Online Supplementary Material

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

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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^{o}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 e^{5} 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 24hours 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. Next, 20µL of CellTiter 96 Aqueous One Solution Reagent (Promega, Southampton, United Kingdom) was added to each well. After 4hours 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 (meanenti).

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 24hours 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,[4] HMC-1 cells were then seeded onto confluent HASM cells in the presence or absence of N/OFQ (300nM) for 48hours. 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 (meana bl).

[^{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 37re cultur$_{2}$ 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 (0.5idine (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 meand. F.

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 (1skolin (1forskolin (1IBMX) and forskolin (1n T125 flasks) were incubeactions were terminated using 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 meanndar.

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_2O. The perfusion medium used was RPMI 1640 lacking phenol red (37irculating fashion through the pulmonary artery at a constant flow o_2O) with 90 breath min^{-1} and a tidal volume of about 200irculating fashion through the pulmonary a_2O) was performed. 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: $P= Vla^{-1} + R_L \cdot Rrou^{-1}$, 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 pneumotacometer and the tracheal cannula of 0.6 cm H_2O 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 acethylcoline (10^{-8}-10^{-3}M). Repetitive dose response curve of acethylcoline was administered as 50sponse curve of or 45 min without any treatment in order to obtain a baseline state. Subse

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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.

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* 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 independant donors), (c) Migration of HLMC towards SCF (n=7 independant 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) [^{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 24 h 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 ± SEM (n=6 independent experiments). Comparisons made by one-way ANOVA. ***p<0.05**.

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