronchopulmonary function, pulmonary tissue and bronchoalveolar lavage (BAL) fluid collection were performed.

**Airway hyperresponsiveness.** AHR to ACh was assessed in an isolated and perfused mouse lung model as described in detail in Roviezzo *et al.* (2007). Mice were anaesthetized with ketamine HCl 40 mg kg<sup>-1</sup> i.p. and medetomidine hydrochloride 0.15 mg kg<sup>-1</sup> i.p. and were exsanguinated after incision of the renal vein as the method required. After 60 min, mean tidal volume was 0.21 ± 0.02 mL ( $n = 61$ ), mean airway resistance 0.23 ± 0.08 cmH<sub>2</sub>O·s·mL<sup>-1</sup> and mean pulmonary artery pressure 2.9 ± 1.4 cmH<sub>2</sub>O. The airway resistance measured was corrected for the resistance of the pneumotachograph and the tracheal cannula of 0.6 cmH<sub>2</sub>O·s·mL<sup>-1</sup>.

**Bronchoalveolar lavage.** Mice were anaesthetized with ketamine HCl 40 mg kg<sup>-1</sup> i.p. and medetomidine hydrochloride 0.15 mg kg<sup>-1</sup> i.p. and then killed by cervical dislocation. 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. BAL 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 analyse the cytokine production. Cell pellets were resuspended in PBS 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 (Reagent, Gentaur, Italy) stained cytopins. At least 200 cells were counted on each cytopin according to standard morphological criteria under light microscopy.

**Cytokine assays.** Measurement of cytokines in BAL fluid was performed taking advantage of the well-established Luminex xMAP technology that allows measurement of a panel of analytes in a small sample volume (100 µL) 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 analyser (Luminex® 200™ System, Invitrogen, Milan, Italy) as detailed previously (Vignali, 2000). All samples were run in duplicate. Data were analysed using XPONENT software (1.9 version, Luminex® 200™ System, Invitrogen).

**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 30 min 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 FITC-conjugated and tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were stained with DAPI. For the assessment of inflammation, sections were stained with haematoxylin–eosin (HE). To facilitate the recognition of eosinophils, a modified HE protocol was used (Meyerholz *et al.*, 2009). The number of eosinophils mm<sup>-2</sup> of the peribronchial tissue was measured. The number of mast cells mm<sup>-2</sup> of the lung tissue was measured after staining with toluidine blue. Mucin production was assessed by immunolabelling with anti-mucin 5 AC antibody (Abcam, Cambridge Science Park, UK). 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 analysed with a Leica fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). 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 *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 3 mL 60% acetonitrile, lyophilized 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<sup>-1</sup>–20 ng·mL<sup>-1</sup>) and OVA-specific IgG (2B Scientific, Oxford, UK, assay range 1.56–100 U·mL<sup>-1</sup>) ELISA was performed according to the manufacturer's protocol.

## Statistical analysis

Analysis between groups was performed (PRISM Version 6 (GraphPad, CA, USA)) 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 (GINA 1–3) and severe asthmatics (GINA 4–5). Between

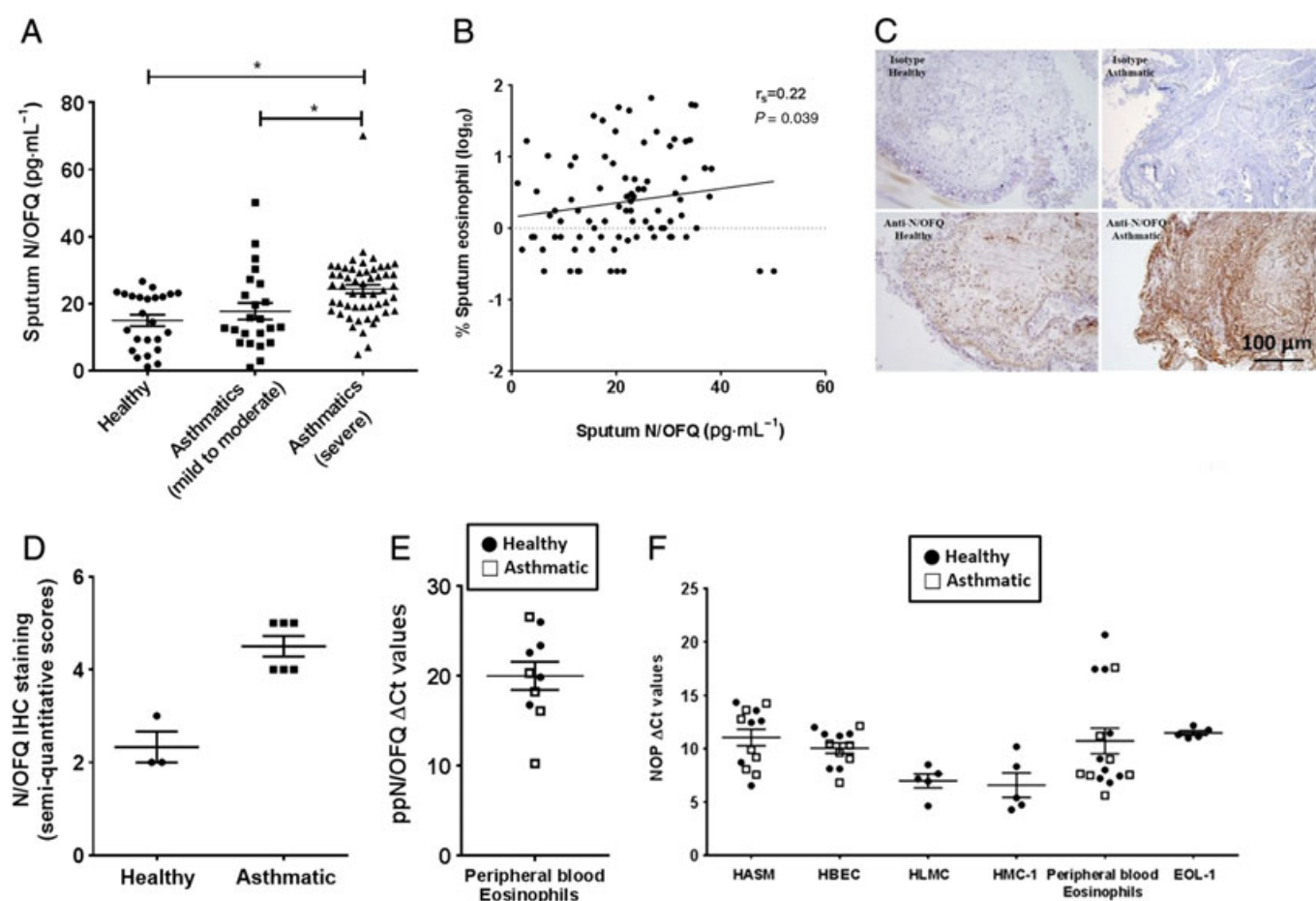
group differences were analysed by unpaired *t*-tests or Fisher's exact test. Correlations were assessed by Spearman rank (*r<sub>s</sub>*) coefficients. Values of *P* < 0.05 were considered significant.

## Results

### N/OFQ expression in asthmatic human airways and blood eosinophils

Severe asthmatics (GINA 4–5) had significantly higher levels of N/OFQ in sputum relative to healthy volunteers and GINA 1–3 subjects (Figure 1A). There was no statistical difference between levels of N/OFQ in the sputum from healthy and asthmatic subjects (mild–moderate). Although N/OFQ and FEV<sub>1</sub>/FVC did not correlate (Supporting Information Fig. SE1),

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 and 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 PBEs, but not in human airway structural cells, from healthy (expression observed in 5/9 independent samples;  $\Delta C_T = 21.73 \pm 1.57$ ) and asthmatic (expression observed in 5/7 independent samples;  $\Delta C_T = 18.31 \pm 2.66$ ) patients with no statistically significant differences (*P* < 0.05; Figure 1E).



### Figure 1

Endogenous N/OFQ expression is increased in human asthmatic airways; the NOP receptor is expressed in human airways. (A) Sputum N/OFQ levels from healthy (*n* = 29 subjects; *n* = 5 below detection limit), mild to moderate asthmatic (GINA 1–3; *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<sup>-1</sup>) 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  $\mu$ m; (D) semi-quantitative IHC staining score (*n* < 5 for healthy human airway tissue); (E) quantitative real-time PCR demonstrating ppN/OFQ mRNA expression on PBEs (*n* = 5 healthy and *n* = 5 asthmatic donors); and (F) quantitative real-time PCR demonstrating NOP receptor mRNA expression in human airway structural and immune cells (*n* numbers represent cells from individual donors for HASM, HBEC, HLME and PBEs and independent experiments for HMC-1 and EOL-1 cell lines). Data are expressed as mean  $\pm$  SEM. Comparisons made by unpaired *t*-test or one-way ANOVA followed by appropriate *post hoc* tests where relevant. \**P* < 0.05.

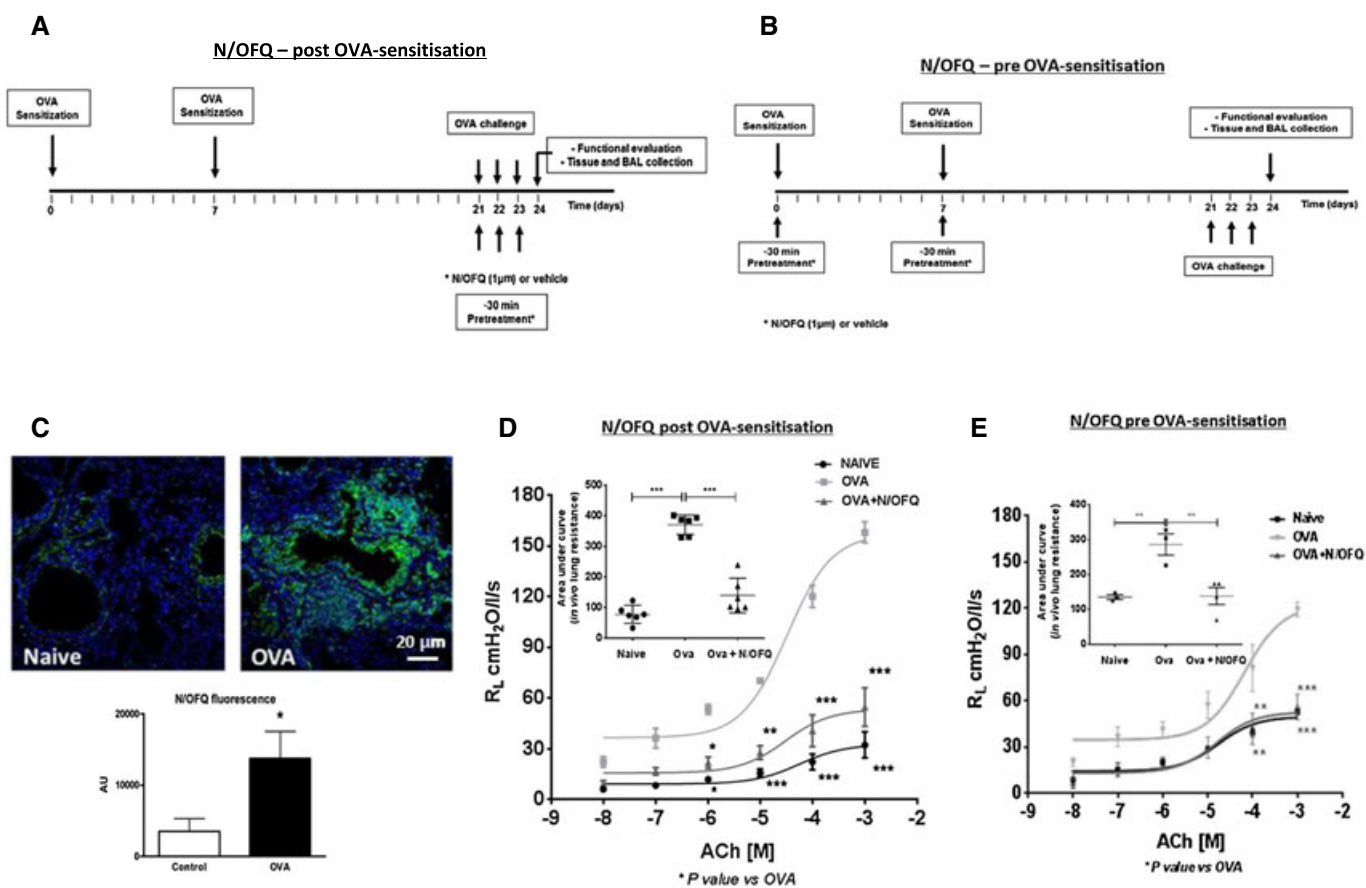
### NOP receptor expression on HLMCs, HMC-1 and airway structural cells

Interestingly, HLMCs and HMC-1 cells expressed higher levels of NOP receptor mRNA transcript than HASM cells and HBECs. NOP receptor mRNA transcript was also detected in EOL-1 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 [<sup>125</sup>I]-N/OFQ radioligand isotope dilution assays, cell membrane NOP receptor density was quantified on HASM ( $B_{max}$ ;  $7.3 \pm 1.1$  fmol·mg<sup>-1</sup> protein,  $n = 9$ ) and HMC-1 cells ( $B_{max}$ ;  $17 \pm 5.9$  fmol·mg<sup>-1</sup> protein,  $n = 3$ ). NOP receptor density was significantly increased in HMC-1 cells compared with HASM ( $P < 0.05$ ; unpaired  $t$ -test). A CHO cell line expressing recombinant human NOP receptors (positive control) expressed  $1321 \pm 60$  fmol·mg<sup>-1</sup> protein ( $n = 4$ ) of NOP receptors. Moreover, N/OFQ treatment of activated HMC-1 cells induced a small inhibition of the signalling messenger cAMP (19.3% at 300 nM N/OFQ; Supporting Information Fig. SE2).

Overall, these data demonstrate that the N/OFQ-NOP receptor system is not only present in human airways but may also have a role in asthma as suggested by increased N/OFQ. The N/OFQ-NOP receptor system may be positively involved in airway pathophysiology; we, therefore, considered whether the concentration was sufficiently 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 and 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-OVA or post-OVA sensitization,



**Figure 2**

N/OFQ inhibits bronchial hyperreponsiveness. (A) *In vivo* experimental protocol: N/OFQ administered 30 min prior to OVA challenge (N/OFQ post-OVA sensitization); (B) *in vivo* experimental protocol: N/OFQ administered 30 min 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  $\mu$ m; (D) measurement of ACh-induced lung resistance in an *in vivo* model (N/OFQ post-OVA sensitization) with AUC (inset,  $n = 6$  mice); and (E) measurement of ACh-induced lung resistance in an *in vivo* model (N/OFQ pre-OVA sensitization) with AUC (inset,  $n = 4$  mice). Data are expressed as mean  $\pm$  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 1  $\mu$ M is equivalent to 15  $\mu$ g·kg<sup>-1</sup>.



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 and 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 and B). There was no significant effect on neutrophils (Figure 3A and B) or alveolar macrophages (data not shown). Histological evaluation revealed an increase in peribronchial inflammatory infiltrates in the lungs of animals sensitized and challenged with OVA, and this was markedly reduced by N/OFQ (Figure 3C and D). Additionally, N/OFQ treatments appeared to reduce peribronchial eosinophil infiltration in OVA-challenged mice (Figure 3E and 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 and H).

Because Th<sub>1</sub> and Th<sub>2</sub> cytokines play an important role in allergic inflammation, to determine if N/OFQ affects inflammatory mediators *in vivo*, cytokine profile was analysed in BAL. When N/OFQ was administered during OVA sensitization and during the OVA challenge phase, the treatment significantly reduced allergen-induced increases in IL-4, IL-5, IL-12 and IL-13 (Supporting Information Fig. SE3a–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 and F). No effect was observed on IFN- $\gamma$  levels (not shown). However, it had no effect on IL-10 and IL-17 levels following administration during the OVA sensitization phase (Supporting Information Fig. SE3e and f).

Finally, OVA-specific IgE in BAL fluid and plasma samples were significantly reduced by N/OFQ (Figure 4G and H). N/OFQ treatment had no effect on OVA-specific IgG levels in either BAL fluid or plasma (Figure 4I and J). These observations suggest a Th<sub>2</sub>-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 min post-stimulation with a maximum effect after 180 min. There was a significant concentration and time-dependent inhibition of carbachol-induced contractility following 24 h pre-treatment with 3 and 300 nM N/OFQ ( $P < 0.05$  following repeated measures by two-way ANOVA). These inhibitory responses were significant from 60 to 180 min post-agonist treatment (Figure 5A–C). We observed a similar effect of N/OFQ on bradykinin-induced gel contraction (Supporting Information Fig. SE4). 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<sup>-1</sup> (8–19 pM) N/OFQ induced a significant increase in EOL-1 and PBE migration. Addition of 30 or 300 nM exogenous N/OFQ significantly inhibited these effects (Figure 6A and B; Supporting Information Fig. SE5a and b).

Chemoattractants including SCF, CXCL10 and supernatants from HASM stimulated with 10 ng·mL<sup>-1</sup> of TNF- $\alpha$  (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; Supporting Information Fig. SE5c). ECM-stimulated eosinophil migration (2.33-fold over control) was also attenuated by 300 nM N/OFQ (Figure 6C; Supporting Information Fig. SE5d). Furthermore, N/OFQ (300 nM) significantly inhibited SCF, CXCL10 and HASM sn-stimulated HMC-1 migration (Figure 6C; Supporting Information Fig. SE5e and f).

### *N/OFQ inhibits the release of chemoattractants that mediate human mast cell and eosinophil migration*

IL-8 and TNF- $\alpha$  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- $\alpha$  release (Supporting Information Fig. SE6).

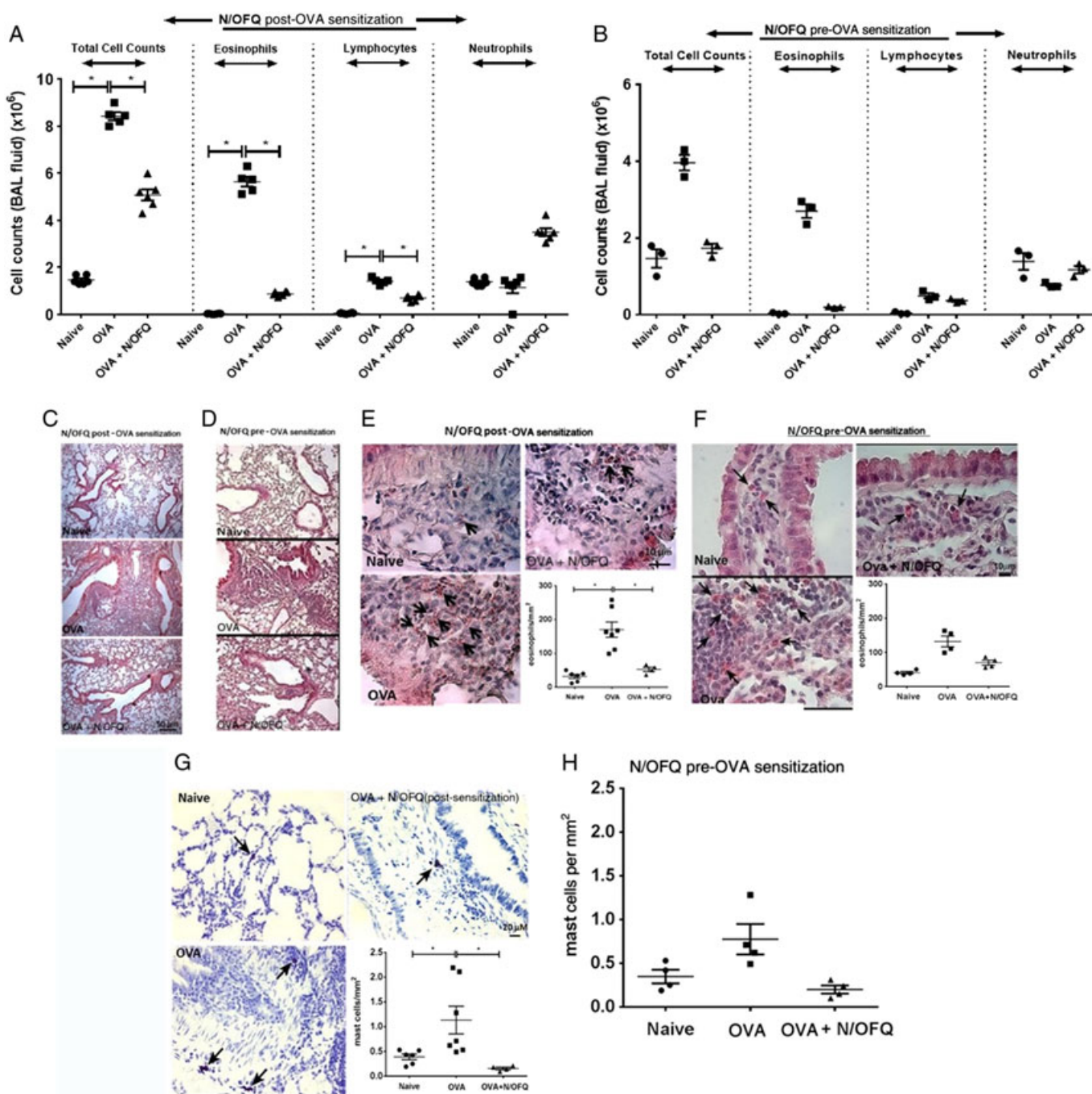
CCL11 and CCL26 are involved in the activation and recruitment of PBEs (Garcia-Zepeda *et al.*, 1996; White *et al.*, 1997). N/OFQ treatment demonstrated a trend towards reduced (increased  $\Delta$ Ct). TNF- $\alpha$ -stimulated CCL11 mRNA expression in HASM (Figure 6E) and TNF- $\alpha$ -stimulated 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 H).

### *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 the proliferation, migration and differentiation of cells adjoining the damaged area (Tam *et al.*, 2011).

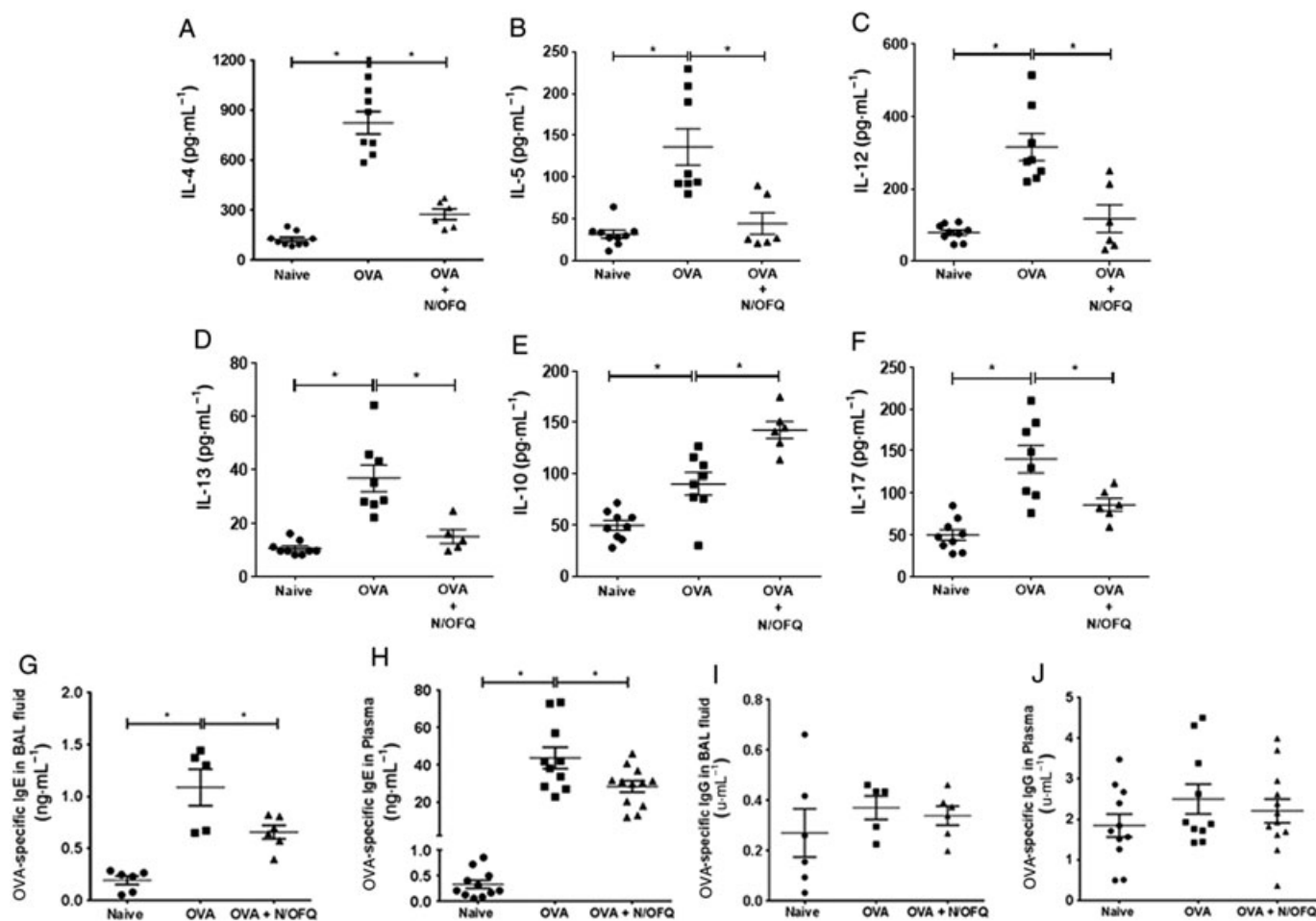
To test the effects of N/OFQ on the repair capacity of bronchial epithelium, confluent monolayers of undifferentiated healthy and asthmatic HBEC were scratch wounded in the presence or absence of the peptide. Although a complete wound closure (a combination of proliferation and migration or chemotaxis) was not achieved, N/OFQ appeared to promote wound repair of healthy HBECs and significantly induced wound closure of asthmatic HBECs (Figure 7A and B); the magnitude of the 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 300 nM N/OFQ, while asthmatic HASM wound closure was markedly increased with 3, 30 and 300 nM N/OFQ (Figure 7C and D). There was a significant





**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 HE staining in a N/OFQ post-OVA sensitized mouse airways ( $n = 6$  mice). Scale bars 50  $\mu\text{m}$ . (D) Representative image of HE staining in a N/OFQ pre-OVA sensitization mouse airways ( $n = 3$  mice). Scale bars, 50  $\mu\text{m}$ . (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, 10  $\mu\text{m}$ . (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, 10  $\mu\text{m}$ . (G) Representative image of mast cells detected by toluidine blue staining,  $n = 7$  mice. Scale bars, 20  $\mu\text{m}$ . (H) Quantitative estimation of mast cells  $\text{mm}^{-2}$  of mice airway tissue in a N/OFQ pre-OVA sensitization mouse airways ( $n = 4$  mice). Data expressed as mean  $\pm$  SEM and analysed by one-way ANOVA followed by appropriate *post hoc* tests where relevant. \* $P < 0.05$ .



**Figure 4**

Administration of N/OFAQ following sensitization 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 are expressed as  $\text{pg}\cdot\text{mL}^{-1}$  (mean  $\pm$  SEM). (G) Measurement of OVA-specific IgE in BAL fluid ( $n = 5$  mice) and (H) in plasma ( $n = 10-12$  mice). Data are expressed as  $\text{ng}\cdot\text{mL}^{-1}$  (mean  $\pm$  SEM). (I) Measurement of OVA-specific IgG levels in mouse BAL fluid and (J) in mouse plasma. Data are expressed as  $\text{U}\cdot\text{mL}^{-1}$  (mean  $\pm$  SEM). Statistical comparisons by one-way ANOVA followed by appropriate *post hoc* tests where relevant. \* $P < 0.05$ .

time ( $P < 0.05$ ) and dose-dependent closure of HASM wound ( $P < 0.05$ ; repeated measures by two-way ANOVA).

Additionally, mitogen-induced proliferation of HASMs, HMC-1 cells alone (Supporting Information Fig. SE7) or when co-cultured with HASMs was not influenced by N/OFAQ (data not shown).

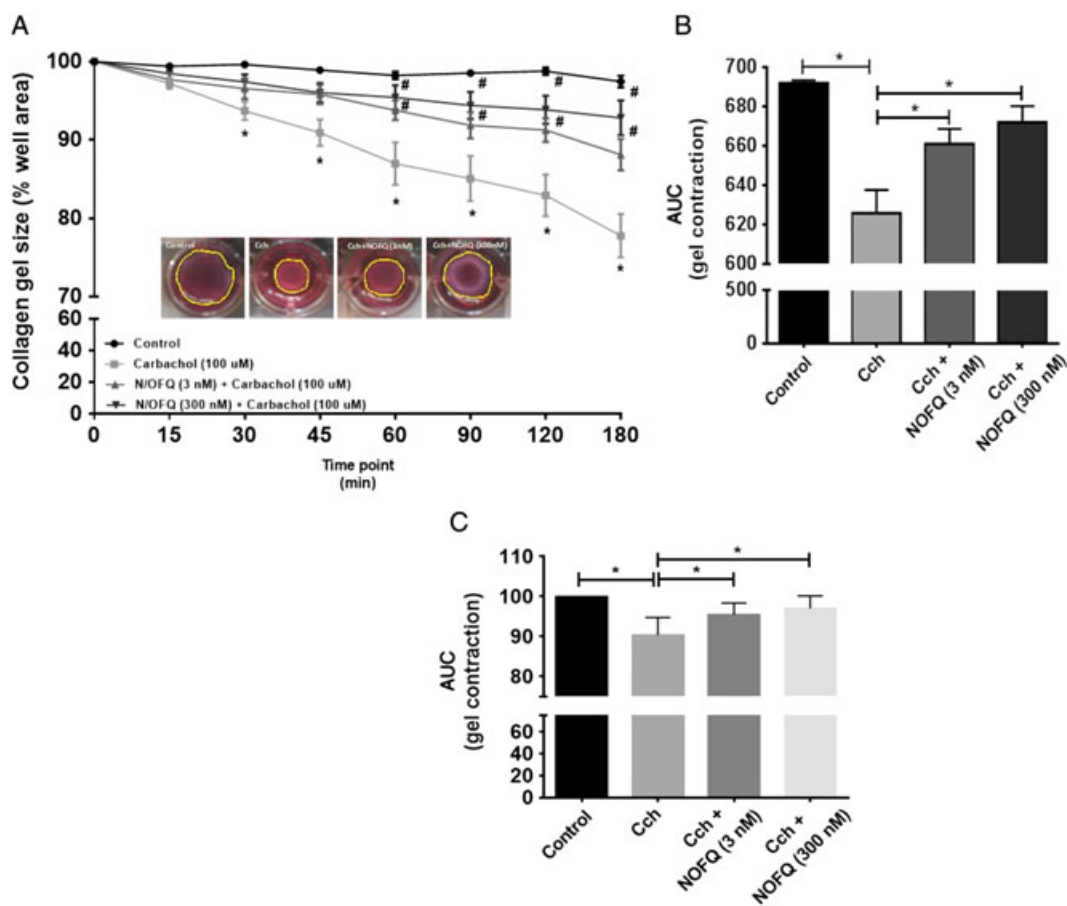
Finally, as the *in vivo* counterpart, the response of airway epithelium to the exogenous N/OFAQ was evaluated. In animals sensitized and challenged with OVA, administration of N/OFAQ reduced by ~50% the extent of epithelial damage (not shown) and decreased the fraction of mucin-labelled epithelial cells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

to report a critical role for this system in asthma and describes a novel agent with combined anti-hyperresponsiveness and immunomodulatory properties.

There is emerging evidence suggesting a generalized immunomodulatory role for the N/OFAQ-NOP receptor system (Miller and Fulford, 2007), and our data showed that asthmatic sputum had significantly elevated levels of N/OFAQ. This may come from the increased eosinophil counts as there was a correlation between increased eosinophils and elevated N/OFAQ. N/OFAQ inhibited eosinophil and mast cell migration and attenuated the release of inflammatory mediators that play key roles in their recruitment. Interestingly, N/OFAQ expression was found to be up-regulated in the lung biopsies from asthmatic patients. In particular, N/OFAQ was increased in the sub-epithelial layer and extracellular matrix. We also demonstrated significant NOP receptor expression in human airway structural and inflammatory cells with ppN/OFAQ expression only in eosinophils. However, we did not detect any significant increase in NOP receptor expression in asthma implying no disease signal. Functional



**Figure 5**

N/OFOQ-NOP activation modulates agonist-induced HASM contraction. (A) Carbachol-induced time-dependent gel contraction ( $n = 7$  HASM cells from seven independent donors); \*statistical comparisons between carbachol and control; #statistical comparisons between N/OFOQ and carbachol. (B) AUC-carbachol response and (C) AUC-carbachol response normalized to control (expressed as 100%). Data are expressed as mean  $\pm$  SEM. Comparisons by one-way ANOVA followed by appropriate *post hoc* tests where relevant. \* $P < 0.05$ ; # $P < 0.05$ .

*in vitro* studies showed that N/OFOQ significantly inhibited agonist-induced HASM-embedded gel contraction, and we hypothesize that this could be an additional effect to its anti-inflammatory role, a response that requires further investigation.

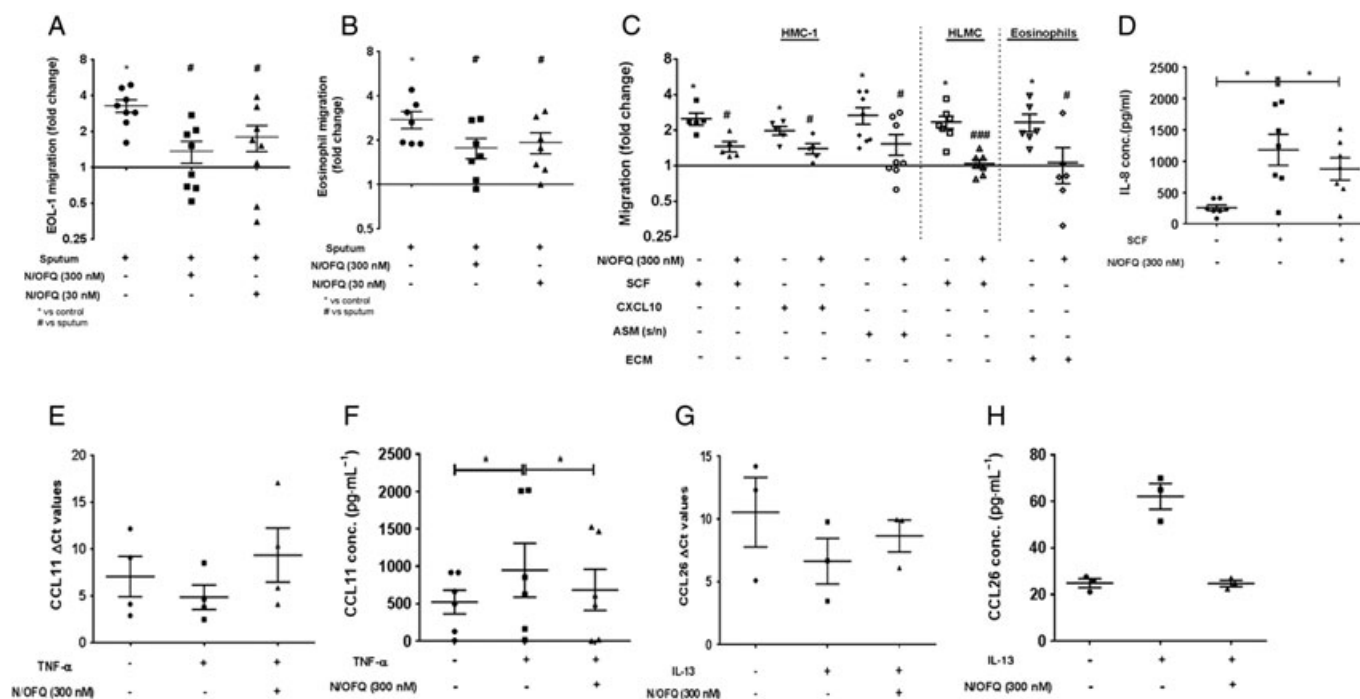
Asthmatic sputum contains several cytokines and chemokines that regulate eosinophil migration including IL-5, IL-8, RANTES, IgA and complexes of IL-8-IgA (Louis *et al.*, 1997). The increase in the levels of endogenous N/OFOQ in sputum ( $\sim 8\text{--}19$  pM;  $15\text{--}35$  pg·mL<sup>-1</sup>) of asthmatic patients was several orders of magnitude lower than that required to exert beneficial effects *in vitro*. We therefore suggest that additional exogenous N/OFOQ 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/OFOQ) with additional N/OFOQ (30 and 300 nM), which is over 2000-fold higher than concentrations measured endogenously, significantly attenuated the migration of eosinophils towards asthmatic sputum.

N/OFOQ is a naturally occurring peptide and does not cross the blood brain barrier (Lambert, 2008), and no significant adverse effects were reported in a clinical trial evaluating the

urodynamic effects of intravesical administration of 1  $\mu$ M N/OFOQ in patients with neurogenic detrusor activity (Lazzeri *et al.*, 2003). Therefore, any systemic/local administration of N/OFOQ is unlikely to induce any unwanted central effects.

As a proof of concept, our *in vivo* experiments confirmed the hypothesis that exogenous N/OFOQ 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 Th<sub>2</sub> cytokine production, goblet cell hyperplasia, mast cell degranulation, IgE production, AHR and airway remodelling (Kumar and Foster, 2001; Gelfand, 2002; Kumar *et al.*, 2008; Han *et al.*, 2013). We found a significant reduction of allergen-induced levels of IL-4, IL-5, IL-12 and IL-13, Th<sub>2</sub> cytokines linked to inflammation (Brightling *et al.*, 2002b). These observations were consistent with previous reports demonstrating inhibition of IL-2 release and T-cell proliferation by N/OFOQ (Miller and Fulford, 2007; Easten *et al.*, 2009). Surprisingly, we did not observe any significant effect of N/OFOQ on IFN- $\gamma$  production, that is the principal effector of Th<sub>1</sub>-mediated inflammation and has a protective effect against Th<sub>2</sub>-driven





**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  $\mu\text{m}$  (pore-size) transwell membrane ( $n = 5$ –7 independent donors). \*Statistical comparisons with control, #statistical comparisons with relevant chemotactic stimuli. (D) SCF-induced IL-8 release from HMC-1 cells ( $n = 7$  replicates). (E) HASM CCL11 mRNA ( $n = 4$  HASM cell samples from 4 independent donors) expression. (F) CCL11 levels in HASM supernatants ( $n = 6$  HASM cell samples from 6 independent donors). (G) HBEC CCL26 mRNA ( $n = 3$  HBEC samples from 3 independent donors) expression. (H) CCL26 levels in HBEC supernatants ( $n = 3$  HBEC samples from 3 independent donors). Data expressed as mean  $\pm$  SEM. Comparisons by one-way ANOVA followed by appropriate *post hoc* tests where relevant. \* $P < 0.05$ ; # $P < 0.05$ .

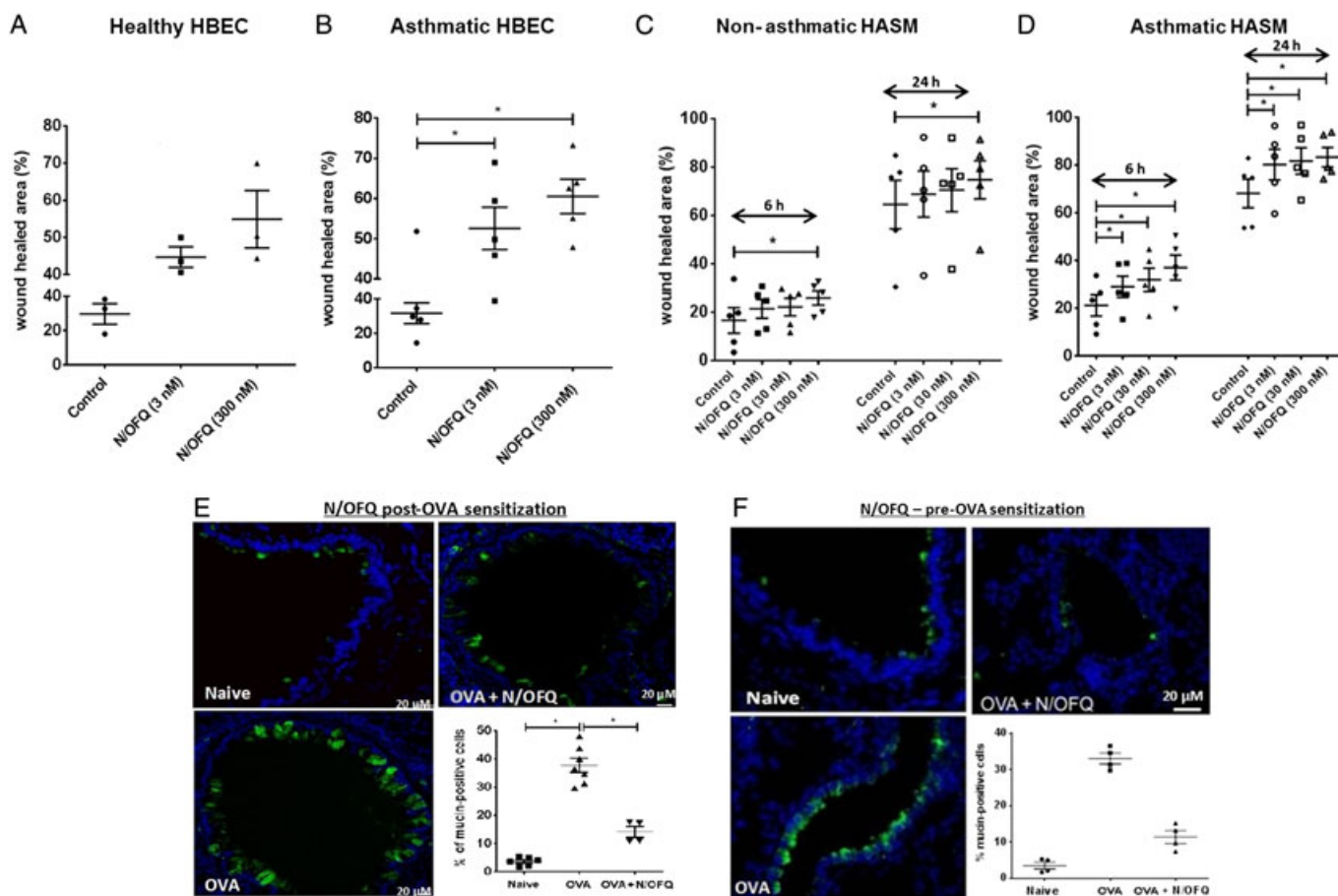
immune responses (Teixeira *et al.*, 2005). Additionally, N/OFQ was able to inhibit levels of OVA-specific IgE in BAL fluid. However, it failed to modulate OVA-specific IgG levels. These observations suggest a Th<sub>2</sub> selective immunomodulatory effect of N/OFQ *in vivo*.

Of note, we 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 remodelling 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 the proliferation, migration and differentiation of cells adjoining the damaged area (Tam *et al.*, 2011). Glucocorticoids, one of the 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 the early-stage migration and proliferation of airway epithelial cells (Wadsworth *et al.*, 2006; Liu *et al.*,

2013). 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 desensitization 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 demonstrated beneficial effects of N/OFQ at 3, 30 and 300 nM following administration of a single-dose for either 4 or 24 h. It would therefore be interesting to investigate whether this N/OFQ administration regimen would result in desensitization of the NOP receptor. Another interesting observation would be to determine whether this would activate any compensatory mechanism(s) to maintain NOP receptor expression.



## 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 ( $n = 3$  HBEC samples from 3 independent donors) and (B) asthmatic HBEC ( $n = 5$  HBEC samples from 5 independent donors). (C) Wound repair of non-asthmatic ( $n = 5$  HASM cell samples from 5 independent donors) and (D) asthmatic HASMs ( $n = 5$  HASM cell samples 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  $\mu\text{m}$ . (F) Mucin-labelled cells (green) in airway epithelium following administration of N/OFQ prior to OVA sensitization ( $n =$  airway tissue from 4 mice). Data are expressed as mean  $\pm$  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 two-way ANOVA).

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 PKC, MAPK, 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 IL-5 neutralization (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 hyperresponsiveness *in vivo* was similar to those described for anti-IL-13 and anti-IL-17 (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 receptor activation has the potential to produce hyperalgesic/antiopioid effects (Schröder *et al.*, 2014), but our observations in human and mouse suggest the main effects on airways are purely peripheral. A simple clinical trial of nebulized N/OFQ both as a prophylactic and during an exacerbation of asthma is clearly warranted. The use of a nebulized formulation would reduce total body dosing, negate the

likelihood of central spread and offer the advantage of a single entity combining anti-hyperresponsiveness and immunomodulatory 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 AHR indicating that 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.

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## Author contributions

S.R.S. is 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 [<sup>3</sup>H]-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. N.S. performed quantitative PCR, migration experiments, *in vitro* ELISA measurements and collagen gel contraction assays; involved in co-ordinating animal experiments in Naples and contributed to writing of the manuscript. M.M. and G.S. performed animal experiments. J.M. performed IL-8 measurements in HMC-1 cells. R.S. and L.W. performed HASM and HBEC wound healing experiments respectively. K.U. and A.D.A. performed histochemistry and immunofluorescence analysis and contributed to writing of the manuscript. R.D.P. performed cytokine assays. R.B. coordinated recruitment of healthy and asthmatic volunteers for sputum and eosinophil collection and collected all relevant clinical patient data. M.P. performed sputum processing and sputum cell counts. V.M. performed immunohistochemistry on human airway

tissues. F.R. made substantial contributions to the final version. G.C. and R.G. provided N/OFQ peptide and made an intellectual contribution to the pharmacology of the manuscript. B.D. 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 N.S.C.E.B. was 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 S.R.S., N.S., R.S., L.W., R.B., M.P. and V.M.D. G.L. was involved in the planning and design of the study, data collection and interpretation; established collaborative links with R.G., G.C. and B.D.; performed and analysed the cAMP and [<sup>3</sup>H]-thymidine incorporation assays; contributed to the design of the animal study and drafting of the manuscript and supervised S.R.S., N.S. and J.M. All authors approved the final draft of the manuscript.

## Conflict of interest

D.G.L. held a consultancy for work on Nociceptin, now lapsed, which was unrelated to the research reported in this paper. The other authors declare no conflicts of interest.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of pre-clinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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**Figure S1** N/OFQ levels in asthmatic sputum do not correlate with FEV1/FVC ratio. Spearman correlation between sputum N/OFQ and FEV1/FVC ratio.

**Figure S2** 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  $\mu$ M) 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 S3** 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 $\pm$ SEM).

**Figure S4** 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 $\pm$ SEM. Comparisons made by two-way ANOVA. Contraction data are a combination of cells harvested from healthy and asthmatic patients.

**Figure S5** 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  $\pm$  SEM. Comparisons by one-way ANOVA.  $*P < 0.05$ .

**Figure S6** N/OFQ had no effect on TNF- $\alpha$  release from HMC-1 cells. SCF-induced TNF- $\alpha$  release from HMC-1 cells ( $n=6$  independent experiments) was not modulated by N/OFQ pre-treatment. Data expressed as pg/ml (mean  $\pm$  SEM) and analysed by paired t-test.

**Figure S7** N/OFQ does not modulate mitogen-induced proliferation of HASMs and HMC-1 cells. (a) [ $^3$ H] 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 24h in the presence or absence of N/OFQ expressed as fold change, (c) [ $^3$ H] 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  $\pm$  SEM ( $n=6$  independent experiments). Comparisons made by one-way ANOVA.  $*P < 0.05$ .