

Nociceptin reduces the inflammatory immune microenvironment in a conventional murine model of airway hyperresponsiveness

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Summary

Background Nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP) are involved in airway hyperresponsiveness (AHR) and inflammation. However, the role of nociceptin at modulating the inflammatory immune microenvironment in asthma is still unclear.

Objective To understand the role of N/OFQ in the regulation of a Th2-like environment, we used a conventional murine model of AHR.

Methods Balb/c and CD1 mice were sensitized to ovalbumin (OVA) and treated with saline solution or N/OFQ, at days 0 and 7. A group of Balb/c mice were killed at 7 and 14 days from the first sensitization for the inflammatory profile evaluation while a group of Balb/c and CD1 mice were aerosol-challenged from day 21 to 23 with OVA and killed 24 h later for functional evaluations.

Results In OVA-sensitized mice, N/OFQ significantly reduced IL-4+ CD4+ T cells in lymph nodes (LN) and IL-13 in the lungs, while it induced IFN- γ increase in the lung. The efflux of dendritic cells (DCs) to the mediastinic LN and into the lung of OVA-sensitized mice was reduced in N/OFQ-treated and sensitized mice. N/OFQ reduced the expression of CD80 on DCs, indicating its ability to modulate the activation of DCs. In a less prone Th2-like environment mice strain, such as CD1 mice, N/OFQ did not modify lung resistances as observed in BALB/c mice. Finally, spectroscopic data showed the N/OFQ was able to interact onto the membrane of DCs obtained from Balb/c rather than CD1 mice, indicating its ability to modulate AHR in a Th2-like environment with a direct activity on DCs.

Conclusions and Clinical Relevance Our data confirmed the capability of N/OFQ to modulate the immune microenvironment in the lung of Th2-biased, OVA-sensitized Balb/c mice, suggesting N/OFQ-NOP axis as a novel pharmacological tool to modulate the inflammatory immune microenvironment in asthma.

Keywords airway hyperresponsiveness, asthma, dendritic cells, Th₂ environment

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Introduction

Asthma is a complex heterogeneous disease during which the establishment of chronic inflammation is at the basis of mucus overproduction and airway-wall remodelling that result in bronchial hyperactivity and variable degree of airflow obstruction [1, 2]. Allergens, some pathogens and some diseases have been implicated in the exacerbation of asthma in both animal

models and humans [3–6]. Lung allergic diseases, such as asthma, are characterized by activated mast cells, increased numbers of eosinophils, increased numbers of invariant natural killer T cells (NKT), T helper 2 (Th2) and T helper 17 lymphocytes (Th17), and neutrophils [7, 8], and increase in sensory neurogenic release [9]. Th2-like cytokines are produced by both Th2 cells (IL-4, IL-5 and IL-13) and type 2 innate lymphoid cells (ILCs) (IL-5 and IL-13). Th2-like cytokines are responsible of the humoral modification in the lung of sensitized, asthma-prone mice and humans. In particular, IL-4

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contributes to both the differentiation/proliferation of Th2 cells but also to the IgE class switching by B cells [10]; IL-5 increases the recruitment of eosinophils [11]; and IL-13 promotes goblet cell hyperplasia at the basis of the bronchial basal membrane modification [12, 13]. Furthermore, in this context, dendritic cells (DCs) and professional antigen-presenting cells (APCs) are essential for the induction and priming of the ensuing Th2-like response during asthmatic conditions [14, 15]. DCs are crucial for both the initiation as well as the maintenance of the inflammatory response in asthma and are implicated in bronchial hyperreactivity (BHR) and goblet cell metaplasia (CGM) [16, 17].

Our previous studies showed that the neuropeptide Nociceptin/Orphanin FQ (N/OFQ), an endogenous heptadecapeptide [18, 19] that selectively binds a G protein-coupled receptor (GPCR) named NOP receptor, is involved in airway hyperresponsiveness (AHR) [20–22], suggesting its potential involvement in the regulation of the inflammatory response into the lung.

NOP receptor and N/OFQ are widely expressed with main actions on pain processing in the brain/spinal cord; at peripheral level, NOP receptor is located in airways and various peripheral immune cells [23]. Activation of the N/OFQ-NOP receptor system leads to the modulation of widespread physiological responses at both central and peripheral levels, such as inhibition of airway smooth muscle contraction or mechanical capsaicin-induced cough and modulation of locomotor activity, learning and memory processes [22, 24]. Although many of the endocrine and central effects of N/OFQ have been clarified, the role of nociceptin at modulating the inflammatory immune microenvironment in asthma is still unclear.

Therefore, the aim of this study was to understand the role of this system in the regulation of a Th2-like environment in a conventional murine model of AHR, typical of asthma.

Methods

Animals and housing

BALB/c and CD1 mice were purchased from Charles River Laboratory (Calco, Lecco, Italy). The experimental protocol was approved by the Animal Care and Use Committee of the Second University of Naples (1966/7.17.2012). Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/1992) as well as with the European Union guidelines for the use of experimental animals (2010/63/EU). The experimental procedures used in the work described in this article were as humane as possible. Mice were housed in the animal facility of the Second University

of Naples. Food and water were supplied *ad libitum*. Room temperature was 22–24°C, relative humidity was 40–50% and the day/night cycle was set at 12/12 h. Mice were acclimatized for 1 week before starting any procedures.

Experimental protocols

Female Balb/c and CD1 mice at 6 weeks of age were used. Balb/c mice were sensitized to ovalbumin (OVA) by subcutaneous (sc) injection with 0.4 mL of 10 µg OVA, absorbed onto 3.3 mg of aluminium hydroxide gel in sterile saline at days 0 and 7 and treated with saline solution or N/OFQ (15 µg/Kg) 30 min before both OVA treatment (Fig. 1a). Animals were divided into three groups: 1. N/OFQ group: unsensitized mice treated with the sole N/OFQ; 2. Vehicle+OVA group: sensitized mice treated with saline solution, considered the vehicle of N/OFQ; 3. N/OFQ+OVA group: sensitized mice treated with N/OFQ. Mice were sacrificed at two different time points (7 and 14 days) from the first allergen exposure, and tissues were collected for flow cytometry analyses.

In a separate set of experiments, both female Balb/c and CD1 mice were used and divided into the same group as of the previous experimental protocol. Animals were sensitized to OVA at day 0 and 7. From day 21 to 23 after the first OVA administration, all animals were aerosol-challenged with 1% OVA in phosphate-buffered saline (PBS) solution using an ultrasonic nebulizer (De Vilbiss Health Care, UK Ltd., Heston, Middlesex, UK) for 7 min per day. Twenty-four hours after the last OVA challenge, animals were killed for functional and cellular evaluation (Fig. 1b).

Bone Marrow-derived DC isolation

Bone Marrow-derived DC (BMDC) were isolated from femurs and tibias and cultured with granulocyte-macrophage colony-stimulating factor (10 ng/mL; Invitrogen, Carlsbad, CA) for 6 to 7 days, as previously described [25]. The purity of BMDCs, as assessed by flow cytometry, was typically greater than 95%. These cells were CD11c positive (DC marker). Cells were used for circular dichroism to prove the presence of N/OFQ receptor.

Flow cytometry analysis

Lungs and lymph nodes (LNs) were isolated and digested with 1–0.5 U/mL of collagenase (Sigma-Aldrich, Rome, Italy). Cell suspensions were passed through 70-µm cell strainers, and red blood cells were lysed. Cell suspensions were used for flow cytometric analyses of different cell subtypes. In another set of

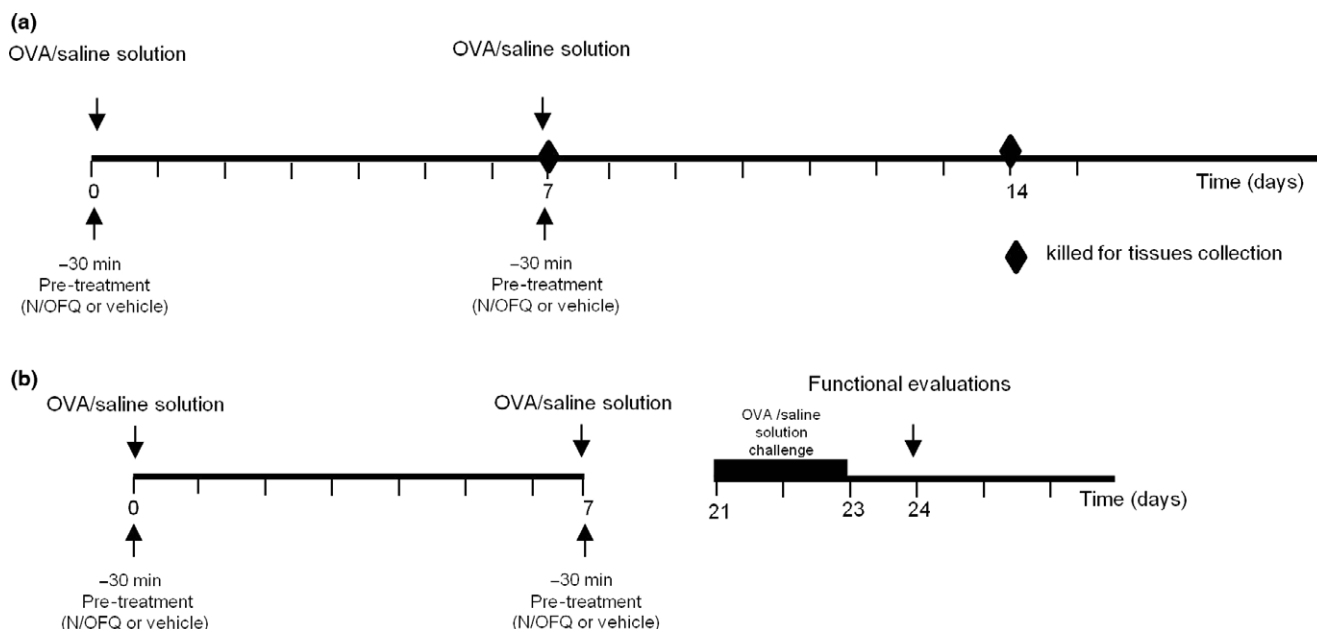


Fig. 1. Schematic representation of treatment regimen. a) Experimental protocol for tissues collection for flow cytometry analyses. b) Experimental protocol for functional and cellular evaluation.

experiments, lung homogenates were used to measure cytokines by means of ELISA. The composition of lung-infiltrated cells was determined by flow cytometry, using several antibodies: CD11c-FITC or allophycocyanin (APC), CD11b-PeCy5.5, F4/80-PE, MHC I-FITC, MHC II-APC, CD4-FITC, IL-4 and CD3-APC (eBioscience, USA). C16/CD32 was used before flow cytometry stainings to block any aspecific Ab bindings.

Intracellular staining was performed for CD4⁺ CD3⁺ IL-4⁺ T cells.

Cytokine measurements

Lung homogenates were used to measure cytokines by means of ELISA. IL-13 and IFN- γ were determined by means of ELISA according to the manufacturer's instructions (eBioscience, CA, USA).

Lung reactivity assay

Lung reactivity was assessed in isolated and perfused mouse lung model prepared essentially as described (21). Briefly, water-jacketed (water temperature, 37°C) perspex chamber was used to accommodate surgery, perfusion and ventilation of murine lungs. Mice were anesthetized with ketamine HCl 40 mg/kg i.p. and medetomidine hydrochloride 0.15 mg/kg i.p. The skin was incised from the abdomen to the throat, and the trachea was exposed, with a small incision, and cannulated. Subsequently, lungs were ventilated with 90 breaths/min room air with a tidal volume (Vt) of approximately 250–300 μ L. 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 fixed with two cannulae at sides on the cork plate. At this point, pulmonary artery was cannulated through a little 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 and ventilated in a non-recirculating manner with constant flow (generated by a peristaltic pump, Ismatec MS Reglo) at 1 mL/min for 45 min without treatment, to obtain a steady baseline. A perfusion medium was RPMI 1640 cell culture medium (Biochrom, Berlin, Germany) supplemented with 4% bovine serum albumin. Successively, a repetitive dose-response curve to acetylcholine (ACh) (10^{-8} to 10^{-3} M), in all experimental groups, was performed.

Circular dichroism

Circular dichroism (CD) spectra were recorded at 278 K on a JASCO J-815 CD spectropolarimeter equipped with Peltier temperature control. Data were collected using a quartz cuvette with a 1 cm path length in the 200–260 nm wavelength range with a data pitch of 1 nm, a bandwidth of 1 nm and a scanning speed of 50 nm/min.

Lyophilized peptide was dissolved in 3 mL of a 0.9% w/v of NaCl, pH = 7.4, to reach a final concentration of 30 μ M. CD1 and Balb/c dendritic cells were pelleted and then resuspended in the final saline solution. Different

aliquots of cells were added into the peptide solution up to two millions of cells. Spectra were recorded after 10 min of incubation of the peptide for each cell addition with both cells type. Circular dichroism spectra were also recorded titrating cells into the saline solution alone, to monitor the contribution of cells to the spectra. Spectra of the cells alone were subtracted with the spectra of the peptide + cells solution to eliminate the contribution of the cells to the CD signal.

All the experiments were carried out at least in duplicate.

Drugs

N/OFQ was synthesized and purified in house according to the procedures previously described [26]. OVA and ACh were purchased from Sigma-Aldrich. All other reagents were of the highest purity available. LPS and GM-CSF were purchased by Microtech s.r.l. (Naples, Italy).

Statistical analysis

All results are reported as means $\pm$ SEM. For statistical analysis, we used one-way or two-way ANOVA followed by Bonferroni post-test or Student's *t*-test where appropriate. A value of $P < 0.05$ was considered as significant.

Results

N/OFQ system on inflammation in OVA-sensitized Balb/c mice

In our previous studies, we demonstrated that the administration of N/OFQ was able to reduce lung resistance [21,

22]. Because the asthmatic symptoms are strictly correlated to the inflammatory environment, we wanted to analyse the ability of N/OFQ to alter lung immune microenvironment in OVA-sensitized mice. Therefore, we first analysed the levels of two Th2-like cytokines involved in inflammatory processes of the airways during asthmatic conditions. At 14 days, N/OFQ treatment caused a significant reduction in IL-13 into the lung of OVA-treated mice (Fig. 2a). Similarly, N/OFQ significantly reduced the percentage of IL-4⁺ CD4⁺ T cells in LNs of OVA-sensitized mice (Fig. 2b). To confirm the role of N/OFQ to inhibit a Th2-like environment, we analysed the levels of a Th1 cytokine, IFN- γ . N/OFQ significantly increased the levels of IFN- γ into the lung of OVA-sensitized mice (Fig. 2c). These data implied that N/OFQ treatment was able to modulate the Th2-like bias in OVA-sensitized mice.

Effects of N/OFQ treatment on lung infiltration of dendritic cells (DCs)

In order to gain insight into the effects of N/OFQ on the recruitment of inflammatory cells to the lung of OVA-treated mice, we went on to analyse the presence of DCs (identified as CD11c⁺CD11b^{int}F4/80⁻ cells) after OVA sensitization with or without N/OFQ treatment. The administration of N/OFQ did not alter the influx of DCs into the lung of OVA-sensitized mice compared to the control mice at 7 and 14 days (Fig. 3a). However, we observed that N/OFQ treatment significantly reduced the efflux of DCs to the mediastinic lymph node at

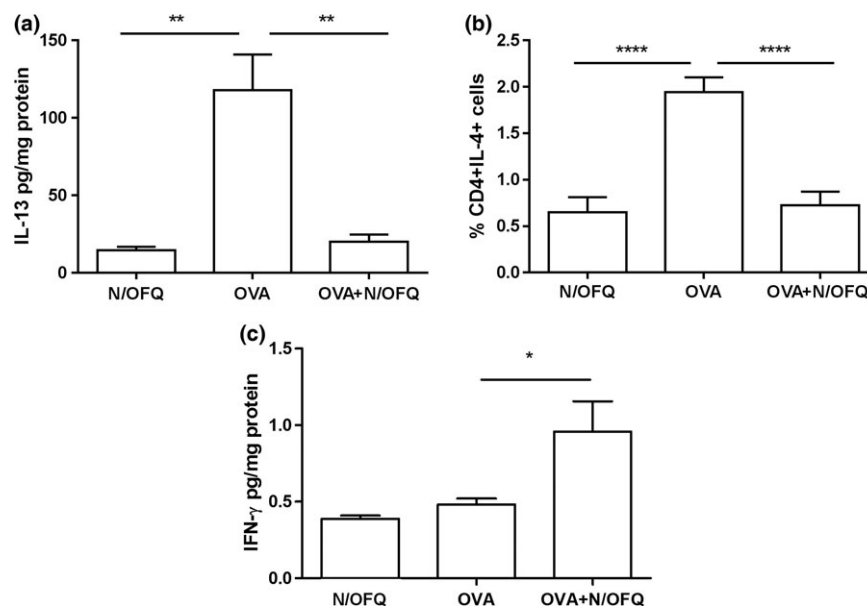


Fig. 2. Effects of N/OFQ treatment on release of inflammatory mediators *in vivo*. N/OFQ treatment caused a significant reduction in IL-13 into the lung OVA-treated mice (a). Similarly, N/OFQ significantly reduced the percentage of IL-4⁺ CD4⁺ T cells in LNs of OVA-sensitized mice (b). On the contrary, N/OFQ caused a significant increase of IFN- γ into the lung of OVA-sensitized mice. Data are means $\pm$ SE ($n = 6$), one-way ANOVA followed by Student's *t*-test was used. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

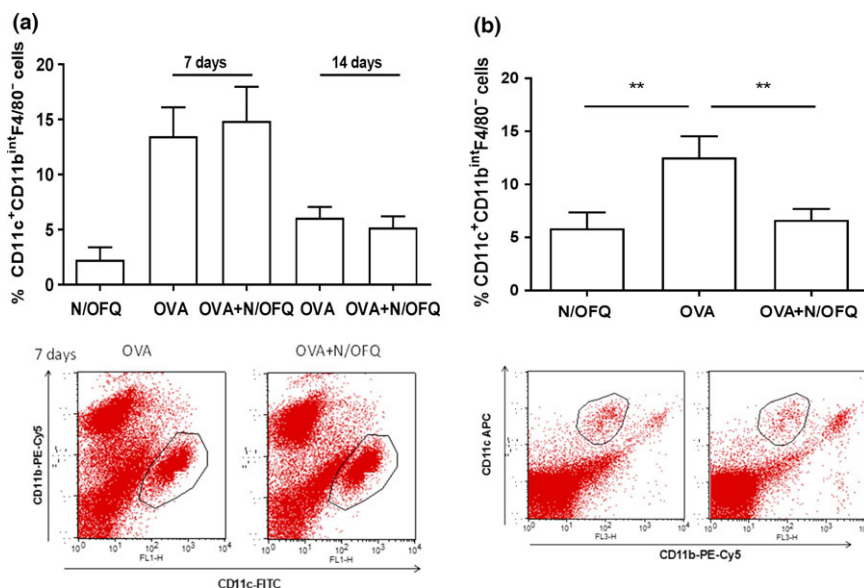


Fig. 3. Effects of N/OFQ treatment on lung infiltration of dendritic cells (DCs) N/OFQ treatment did not alter the influx of DCs into the lung of OVA-sensitized mice compared to the control mice at 7 and 14 days (a). However, N/OFQ treatment significantly reduced the efflux of DCs to the mediastinic lymph node at 7 days (b). Data are means $\pm$ SE ($n = 6$), one-way ANOVA followed by Student's *t*-test was used. $**P < 0.01$.

7 days (Fig. 3b). These latter data well correlate with the lower Th2 polarization of CD4⁺ T cells in the LN at 7 days (Fig. 3b).

Effect of N/OFQ administration on cellular apoptosis

In order to explain the mechanism of how N/OFQ modulated the inflammatory response, validating the thesis of its immunoregulatory role, we performed a flow cytometry analysis to verify whether the immunomodulation by N/OFQ in OVA-treated BALB/c mice was related to cell death of DCs. The administration of N/OFQ did not induce lung-infiltrated DCs to die as very low levels of Annexin V was observed on these cells in all treatments performed (Fig. 4).

Effect of N/OFQ on DCs maturation and activation

Because of the pivotal role of DCs in the induction of asthma and because N/OFQ proved beneficial activity against lung resistance in OVA-sensitized mice [27], we went on to understand the DC phenotype. We analysed, 7 days from the first allergen exposure, the expression of MHC class I/II and CD80, well-known markers for maturation and activation, respectively [28]. The administration of N/OFQ did not alter MHC I (Fig. 5a) and MHC II (Fig. 5b) expression, but instead, it increased CD80 expression (Fig. 5c), implying that N/OFQ induced the activation of the mature MHC I⁺ and MHC II⁺ DCs to exert their APC activity via the overexpression of CD80.

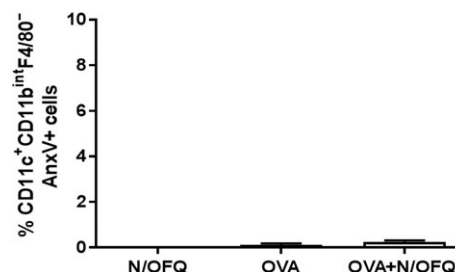


Fig. 4. Effect of N/OFQ administration on cellular apoptosis. The administration of N/OFQ did not induce lung infiltrated DCs to die as very low levels of Annexin V was observed on these cells in all treatments performed. Data are means $\pm$ SE ($n = 6$), one-way ANOVA followed by Student's *t*-test was used.

Effect of N/OFQ system on lung reactivity of OVA-sensitized CD1 mice

The data so far shown suggested that N/OFQ could alter a Th2-like microenvironment in the lung of OVA-treated Balb/c mice. Therefore, we studied whether the same effect of N/OFQ system on lung reactivity was reproducible in another mouse strain (CD1), which is less asthma prone compared to BALB/c. As hypothesized, we observed that the administration of Ach (10 nM to 1 mM) onto the lungs of OVA+N/OFQ-treated CD1 mice did not alter lung resistance (Fig. 6a) as instead observed for BALB/c mice (Fig. 6b), confirming its specific activity in a Th2-like environment.

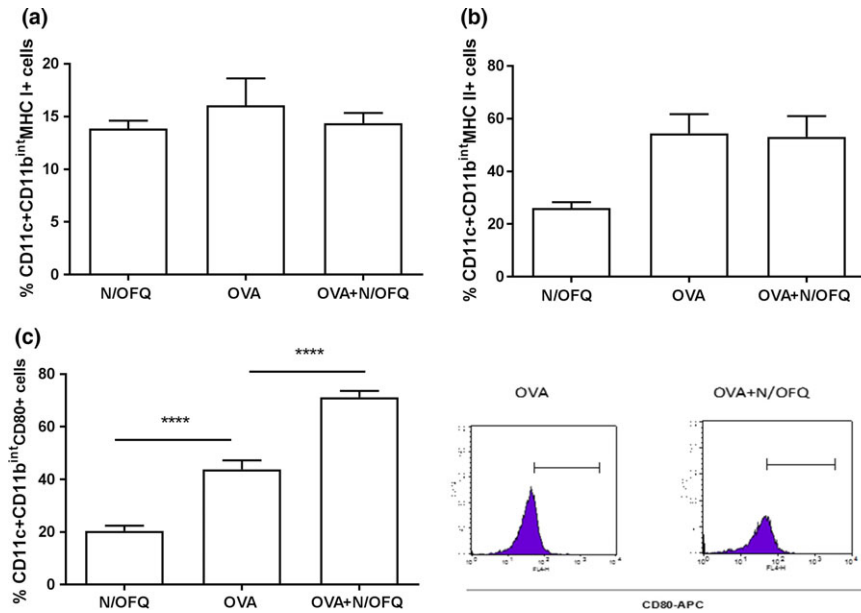


Fig. 5. Effect of N/OFQ on DCs maturation and activation. The administration of N/OFQ did not alter MHC I (a) and MHC II (b) expression, but instead it increased CD80 expression (c). Data are means $\pm$ SE ($n = 6$), one-way ANOVA followed by Student's t -test was used. **** $P < 0.0001$.

N/OFQ titration with DCs by circular dichroism

To support the data obtained on the role of N/OFQ in DCs activation, we went on to analyse the presence of NOP receptor via circular dichroism. BMDCs from Balb/c and CD1 mice were used. The spectroscopic data strongly indicated that N/OFQ is able to interact with the cell membrane of DCs obtained from Balb/c (Fig. 7a) mice rather than CD1 mice (Fig. 7b), confirming its specific activity in a Th2-like environment as observed *in vivo*. We monitored via CD the changes in secondary structure of N/OFQ during titration experiments with DCs obtained from Balb/c or CD1 mice. As expected, N/OFQ in solution adopts a random coil conformation [26,29], as shown also by its CD spectrum. Titration of N/OFQ with aliquots of DCs obtained by Balb/c mice caused an increase of CD band centred around 222 nm (Fig. 7a), which

intensity depends on the number of cells added: at low cell concentration, a high content of random coil, over-structured peptide is present. These data suggest that N/OFQ peptide changes its secondary structure upon interacting with this type of DCs cells. On the other hand, addition of CD1 cells did not cause such significant changes in the spectra (Fig. 7b), suggesting a lower interaction affinity of N/OFQ with these cells. We performed the experiments at least three times with different batches of cells isolated from different animals. Even though the different batches from the different individual produced CD spectra that are not superimposable, the data obtained upon cell additions resulted very similar in trend and shape, strongly suggesting that the changes of the CD spectra are due to the interaction between N/OFQ and its receptor expressed on the membrane of Balb/c rather than CD1 DCs.

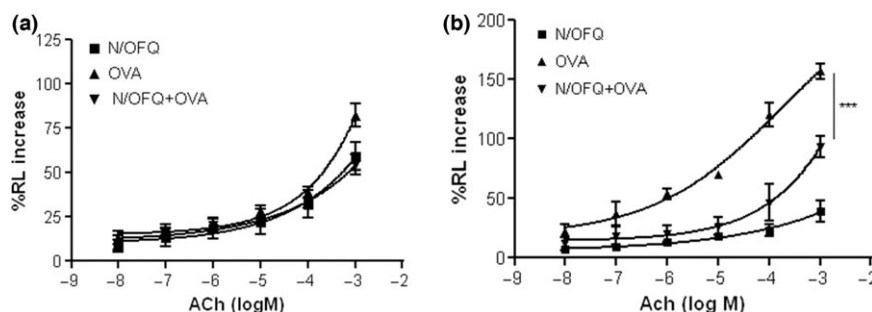


Fig. 6. Effect of N/OFQ system on lung reactivity of OVA-sensitized CD1 mice. The administration of ACh (10 nM to 1 mM) onto the lungs of OVA+ N/OFQ-treated CD1 mice did not alter lung resistance (a) as instead observed for BALB/c mice (b). Data are means $\pm$ SE ($n = 5$). *** $P < 0.001$.

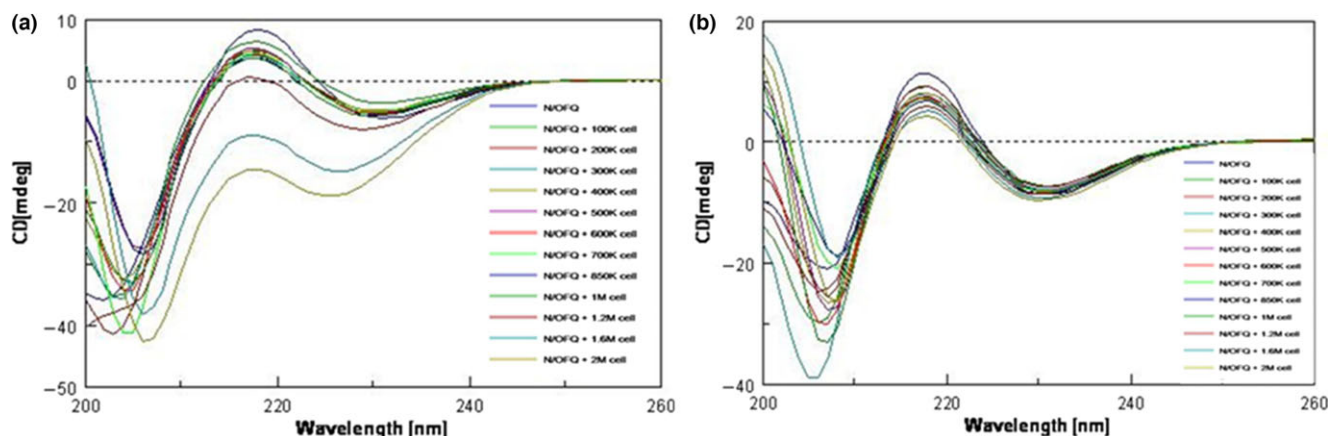


Fig. 7. N/OFQ titration with DCs by circular dichroism. Titration of N/OFQ with aliquots of DCs obtained by Balb/c mice caused an increase of CD band centred around 222 nm (a). Addition of CD1 mice cells did not cause such significant changes in the spectra (b). Spectra are representative of one set of experiment and are net of cells contribution.

Discussion and conclusions

The results obtained in the present study show that N/OFQ reduces the inflammatory immune Th2-like microenvironment in a conventional murine model of asthma. We previously reported that N/OFQ receptor activation decreases airway hyperresponsiveness induced by allergen in sensitized mice and modulated immunopathology [22, 27]. These effects, linked to a reduction of Th2 cytokines and OVA-specific IgE levels in BAL [22, 27], led us to hypothesize a N/OFQ Th2-selective immunomodulatory effect *in vivo*. Th2 cells orchestrate the asthmatic inflammation through the secretion of a series of cytokines [30], and a key role is played by IL-4 [31,32]. This cytokine promotes the development of Th2 responses by modulating the differentiation of T cells [33], and data from Galli et al. showed that in the presence of early IL-4 in the LN, naïve T cells acquire the characteristics of Th2 cells [34]. In this direction, in the present study, we observed a significant reduction of IL-4+ CD4+ T cells in LNs from N/OFQ-treated compared to OVA-sensitized mice. In support, in the lung tissues, we found a modulation of the levels of IL-13, a cytokine closely related to IL-4, able to bind to IL-4 receptor α and also expressed by Th2 cells from patients with asthma [35]. Moreover, we determined the levels of IFN- γ , a Th1 cytokine, and we observed a significant increase of this cytokine in pulmonary tissues obtained from OVA-sensitized Balb/c mice treated with N/OFQ. This result well fit with a previous finding in which N/OFQ has been shown an inhibitory effect on T cell proliferation that involves IFN- γ [36]. These results confirm our previous data on immunomodulatory activity of N/OFQ and support our hypothesis of a Th2 polarization of N/OFQ immunomodulation in the early phase of inflammation. Although Th cells are widely believed to have a direct role in asthma

disease, a key role during both initiation and progression of the immune response is played by APCs [37,38]. In fact, the polarization of such effector T cells is critically dependent upon cytokines production by APCs [39]. In particular, DCs can influence polarization of T cells by a specific expression and secretion of cytokine pattern [40] depending on their phenotype [41,42], their maturation status and the consequent costimulatory molecule pattern [43] and the environment they encounter [44]. In this regard, the present study showed that the treatment with N/OFQ facilitated the recruitment of DCs which were in their active phenotype (CD80+ cells), effect that was concomitant to the lower percentage of IL-4+ CD4+ T cells in the mediastinic LN. These data imply the capability of N/OFQ to modulate a Th2-like polarization.

N/OFQ did not induce DCs to apoptosis, indicating that the modulation of these cells was linked to other mechanisms not related to the number reduction. Therefore, we analysed the expression of MHC class I/II and CD80, well-known markers for maturation and activation, respectively [28]. The data obtained showed that N/OFQ is able to modify the sole CD80 expression, suggesting that N/OFQ modulated the activation of DCs in that to exert their APC activity. It is well known that during asthmatic conditions, DCs are in their mature (MHC I+/MHC II+ cells), but not active (CD80+) status. In this study, we demonstrated that N/OFQ reduces allergen-induced airway hyperreactivity. As previously documented by our research group [21], N/OFQ may be involved in the sensitization phase to allergen. In particular, in the present study, we documented a strictly correlation to the activation of DCs that was strictly dependent on the Th2 environment. Indeed, to support the immunomodulatory effect of the N/OFQ in a Th2 environment, we evaluated the pulmonary reactivity of N/OFQ-treated CD1 mice vs.

BALB/c. N/OFQ system was able to modulate lung reactivity [22, 27] of Th2-prone BALB/c mice [45,46] compared to the Th1-prone mouse strain CD1 [45,47]. Indeed, we did not obtain the same results in CD1 mice as those observed in BALB/c mice on pulmonary resistances, indicating the ability of N/OFQ to modulate AHR, a key aspect of the asthmatic disease only in a Th2-like environment.

To further support these data, we performed via circular dichroism (CD) an *in vitro* study of the interaction between the peptide and different DCs from Balb/c and CD1 mice. The CD experimental analysis has been performed to follow structural changes of N/OFQ upon interaction with DCs. The spectroscopic data strongly indicated that N/OFQ was able to interact on the cell membrane of DCs obtained from Balb/c mice rather than CD1 mice. The obtained results strongly indicate that N/OFQ interacts with DCs from Balb/c but not with those from CD1 mice, but correlations with the pathogenesis of asthma, in the

context of DCs and T cells, cannot be drawn on the basis of this spectroscopic analysis. Therefore, these data further indicate a Th2 modulation of N/OFQ and at the same time show its direct activity on DCs.

In conclusion, our data confirmed our previous results on the capability of N/OFQ to modulate the immune microenvironment in the lung of Th2-biased, OVA-sensitized Balb/c mice. This effect was accompanied by the reduction of IL-13 lung levels and IL-4+ CD4+ T cells in the mediastinic lymph node due to the mature/active DCs into the lung of OVA-sensitized mice.

Therefore, N/OFQ-NOP axis may represent a novel pharmacological tool to modulate the immune microenvironment during asthmatic conditions.

Conflict of interests

The authors declare no conflict of interest.

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