Immunology and Cell Biology (2011) 89, 882–888

www.nature.com/icb

ORIGINAL ARTICLE

Y1 signalling has a critical role in allergic airway inflammation

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Asthma affects 300 million people worldwide, yet the mechanism behind this pathology has only been partially elucidated. The documented connection between psychological stress and airway inflammation strongly suggests the involvement of the nervous system and its secreted mediators, including neuropeptides, on allergic respiratory disease. In this study, we show that neuropeptide Y (NPY), a prominent neurotransmitter, which release is strongly upregulated during stress, exacerbates allergic airway inflammation (AAI) in mice, via its Y1 receptor. Our data indicate that the development of AAI was associated with elevated NPY expression in the lung and that lack of NPY-mediated signalling in NPYKO mice or its Y1 receptor in Y1KO mice significantly improved AAI. *In vivo*, eosinophilia in the bronchoalveolar fluid as well as circulating immunoglobulin E in response to AAI, were significantly reduced in NPY- and Y1-deficient compared with wild-type mice. These changes correlated with a blunting of the Th2 immune profile that is characteristic for AAI, as shown by the decreased release of interleukin-5 during *ex vivo* re-stimulation of T cells isolated from the thoracic draining lymph nodes of NPY- or Y1-deficient mice subjected to AAI. Taken together this study demonstrates that signalling through Y1-receptors emerges as a critical pathway for the development of airway inflammation and as such potentially opens novel avenues for therapeutic intervention in asthma.
Immunology and Cell Biology (2011) **89,** 882–888; doi:10.1038/icb.2011.6; published online 8 March 2011

Keywords: allergic airway inflammation; neuropeptide Y; Y1 receptor

Asthma is a chronic inflammation of the airways characterized by recurrent episodes of airway obstruction and associated with an inappropriate immune response to harmless environmental antigens. It is initiated and perpetuated by CD4 positive Th2 lymphocytes that secrete interleukin (IL)-4, 5 and 13, which are involved in the remodelling of airway epithelium, the hypereosinophilia and the secretion of Immunoglobulin E (IgE) by B lymphocytes.[1]

The exact mechanisms involved in this immune response initiation still remains unclear. Recent evidence has highlighted the 'neuro-immune' crosstalk as a contributor to the development of the airway inflammation.[2] Indeed, a dysfunction of airway innervation can lead to asthma symptoms like breathlessness and cough. Neurotrophin and tackinin like bradykinin A and substance P, secreted by sensory nerves innervating the lung, directly contribute to the immune cell activation, bronchoconstriction and vasodilatation eventually leading to asthma development.[3] On the other hand, neural mediators can also have a protective role as levels of α-melanocyte-stimulating hormone, which is known to have an anti-inflammatory role and protect mice from allergic airway inflammation (AAI), is decreased in the lung of asthmatic patients.[4] However, the most convincing circumstantial evidence that the nervous system has a key role on asthma development is the fact that psychological stress worsens asthmatic syndrome.[2] Stress causes a highly complex response and thus what causes its worsened effects on asthma remains elusive. Stress is also known to stimulate the release of certain neuropeptides by sympathetic nerves, with neuropeptide Y (NPY) being the most prominent one.[5] Importantly, human studies have revealed increased NPY levels in the serum of asthmatic patients under resting conditions and during severe acute asthmatic crises.[6] Moreover, immunohistochemistry of lung biopsies have shown that lung tissue is highly innervated with NPY-positive nerve fibres entering the bronchus-associated lymphoid tissue and the branches of the pulmonary artery.[7] Furthermore, NPY can exert a significant contractile effect on trachea[8] suggesting that NPY could have a direct aggravating effect on airways during asthma.

NPY signals through at least 5 G-protein coupled receptors (Y1, Y2, Y4, Y5 and Y6) and is widely expressed in the brain to regulate a broad range of functions such as feeding, anxiety, memory and circadian rhythms.[9] In the periphery, NPY is important for the regulation of blood pressure and energy homeostasis but also influences immune function especially via its Y1 receptor.[10] During sympathetic stimulation, NPY is co-released with norepinephrine in the lymph node in close proximity to immune cells.[11,12] It has been demonstrated that most of the innate and adaptive immune cells are potential targets of

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Received 1 July 2010; revised 12 January 2011; accepted 13 January 2011; published online 8 March 2011

NPY, including B lymphocytes, CD4 and CD8 positive T lymphocytes, macrophages, dendritic cells (DCs), natural killers and mast cells, all of which express the Y1 receptor.[10] NPY also has an active role on the adaptive immune response by skewing the T-cell profile towards Th2 effector by directly promoting the release of IL-4 and inhibiting interferon-γ secretion *in vitro*.[13] Moreover, NPY modulates the DC functionality, initiator of the Th2 orientation, ass lack of Y1 signalling impairs DC's capacity to induce an adaptive immune response.[10] Finally, like in the airway allergic reaction, the delayed type hypersensitivity model requires both a challenge and sensitization with the antigen, contributing to an exacerbated inflammatory response. We have shown that lack of Y1 receptor signalling protected the mice against delayed type hypersensitivity suggesting that NPY has a key role in the control of inflammation.[10]

Based on this evidence, we hypothesize that NPY may also have a critical role in AAI development. To test this hypothesis, we studied the immune response profile and the airway inflammation in mice lacking NPY as well as mice lacking the major Y-receptor, Y1, on immune cells during AAI development.

RESULTS

NPY deficiency improves lung inflammation associated with AAI

AAI is characterized by the exacerbated recruitment of immune cells mostly eosinophils, in the bronchoalveolar fluid (BALF). As NPY is also locally expressed in the lung and is known to modulate the inflammatory response, we first determined if its level of expression was changed during AAI. Interestingly, we found that allergic mice had a trend to increased NPY (P=0.08) in their lung compared with non-allergic mice (Figure 1a). To determine if NPY might has a detrimental role on the development of AAI, we then studied the impact of the lack of NPY on the airway immune cell recruitments during AAI. BALF cells from control mice (WT) and from NPY knockout (NPYKO) mice were analysed both under non-allergic and allergic conditions. In both groups, under allergic conditions the total number of cells in the BALF was significantly increased compared with basal conditions (Figure 1b). However, NPYKO mice had significantly reduced numbers of BALF cells recruited compared with WT mice (Figure 1b). This reduced cell recruitment in the NPYKO mice is correlated with a significant decreased hypereosinophilia compared with WT mice whereas the neutrophils, macrophages and lymphocytes were found in similar amounts in both groups (Figure 1c). Importantly, histological analysis of lungs from NPYKO mice revealed that the lack of NPY showed reduced lung inflammation under allergic conditions (Figures 1d–e).

NPY modulates the Th2 response associated with AAI

AAI results from an exacerbated Th2 immune response, characterized notably by the increase of cytokines like IL-5 and IL-10, which are directly involved in the recruitment of eosinophils.[1] As NPY is known to favour the T-cell orientation toward a Th2 profile[13] and that the lack of NPY leads to a decreased hypereosinophilia in the BALF, we hypothesized that the lack of NPY might impair the typical Th2 response in AAI. To assess the immune response profile in the absence of NPY during AAI, we measured the cytokines released by immune cells from the mediastinal lymph node isolated from WT compared with NPYKO mice after *ex vivo* re-stimulation with ovalbumin (OVA). Although the release of IL-5 was significantly increased in both allergic groups after OVA restimulation, the cells isolated from NPYKO mice secreted significantly less IL-5 than the cells from WT mice (Figure 2a). Moreover, IL-10 levels were also significantly increased in both allergic groups although the increase was not as high in the NPYKO mice compared with the WT mice (Figure 2b).

In order to determine whether the decreased Th2 responses seen in the NPYKO mice were due to an immune response skewed toward the Th1 effector response, we measured *ex vivo* release of interferon-γ under the same conditions used to measure IL-5 and IL-10. Consistent with the lack of an increased Th1 response in the absence of NPY, the levels of interferon-γ were not increased under allergic conditions in the WT or the NPYKO mice (Figure 2c). As Th2 cytokines participate to the OVA specific B cell antibody isotype switch towards the IgE subtype, we measured the level of IgE under non-allergic and allergic conditions in the NPYKO and WT mice. Under allergic conditions, the WT mice displayed a significant increase in serum IgE. In contrast, serum levels of IgE in NPYKO mice were maintained at similar levels whether mice were under non-allergic or allergic conditions (Figure 2d). The significant lower levels of IgE in the allergic NPYKO mice compared with allergic WT mice (Figure 2d), confirmed the altered Th2 responses in the NPYKO mice. Intriguingly, the levels of OVA-specific IgE were increased in both allergic groups and reached similar levels in the WT and NPYKO mice (Figure 2e).

Y1 receptor signalling controls BALF cell recruitment

We have previously shown that Y1 is the major Y-receptor mediating the effects of NPY on the immune system.[10] Therefore, we investigated if it also contributed to the development of AAI. As observed in the NPYKO model, we found that the increased BALF immune cell numbers was significantly less in the Y1KO mice under allergic conditions compared with the WT mice (Figure 3a) consistent with a key role of this receptor in triggering the inflammation effect of NPY in AAI. This decreased cell number is mostly due to the reduction of eosinophils but also the significant decrease of neutrophils recruited in the BALF compartment (Figure 3b). Interestingly, the histological analyses of the lung sections revealed that the absence of Y1 signalling reduced lung inflammation in AAI (Figure 3c) with a significantly decrease of the inflammatory score in the Y1KO mice under allergic conditions (Figure 3d).

Y1 receptor signalling modulates the Th2 response in AAI

In order to determine why the recruitment of immune cells in the BALF was reduced in the Y1KO mice, we studied *in vitro* the impact of lack of Y1 signalling on the Th2 immune response.

The *ex vivo* re-stimulation of lymphocytes isolated from the mediastinal lymph node of Y1KO and WT mice with OVA revealed that a lack of Y1 signalling totally prevented the initiation of a Th2 profile. Indeed, compared with cells isolated from non-allergic Y1KO mice, the cells from the allergic Y1KO mice showed unchanged IL-5 levels (Figure 4a). Thus the release of IL-5 was significantly decreased in allergic Y1KO mice compared with WT allergic mice (Figure 4a). The decrease in Th2 specific cytokines in the Y1KO mice was also correlated with decreased total serum IgE levels in the Y1KO allergic mice (Figure 4b) as well as with a significant decrease of serum OVA specific IgE (Figure 4c).

DISCUSSION

Our results from this study demonstrate for the first time that NPY has a critical role in the development of AAI and that this is specifically mediated through Y1 receptor signalling.

Indeed, although we show that mice developing AAI have a trend (P=0.08) to increased expression of NPY in their lungs, the complete absence of NPY in mice alleviates the symptoms of AAI consistent with an aggravating role of NPY in this condition. Among the five identified receptors for NPY, the Y1 receptor appears to be the major one through which NPY mediates its immune function.[10] We show

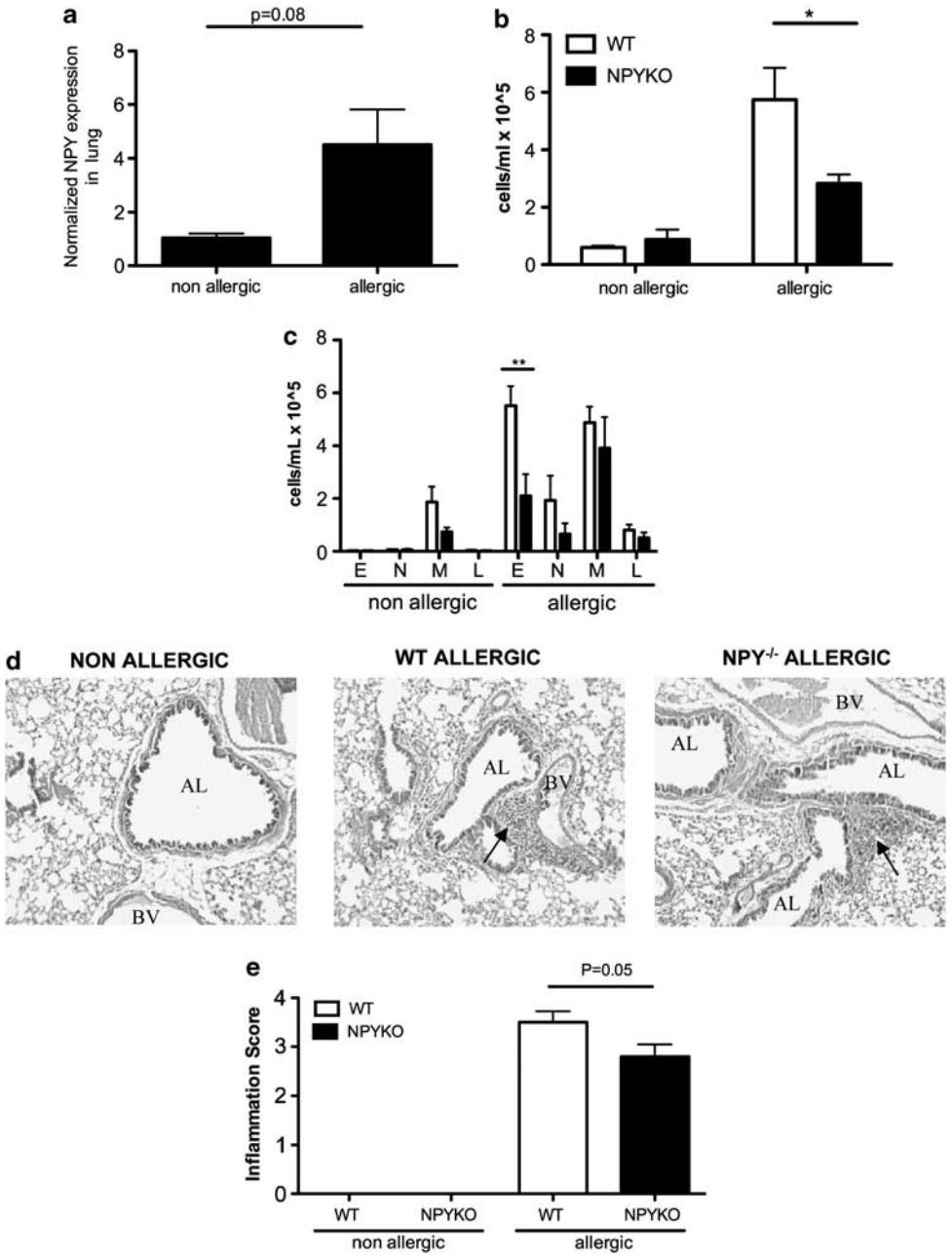

Figure 1 Absence of NPY improves BALF cell recruitment and lung inflammation in AAI. (**a**) Measurement of NPY expression in the lung of C57/BL6 mice under basal conditions (non-allergic mice, $n=4$), or after AAI development (allergic mice $n=7$), by real time PCR. These are represented by the level of NPY in each mouse as well as the mean ± s.e.m. (**b**) Counting of BALF cell number in wild-type mice (WT, white column) under basal (non-allergic, $n=6$) or after AAI development (allergic, $n=13$) conditions, and in NPYKO mice (black column, with $n=8$ NPYKO non-allergic and $n=11$ NPYKO allergic). These are represented by the means ± s.e.m. with $^*P<0.05$. (**c**) Differential counting of the BALF cell populations with E standing for eosinophils, N for polymorphonuclear neutrophils, M for macrophages and L for lymphocytes in WT mice (white columns) under basal conditions (non-allergic, $n=6$) or during AAI (allergic, $n=11$) and in NPYKO mice (black columns with $n=8$ NPYKO non-allergic and $n=11$ NPY KO allergic). These are represented by the means ± s.e.m. with $^{**}P<0.01$. (**d**) Representative pictures of lung inflammation after haematoxylin and eosin staining under basal conditions in WT mice (non-allergic) or during AAI in WT mice (WT allergic) and in NPYKO mice (NPYKO allergic). Pictures have a ×100 magnification and BV stands for blood vessel and AL for airway lumen. (**e**) Inflammatory score measured based on the haematoxylin and eosin staining of lungs of WT (white columns) under basal (non-allergic $n=7$) and AAI conditions (allergic $n=10$) and of NPYKO mice (black columns) under basal (non-allergic $n=6$) and AAI conditions (allergic $n=10$). These are represented by the mean ± s.e.m.

that mice lacking Y1 receptor signalling have significantly reduced symptoms of AAI suggesting that NPY exerts its aggravating effects at least partly via Y1 receptor and possibly by directly targeting these immune cells.

Furthermore, this is consistent with an even greater protective effect from AAI in Y1KO mice, compared with mice lacking NPY, which have a higher lung inflammatory score. This result could be explained by the fact that the NPYKO mice still express PYY, another ligand for

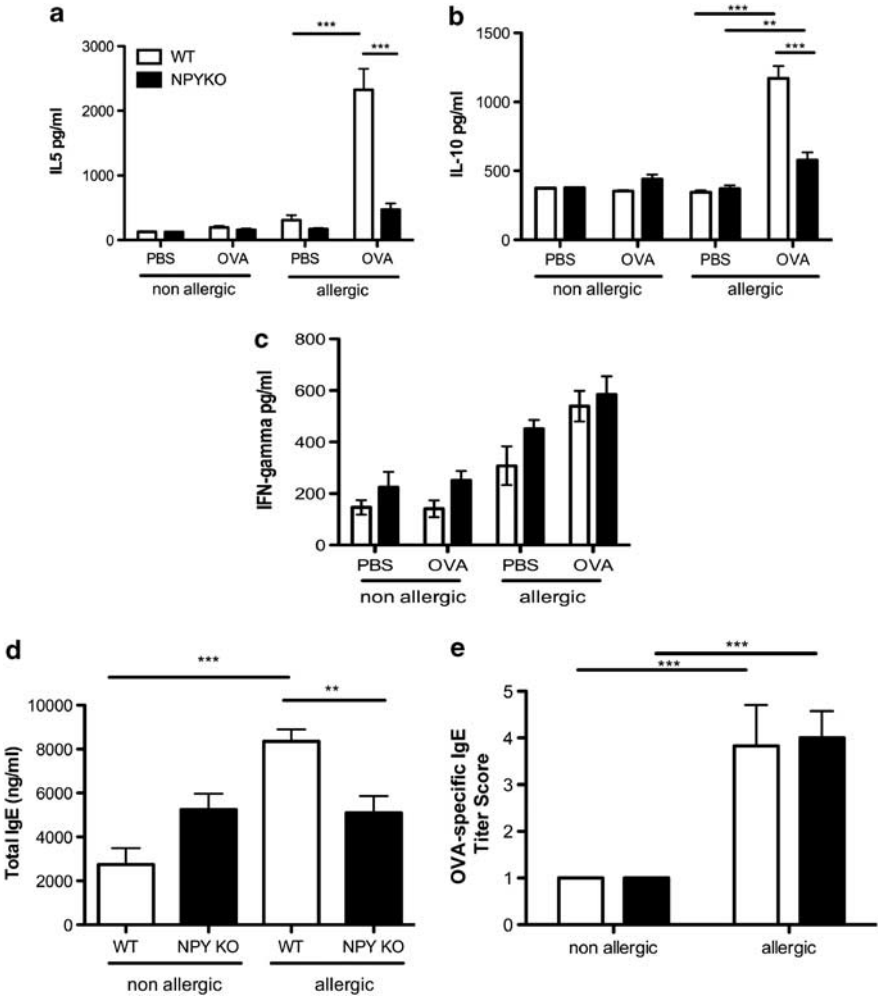

Figure 2 Absence of NPY blunts the Th2 immune response without promoting a Th1 immune response. (**a**) Measurement of IL-5 concentration (pg ml^{-1}) in the supernatant of cultured mediastinal lymph node cells either unstimulated (PBS) or stimulated with 100 μg of OVA. Cells were isolated from either WT (white columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6) or from NPYKO mice (black columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6). These are represented by the mean ± s.e.m. with ***P<0.001. (**b**) Measurement of IL-10 concentration (pg ml^{-1}) in the supernatant of cultured mediastinal lymph node cells either unstimulated (PBS) or stimulated with 100 μg of OVA. Cells were isolated from either WT (white columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6) or from NPYKO mice (black columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6). These are represented by the mean ± s.e.m. with **P<0.01 and ***P<0.001. (**c**) Measurement of interferon-γ concentration (pg ml^{-1}) in the supernatant of cultured mediastinal lymph node cells either unstimulated (PBS) or stimulated with 100 μg of OVA. Cells were isolated from either WT (white columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6) or from NPYKO mice (black columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=6). These are represented by the mean ± s.e.m. (**d**) Total serum IgE concentration (ng ml^{-1}) measured in WT (white columns) under basal (non-allergic, n=4) or during AAI (allergic, n=6) and in NPYKO mice (black columns) either under non-allergic (n=4) or allergic (n=6) conditions. These are represented by the mean ± s.e.m. with **P<0.01 and ***P<0.001. (**e**) OVA specific serum IgE (titre score) measured in WT (white columns) under basal (non-allergic, n=4) or during AAI (allergic, n=6) and in NPYKO mice (black columns) either under non-allergic (n=4) or AAI (n=6) conditions. These are represented by the mean ± s.e.m. with ***P<0.001.

the Y1 receptor. Thus, the remaining Y1 signalling in the NPYKO mice might account for the partial development of AAI.

In both Y1KO and NPYKO mice, the recruitment of cells in the BALF is significantly decreased, which is consistent with the decreased eosinophilia, also observed in the blood (Supplementary Figure 1), as well as the lower level of IgE compared with allergic WT mice. Surprisingly, although the total IgE level were comparable between the NPY-/- and the WT mice, the level of OVA-specific IgE were significantly decreased in the first group (data not shown). NPY might act as a survival factor on memory B cells by signalling through a Y-receptor other than the Y1 as the Y1KO mice do not have this phenotype. Both of the decreased recruitment of BALF cells, the decreased eosinophilia and specific IgE in both the NPYKO and Y1KO mice are directly linked to the Th2 immune cytokines. Indeed, the decrease of IL-5, the cytokine known to promote the differentiation of eosinophils from bone marrow progenitors[14] might explain the improved AAI in NPYKO and Y1KO mice, also illustrated by their lower levels of IL-10 as published before.[15] We show that both, lack of NPY or specific Y1-mediated signalling blunts the Th2 immune profile of restimulated lymphoid cells isolated from allergic NPY KO and Y1KO mice *ex vivo*. The two major cell types involved in this immune profile are the T lymphocytes themselves and the antigen presenting cells, mostly the DCs, instructing T cells to become Th2 T cells. Both of these cells types express functional Y1 receptors[10] and are thus able to respond to NPY. Possible mechanisms explaining how NPY-Y1 signalling might affect the Th2 immune profile, could be either via

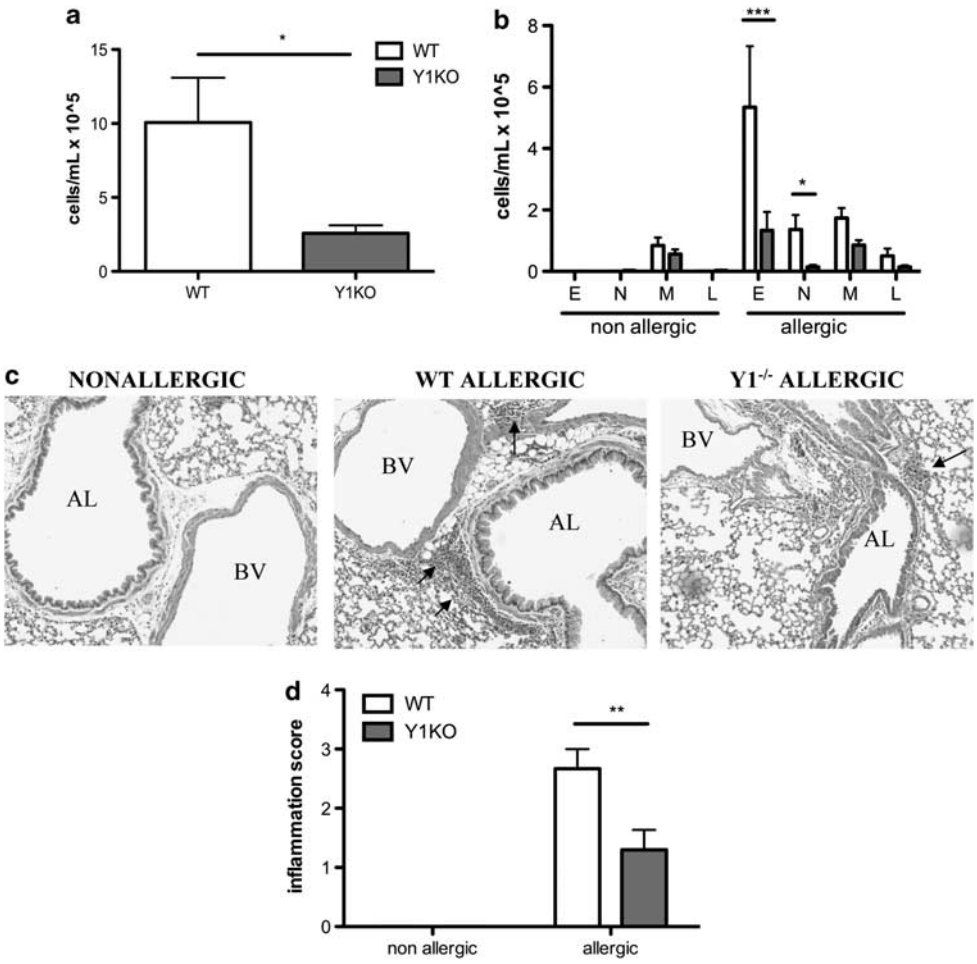

Figure 3 Absence of Y1 receptor signalling protects from BALF cell recruitments and lung inflammation during AAI. (**a**) Counting of BALF cell number in wild-type mice (WT, white column) under basal (non-allergic, $n=7$) or after AAI development (allergic, $n=9$) conditions, and in Y1KO mice (grey column, with $n=6$ Y1KO non-allergic and $n=10$ Y1KO allergic). These are represented by the means ± s.e.m. with $^*P<0.05$. (**b**) Differential counting of the BALF cell populations with E standing for eosinophils, N for polymorphonuclear neutrophils, M for macrophages and L for lymphocytes in WT mice (white columns) under basal conditions (non-allergic, $n=6$) or during AAI (allergic, $n=10$) and in Y1KO mice (grey columns) with $n=6$ non-allergic and $n=10$ allergic mice. These are represented by the means ± s.e.m. with $^*P<0.05$ and $^{***}P<0.001$. (**c**) Representative pictures of lung inflammation after haematoxylin and eosin staining under basal conditions in WT mice (non-allergic) or during AAI in WT mice (WT allergic) and in Y1KO mice (Y1KO allergic). Pictures have a ×100 magnification and BV stands for blood vessel and AL for airway lumen. (**d**) Inflammatory score measured based on the haematoxylin and eosin staining of lungs of WT (white columns) under basal (non-allergic $n=7$) and AAI conditions (allergic $n=9$) and of Y1KO mice (grey columns) either non-allergic ($n=6$) or allergic ($n=10$) conditions. These are represented by the mean ± s.e.m. with $^{**}P<0.01$.

NPY directly targeting Y1 receptors on the DCs and/or the T cells to promote a Th2 immune response. We have previously shown that lack of Y1 signalling in the DCs impaired their capacity to uptake the antigen and to efficiently stimulate the T cells.[10] Potentially, this effect of NPY on the DCs may be mediated by an autocrine process consistent with the expression of NPY in DCs.[16] During AAI, the possible inability of the DCs to properly mount an immune response might explain the protection seen in the Y1KO and NPYKO mice. On the other hand it has also been demonstrated that NPY has a direct effect on T-cell function. Indeed, the addition of NPY on isolated splenocytes and T-cell clones, was shown to promote the release of IL-4 and to inhibit the release of interferon-γ.[13] Thus, in AAI NPY-Y1 signalling might actively orientate the T cells toward a Th2 immune response and its absence would blunt it. Interestingly, in AAI the absence of NPY-Y1 signalling in the NPYKO and Y1KO mice does not lead to a reorientation towards a Th1 immune profile as the level of interferon-γ is not increased in the restimulated cells *ex vivo*. This suggests that the absence of NPY-Y1 signalling seems beneficial to protect against AAI and does not lead to an improper or uncontrolled immune response.

However, the effect of NPY on AAI might not be exclusively immune in origin, as studies have also suggested a direct effect of NPY on airway muscle tone and possibly lung vascular resistance. More precisely, NPY is known to directly target airway smooth muscle cells, inducing a dose dependent contraction in trachea, bronchi and lung parenchyma strips of guinea pigs.[8,17] Thus, in the absence of NPY signalling the opposite may occur leading to reduced airway resistance in AAI. On the other hand, a study has shown that systemic injection of NPY induces vasoconstriction of bronchial and pulmonary vessels, which was mimicked by stimulation of sympathetic perivascular nerves.[18] This suggests a possible direct effect of NPY on airway vasotonicity, however, further investigation will be necessary to fully determine the exact mechanism involved.

Taken together, this work demonstrates that NPY signalling through Y1 receptors is critical for the regulation of the Th2 immune response

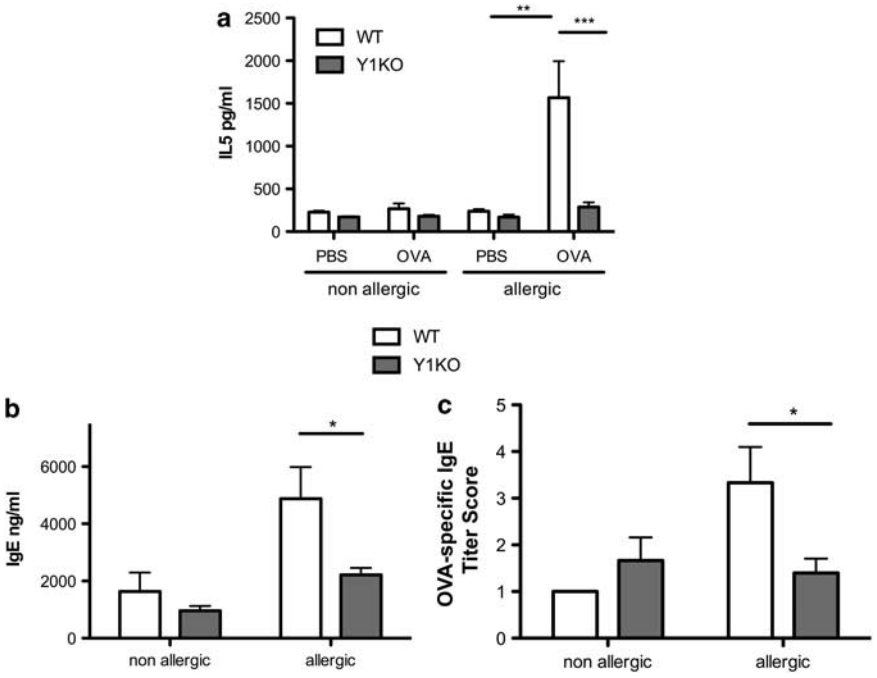

Figure 4 Lack of Y1 signalling inhibits the Th2 immune response. (**a**) Measurement of IL-5 concentration (pg ml^{-1}) in the supernatant of cultured mediastinal lymph node cells either unstimulated (PBS) or stimulated with 100 μg of OVA. Cells were isolated from either WT (white columns) under basal conditions (non-allergic n=7) and during AAI (allergic, n=9) or from Y1KO mice (grey columns) under basal conditions (non-allergic n=6) and during AAI (allergic, n=10). These are represented by the mean ± s.e.m. with $^{**}P<0.01$ and $^{***}P<0.001$. (**b**) Total serum IgE concentration (ng ml^{-1}) measured in WT (white columns) under basal (non-allergic, n=7) or during AAI (allergic, n=9) and in Y1KO mice (grey columns) either under non-allergic (n=6) or allergic (n=10) conditions. These are represented by the mean ± s.e.m. with $^{*}P<0.05$. (**c**) OVA specific serum IgE (titre score) measured in WT (white columns) under basal (non-allergic, n=7) or during AAI (allergic, n=9) and in Y1KO mice (grey columns) either under non-allergic (n=6) or AAI (n=10) conditions. These are represented the by mean ± s.e.m. with $^{*}P<0.05$.

necessary for AAI development. It also shows that targeting NPY signalling might be a promising strategy to improve AAI and potentially also helpful for the treatment of asthma.

METHODS

Animals

Generation of the Y1KO and NPYKO mice were published previously.[19,20] Mice were backcrossed to Bl6 and wild-type littermates were used where possible. All research and animal care procedures were approved by the Garvan Institute/ St Vincent's Hospital Animal Experimentation Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 °C) and illumination (12-h light cycle, lights on at 0700 h). Mice were fed a normal chow diet *ad libitum* (Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia).

AAI model

Female NPYKO, Y1KO and WT mice, aged 8–12 weeks, were sensitized by intraperitoneal injection of 100 μg OVA (Sigma-Aldrich, Sydney, NSW, Australia), or phosphate-buffered saline (PBS), adsorbed on 100 μl aluminium hydroxide (alum) (Pierce, Rockford, IL, USA) on day 0. On days 12, 14, 16 and 18, mice were re-challenged via exposure to aeroallergen consisting of either 1% OVA in PBS (OVA group) or PBS alone (PBS group) for 30 min using a nebulizer and secure container. The mice were sacrificed on day 19.

Quantitative PCR

Total RNA was isolated from the left lung of allergic (OVA treated) versus non-allergic (PBS treated) mice using TRIzol reagent (Invitrogen, Life Technologies, Mulgrave, VIC, Australia) and complementary DNA synthesis using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR was conducted using the SensiMix Probe (Bioline (Aust) Pty Ltd, Alexandria, NSW Australia) according to the manufacturer's specifications. The following primers were used: NPY forward: 5′-CCGCTCTGCGACACTACAT-3′, reverse 5′-TGTCTCAGGGCTGG ATCTCT-3′; rpl13a forward: 5′-ATCCCTCCACCCTATGACAA-3′, reverse: 5′- GCCCCAGGTAAGCAAACTT-3′. The results were normalized with the housekeeping gene *RPL13a* and expressed as fold increased over control values (PBS mice) using a previously described method (2^{-CT}; CT, cycle threshold).[21]

BALF cytology

After cannulating the trachea of the mice on day 19, bronchoalveolar lavage fluid (BALF) was collected by washing the lung lumen once with 800 μl cold PBS per mouse and then immediately centrifuged at 300 *g* for 10 min at 4 °C. The pellet was resuspended in 400 μl of cold PBS and 1×10^5 cells were spun in cytospin for 5 min at 1500 r.p.m. To do the differential leukocyte counts, the cytospin preparations of BALF were stained with modified Wright-Giemsa (Sigma-Aldrich).

Blood eosinophilia

Tail blood was treated with red blood cell lysis buffer and spun as described in the section *BALF cytology*. To count the eosinophils, the cytospin preparations of blood were stained with modified Wright-Giemsa (Sigma-Aldrich). The ratio of eosinophils on the number of total leukocyte counted was used to compare groups. An average of 340 leukocytes were counted per mouse.

Lymph node cells re-stimulation *in vitro*

Cells isolated from the mediastinal lymph nodes for each mouse were cultured at the concentration of 4×10^6 cells per ml (in a volume of 100 μl) in 96-well U-bottom plates (Corning, Lowell, MA, USA) in complete RPMI medium. Two conditions of culture were used first, the condition OVA where 100 μl of

complete medium containing 1000 μg ml^{-1} of OVA (Sigma-Aldrich) was used or the condition PBS in which 100 μl of complete medium with PBS was added to the cells. The cells were incubated for 72 h at 37 °C and each condition was made in triplicate.

Cytokine and IgE measurement

The cytokine concentration were measured by enzyme-linked immunosorbent assay according to the manufacturer's protocols of BD Bioscience (Rockville, MD, USA) for the IL-4, IL-5, interferon-γ and IL-10 and Peprotech (Rocky Hill, NJ, USA) for IL-13. The measurements were made from the supernatants of the mediastinal lymph node cultures as described above.

Total IgE concentration and anti-OVA IgE titre were measured in the serum isolated from the blood obtained by exsanguination from the mouse left underarm. Total IgE were measured by enzyme-linked immunosorbent assay according to the manufacturer's procedure (BD Bioscience). For the evaluation of specific anti-OVA IgE, plates were coated with 20 μg ml^{-1} of OVA and the specific IgE concentration measured with an IgG anti IgE.

Lung histology

The lung inflammation was assessed after staining with hematoxylin eosin of paraffin-embedded lung sections. The extent of the haematoxylin and eosin inflammatory response was graded in a blinded fashion as follows: 0=minimal or no inflammation; 1=mild inflammation, only perivascular or peribronchiolar; 2=moderate inflammation, some parenchymal involvement; 3=marked inflammation, widespread parenchymal involvement; 4=severe inflammation, little normal parenchyma.

Statistical analyses

Statistical analysis was performed using the Student's *t*-test. *P* values less than 0.05 were considered statistically significant. *P* values are shown in each figure as follows: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. All analyses were performed using Prism 3.0 (GraphPad Software, La Jolla, CA, USA) software.

ACKNOWLEDGEMENTS

We thank the staff of the Garvan Institute Biological Testing Facility for facilitation of these experiments. This research was supported by a grant from the National Health and Medical Research Council (NHMRC) of Australia and NHMRC Fellowships to HH and FM.

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