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The Neutrophil Activating Protein of Helicobacter pylori down-modulates Th2 inflammation in allergic asthma

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The Neutrophil Activating Protein of *Helicobacter pylori* down-modulates Th2 inflammation in allergic asthma

Running title: **HP-NAP prevents allergy development**

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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 interacion with TLR2, and to promote Th1 polarization of allergen-specific T-cell responses. This study was aimed to assess whether systemic/i.p. and/or mucosal HP-NAP administration inhibited the Th2-mediated bronchial inflammation using a mouse model of allergic asthma induced by inhaled OVA. Systemic HP-NAP delivery markedly reduced the lung eosinophilia in response to repeated challenge with aerosolized ovalbumin (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 resulted in significant reduction of total serum IgE. Mucosal administration of HP-NAP was equally successful as the systemic delivery in reducing eosinophilia and Th2 cytokine levels in bronchoalveolar lavage. However, no suppression of lung eosinophilia and bronchial Th2 cytokines was observed in Toll-like receptor (TLR)-2 -knock-out mice following HP-NAP treatment. These results suggests that HP-NAP is a microbial product able to down-modulate Th2 inflammation in allergic asthma.

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 sub-basement membrane area (Kay, 2001; Larché *et al.*, 2003).

The reason why the severity and incidence of asthma has 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 Neutrophil Activating Protein (HP-NAP) produced by the Gram negative bacterium *Helicobacter pylori*, by acting on both neutrophils and monocytes via TLR2 agonistic interaction, significantly contributes to induce an IL-12 and IL-23 enriched milieu, (Amedei *et al.*, 2006) which 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 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 levels IgE 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.

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, one week before the first airway challenge with OVA (Fig. 1). Negative control mice received saline alone. On day 18, differential WBC counts were obtained in bronchoalveolar lavage (BAL) samples from saline-, OVA-, or systemic HP-NAP-treated mice. As depicted in Figure 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.

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 mice. However, co-administration of systemic HP-NAP and OVA significanty dropped down both the eosinophil counts ($P < 0.01$) and IgE levels ($P < 0.03$) (Table 1), whereas no significant differences were found in the counts of blood lymphocytes or neutrophils between systemic HP-NAP and OVA mice.

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

Tissue eosinophil recruitment and accumulation in allergic inflammation depends not only on the release of selective chemokines, but also on cell activation induced by different 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 suggests that the Th2 response to allergen/OVA was strongly down-regulated in OVA mice by systemic HP-NAP treatment.

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 day 7 and 8, just before the first airway challenge with OVA (Fig. 1B). Negative control mice received saline alone. As shown in Figure 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 4B). 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 ($P < 0.05$). 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 IgE (Fig. 5) **(**$P < 0.035$), 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. However, 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 (Table 2).

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 nonatopic 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.

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 (ODNs) 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; Rothemberg, 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 synthetic lipopeptides administered immediately before airway allergen challenge inhibit Th2-type responses and IgE production (Akdis *et al.*, 2003). 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).

In this sudy, we have demonstrated that HP-NAP, a TLR2 agonist, was unable to inhibit lung eosinophila 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.

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, 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 administration inhibits *in vivo* lung eosinophil infiltration and Th2 inflammation in a mouse model of allergic asthma.

Experimental procedures

Mice and treatment protocol

C57BL/6j, wild-type mice (Harlan, Italy), and *tlr2-/-* mice (kind gift of Prof. A. Zychlinsky, Berlin Germany) (Takeuchi *et al.*, 1999), six to eight weeks-old mice, were used in the study, following the rules of local veterinary ethical commitee. 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: a) saline, or b) OVA, or c) OVA plus intraperitoneal (i.p.) HP-NAP, or d) OVA plus HP-NAP delivered in the nasal mucosa. Mice indicated as OVA were sensitized with i.p. OVA (100 µg/mouse); mice designated as systemic (Sy) HP-NAP were sensitized with i.p. OVA plus i.p. HP-NAP (10 µg/mouse) on day 1 (Fig. 1A); mice indicated as mucosal (Mu) HP-NAP received i.p. OVA on day 1 and intranasal HP-NAP (10 µg/mouse) on days 7 and 8 (Fig. 1B). Then all animals were exposed to aerosolized OVA (2% in PBS) for five min on day 8, and finally exposed to aerosolized antigen (1% in PBS) for twenty min daily from day 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 anesthetized 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 ice-cold PBS and, after centrifugation, bronchoalveolar lavage (BAL) supernatants were divided in aliquots and frozen immediately at -80°C. Cell pellets were resuspended in PBS and total cells were counted and characterized. Lungs were excised, rolled in Tissue Tek OCT (Raymond Lamb, London, U.K.), frozen in liquid nitrogen, and stored at -80°C.

HP-NAP protein preparation

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±20Da.

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 hematoxylin. 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 analyzed using Bio-Plex Manager software (Bio-Rad).

Measurement of serum IgE

Serum and BAL IgE levels were measured using a specific sandwich ELISA (Alpha Diagnostic, San Antonio, TX) according to the manufacturer's instructions.

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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References

Akdis, C.A., Kussebi, F., Pulendran, B., Akdis, M., Lauener, R.P., and Schmidt-Weber, C.B. (2003) Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. *Eur J Immunol* **33**: 2717-2726.

Amedei, A., Cappon, A., Codolo, G., Cabrelle, A., Polenghi, A., Benagiano, M., *et al* (2006) The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. *J Clin Invest* **116**: 1092-1101.

Atkins, M.B., Robertson, M.J., Gordon, M., Lotze, M.T., De Coste, M., DuBois, J.S., *et al* (1997) Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. *Clin Cancer Res* **3**: 409-417.

Banerjee, B., Kelly, K.J., Fink, J.N., Henderson, Jr. J.D., Bansal, N.K., and Kurup, V.P. (2004) Modulation of airway inflammation by immunostimulatory CpG oligonucleotides in a murine model of allergic aspergillosis. *Infect Immun* **72**: 6087–6094.

Cates, E.C., Fattouh, R., Wattie, J., Inman, M.D., Goncharova, S., Coyle, A.J., *et al* (2004) Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* **173**: 6384-6392.

Chen, Y., and Blaser, M.J. (2007) Inverse associations of *Helicobacter pylori* with asthma and allergy. *Arch Intern Med* **167**: 821-827.

Chilosi, M., Pizzolo, G., Fiore-Donati, L., Bofill, M., and Janossy, G. (1983) Routine immunofluorescent and histochemical analysis of bone marrow involvement of lymphoma/leukaemia: the use of cryostat sections. *Br J Cancer* **48**: 763-775.

Chisholm, D., Libet, L., Hayashi, T., and Horner, A.A. (2004) Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities. *J Allergy Clin Immunol* **113**: 448–454.

Colombo, M.P., and Trinchieri, G. (2002) Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* **13**: 155-168.

Corry, D.B., and Kheradmand, F. (1999) Induction and regulation of the IgE response. *Nature* **402**: B18-B23.

Del Prete, G., De Carli, M., D'Elios, M.M., Maestrelli, P., Ricci, M., Fabbri, L., and Romagnani, S. (1993) Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* **23**: 1445-1449.

D'Elios, M.M., Amedei, A., Cappon, A., Del Prete, G., and de Bernard, M. (2007) The neutrophil-activating protein of *Helicobacter pylori* (HP-NAP) as an immune modulating agent. *FEMS Immunol Med Microbiol* **50**: 157-164.

Drazen, J.M., Arm, J.P., and Austen, K.F. (1996) Sorting out the cytokines of asthma. *J Exp Med* **183**: 1-5.

Estelle, F., Simons, R., Shikishima, Y., Van Nest, G., Eiden, J.J., and HayGlass, K.T. (2004) Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J Allergy Clin Immunol* **113**: 1144–1151.

Foster, P.S., Hogan, S.P., Ramsay, A.J., Matthaei, K.I., and Young, I.G. (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* **183**: 195-201.

Freytag, L.C., and Clements, J.D. (2005) Mucosal adjuvants, *Vaccine* **23**: 1804–1813.

Gangur, V., Simons, F.E.R., and HayGlass, K.T. (1999) IP-10 mediated reinforcement of human type 1 cytokine synthesis to environmental allergens among non-atopic subjects. *Int Arch Allergy Immunol* **118**: 387–390.

Gonzalo, J.A., Lloyd, C.M., Kremer, L., Finger, E., Martinez-A.C., Siegelman, M.H., *et al* (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* **98**: 2332-2345.

Herz, U., Lacy, P., Renz, H., and Erb, K. (2000) The influence of infections on the development and severity of allergic disorders. *Curr Opin Immunol* **12**: 632-640.

Hirota, K., Kazaoka, K., Niimoto, I., Kumihara, H., Sajiki, H., and Isobe, Y. (2002) Discovery of 8-hydroxyadenines as a novel type of interferon inducer. *J Med Chem* **45**: 5419–5422.

Jacobsen, E.A., Ochkur, S.I., Pero, R.S., Taranova, A.G., Protheroe, C.A., Colbert, D.C., *et al* (2008) Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells *J Exp Med* **205**: 699-710.

Kay, A.B. (2001) Allergy and allergic diseases. *N Engl J Med* **344**: 109-113.

Lampinen, M., Carlson, M., Hakansson, L.D., and Venge, P. (2004) Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* **59**: 793-805.

Larché, M., Robinson, D.S., and Kay, A.B. (2003) The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* **111**: 450-463.

Leonard, J.P., Sherman, M.L., Fisher, G.L., Buchanan, L.J., Larsen, G., Atkins, M.B., *et al* (1997) Effects of single-dose interleukin-12 exposure on interleukin-12- associated toxicity and interferon-γ production. *Blood* **90**: 2541-2548.

Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., and Kasper, D.L. (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122**: 107–118.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., *et al* (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**: 715-725.

Patel, M., Xu, D., Kewin, P., Choo-Kang, B., McSharry, C., and Thomson, N. C. (2005) TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol* **174**: 7558-7563.

Redecke, V., Hacker, H., Datta, S., Fermin, A., Pitha, P., and Broide, D. (2004) Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J Immunol* **172**: 2739–2743.

Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., *et al* (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* **326**: 298-304.

Rothenberg, M.E. (1998) Eosinophilia. *N Engl J Med* **338**: 1592-1600.

Sanderson, C.J. (1992) Interleukin-5, eosinophils, and disease. *Blood* **79**: 3101-3109.

Simson, L., and Foster, P.S. (2000) Chemokine and cytokine cooperativity: eosinophil migration in the asthmatic response. *Immunol Cell Biol* **78**: 415-422.

Strachan, D.P. (1989) Hay fever, hygiene and household size. *BMJ* **299**: 1259-1260.

Straus, W. (1979) Peroxidase procedures. Technical problems encountered during their application. *J Histochem Cytochem* **27**: 1349-1351.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., *et al* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**: 443-451.

Taylor, R.C., Richmond, P., and Upham, J.P. (2006) Toll-like receptor 2 ligands inhibit Th2 responses to mite allergen. *J Allergy Clin Immunol* **117**: 1148-1154.

Tighe, H., Takabayashi, K., Schwartz, D., Van Nest, G., Tuck, S., Eiden, J.J., *et al* (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* **106**: 124–134.

Till, S.J., Francis, J.N., Nouri-Aria, K., and Durham, S.R. (2004) Mechanisms of immunotherapy. *J Allergy Clin Immunol* **113**: 1025–1034.

Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immuno-regulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* **13**: 251-276.

Trinchieri, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**: 133-146.

Trujillo-Vargas, C.M., Mayer, K.D., Bickert, T., Palmetshofer, A., Grunewald, S., Ramirez-Pineda, J.R., *et al* (2005) Vaccinations with T-helper type 1 directing adjuvants have different suppressive effects on the development of allergen-induced T-helper type 2 responses. *Clin Exp Allergy* **35**: 1003-1013.

Tulic, M.T., Fiset, P-O., Christodoulopoulos, P., Vaillancourt, P., Desrosiers, M., and Lavigne, F. (2004) Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol* **113**: 235–241.

Turcanu, V., Maleki, S.J., and Lack, G. (2003) Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest* **111**: 1065–1072.

Vicentini, L., Mazzi, P., Caveggion, E., Continolo, S., Fumagalli, L., Lapinet-Vera, J.A., *et al* (2002) Fgr deficiency results in defective eosinophil recruitment to the lung during allergic airway inflammation. *J Immunol* **168**: 6446-6454.

Wohlleben, G., and Erb, K.J. (2001) Atopic disorders: a vaccine around the corner? *Trends Immunol* **22**: 618-626.

Wohlleben,. G, and Erb, K.J. (2006) Immune stimulatory strategies for the prevention and treatment of asthma. *Curr Pharm Des* **12**: 3281-3292.

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

Treatment of mice	Blood WBC differential count/µl (mean ± SE)			Total serum IgE levels
	Lymphocytes	Neutrophils	Eosinophils	ng/ml (mean ± SD)
Saline	7,190 ±700	1,298 ± 105	631 ± 79	44 ± 4
OVA alone	5,438 ± 528	1,333 ± 140	1,079 ± 131	561 ± 193
OVA + Sy HP-NAP	4,035 ± 350	842 ± 75	131 ± 27 ***	88 ± 35 **

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. ** $P < 0.03$, *** $P < 0.01$ OVA plus Sy HP-NAP versus OVA alone. OVA: ovalbumin; Sy: systemic; WBC: white blood cells.

Table 2. HP-NAP reduces the Th2 cytokine release in the bronchoalveolar lavage of OVA

Treatment of mice	BAL cytokine levels (mean ± SD)		
	IL-4 (pg/ml)	**IL-5 (pg/ml)**	**GM-CSF (pg/ml)**
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**
	IL-4 (pg/ml)	**IL-5 (pg/ml)**	**GM-CSF (pg/ml)**
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

sensitized wild-type, but not *tlr2*$^{-/-}$ mice.

On day 18, bronchoalveolar lavage 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. * $P< 0.05$, ** $P< 0.04$ OVA plus Sy or Mu HP-NAP versus OVA alone mice.

Figure legends

Figure 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) and a second phase of induction of the allergic response with aerosolized OVA (2% in PBS) for five minutes on day 8, and finally exposed to aerosolized antigen (1% in PBS) for twenty minutes 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) on day 1, whereas in the mucosal protocol (B) mice received intranasal HP-NAP (10 μg/mouse) on days 7 and 8.

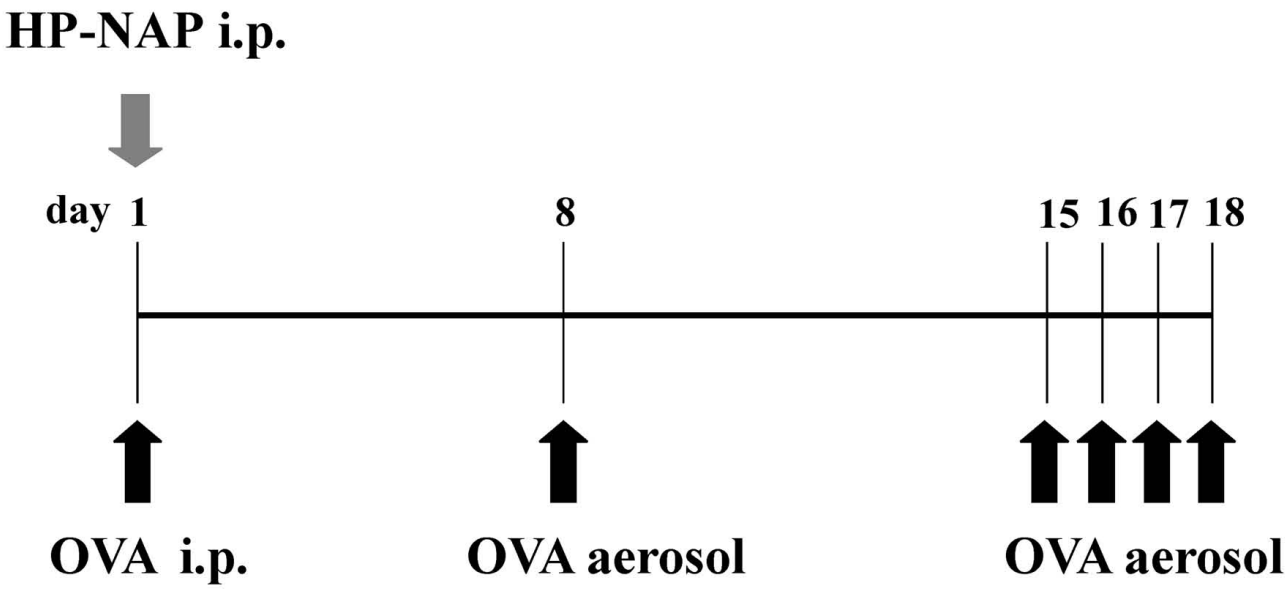
Figure 2. Intraperitoneal administration of HP-NAP inhibited the development of airway eosinophilia in OVA-sensitized animals. (A) On day 1, mice were sensitized with OVA alone or with OVA plus systemic HP-NAP (Sy HP-NAP), and then exposed to aerosolized OVA. Control animals, coded as saline, were injected 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 ($\pm$ 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.

Figure 3. Systemic HP-NAP administration results in reduced Th2 cytokines accumulation in the airways lumen. Bronchoalveolar lavage 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) in samples from saline mice. * $P < 0.05$ versus OVA alone mice.

Figure 4. Mucosal administration of HP-NAP inhibits the development of airway eosinophilia in OVA-sensitized animals. (A) OVA-sensitized mice were treated intranasally with aerosolized HP-NAP (Mu HP-NAP) on day 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 day 15 to day 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.

Figure 5. Mucosal HP-NAP administration results in reduced Th2 cytokine accumulation in the airways lumen and reduced serum IgE levels. (A) On day 18, bronchoalveolar lavage 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. * $P < 0.01$ versus mice treated with OVA alone. (B) Mean (± SD) levels of serum IgE assessed by a specific ELISA method in OVA-sensitized mice treated with OVA alone or OVA plus Mu HP-NAP. ** $P < 0.035$ versus mice treated with OVA alone.

A


HP-NAP i.p.
day 1
8
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OVA i.p.
OVA aerosol
OVA aerosol

B

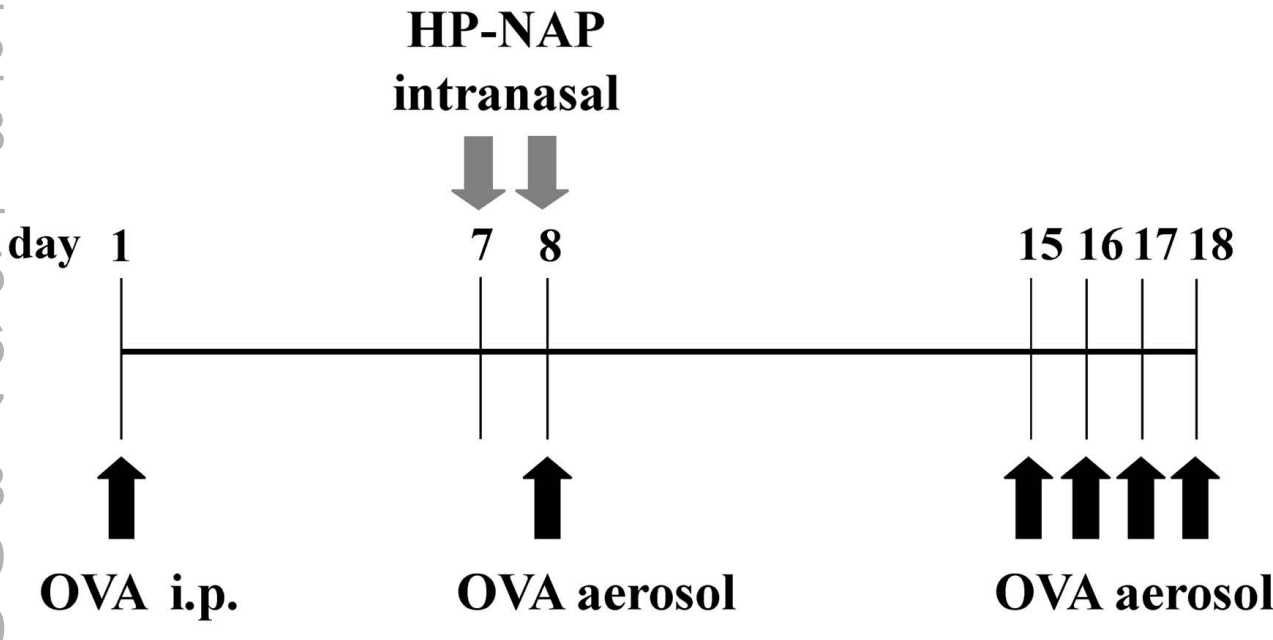
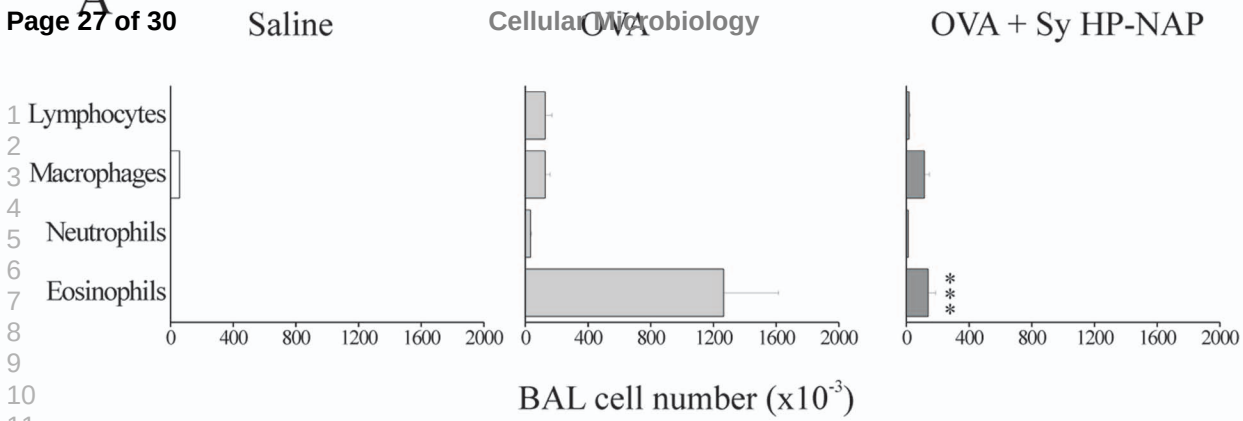

HP-NAP
intranasal
day 1
7 8
15 16 17 18
OVA i.p.
OVA aerosol
OVA aerosol

Fig 1

A


Saline
OVA
OVA + Sy HP-NAP
Lymphocytes
Macrophages
Neutrophils
Eosinophils
0
400
800
1200
1600
2000
***
BAL cell number (x10-3)

B

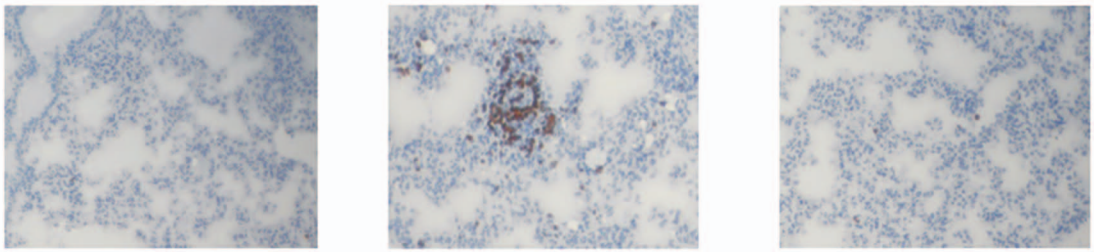


Fig 2

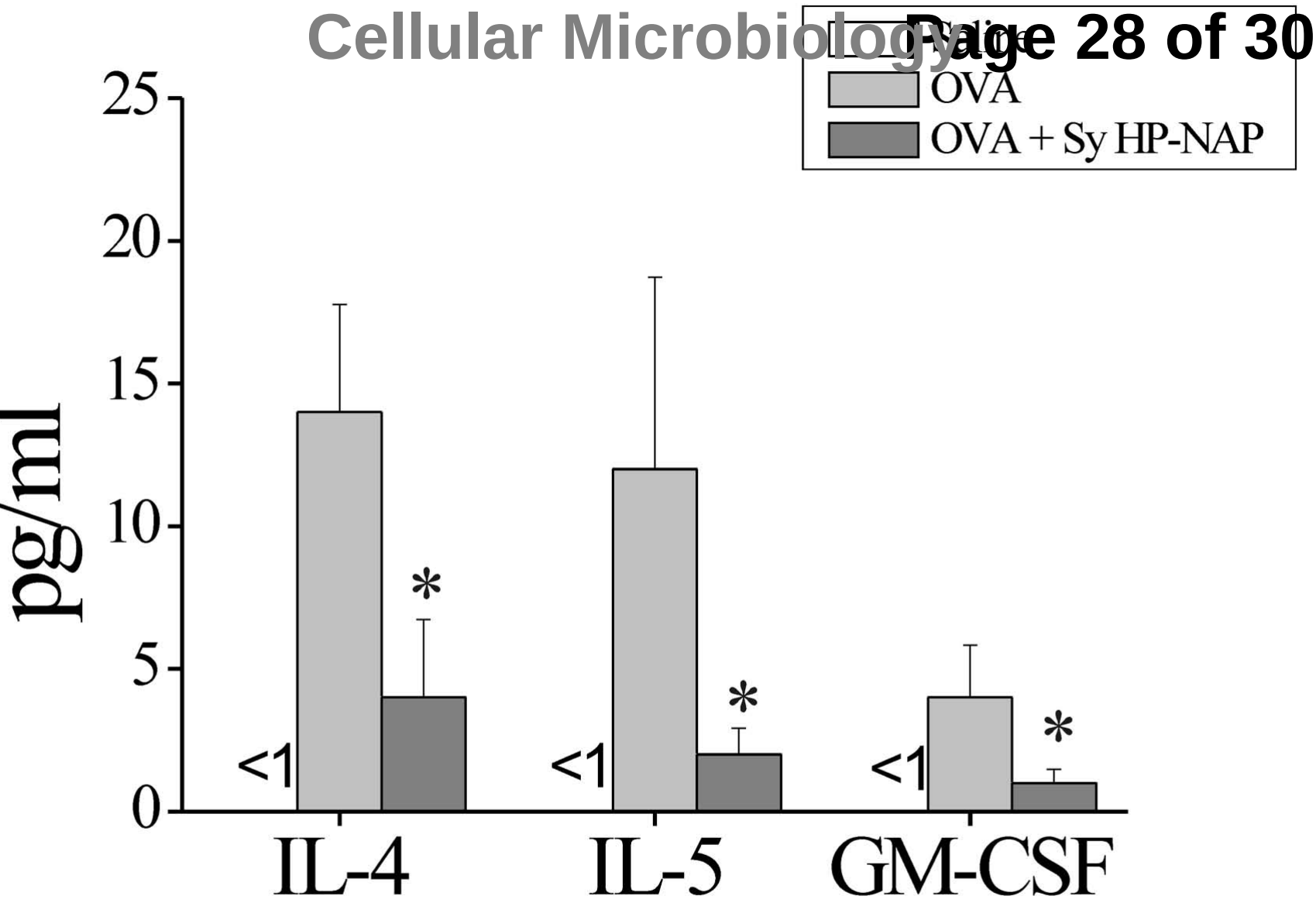
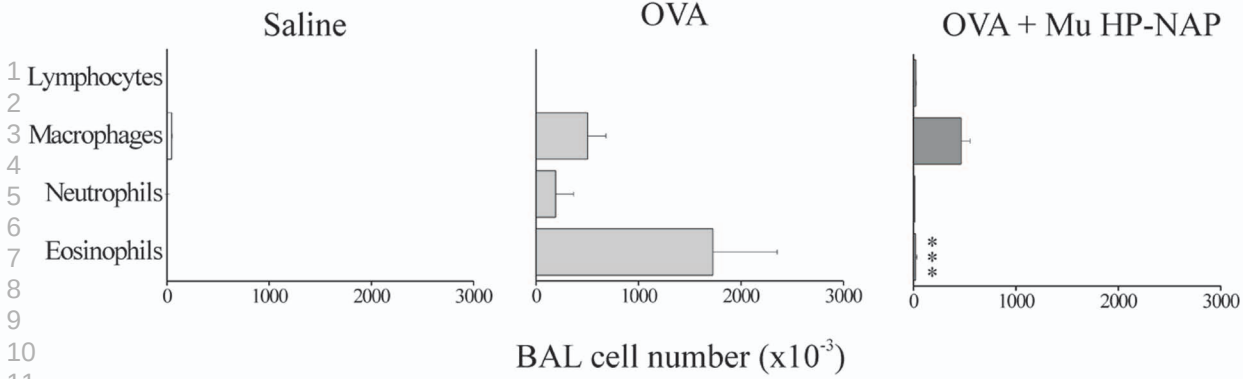


Fig 3

A


Saline
OVA
OVA + Mu HP-NAP
Lymphocytes
Macrophages
Neutrophils
Eosinophils
0
1000
2000
3000
***
BAL cell number (x10-3)

B

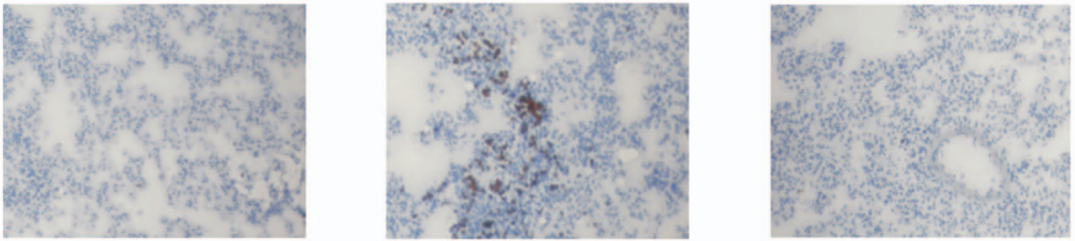
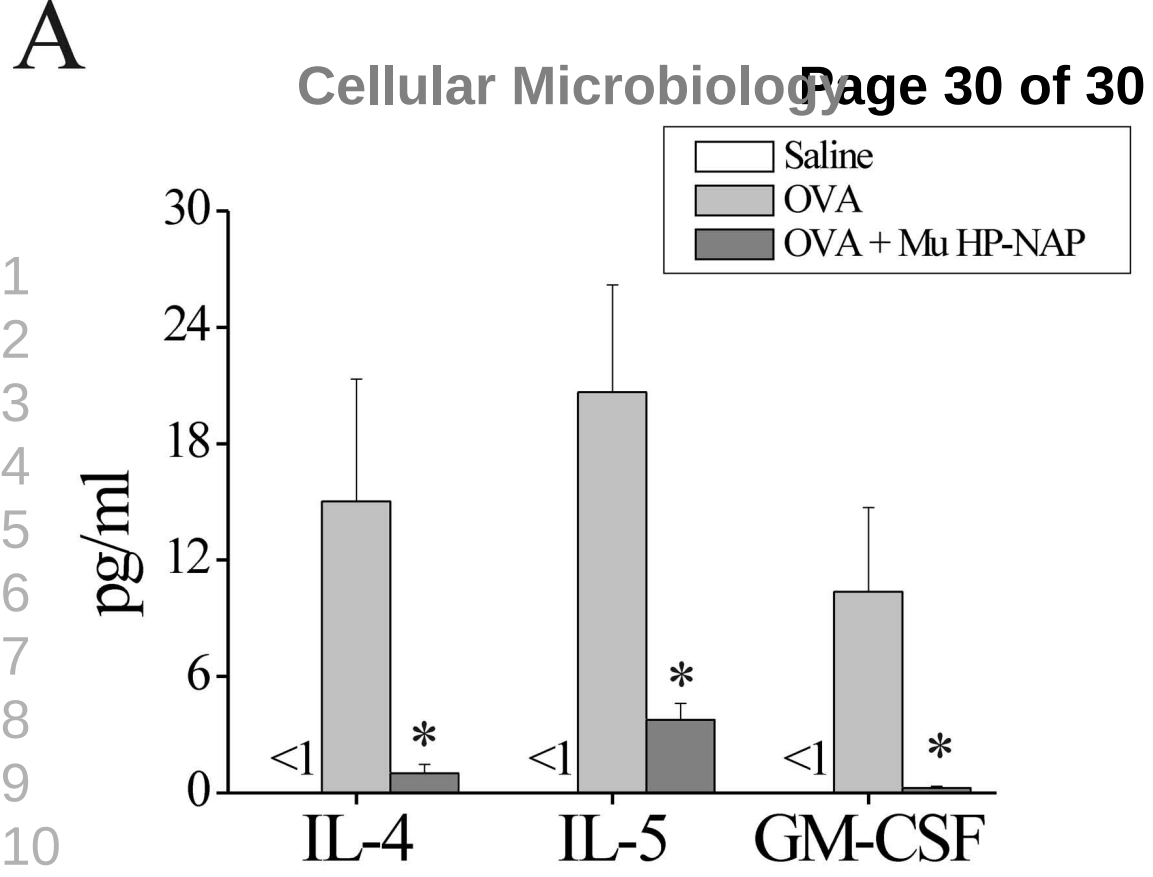


Fig 4


A
Saline
OVA
OVA + Mu HP-NAP
pg/ml
30
24
18
12
6
0
<1
<1
<1
*
*
*
IL-4
IL-5
GM-CSF

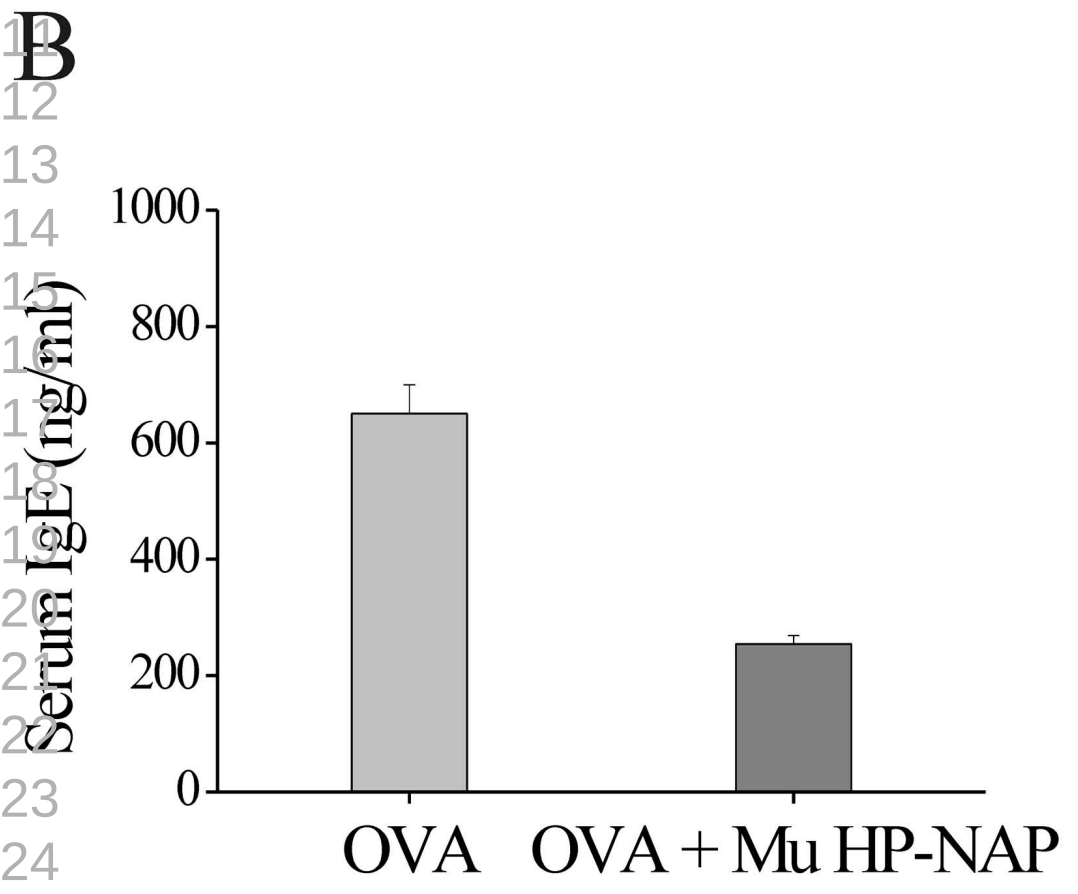

B
Serum IgE (ng/ml)
1000
800
600
400
200
0
OVA
OVA + Mu HP-NAP

Fig 5