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The neutrophil-activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in ovalbumin-induced allergic asthma

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Summary

The *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is able *in vitro* to elicit IL-12 and IL-23 production via agonistic interaction with toll-like receptor 2, and to promote Th1 polarization of allergen-specific T-cell responses. This study was aimed to assess whether systemic/intraperitoneal and/or mucosal HP-NAP administration inhibited the Th2-mediated bronchial inflammation using a mouse model of allergic asthma induced by inhaled ovalbumin (OVA). Systemic HP-NAP delivery markedly reduced the lung eosinophilia in response to repeated challenge with aerosolized OVA. Likewise, the production of IL-4, IL-5 and GM-CSF was significantly lower in the bronchoalveolar lavage of animals treated with systemic HP-NAP plus OVA than that of animals treated with OVA alone. Systemic HP-NAP also significantly resulted in both reduction of total serum IgE and increase of IL-12 plasma levels. Mucosal administration of HP-NAP was equally successful as the systemic delivery in reducing eosinophilia, IgE and Th2 cytokine levels in bronchoalveolar lavage. However, no suppression of lung eosinophilia and bronchial Th2 cytokines was observed in toll-like receptor 2-knock-out mice following HP-NAP treatment. These results identify HP-NAP as a candidate for novel strategies of prevention and treatment of allergic diseases.

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†Conflict of interest: these authors are within the applicants of EU Patent 05425666.4 for HP-NAP as potential therapeutic agent in cancer, allergic and infectious diseases. The remaining authors have declared that no conflict of interest exists.

Introduction

Asthma is one of the most common chronic diseases in western countries and is characterized by airway obstruction, bronchial hyper responsiveness and airway inflammation. Typical pathological features include infiltration of the airways by activated lymphocytes, particularly Th2 cells and eosinophils, damage of the bronchial epithelium, mucous gland hyperplasia and collagen deposition in the epithelial subbasement membrane area (Kay, 2001; Larché *et al*., 2003).

The reason why the severity and incidence of asthma have dramatically increased in the developed nations over the last decades is unknown; however, epidemiological studies and experimental data provided evidence suggesting that infectious diseases can influence the development of allergic disorders (Strachan, 1989). It has been demonstrated an inverse correlation between the onset of allergic disorders and the incidence of infections (Herz *et al*., 2000). This phenomenon can be explained with the inhibition of the allergic Th2 inflammation by Th1 responses elicited by infectious agents, able to induce the production of IFN-γ, IL-12, IL-18 and IL-23 (Herz *et al*., 2000; Wohlleben and Erb, 2001). This view is supported by studies showing that animals can be protected from developing asthma by using live or killed bacteria or their components, which induce Th1 responses (Wohlleben and Erb, 2006).

We have recently shown that the *Helicobacter pylori* neutrophil-activating protein (HP-NAP) produced by the Gram-negative bacterium *H. pylori*, by acting on both neutrophils and monocytes via toll-like receptor (TLR)2 agonistic interaction, significantly contributes to induce an IL-12 and IL-23-enriched milieu (Amedei *et al*., 2006) that has the potential of driving the differentiation of antigen-stimulated T cells towards a polarized Th1 phenotype (Oppmann *et al*., 2000; Trinchieri, 2003). Accordingly,

addition in culture of HP-NAP to allergen-induced T cell lines resulted in a remarkable increase of IFN-γ-producing T cells and decrease of IL-4-secreting cells (Amedei *et al*., 2006). For this reason, it has been suggested that HP-NAP may represent a novel immune modulating agent (D'Elios *et al*., 2007).

In order to evaluate *in vivo* whether HP-NAP might be a possible new tool for therapeutic strategies aimed to redirect Th2 into Th1 responses, herein we address the hypothesis that the administration of HP-NAP can suppress the development of OVA-induced asthma in mice. After intraperitoneal (i.p.) OVA priming, Th2 responses in the mouse lung were induced by repeated OVA aerosol challenge (Gonzalo *et al*., 1996). Following this OVA treatment, eosinophils were recruited and activated in bronchial airways and serum IgE levels increased. Furthermore, the elicited Th2 response correlated with the appearance of airway hyper responsiveness (Foster *et al*., 1996). Here we show that both systemic and mucosal administration of HP-NAP strongly inhibit the development of airway eosinophilia and bronchial inflammation. Likewise, HP-NAP treatment strongly affected the lung cytokine release, reducing the production of IL-4, IL-5 and GM-CSF. These findings provide the indication that both systemic administration and mucosal administration of HP-NAP are effective in preventing allergic asthma.

Results

HP-NAP strongly suppresses the development of OVA-induced airway eosinophilia

To investigate the influence of i.p. (systemic) administration of HP-NAP on the development of an OVA-specific Th2 response in the lung, mice were injected i.p. with PBS or with HP-NAP at the same time of OVA sensitization, 1 week before the first airway challenge with OVA (Fig. 1). In a preliminary dose–response experiment the amount of HP-NAP needed to be administered in order to detect anti-Th2 effects was assessed (Fig. S1). Negative control mice received saline alone. On day 18, differential white blood cell (WBC) counts were obtained in bronchoalveolar lavage (BAL) samples from saline-, OVA- or systemic HP-NAP-treated mice. As depicted in Fig. 2A, the few BAL cells of saline-treated mice were mainly macrophages, whereas in OVA-treated mice, eosinophils represented the major BAL cell population. The i.p. co-administration of HP-NAP and OVA resulted in a significantly reduced number ($P < 0.01$) of BAL eosinophils (Fig. 2A) and prevented OVA-induced airway eosinophilia (Fig. 2B). The numbers of macrophages, neutrophils and lymphocytes in systemic HP-NAP-treated mice were similar to those of OVA-treated mice (Fig. 2A). Taken together, these data suggest that systemic HP-NAP administration resulted in a significant down-modulation of the eosinophilic airway inflammation induced by aerosolized OVA.

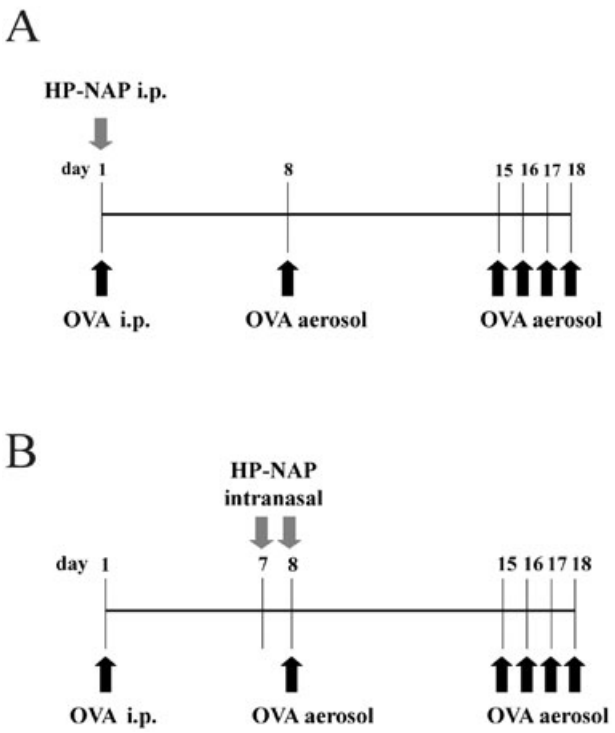

Fig. 1. Experimental protocol of the study. HP-NAP has been given systemically (A) or via mucosal route (B). Groups of nine C57BL/6j mice were treated with saline, with OVA alone, with OVA plus i.p. HP-NAP or with OVA plus mucosal HP-NAP. In both systemic and mucosal protocols, mice were treated with OVA according to a standardized procedure consisting of a first phase of sensitization with OVA i.p. (100 μg mouse^{-1}) and a second phase of induction of the allergic response with aerosolized OVA (2% in PBS) for 5 min on day 8, and finally exposed to aerosolized antigen (1% in PBS) for 20 min daily on days 15–18. Control animals, designed as saline, were injected with PBS alone and then exposed to aerosolized PBS. In the systemic protocol (A) mice were treated with i.p. HP-NAP (10 μg mouse^{-1}) on day 1, whereas in the mucosal protocol (B) mice received intra-nasal HP-NAP (10 μg mouse^{-1}) on days 7 and 8.

HP-NAP reduces serum IgE levels and eosinophil count in the blood

We next asked whether systemic HP-NAP delivery had any effect on either total serum IgE or blood eosinophilia. On day 18 of the experimental protocol, blood samples were collected from the animals belonging to the three groups of treatment (saline, OVA, systemic HP-NAP). Mice treated with OVA alone showed blood eosinophilia and increased total serum IgE, as compared with saline-treated mice. However, co-administration of systemic HP-NAP and OVA significantly dropped down both the

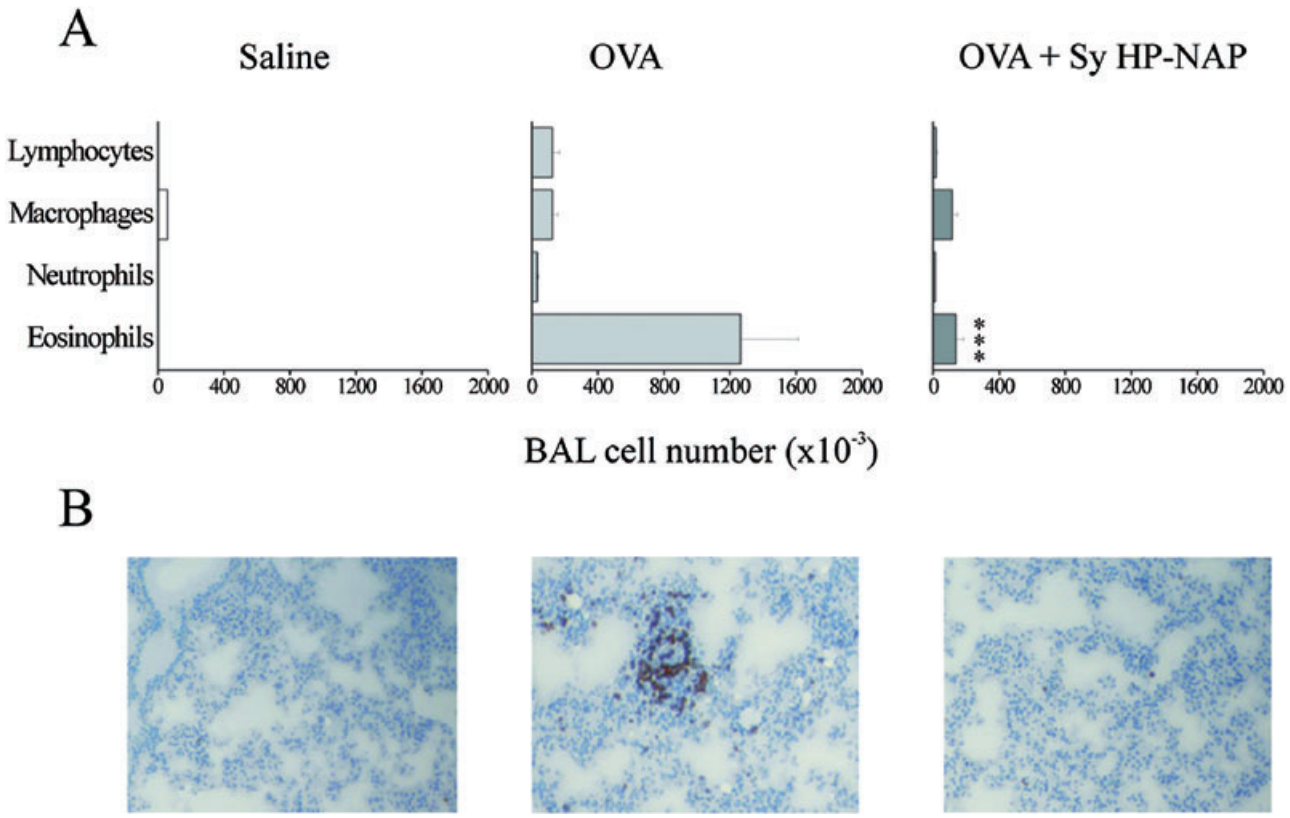

Fig. 2. Intraperitoneal administration of HP-NAP inhibited the development of airway eosinophilia in OVA-sensitized animals.
A. On day 1, mice were sensitized with OVA alone or with OVA plus systemic HP-NAP (Sy HP-NAP), and then exposed to aerosolized OVA. Control animals, coded as saline, were injected with PBS alone and then exposed to aerosolized PBS. Cytocentrifuge preparations from BAL of the three groups of animals on day 18 were stained to calculate the proportions of eosinophils, macrophages, neutrophils and lymphocytes. Absolute counts (± SD) for each cell type were calculated from the number of total cells in the BAL. ***$P < 0.01$ versus OVA-treated mice.
B. Frozen sections of lungs from PBS-treated (left), OVA-sensitized (centre) and Sy HP-NAP-treated OVA-sensitized animals (right). Eosinophil accumulation in the lung parenchyma is virtually absent in OVA-sensitized mice treated with Sy HP-NAP.

eosinophil counts and IgE levels (Table 1), whereas no significant differences were found in the counts of blood lymphocytes or neutrophils between systemic HP-NAP- and OVA-treated mice. As the Th1 and Th2 developmental programmes are mutually inhibitory, it was investigated whether systemic HP-NAP treatment would enhance Th1 responses resulting in increased IL-12 plasma levels. Interestingly, mice treated with systemic HP-NAP and OVA had significantly higher plasma levels of IL-12 than mice treated with OVA alone (259 ± 87 pg ml^{-1} versus 13 ± 12 pg ml^{-1} respectively, $P < 0.05$).

Reduction of BAL Th2 cytokine levels following systemic HP-NAP treatment

Tissue eosinophil recruitment and accumulation in allergic inflammation depend not only on the release of selective chemokines, but also on cell activation induced by different

Table 1. Systemic HP-NAP reduces both serum IgE levels and eosinophil count in the blood of OVA-sensitized mice.

	WBC differential count µl^{-1} (mean ± SE)			
Treatment of mice	Lymphocytes	Neutrophils	Eosinophils	Total serum IgE levels ng ml^{-1} (mean ± SD)
Saline	7190 ± 700	1298 ± 105	631 ± 79	44 ± 4
OVA alone	5438 ± 528	1333 ± 140	1079 ± 131	561 ± 193
OVA + Sy HP-NAP	4035 ± 350	842 ± 75	131 ± 27***	88 ± 35**

**$P < 0.04$; ***$P < 0.01$ OVA plus Sy HP-NAP versus OVA alone.
On day 18, blood was taken from saline-, OVA- and OVA plus Sy HP-NAP-treated animals. Blood smears were stained to calculate the proportions and the mean (±SD) absolute counts of lymphocytes, neutrophils and eosinophils. Levels of total serum IgE were assessed by a specific ELISA assay.

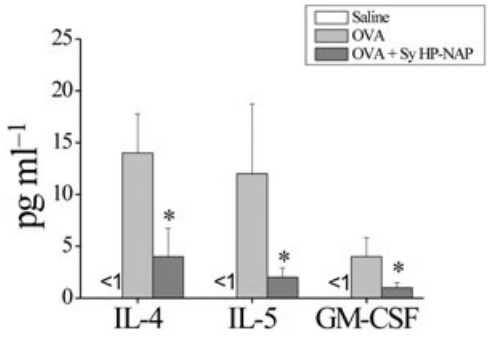

Fig. 3. Systemic HP-NAP administration results in reduced Th2 cytokine accumulation in the airway lumen. BAL samples were collected from saline-, OVA- and OVA plus Sy HP-NAP-treated animals on day 18, and cytokines were assayed in the cell-free supernatants with a Bio-Plex cytokine assay. Mean values (±SD) are reported. Cytokines were undetectable (< 1 pg ml^{-1}) in samples from saline mice. *$P < 0.05$ versus OVA alone-treated mice.

cytokines, such as IL-5 and GM-CSF. Both IL-5 and GM-CSF stimulate the eosinophil response to specific chemoattractants and induce eosinophil differentiation and activation (Lampinen *et al*., 2004). Therefore, it was investigated whether the HP-NAP-induced suppression of airway eosinophilia was associated with reduction of lung IL-4, IL-5 and GM-CSF. To this aim, BAL fluids were collected from saline-, OVA- and systemic HP-NAP-treated mice and the cytokine levels were measured in each BAL sample. In the BAL of saline-treated mice, none of the Th2 cytokines was detectable. In contrast, IL-4, IL-5 and GM-CSF were significantly increased in the BAL fluid of OVA-treated mice, whereas in systemic HP-NAP plus OVA-treated animals the levels of BAL IL-4, IL-5 and GM-CSF were significantly lower ($P < 0.05$) (Fig. 3). The reduced BAL levels of IL-5 and GM-CSF detected in systemic HP-NAP-treated animals correlated with the significant reduction of blood eosinophilia (Table 1) ($R = 0.805$). Finally, the strong reduction of IL-4 expression and the lower serum IgE levels in HP-NAP-treated mice suggest that the Th2 response to allergen/OVA was strongly downregulated in OVA mice by systemic HP-NAP treatment. Furthermore, HP-NAP treatment resulted in significant inhibition of the Th2 allergic response also in BALB/c mice, which have a propensity towards mounting Th2 response, serum IgE and IL-5 levels being 159 ± 48 ng ml^{-1} and 215 ± 39 pg ml^{-1} in OVA plus HP-NAP-treated mice whereas 952 ± 254 ng ml^{-1} and 1362 ± 208 pg ml^{-1} in OVA-treated mice respectively ($P < 0.02$).

Mucosal HP-NAP administration inhibits OVA-induced Th2 airway inflammation

We then moved to address the hypothesis that not only systemic, but also mucosal administration of HP-NAP could suppress the development of OVA-induced asthma. To this aim, an experimental protocol was developed in which mice were i.p. injected with OVA on day 1, and HP-NAP was administered via nasal route on days 7 and 8, just before the first airway challenge with OVA (Fig. 1B). Negative control mice received saline alone. As shown in Fig. 4A, treatment with OVA alone resulted, on day 18, in high numbers of eosinophils in the BAL samples. In contrast, the co-administration of HP-NAP and OVA significantly reduced ($P < 0.01$) or abrogated BAL eosinophilia and prevented OVA-induced airway eosinophil accumulation (Fig. 4A and B). To assess whether the mucosal administration of HP-NAP had any effect on the BAL levels of IL-4, IL-5 and GM-CSF, BAL fluids were collected from saline-, OVA- and Mu HP-NAP-treated mice and the cytokine levels were measured in each BAL sample. In mice treated with HP-NAP, BAL levels of IL-4, IL-5 and GM-CSF did not increase upon repeated OVA challenge (Table 2), as occurred in the BAL fluids of mice treated with OVA alone. The low BAL levels of IL-5 and GM-CSF detected in mucosal HP-NAP-treated animals well correlated ($R = 0.805$) with the reduction of lung eosinophilia. Finally, the mucosal co-administration of HP-NAP with OVA reduced the levels of serum and BAL IgE (Fig. 5), suggesting that the mucosal route of administration of HP-NAP was as effective as the systemic one in inhibiting both OVA-induced airway inflammation and the associated predominant Th2 response.

HP-NAP inhibition of Th2 airway inflammation requires the TLR2

Previous studies demonstrated that HP-NAP is a TLR2 agonist able to stimulate IL-12 production by monocytes and is able to promote Th1 polarization of allergen-specific T cell responses *in vitro* (Amedei *et al*., 2006;

Table 2. HP-NAP reduces the Th2 cytokine release in the BAL of OVA-sensitized wild-type, but not *tlr2*$^{-/-}$ mice.

	BAL cytokine levels (mean ± SD)		
Treatment of mice	IL-4 (pg ml^{-1})	IL-5 (pg ml^{-1})	GM-CSF (pg ml^{-1})
Wild-type			
OVA alone	14.4 ± 5.0	13.1 ± 6.0	7.2 ± 2.9
OVA + Sy HP-NAP	4.0 ± 3.3*	2.9 ± 0.8*	1.6 ± 1.0*
OVA + Mu HP-NAP	0.7 ± 0.6**	2.8 ± 1.6**	0.4 ± 0.3**
tlr2$^{-/-}$			
OVA alone	16.3 ± 5.0	14.1 ± 6.0	7.8 ± 3.4
OVA + Sy HP-NAP	16.6 ± 5.3	13.7 ± 5.4	7.5 ± 3.2
OVA + Mu HP-NAP	16.8 ± 4.9	14.8 ± 6.2	7.9 ± 3.3

*$P < 0.05$, **$P < 0.04$, OVA plus Sy or Mu HP-NAP versus OVA alone-treated mice.
On day 18, BAL samples were collected from OVA- and OVA plus Sy or Mu HP-NAP-treated wild-type or TLR2^{-}OVA-sensitized mice. Cytokines were assayed in the cell-free supernatants with the Bio-Plex cytokine methods. Mean values (±SD) are reported.

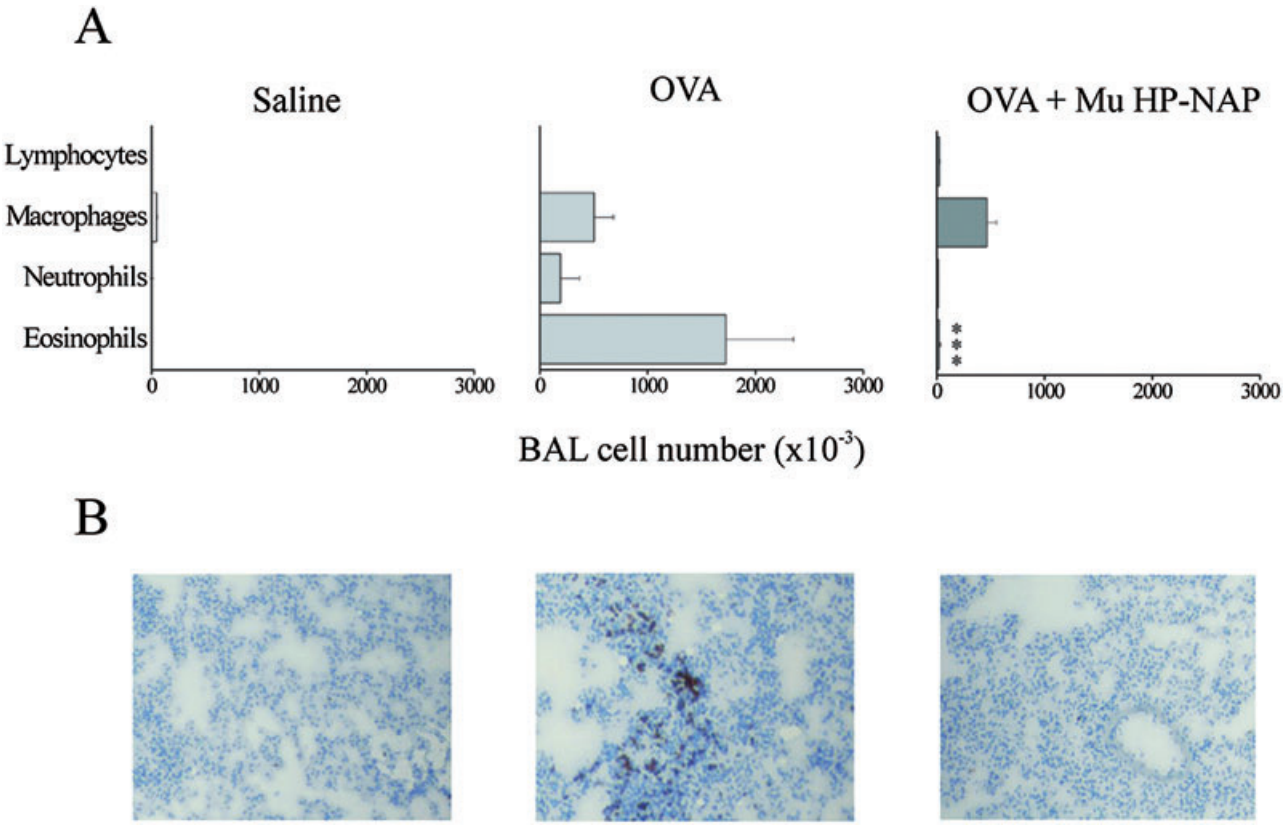

Fig. 4. Mucosal administration of HP-NAP inhibits the development of airway eosinophilia in OVA-sensitized animals.
A. OVA-sensitized mice were treated intra-nasally with aerosolized HP-NAP (Mu HP-NAP) on days 7 and 8. On day 8, both Mu HP-NAP mice and OVA mice were exposed to aerosolized OVA, followed by repeated aerosol challenge from days 15 to 18. Control animals, coded as saline, were injected with PBS alone and then exposed to aerosolized PBS. On day 18, cytocentrifuge preparations from BAL of the three groups of animals were stained to calculate the proportions of eosinophils, macrophages, neutrophils and lymphocytes. Absolute counts (±SD) for each cell type were calculated from the number of total cells in the BAL. ***$P < 0.01$ versus mice treated with OVA alone.
B. Frozen sections of lungs from PBS-treated (left), OVA-sensitized (centre) and Mu HP-NAP-treated OVA-sensitized animals (right). Eosinophil infiltration in the lung parenchyma was virtually absent in OVA-sensitized mice treated with Mu HP-NAP.

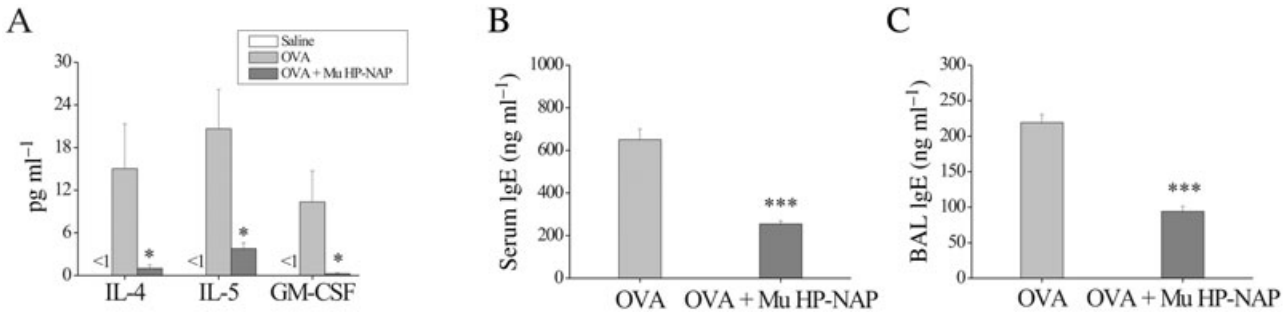

Fig. 5. Mucosal HP-NAP administration results in reduced Th2 cytokine accumulation in the airway lumen and reduced serum and BAL IgE levels.
A. On day 18, BAL samples were collected from OVA-sensitized mice treated with OVA alone or OVA plus Mu HP-NAP, and IL-4, IL-5 and GM-CSF mean (± SD) levels were measured in the cell-free supernatants with a Bio-Plex cytokine assay. *$P < 0.05$ versus mice treated with OVA alone.
B and C. Mean (± SD) levels of serum and BAL IgE assessed by a specific ELISA method in OVA-sensitized mice treated with OVA alone or OVA plus Mu HP-NAP. ***$P < 0.01$ versus mice treated with OVA alone.

D'Elios *et al*., 2007). On the basis of these results, we moved to evaluate the potential immunomodulating activity of TLR2 and HP-NAP in Th2 airway inflammation *in vivo*. To this purpose, Th2 cytokines were measured from BAL of wild-type and *tlr2*$^{-/-}$ mice exposed to HP-NAP. As depicted in Table 2, the reduction of Th2 cytokines in the BAL fluids was not observed in TLR2-knock-out mice after systemic or mucosal treatment with HP-NAP. These results, obtained in mice deficient for TLR2, suggest that TLR2 expression is required for the beneficial effects of HP-NAP in allergic asthma.

Discussion

Bronchial asthma is characterized by lung infiltration of different leukocytes, such as Th2 cells and eosinophils that play a major role in the development of allergic inflammation (Drazen *et al*., 1996). The mechanisms responsible for successful immunotherapy in allergic disorders are still only partially understood. On the other hand, CD4^{+} T cells from non-atopic subjects produce IFN-γ and little or no IL-4 in response to common environmental allergens (Gangur *et al*., 1999; Turcanu *et al*., 2003). Therefore, in order to hamper the pathogenetic mechanism of allergy, most studies pointed at immune deviation from Th2 towards a less pathogenic Th1 response (Till *et al*., 2004). Even if recombinant or modified allergens have been proposed as safe treatments of allergic subjects, a promising strategy seems to be the use of novel adjuvants or immunomodulators to induce immune deviation, and several compounds have been tested over the years (Tighe *et al*., 2000; Freytag and Clements, 2005).

IL-12 is the major cytokine driving Th1 responses both *in vivo* and *in vitro* and its use in therapy has been proposed (Trinchieri, 1995). However, its side-effects and toxicity in humans raised important concerns (Atkins *et al*., 1997; Leonard *et al*., 1997; Colombo and Trinchieri, 2002). Thus, a safer approach might be the use of adjuvants able to induce a gradual moderate production of endogenous IL-12 that might result in efficient immune deviation to Th1 of allergen-specific Th2 responses.

The HP-NAP is a *H. pylori* product that promotes Th1 immune responses (Amedei *et al*., 2006; D'Elios *et al*., 2007). The present study reports findings that suggest its potential benefit in the treatment of allergic asthma as a Th2-inhibiting immune modulator, via both systemic and mucosal routes of administration.

Several studies were devoted to the definition of new immune modulating factors able to inhibit Th2 responses and different compounds have been proposed for the treatment and prevention of allergic asthma, including several TLR ligands mimicking the effects of microbial components, such as dsRNA, CpG-oligodeoxynucleotides and imidazoquinolines (Hirota *et al*., 2002; Banerjee *et al*., 2004; Estelle *et al*., 2004; Tulic *et al*., 2004; Mazmanian *et al*., 2005; Trujillo *et al*., 2005). It has been shown *in vitro* that HP-NAP is able to inhibit the development of allergen-specific Th2 responses and to stimulate the production of IL-12 and IL-23 via TLR2 pathway (Amedei *et al*., 2006).

Th2 cells direct allergic inflammation in asthma, and the mediators produced during allergic responses have been elucidated in great detail (Robinson *et al*., 1992; Del Prete *et al*., 1993). IL-4 produced by Th2 cells is essential to promote IgE synthesis as well for Th2 differentiation and recruitment (Corry and Kheradmand, 1999). Upon allergen recognition, activated Th2 cells orchestrate lung inflammation through the release of different cytokines, chemokines and mast cell products that induce alteration of endothelial permeability, oedema formation and recruitment of different inflammatory cells. Among these, eosinophils are very important for further amplification of asthma inflammation due to their ability to release molecules that induce tissue damage and remodelling (Sanderson, 1992; Rothenberg, 1998; Simson and Foster, 2000; Till *et al*., 2004; Jacobsen *et al*., 2008). In this context, IL-5 plays an essential role in eosinophil differentiation, recruitment and priming.

Here we report that systemic administration of HP-NAP results in marked reduction of lung eosinophilia, as well as in the decreased production of Th2 cytokines, such as IL-4, IL-5 and GM-CSF. Interestingly, a large epidemiological study has recently demonstrated consistent inverse association between *H. pylori* infection and the presence of allergic disorders, such as asthma and rhinitis (Chen and Blaser, 2007).

It has been demonstrated that mucosal exposure of mice to allergen elicits allergic airway inflammation via a GM-CSF-mediated mechanism (Cates *et al*., 2004). Here we demonstrate that HP-NAP treatment of OVA-induced asthma induces not only the reduction of IL-4 and IL-5 but also strong reduction of GM-CSF at bronchial level. Moreover, the poor eosinophil accumulation in the lung of HP-NAP-treated OVA-sensitized mice correlated with the marked reduction of BAL GM-CSF and IL-5, both promoting tissue eosinophilia.

Previous reports looking at the interaction between TLR2 stimulation and allergy have provided rather contradictory data. Initial studies using murine models of allergic asthma reported that TLR2 ligands administered during the sensitization period led to enhancement of Th2-mediated allergic inflammation (Chisholm *et al*., 2004; Redecke *et al*., 2004). Conversely, more recent studies found that TLR2 agonist synthetic lipopeptides administered immediately before airway allergen challenge inhibit Th2-type responses, IgE production and eosinophilia (Akdis *et al*., 2003). In this elegant study, stimulation of TLR2, but not of TLR4, was reported to suppress IL-4

production by Th2 cells by promoting the differentiation of IL-10- and IFN-γ-producing T cells. Other studies in mice and humans also suggest that TLR2 stimulation inhibits established Th2 allergic airway inflammation (Patel *et al*., 2005; Taylor *et al*., 2006), and that inhibition of Th2 allergic response was mediated by an augmented Th1 response promoted by TLR2 ligands (Patel *et al*., 2005).

In this study, we have demonstrated that HP-NAP, a TLR2 agonist, was unable to inhibit lung eosinophilia and Th2 inflammation in TLR2-knock-out mice. Moreover, it is of note that HP-NAP, as other TLR2 ligands, does not only inhibit the production of Th2 cytokines, but also reduces the levels of serum IgE and the degree of peripheral blood eosinophilia. Thus, although further investigations will be required to fully elucidate the complex mechanisms by which TLR2 ligands shape the activation of innate and adaptive responses, these results strengthen the protective role of TLR2 in Th2-mediated allergic inflammation, and suggest that TLR2 is essential for the beneficial effects of HP-NAP in OVA-induced allergic asthma.

Furthermore, the potential beneficial effect of HP-NAP for asthma treatment has been tested in mice by using mucosal administration. When delivered via nasal route, HP-NAP induced a remarkable reduction of lung allergic inflammation, being able to reduce both mucosal eosinophilia and BAL levels of IL-4, IL-5, GM-CSF and IgE, as well as the systemic hyper-IgE response. No adverse effects were observed in any of the mice treated with HP-NAP, via either systemic or mucosal route.

Taken together, this study demonstrates that both mucosal and systemic HP-NAP administrations inhibit *in vivo* lung eosinophil infiltration and Th2 inflammation in a mouse model of allergic asthma. We would like to propose HP-NAP as a candidate for novel strategies of prevention and treatment of allergic diseases.

Experimental procedures

Mice and treatment protocol

C57BL/6j, wild-type mice (Harlan, Italy), *tlr2*$^{-/-}$ mice (kind gift of Prof A. Zychlinsky, Berlin, Germany) (Takeuchi *et al*., 1999) and BALB/c (Harlan, Italy) 6- to 8-week-old mice were used in the study, following the rules of local veterinary ethical committee. In a preliminary dose–response experiment the amount of HP-NAP needed to be administered in order to detect anti-Th2 effects was assessed (Fig. S1). In subsequent experiments, mice were treated with OVA according to a standardized procedure consisting of a first phase of sensitization and a second phase of induction of the allergic response (Gonzalo *et al*., 1996; Vicentini *et al*., 2002). Groups of nine, wild-type or TLR2-knock-out, C57BL/6j mice were treated with: (i) saline, or (ii) OVA, or (iii) OVA plus i.p. HP-NAP, or (iv) OVA plus HP-NAP delivered in the nasal mucosa. Mice indicated as OVA were sensitized with i.p. OVA (100 μg mouse^{-1}); mice designated as systemic (Sy) HP-NAP were sensitized with i.p. OVA plus i.p. HP-NAP (10 μg mouse^{-1}) on day 1 (Fig. 1A); mice indicated as mucosal (Mu) HP-NAP received i.p. OVA on day 1 and intra-nasal HP-NAP (10 μg mouse^{-1}) on days 7 and 8 (Fig. 1B). Then all animals were exposed to aerosolized OVA (2% in PBS) for 5 min on day 8, and finally exposed to aerosolized antigen (1% in PBS) for 20 min daily from days 15 to 18 (Fig. 1). Control animals, indicated as saline, were injected with PBS alone and then exposed to aerosolized PBS.

On day 18, within 1 h after the last antigen challenge, animals were anaesthetized and peripheral blood was collected from the retroorbital venous plexus to prepare smears for differential WBC count and serum samples. Mice were then sacrificed and the tracheas were cannulated. Airways were washed four times with 0.5 ml of ice-cold PBS and, after centrifugation, BAL supernatants were divided in aliquots and frozen immediately at –80°C. Cell pellets were re-suspended in PBS and total cells were counted and characterized. Lungs were excised, rolled in Tissue Tek OCT (Raymond Lamb, London, UK), frozen in liquid nitrogen and stored at –80°C.

HP-NAP protein preparation

The HP-NAP was cloned, expressed and purified from *Bacillus subtilis* to avoid lipopolysaccharyde contamination, as described (Amedei *et al*., 2006). The recombinant protein was pure as judged from overloaded gels composed of different percentages of polyacrylamide. Mass spectrometry analysis, performed with a Maldi Reflex (Brucker Analytik), confirmed that the protein consisted of a single molecule of 16 875 ± 20 Da.

Lung histochemistry and differential cell count

Lung cryostat sections (7 μm thickness), BAL cytocentrifuge preparations and blood smears were stained for phenylhydrazine-resistant peroxidase, which stains specifically eosinophil granulocytes (Straus, 1979; Chilosi *et al*., 1983; Lampinen *et al*., 2004). Myeloperoxidase was first inhibited with 0.1% phenylhydrazine in PBS for 30 min, before peroxidase staining with 3′-3-diaminobenzidine. Nuclei were counterstained with haematoxylin. Pulmonary eosinophilia in lung sections was semiquantitatively assessed by grading the severity of eosinophil infiltration as follows: grade 1, absence of positive cells (or rarely detectable in the parenchyma); grade 2, few scattered groups of positive cells, mostly parenchymal or perivascular; grade 3, moderate perivascular and peribronchial infiltration of eosinophils in most fields; grade 4, diffuse, heavy eosinophil infiltration. At least 200 cells were counted in BAL cell preparations and the different cell types were expressed as a percentage of total cells. The number of eosinophils in blood smears was expressed as a percentage of WBC. At least 300 cells were counted in each slide.

Cytokine assays in cell-free BAL supernatants

IL-4, IL-5 and GM-CSF levels were measured in BAL fluid samples with a Bio-Plex Th1/Th2 cytokine panel (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Cytokine levels were determined by comparison with standard curves generated with murine recombinant cytokines and analysed using Bio-Plex Manager software (Bio-Rad).

Measurement of IgE, IL-5 and IL-12 plasma levels

Serum and BAL IgE, IL-5 and IL-12 plasma levels were measured using specific sandwich ELISA (Alpha Diagnostic, San Antonio, TX, and R and D Systems GmbH, Wiesbaden, Germany) according to the manufacturers' instructions. In some experiments BALB/c mice were i.p. treated with the same protocol (saline or OVA, or with OVA plus i.p. HP-NAP) used for C57BL/6j mice, and IgE and IL-5 plasma levels were measured.

Statistical analysis

Data were expressed as mean values ± SD. Statistical significance between different groups of mice was calculated by unpaired Student's *t*-test. A probability (*P*) of less than 0.05 was considered significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Dose–response effect of graded concentrations of HP-NAP on the BAL eosinophil counts. On day 1, groups of six C57BL/6j mice were treated with saline or with OVA alone or with OVA plus systemic HP-NAP (Sy HP-NAP) at the respective indicated dose, and then exposed to aerosolized OVA. On day 18, cytocentrifuge preparations from BAL of all groups of animals were stained to calculate the number of eosinophils. Values are presented as percentage (±SD) of eosinophil counts from BAL of Sy HP-NAP-treated mice compared with BAL eosinophils from OVA alone-treated mice.

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