

Nociceptin/orphanin FQ receptor activation decreases the airway hyperresponsiveness induced by allergen in sensitized mice

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Sullo N, Roviezzo F, Matteis M, Ianaro A, Calò G, Guerrini R, De Gruttola L, Spaziano G, Cirino G, Rossi F, D'Agostino B. Nociceptin/orphanin FQ receptor activation decreases the airway hyperresponsiveness induced by allergen in sensitized mice. *Am J Physiol Lung Cell Mol Physiol* 304: L657–L664, 2013. First published March 15, 2013; doi:10.1152/ajplung.00358.2012.—Several studies suggest that the N/OFQ (nociceptin/orphanin FQ)-NOP (N/OFQ peptide) receptor pathway is involved in airway physiology. We previously demonstrated a modulation of the endogenous N/OFQ levels in allergen-sensitized mice. Here, we investigated the effects of NOP receptor activation in allergen sensitization using a murine model of allergen-induced airway hyperresponsiveness (AHR). BALB/c mice were intraperitoneally treated with the NOP receptor agonist UFP-112, either during the sensitization phase (30 min before ovalbumin administration) or at the end of sensitization process (15 min before bronchopulmonary reactivity evaluation). At day 21 from the first allergen exposure, bronchopulmonary reactivity and total and differential cell count in bronchoalveolar lavage fluid were evaluated. In a separate set of experiments cell proliferation in lymphocytes, cytokine levels, IgE serum levels, and the effect of UFP-112 on IL-13-induced AHR were evaluated. Pretreatment with UFP-112, during the sensitization phase, caused a significant reduction in allergen-induced AHR and total cell lung infiltration. No effect on allergen-induced AHR was observed when the treatment was performed at the end of sensitization process, on tissues harvested from OVA-sensitized mice and on IL-13-induced AHR. The *in vitro* proliferative response of lymphocytes was significantly reduced by pretreatment during the sensitization phase with UFP-112. This effect was paralleled by a significant modulation of cytokine secretion in pulmonary tissues and lymphocytes. In conclusion, we demonstrated a role for the NOP receptor and N/OFQ pathway in the AHR induced by allergen, probably through a modulation of the immune response that triggers the development of AHR that involves pro- and anti-inflammatory cytokines.

nociceptin/orphanin FQ; allergen sensitization; airway hyperresponsiveness; immune response; allergic asthma

THE NEUROPEPTIDE nociceptin/orphanin FQ (N/OFQ) and its receptor N/OFQ peptide (NOP) are closely related to the opioid system (20, 26). They were originally discovered within the central nervous system but have also been located throughout the periphery (16). Although some of the complex central and endocrine effects of N/OFQ pathway are beginning to be elucidated, the role of N/OFQ in the periphery remains poorly understood. In the

airway NOP agonists inhibit the cough reflex and acetylcholine (ACh) release from guinea pig trachea (23, 18). Moreover, NOP agonists reduce bronchoconstriction and airway inflammation induced by esophageal instillation of hydrochloric acid (6). Collectively, these studies suggest that the N/OFQ-NOP receptor pathway is involved in airway physiology.

Airway inflammation is a central component of most manifestations of allergic asthma. It may result from a reaction to an inhaled environmental substance (e.g., an allergen) that triggers a series of inflammatory events (3, 17). Sensitization to an allergen reflects the allergen's ability to elicit a TH2-cell response. Several studies indicate the capacity of various immunocompetent cells to synthesize mRNA for the precursor of N/OFQ, preproN/OFQ. Moreover, immune cell types such as normal circulating lymphocytes, polymorphonuclear cells, and monocytes in addition to T, B, and monocytic cell lines express the full-length NOP receptor mRNA (25, 30). Although some evidence shows that the NOP receptor modulates proliferation of human lymphocytes *in vitro* (24, 32) and regulates antibody production (13) and neutrophil chemotaxis (30), the functional significance of the immune-derived N/OFQ pathway is still unclear.

Active sensitization with ovalbumin is widely used as a pre-clinical model of allergic airway diseases. Although this model may not entirely reflect the human pathology, this model has many similarities with human allergic asthma such as histological features, allergen-induced eosinophilia, and early and late-phase airway obstruction after allergen challenge (10). Using this model we have recently shown a differential airway responsiveness to capsaicin between naive and ovalbumin-sensitized mice that was related to a modulation of the endogenous N/OFQ (7).

Since it is still unclear why the immune system in allergic asthmatic and healthy individuals reacts differently to an inhaled allergen, understanding the mechanisms involved in respiratory tolerance to inhaled allergens could potentially lead to the development of new and/or better therapeutic approaches to asthma and allergic diseases. Therefore the aim of this study was to investigate, in a well-known and characterized murine model of allergen-induced airway hyperresponsiveness (AHR), the effects of NOP receptor activation in the mechanisms of allergen sensitization.

METHODS

Experimental Protocol

Female BALB/c mice were used through the study. A schematic representation of the study protocol is shown in Fig. 1. The animals were sensitized to ovalbumin (OVA) by subcutaneous injection of 0.2

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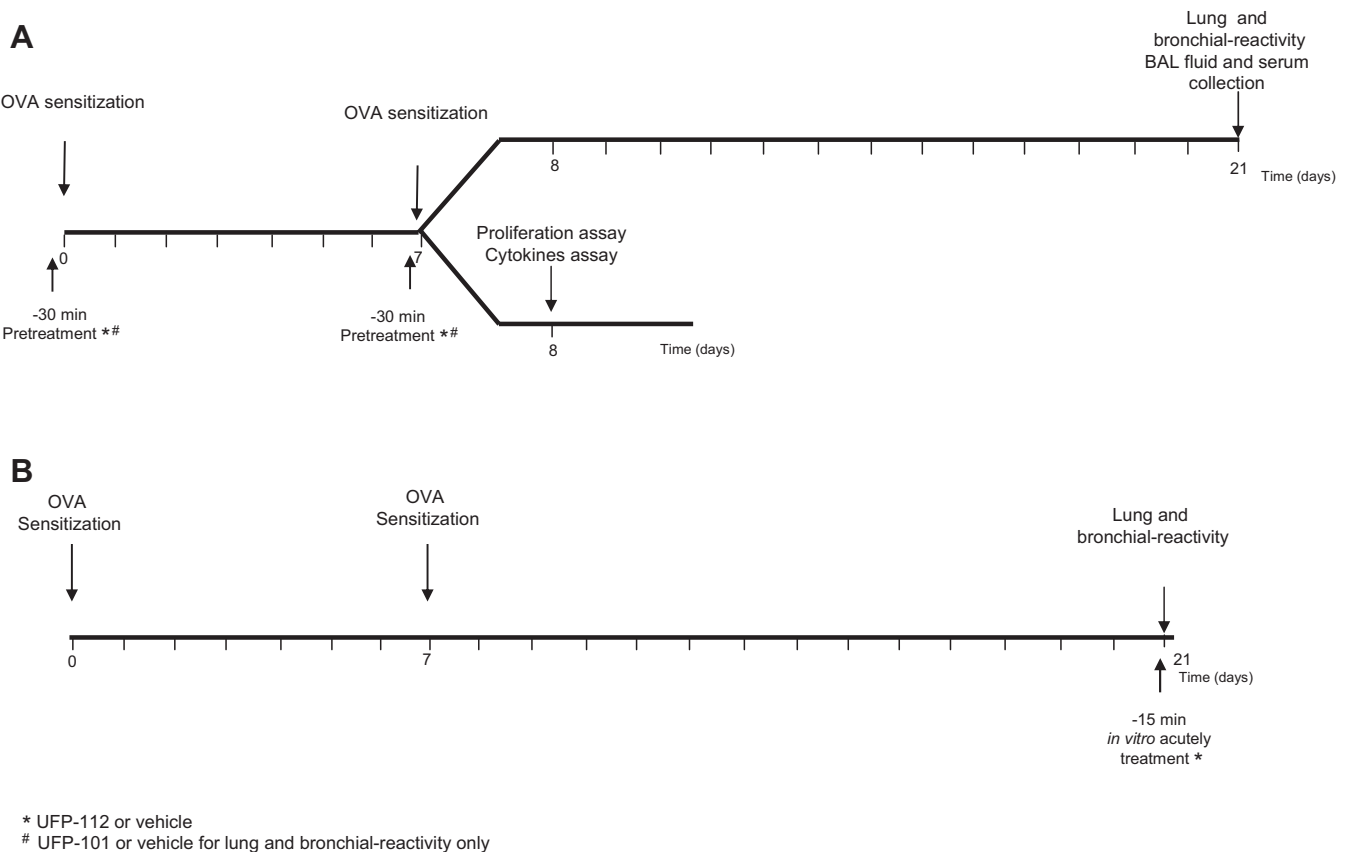


Fig. 1. Schematic representation of treatment regimen. Functional study and cell proliferation assay on sensitized animals treated with NOP agonist, antagonist, or vehicle during the sensitization phase, 30 min before ovalbumin (OVA) administration (A) or acutely at the end of the sensitization process, 15 min before functional evaluations (B). BAL, bronchoalveolar lavage.

ml of 10 μ g OVA, absorbed to 3.3 mg of aluminum hydroxide gel in sterile saline at *days 0* and *7*. The sensitized animals were divided into three groups, with eight mice for each experimental group. The first three groups were treated intraperitoneally with UFP-112 (1.5 ng/g), a selective NOP receptor agonist, alone or in combination with UFP-101 (15 ng/g), a selective NOP receptor antagonist, or with saline (vehicle), respectively, 30 min before the initial systemic administration of OVA, at *days 0* and *7* (Fig. 1A). In a separate set of experiments, isolated tissues harvested by OVA-sensitized mice were *in vitro* acutely treated, on the 21st day, with NOP receptor agonist (0.1 μ M) or vehicle, 15 min before bronchopulmonary reactivity evaluation (Fig. 1B). The third group (sham) not sensitized and not treated was used as control. On the 21st day after the first allergen administration, bronchopulmonary reactivity in isolated lung and isolated bronchi, bronchoalveolar lavage (BAL) fluid and serum collection were performed in each group (Fig. 1, A and B). Moreover, we evaluated the effect of NOP receptor agonist on IL-13-induced airway sensitization. For this reason, as previously described (22), animals received an intratracheal instillation of 0.5 μ g recombinant murine IL-13 solution (Sigma, St. Louis, MO) or phosphate-buffered saline on *days 1*, *3*, and *5*, 30 min after UFP-112 (1.5 ng/g) treatment. Measurements of bronchopulmonary reactivity in isolated lung were performed on *day 6*, 24 h after the last instillation.

In another set of experiments at the 8th day from the initial systemic administration, mice were euthanized and lymphocytes harvested were used to evaluate proliferation. An aliquot of the immune cells harvested at *day 8* was cultured and challenged *in vitro* with OVA (300 μ g/ml), and cytokine levels were evaluated (Fig. 1A). Moreover, at the 8th day cytokine levels were also measured in pulmonary tissues.

All experimental procedures were in accordance with Italian D.Lgs. 116/92. The protocol was approved by the Veterinary Animal Care and Use

Committee of the Second University of Naples and conformed to the National Ethical Guidelines of the Italian Ministry of Health.

Lung Reactivity Assay

Lung reactivity was assessed in an isolated and perfused mouse lung model. As previously described (28), a water-jacketed (water temperature, 37°C) Perspex chamber was used to accommodate surgery, perfusion, and ventilation of murine lungs. Mice were anesthetized with 160 mg/kg body wt pentobarbital sodium. The skin was incised from the abdomen to the throat, and the trachea was exposed with a small incision and cannulated. The abdomen was opened, the diaphragm resected, and 50 μ l of heparin injected into the heart. The mouse was exsanguinated after incision of the renal vena, the thorax was opened, and the two thoracic halves were fixed at the sides of the cork plate with two cannulas. At this point the pulmonary artery was cannulated through a small piece of skin on the right atrium, so that the arterial cannula was inserted into the pulmonary artery and fixed by the ligature. Drugs were administered through the arterial cannula into the pulmonary artery. After preparation, the lungs were perfused in a nonrecirculating fashion through the pulmonary artery at a constant flow of 1 ml/min, resulting in a pulmonary artery pressure of 2–3 cmH₂O. As a perfusion medium we used RPMI 1640 medium lacking phenol red (37°C) that contained 4% low-endotoxin-grade albumin. The lungs were ventilated by negative pressure (–3 to –9 cmH₂O) with 90 breaths/min and a tidal volume of ~200 μ l. Every 5 min a hyperinflation (–20 cmH₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45-24) and air flow velocity with a 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) that was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analyzed by the Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). For lung mechanics, the data were analyzed by applying the following formula: $P = V \cdot C^{-1} + R_L \cdot dV \cdot dt^{-1}$, where P is chamber pressure, C pulmonary compliance, V tidal volume, and R_L airway resistance. After 60 min, mean tidal volume was 0.21 ± 0.02 ml ($n = 61$), mean airway resistance 0.23 ± 0.08 cmH₂O·s·ml⁻¹, and mean pulmonary artery pressure 2.9 ± 1.4 cmH₂O. The measured airway resistance was corrected for the resistance of the pneumotachometer and the tracheal cannula of 0.6 cmH₂O·s·ml⁻¹. Successively, a repetitive dose-response curve to ACh (10^{-8} to 10^{-3} M) in all experimental groups treated either in vivo during the sensitization phase or acutely in vitro, on day 21 of OVA sensitization.

Tissue Preparation and Bronchial Reactivity Assay

Bronchial reactivity was assessed in isolated bronchial rings, obtained as previously described (29). Briefly, mice were euthanized and bronchial tissue was rapidly dissected and cleaned from fat and connective tissue. Rings 1–2 mm in length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O₂-5% CO₂) Krebs solution at 37°C and mounted to isometric force transducers (type 7006, Ugo Basile, Comerio, Italy) and connected to a Powerlab 800 (ADInstruments). The composition of the Krebs solution was as follows (mol/l): 0.118 NaCl, 0.0047 KCl, 0.0012 MgCl₂, 0.0012 KH₂PO₄, 0.0025 CaCl₂, 0.025 NaHCO₃, and 0.01 glucose. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to 0.5 g, and bathing solution was periodically changed. In a preliminary study a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experimental group was performed a cumulative concentration-response curve to ACh (10^{-8} to 3×10^{-5} mol/l).

Bronchoalveolar Lavage and Cell Count

BAL fluid from animals 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 1,000 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 cell count was performed with the Countess automated cell counter (Invitrogen), which evaluates count and cell viability, by using Trypan blue stain according to the manufacturer's instructions. Differential counting was performed on Diff-Quik (Reagent, Gentaur, Italy)-stained cytopspins. At least 200 cells were counted on each cytopspin according to standard morphological criteria under light microscopy.

Preparation of Cell Suspension and Proliferation Assay

Cell suspension was obtained as previously described (14). Briefly, peripheral lymph nodes (axillary, brachial, inguinal, cervical) and mesenteric lymph nodes from BALB/c mice were taken at day 7, after the allergen exposure. Subsequently, these were pooled and prepared as single-cell suspensions, by passing through a Nitex sieve (Cadisch Precision Meshes, London, UK) by use of a syringe plunger, and washed in sterile RPMI-1640 (Invitrogen Life Technologies, Paisley, UK). Monocytes were obtained by separation with Histopaque 10771 (Sigma). The cell suspensions were used for the evaluation of the proliferative response to OVA. The MTT colorimetric assay, based on the ability of viable cells to reduce the yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) salt to the blue MTT formazan product, was used to assess lymphocyte and spleno-

cyte proliferation in vitro. Preliminary experiments determined that splenocyte number and absorbance (570 nm) formed a linear relationship, thereby providing a reliable assay for measurement of cell proliferation. In each study group, cells were aliquoted into 96-well plates with 2×10^6 cells per well in RPMI media containing streptomycin-penicillin (1%), sodium pyruvate (0.1%), 2-mercaptoethanol (0.1%), and fetal calf serum (10%). Samples recovered from all experimental animal group were cultured in the absence or presence of OVA (300 µg/ml) in a final volume of 150 µl for 96 h in an incubator (37°C, 5% CO₂ + 95% air). In the last 4 h of incubation, 25 µl of MTT reagent was added to each well. This step was followed by the addition of detergent reagent (100 ml per well) for 2 h to enable complete solubilization of formazan product (plates kept in the dark).

The cell suspensions were used for the evaluation of the proliferative response to OVA. Each treatment/assay was based on $n = 6$ replicate samples.

IgE Serum Levels Assay

Blood was collected from heart. Total serum IgE levels were measured by ELISA using matched antibody pairs (BD Pharmingen, Franklin Lakes, NJ).

Cytokine Assay

Amounts of cytokines (IFN-γ, IL-2, IL-4, and IL-5) in the supernatants obtained either from lymphocytes or from lung tissues were determined by using an ELISA kit (DuoSet ELISA Development System, R&D) according to the manufacturer's instruction. Lung tissues were homogenized in 500 µl of lysis buffer (20 mM HEPES, 0.4 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 20% glycerol) with protease inhibitors (1 mM DTT, 0.5 mM PMSF, 15 µg/ml Tryp-inhibitor, 3 µg/ml pepstatin-A, 2 µg/ml leupeptin, and 40 µM benzamide). The supernatant recovered after centrifugation at 12,000 g at 4°C for 15 min was used for IFN-γ, IL-2, IL-4, and IL-5 measurement.

Drugs

UFP-112 (NOP receptor agonist) and UFP-101 (NOP receptor antagonist) were prepared and purified in house (University of Ferrara). OVA, recombinant murine IL-13, and ACh were purchased from Sigma-Aldrich.

Statistical Analysis

All results are reported as means $\pm$ SE. For statistical analysis we have used the two-way ANOVA followed by Bonferroni posttest. A value of $P < 0.05$ was taken as significant.

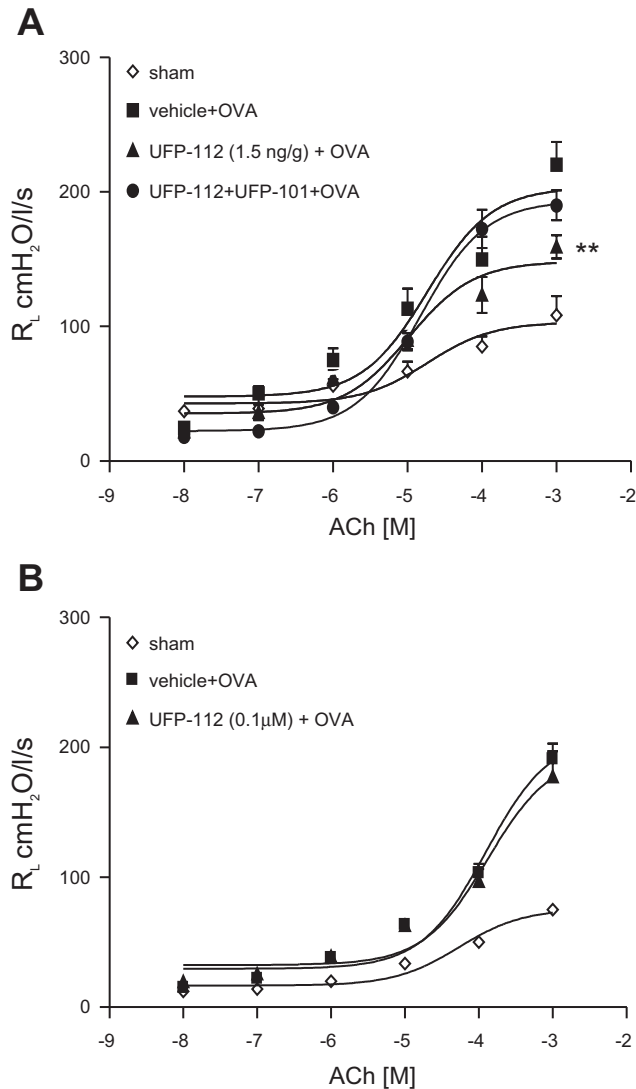
RESULTS

Effect of Selective NOP Agonist on Lung and Bronchial Reactivity

Isolated and perfused lung. Pretreatment with the selective agonist, UFP-112, 30 min prior to allergen administration, during the sensitization phase, caused a significant reduction in allergen-induced hyperreactivity to ACh (Fig. 2A). Pretreatment with UFP-101 abolished the inhibitory effects of UFP-112 on ACh-induced bronchoconstriction (Fig. 2A).

No effect on allergen-induced airway AHR was observed when the treatment with selective NOP receptor agonist was performed acutely at the 21st day on tissues harvested by OVA-sensitized mice, 15 min before measurement of pulmonary reactivity (Fig. 2B).

Isolated bronchi. The experiments performed on bronchial tissues harvested by mice sensitized with OVA and pretreated in vivo during the sensitization phase with selective NOP receptor



* P UFP-112+OVA vs vehicle+OVA

Fig. 2. Effect of UFP-112 on acetylcholine (ACh)-induced bronchoconstriction. Intraperitoneal pretreatment with UFP-112, 30 min before allergen exposure, causes a significant reduction in ACh-induced bronchoconstriction respect to vehicle-treated mice (A). No effect on ACh-induced bronchoconstriction is observed when UFP-112 treatment is performed 21 days from the first allergen exposure (B). Data are means $\pm$ SE ($n = 8$). UFP-112 vs. OVA $**P < 0.01$. R_L , airway resistance.

agonist, as described above, confirmed the results obtained in the lung. Indeed, there was a significant decrease in bronchial reactivity to ACh in vitro following UFP-112 treatment in vivo and pretreatment with UFP-101 abolished the inhibitory effects of UFP-112 (Fig. 3A). Similar experiments were performed on bronchial tissues harvested from sensitized animals treated acutely in vitro at 21st day with UFP-112, 15 min before bronchial reactivity measurements. The selective NOP receptor agonist did not modify in vitro the response to ACh (Fig. 3B).

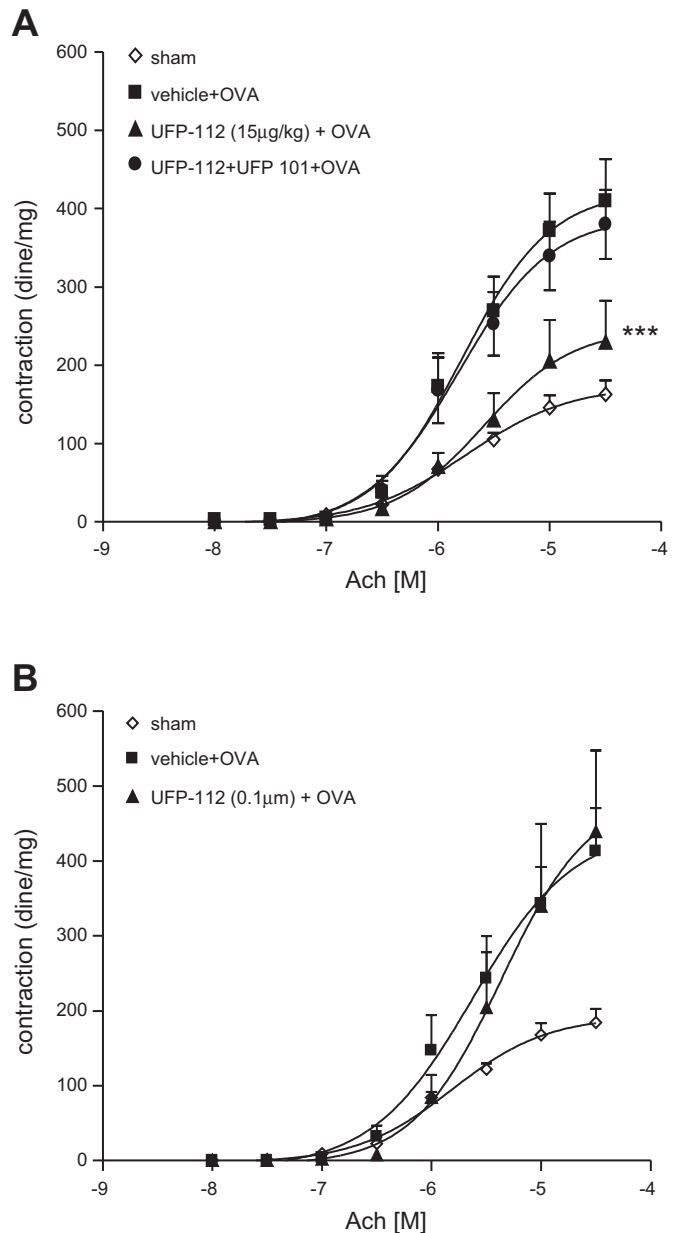
IL-13-Induced AHR

IL-13 was able to induce AHR to ACh in control mice. The treatment with the NOP receptor agonist UFP-112 was unable to modify the IL-13-induced AHR to ACh (Fig. 4).

Total and Differential Cell Count

The analysis of inflammatory cells in the BAL fluid samples revealed that, compared with the sham group, OVA sensitization induced a significant increase in total cell numbers. Treatment with UFP-112 reduced in a significant manner the total number of inflammatory cells (Fig. 5A).

With respect to sham mice, the exposure to OVA resulted in a significant increase in the percentage of eosinophils. Conversely, in the mice treated with UFP-112, the percentage of eosinophils was lower compared with OVA-treated mice (Fig. 5B).



* P UFP-112+OVA vs vehicle+OVA

Fig. 3. Effect of UFP-112 on bronchial reactivity. Animals sensitized with OVA and pretreated with UFP-112 show a significant decrease of ACh-induced hyperreactivity (A). UFP-112 is unable to modify ACh-induced hyperresponsiveness when the treatment is performed at the end of the sensitization process (B). Data are means $\pm$ SE ($n = 8$). $***P < 0.001$.

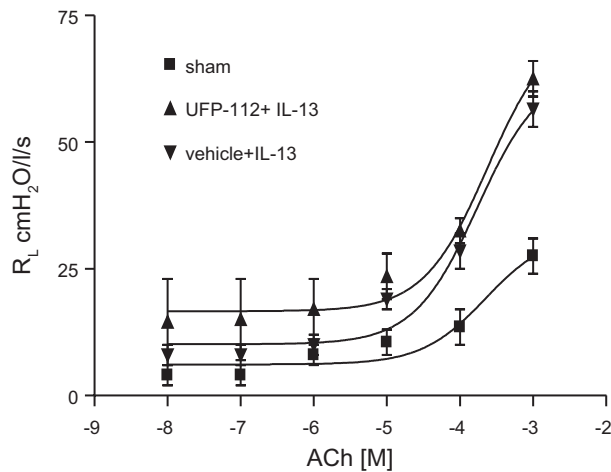


Fig. 4. Effect of UFP-112 on IL-13-induced change in airway function. IL-13 was able to induce airway hyperresponsiveness to ACh in control mice. Treatment with the nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor agonist UFP-112 was unable to modify the IL-13-induced airway hyperresponsiveness to ACh. Data are means $\pm$ SE ($n = 8$).

Proliferation Assays

The in vitro proliferative response of lymphocytes was significantly reduced by the in vivo pretreatment during the sensitization phase with UFP-112 (Fig. 6A). When lympho-

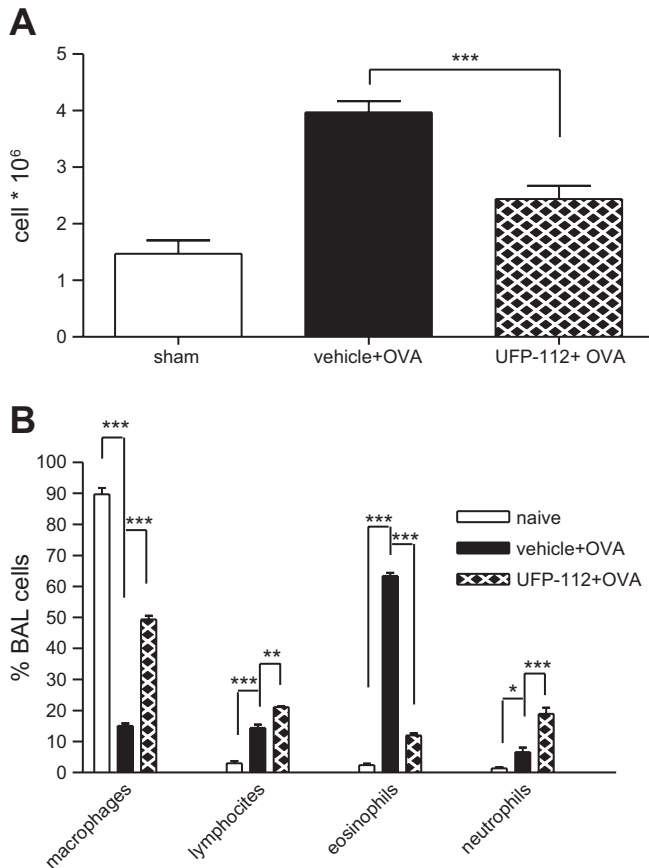


Fig. 5. Effect of UFP-112 on inflammatory cells. In the BAL fluid samples treatment with UFP-112 reduces in a significant manner the total number of inflammatory cells (A) and the percentage of eosinophils (B), compared with vehicle group. Data are means $\pm$ SE ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

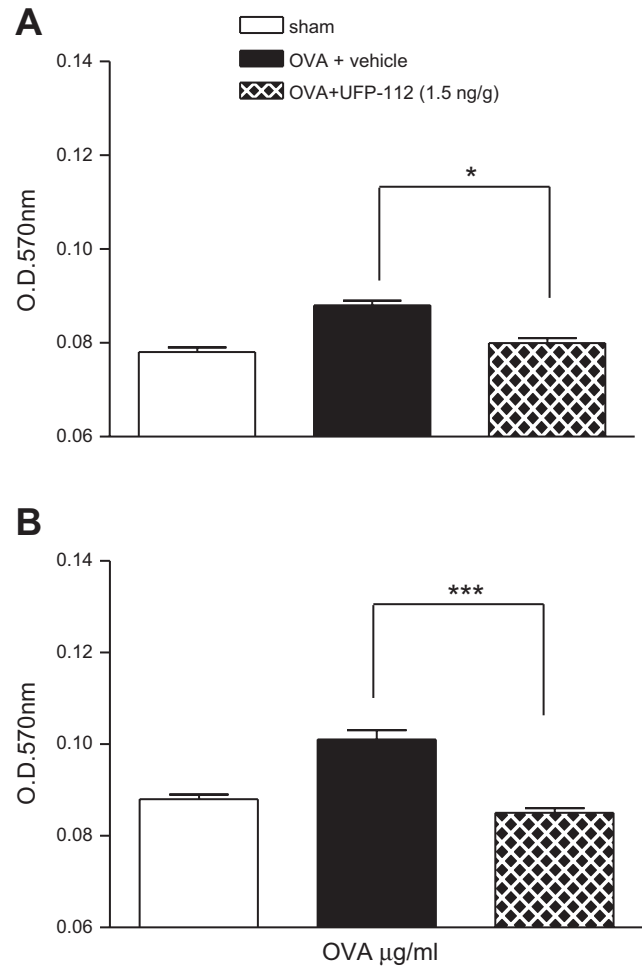


Fig. 6. Proliferation assay for lymphocytes. In vivo pretreatment with UFP-112 significantly reduce the in vitro proliferative response of lymphocytes obtained from OVA-sensitized mice (A). In the challenge in vitro with OVA there is a more marked effect of NOP receptor agonist (B). Data are means $\pm$ SE ($n = 8$). * $P < 0.05$, *** $P < 0.001$.

cytes harvested from mice treated in vivo with UFP-112 were challenged in vitro with OVA (300 μ g/ml), there was a more marked effect of selective NOP receptor agonist (Fig. 6B).

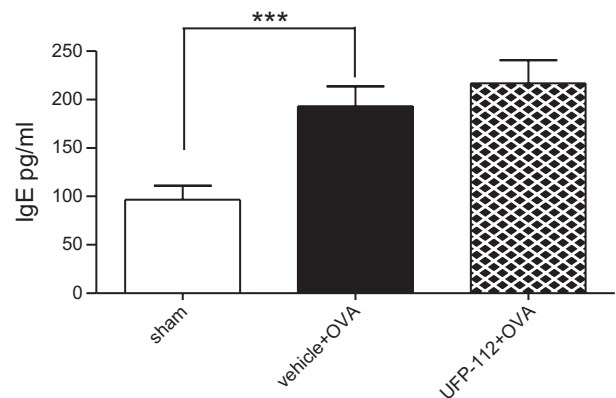


Fig. 7. Measurement of IgE serum levels. UFP-112 treatment was unable to modify the IgE serum levels with respect to OVA-sensitized mice. Data are means $\pm$ SE ($n = 8$). *** $P < 0.001$.

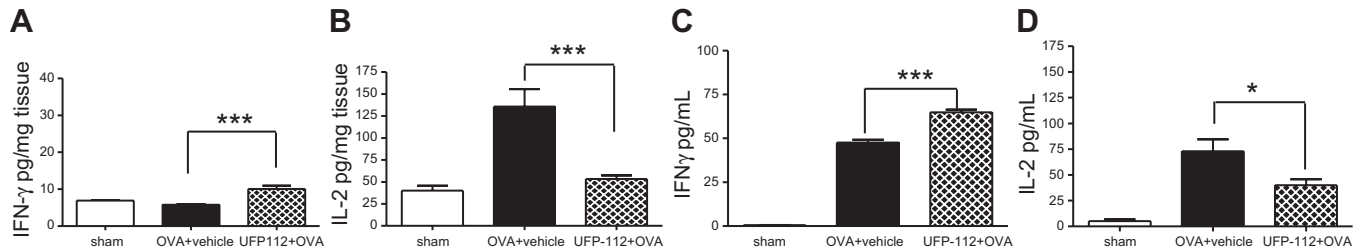


Fig. 8. Measurement of IFN- γ and IL-2 levels. Treatment with UFP-112 significantly increases/reduces the IFN- γ and IL-2 levels, respectively, both in pulmonary tissues (A and B) and in lymphocyte supernatant (C and D). Data are means $\pm$ SE ($n = 8$). * $P < 0.05$, *** $P < 0.001$.

IgE Serum Levels

OVA sensitization caused a significant increase of IgE serum levels with respect to sham mice. NOP receptor agonist treatment was unable to modify the IgE serum levels with respect to OVA-sensitized mice (Fig. 7).

Cytokine Levels

OVA sensitization caused a significant increase of cytokine levels with respect to sham group. The cytokine levels in pulmonary tissues and in supernatant of lymphocyte cells harvested from each experimental group, pretreated in vivo during the sensitization phase with UFP-112, were significantly modulated. In particular, concerning the Th₁ pathway, the treatment with UFP-112 significantly increased/reduced the IFN- γ and IL-2 levels, respectively, both in pulmonary tissues (Fig. 8, A and B) and in lymphocyte supernatant (Fig. 8, C and D). Conversely, concerning the Th₂ pathway, NOP receptor activation induced a significant reduction of IL-4 and IL-5 levels both in pulmonary tissues (Fig. 9, A and B) and in lymphocyte supernatant (Fig. 9, C and D).

DISCUSSION AND CONCLUSIONS

In the present study we demonstrate that NOP receptor activation, by its selective agonist UFP-112, significantly reduces the AHR and lung eosinophilic infiltration, induced by allergen sensitization in mice. Indeed, administration of the NOP receptor agonist during the sensitization phase significantly reduces the AHR both in vivo and in vitro. We have selected UFP-112 as NOP agonist since it has been shown in several studies that UFP-112 is a highly potent and selective full agonist of the NOP receptor (4, 5, 9, 27). Moreover, in a previous paper we have demonstrated that UFP-112 was able to reduce the AHR induced by capsaicin with doses 10 times lower than the physiological agonist N/OFQ (7). Moreover, the NOP receptor antagonist UFP-101 was able to reduce the

inhibitory effects of UFP-112 on ACh-induced bronchoconstriction, showing a direct involvement of the NOP receptor in the inhibitory effect of UFP-112. This result, taken together with the findings that endogenous N/OFQ lung levels were significantly elevated in sensitized mice and that UFP-112 failed to modulate the bronchoconstriction induced by acetylcholine in absence of OVA sensitization (7), led us to hypothesize that the N/OFQ pathway could be involved in the sensitization phase to allergen. An essential aspect of the effect of the NOP receptor activation on AHR was the time frame of agonist administration. Indeed, on *day 21* following allergen sensitization, acute administration of agonist in the isolated lung harvested from sensitized animals was ineffective in modulating ACh-induced bronchial contraction. Similarly, if the agonist, on *day 21*, was added in vitro to bronchi harvested from sensitized mice, it did not modify ACh-induced contraction. Therefore, the role of the NOP receptor activation relies on a mechanism triggered following in vivo administration during the sensitization phase. Indeed, reduction of AHR was observed only when the NOP receptor was activated in vivo by administering it to mice at *days 0* and *7*, 30 min prior to the allergen administration. Moreover, in our model, in agreement with several studies (15, 19, 31), IL-13 was able to induce AHR. UFP-112 treatment 30 min before IL-13 administration was unable to modify IL-13-induced AHR, further confirming the role of the NOP receptor agonist in the early phases of the sensitization process. Sensitization to an allergen may reflect the allergen's ability to elicit a Th₂-cell response, and thus several factors are involved in the likelihood for development of a clinically significant sensitization (11). Therefore, to gain further insights into the mechanism(s) involved in the N/OFQ pathway activation we performed a series of matched experiments on lymphocytes, obtained from peripheral lymph nodes. The lymphocytes harvested from animals pretreated in vivo during the sensitization phase with the NOP receptor agonist had a proliferation index significantly lower compared with

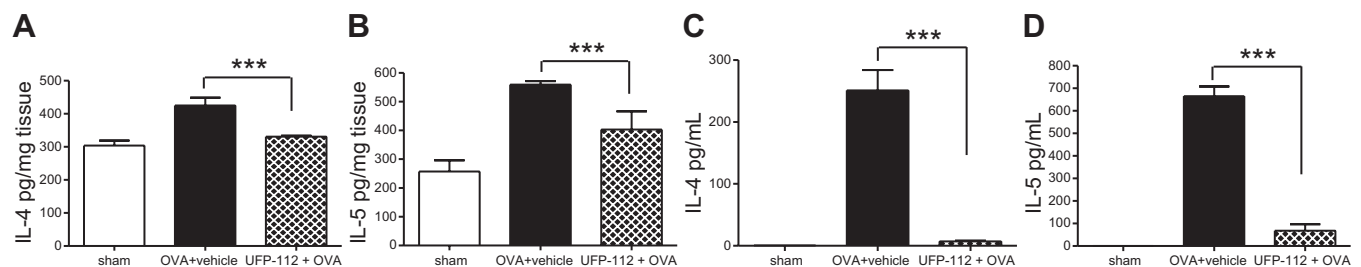


Fig. 9. Measurement of IL-4 and IL-5 levels. NOP receptor activation induced a significant reduction of IL-4 and IL-5 levels both in pulmonary tissues (A and B) and in lymphocyte supernatant (C and D). Data are means $\pm$ SE ($n = 8$). *** $P < 0.001$.

lymphocytes harvested from vehicle-treated mice. Thus the studies on cell proliferation confirmed a role for the NOP receptor activation on mechanisms triggered by allergen exposure in modulating the early phase of T cell activation.

In the last decade there have been several articles in the literature documenting the ability of the N/OFQ pathway activation to modulate immune cell function. In fact, as early as 2002, was shown that mRNA for the NOP receptor is expressed in CD4⁺ T cells isolated from human peripheral blood mononuclear cells (2). Successively, Waits et al. (32) showed that N/OFQ modulates T cell activation by upregulating the costimulatory molecule CD28, the α -chain of the IL-2 receptor, CD25, and the early activation molecule CD69 in staphylococcal enterotoxin B-activated T cells. Finally, very recent work from Anton et al. (1) added new information to the role of N/OFQ pathway in the immune system. Since in the present study, UFP-112 was unable to interfere with the IgE serum levels, it is likely that NOP receptor activation in our experimental conditions triggers an immunomodulatory activity, independently of IgE modulation.

An important role in orchestrating the allergic inflammation is played by cytokines of both the Th₁ (such as INF- γ and IL-2) and Th₂ (such as IL-4 and IL-5) pathways. In the present study, in the pulmonary tissues and in the supernatant of lymphocyte cells harvested from animals pretreated in vivo during the sensitization phase with NOP receptor agonist, we found a significant modulation of the cytokine levels. In particular, we observed a significant reduction of the proinflammatory cytokines IL-2, IL-4, and IL-5, coupled to a significant increase of IFN- γ levels both in pulmonary tissues and in lymphocytes. This result well fit with a previous finding in which N/OFQ has been shown to promote an anti-inflammatory action via suppression of IL-2 in vivo (21). The same applies to IFN- γ . Indeed, N/OFQ has an inhibitory effect on T cell proliferation that involves IFN- γ (8). In addition, the mice lacking the N/OFQ precursor gene show a diminished IFN- γ mRNA induction (12). Therefore these data, taken together with the finding that NOP receptor activation in vivo in the early phase of allergen sensitization significantly modulates the response, support our hypothesis of an immunoregulatory role for the N/OFQ pathway. In conclusion, we demonstrated for the first time a role for the NOP receptor and thus of the N/OFQ pathway in the AHR induced by allergen. In particular, we showed that following NOP receptor activation there is a modulation of the immune response that triggers the development of AHR that involves pro- and anti-inflammatory cytokines.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

N.S., F. Roviezzo, M.M., A.I., R.G., L.D.G., and G.S. performed experiments; N.S. and F. Roviezzo interpreted results of experiments; M.M. and G.S. prepared figures; A.I. and B.D. drafted manuscript; G. Calo and G. Cirino edited and revised manuscript; R.G. analyzed data; F. Rossi approved final version of manuscript; B.D. conception and design of research.

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