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Helicobacter pylori, asthma and allergy

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Running title: ***Helicobacter pylori*, asthma and allergy**

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Abstract

Bronchial asthma and allergic diseases are orchestrated by T cells producing T helper (Th) 2 cytokines, such as interleukin (IL)-4, IL-5, and are inhibited by Th1 responses. *Helicobacter pylori* chronically infects the human population from approximately 100,000 years and preferentially elicits a Th1 mucosal immune response with production of interferon-γ, and IL-12. Among several bacterial factors the neutrophil-activating protein of *H. pylori* (HP-NAP) not only exerts a key role driving Th1 inflammation but it is also able *in vitro* and *in vivo* to inhibit Th2 responses in allergic bronchial asthma, in humans and mice. Both systemic and mucosal administration of HP-NAP are successful in reducing eosinophilia, IgE and systemic Th2 cytokines at bronchial level. Thus, these results identify HP-NAP as a candidate for novel strategies of prevention and treatment of allergic diseases.

Introduction

Helicobacter pylori is a Gram-negative bacterium that chronically infects the stomach of more than 50% of the human population and represents the major cause of gastroduodenal pathologies (Warren & Marshall, 1983; Parsonnet *et al.*, 1991; Wotherspoon *et al.*, 1991; Blaser, 1993). *Helicobacter pylori* gastric colonization is typically followed by mucosa infiltration of polymorphonuclear leukocytes (PMNs), macrophages and T helper (Th) 1 lymphocytes, with active production of interleukin (IL)-12 and interferon (IFN)-γ (D'Elios *et al.*, 1997).

It has been recently reported an inverse association between the *H. pylori* infection and the frequency of allergic asthma (Blaser *et al.*, 2008); however the absence of a convincing molecular mechanism, beside the epidemiological data, in support to such an observation, represented the weak point of this study and raised several criticisms (Wjst, 2008).

Bronchial asthma and allergic diseases are characterized by Th2 inflammation, that is strongly inhibited by IL-12 and IFN-γ. We recently demonstrated that in allergic asthmatic patients the typical Th2 responses can be redirected into Th1 by the neutrophil activating protein of *H. pylori* (HP-NAP) (Amedei *et al.*, 2006). Furthermore, the *in vivo* administration of HP-NAP prevents the typical eosinophil accumulation in the lung and the increase of serum IgE in a mouse model of allergic asthma (Codolo *et al.*, 2008). These results open the possibility that HP-NAP might be part of the molecular mechanism underlying the negative association between *H. pylori* infection and allergy, corroborating the epidemiological observations with a plausible scientific explanation. Such an intriguing issue will be discussed in the present review aimed to focus on the immunopathological basis of bronchial asthma, allergy and *H. pylori* infection. Finally, the potential benefit of HP-NAP as a new tool for the prevention and treatment of asthma and allergy will also be considered.

Th1/Th2 responses in health and diseases

In the course of evolution, the immune system had to continuously shape and refine its mechanisms of defense against pathogens. In response to different microorganisms, specialized types of specific responses allow the recognition and elimination of infectious agents. Viruses, which grow within the infected cell, can be successfully eliminated only by killing their host cells by $CD8^+$ class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes, which recognize viral antigens synthesized within infected cells and presented on their surface in the context of class I MHC molecules. In contrast, most of microbial antigens are endocytosed by antigen-presenting cells (APC), processed and presented preferentially in association with MHC class II molecules to $CD4^+$ class II MHC-restricted Th cells. $CD4^+$ T cells help B cells for the production of antibodies, which challenge extracellular microbes or neutralize their exotoxins (humoral immunity). Some microbes such as mycobacteria, however, can survive within macrophages in spite of the microbicidal activity of these cells unless $CD4^+$ Th cells reactive to mycobacterial antigens activate macrophage production of reactive oxygen intermediates, nitric oxide, and tumor necrosis factor (TNF)-α leading to the microbes' destruction (cell-mediated immunity). Most immune responses against pathogens involve both arms of the immune system (humoral and cell-mediated immunity) acting in concert.

During the effector specific immune response, different patterns of cytokine profiles are characteristic of certain Th cell subsets, whose polarized forms are Th1 and Th2 cells (Mosmann *et al.*, 1986; Del Prete *et al.*, 1991). Th1 cells producing IFN-γ and TNF-β elicit macrophage activation and B-cell production of opsonizing and complement-fixing antibodies, whereas Th2 cells producing IL-4, IL-5, IL-10, and IL-13 induce the production of high levels of antibodies, including IgE, and eosinophilia. The two types of effector responses are usually distinct and mutually exclusive. Between polarized Th1 and Th2 cells, a heterogenous population of T-cell

subsets producing at the same time peculiar combination of Th1/Th2 cytokines (collectively defined as Th0 cells) take part into the immune response (D'Elios & Del Prete, 1998). A new subset of Th cells, named Th17 cells, producing IL-17 alone or in combination with IFN-γ has been recently identified (Weaver *et al.*, 2006). Th17 cells play a critical function in protection against microbial challenges, particularly extracellular bacteria and fungi (Bettelli et al., 2007). Further, some of the autoimmune responses formerly attributed to Th1 cells, such as experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA), Lyme arthritis and some forms of inflammatory bowel disease (IBD), have now been shown to be mediated, at least in part, by Th17 cells (Codolo *et al.*, 2008; Romagnani, 2008).

The factors responsible for the Th cell polarization into a predominant Th1 or Th2 profile have been extensively investigated. Several evidence suggests that Th1 and Th2 cells develop from the same Th-cell precursor under the influence of mechanisms associated with antigen presentation (Kamogawa *et al.*, 1993). Both environmental and genetic factors influence the Th1 or Th2 differentiation by determining the "leader cytokine" in the microenvironment of the responding Th-cell. IL-4 is the most powerful stimulus for Th2 differentiation, whereas IL-12 and IFNs favor Th1 development (Swain *et al.*, 1990; Trinchieri 2003). A role has been demonstrated for the site of antigen presentation, the physical form of the immunogen, the type of adjuvant, and the dose of antigen (Constant & Bottomly, 1997). Potent adjuvants and microbial products induce Th1-dominated responses because they stimulate IL-12 production. IFN-γ and IFN-α favor the Th1 development by enhancing IL-12 secretion by macrophages and maintaining the expression of functional IL-12 receptors on Th cells (Szabo *et al.*, 1995).

Th1- and Th2-dominated responses not only provide different strategies of protection against pathogens, but also play a role in some pathological conditions (Table 1). Studies in humans and gene-targeted mice have clearly shown that Th1-dominated responses are effective in protection against several microbes and usually drive their clearance. However, if the pathogen persists,

chronically ongoing Th1 response may result in inflammatory tissue damage. Extracellular infectious agents would be more efficiently counteracted by combination of Th2 and Th1 cytokines, as in Th0 cell activation. Th2-dominated responses are usually observed during infections by intestinal nematodes. Since IL-4, IL-10, and IL-13 inhibit the development of Th1 cells and macrophage activation, Th2 response avoid the extensive inflammatory tissue injury that would eventually result from a Th1- and macrophage-mediated responses to such complex parasites. A switch toward a Th2-dominated response can also occur in some immune responses against microbes, when the Th1 response fails to rapidly clear the infection (D'Elios & Del Prete, 1999).

Bronchial asthma and Th2-driven inflammation

Asthma, defined by the World Health Organization as a "chronic inflammatory disease of the airways", is a complex disorder characterized by airway hyper-resposiveness to a variety of specific and nonspecific stimuli, and mucus hypersecretion by goblet cells. The increased usage of bronchoscopy associated with bronchoalveolar lavage (BAL) and bronchial biopsies provided important tools for research on asthma. The histopathological hallmark of bronchial asthma, even a mild one, is represented by epithelial shedding, basal membrane thickening, inflammatory infiltrates consisting of T lymphocytes, and accumulation of activated eosinophils.

Immunological and molecular studies of bronchial biopsies and BAL samples obtained in baseline disease or taken after natural or "experimentally" induced asthma exacerbations have shown the *in vivo* relevance of T cells, inflammatory cells, and related cytokine network in the pathogenesis of different variants of bronchial asthma, allergic, occupational or non-allergic. Asthma is driven and maintained by bronchial persistence of a subset of chronically activated memory T cells, previously sensitized against allergenic, occupational, viral, or other "alien". Limiting dilution T-cell cloning techniques and in situ hybridization studies demonstrated that

activated T cells and related cytokines could be indentified in biopsies derived from all major variants of asthma.

In allergic asthmatic patients allergen exposure induces in the airways a predominant activation of $CD4^+$ Th2 lymphocytes, able to over-express several Th2 cytokines, including IL-4 and IL-5 (Robinson *et al.*, 1992; Del Prete *et al.*, 1993). Moreover, the degree of IL-5 expression at bronchial level is associated with disease severity in both atopic and non-atopic asthma (Kon & Kay, 1999). IL-5 and GM-CSF can be considered the most important cytokines for eosinophil accumulation in asthmatic inflammation. Th2 cytokines in bronchial asthma are produced not only by $CD4^+$ but also by $CD8^+$ T cells, which contribute to the genesis of asthma and to the clinical expression of the disease (Betts & Kemeny, 2008).

***H. pylori*-driven Th1 response and HP-NAP**

In *H. pylori* infection, a predominant activation of Th1 cells, with production of IFN-γ, IL-12, IL-18, IL-23 and TNF-α, occurs in vivo in the stomach in humans and animal models (D'Elios *et al.*, 1997; Mohammadi *et al.*, 1996; Luzza *et al.*, 2000; Mattapallil *et al.*, 2000; Rossi *et al.*, 2000; Tomita *et al.*, 2001; Vivas *et al.* 2008). Complex and fascinating mechanisms are responsible for the mucosal Th1 polarization (D'Elios et al., 1997; Del Giudice *et al.*, 2001; D'Elios *et al.*, 2005). Stimulation of neutrophils, monocytes, and dendritic cells with HP-NAP resulted in prompt and remarkable up-regulation of IL-12 and IL-23 mRNA expression and protein secretion, via TLR2 activation. In the gastric mucosa of *H. pylori*-infected patients, a remarkable proportion of Th cells shows a significant proliferation to different *H. pylori* antigens, including HP-NAP, CagA, urease, VacA and heat shock proteins. HP-NAP drives production of high levels of IFN-γ and TNF-α by antigen-specific gastric Th cells and elicits a powerful cytotoxic activity, thus promoting a polarized Th1 response (Amedei *et al.*, 2006).

We recently investigated the immune-modulating activities of HP-NAP in relation to the structure of the protein. HP-NAP structurally belongs to the *Dps* (**D**NA **p**rotecting protein under **s**tarved conditions) family (Grant *et al.*, 1998). It consists of twelve identical subunits arranged in a dodecameric shell with 32 symmetry (Fig. 1A) (Zanotti *et al.*, 2002). The resulting complex, whose size is of about 90 Å in diameter, surrounds a large central cavity, whose function is to host Fe atoms in the form of mixed iron oxides (Tonello *et al.*, 1999). In addition, 12 Fe ions are structurally bound in the inner part of the shell, contributing to its structural stability and possibly representing the sites for iron oxidation. Since each subunit in the dodecamer makes strong interactions with the neighboring subunit, this quaternary arrangement is very stable and resistant to denaturation. For this reason it can be assumed that the immunogenic properties of this protein, which are in general not shared by other members of the Dps family, depend on the characteristics of the protein surface. Since the surface of the dodecamer is characterized by a large presence of positively charged residues (Fig. 1B), a property which is peculiar of HP-NAP within the Dps family, it has been hypothesized (Zanotti *et al.*, 2002) that this basic character could be responsible for neutrophil activation, in a way similar to other chemokines (Laurence *et al.*, 2001). Moreover it has been recently demonstrated that neutrophil activation is stimulated by structural elements that are localized within the C-terminal region of the HP-NAP subunit (Kottakis *et al.*, 2007).

The crystal structure of the extracellular domain of TLR2 (the receptor engaged by HP-NAP upon the surface of cells of innate immunity) in association with TLR1 and a lipopeptide has been recently resolved (Jin *et al.*, 2007), and also that of TLR4 in complex with MD-2 (Kim *et al.*, 2007). The extracellular domain of TLRs shares a common structural feature, represented by a repetition of 16 to 28 LRR modules (Matsushima *et al.*, 2007). The binding of ligands to these extracellular domains triggers a rearrangement of the receptor that, in turn, induces the recruitment of specific adaptor proteins to the intracellular domains (O'Neil & Bowie, 2007). The surface of both TLR2 and TLR4 is heavily charged and the complex of TRL4 with MD-2 is stabilized by electrostatic and hydrogen bonds interactions (Kim *et al.*, 2007). Since the surface of HP-NAP is also heavily

charged, the TLR4-MD2 complex could possibly represent a prototype of the complex of TLR2 with HP-NAP.

H. pylori and bronchial asthma: is HP-NAP the molecular explanation of the inverse association?

The severity and incidence of asthma have dramatically increased in the developed nations over the last decades. Although the underlain reason is still unknown, 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 & Erb, 2001). This view is supported by studies showing that animals can be protected from developing asthma by administration of alive or killed bacteria or their components, which induce Th1 responses (Wohlleben & Erb, 2006).

Interestingly, on the basis of large epidemiological studies, it has been recently purposed a consistent negative association between *H. pylori* infection and the presence of allergic disorders, such as asthma and rhinitis (Chen & Blaser, 2007; Blaser *et al.*, 2008; Chen & Blaser, 2008). Although it is undoubtedly a fascinating theory, no convincing molecular mechanism has been proposed to support it, and this was the principal reason for the criticism raised.

Our recent studies carried on with the Neutrophil Activating Protein HP-NAP may help in understanding such a complex issue. We have shown that addition in culture of HP-NAP to allergen-induced T cell lines derived from allergic asthmatic patients resulted in a remarkable increase of IFN-γ-producing T cells and decrease of IL-4-secreting cells, thus in a redirection of the immune response from a Th2 to a Th1 phenotype (Amedei *et al.*, 2006). These results suggest the

that HP-NAP might be the key element responsible for the decrement of allergy frequency in *H. pylori*-infected patients.

HP-NAP as candidate for prevention and treatment of bronchial asthma

To address whether HP-NAP, on the basis of its immune-modulating activity, could be beneficial for prevention and treatment of bronchial asthma, HP-NAP was administered via intra-peritoneal (i.p.) or intra-nasal route using a mouse model of allergic asthma induced by inhaled ovalbumin (OVA). To this aim, groups of nine C57BL/6j, wild type or tlr2$^{-/-}$, mice were treated with saline, or with OVA alone, or 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. and a second phase of induction of the allergic response with aerosolized OVA on day 8, and finally exposed to aerosolized antigen on days 15-18 (Gonzalo *et al.*, 1996). Control animals were injected with PBS alone and then exposed to aerosolized PBS. In the systemic protocol mice were treated with i.p. HP-NAP on day 1, whereas in the mucosal protocol mice received intranasal HP-NAP on days 7 and 8 (Codolo *et al.*, 2008).

After priming and repeated aerosol challenge with OVA, Th2 responses in the mouse lung were induced. Moreover, following OVA treatment, eosinophils were recruited and activated in bronchial airways and serum levels IgE increased, and the elicited Th2 response correlated with the appearance of airway hyper responsiveness. Both systemic and mucosal administration of HP-NAP strongly inhibit the development of airway eosinophilia and bronchial inflammation (Fig.2). Likewise, HP-NAP treatment strongly affected the lung cytokine release, reducing the production of IL-4, IL-5, and GM-CSF (Fig. 3). Systemic HP-NAP also significantly resulted in both reduction of total serum IgE and increase of IL-12 plasma levels. However, no suppression of lung eosinophilia

and bronchial Th2 cytokines was observed in TLR-2 -knock-out mice following HP-NAP treatment (Codolo *et al.*, 2008).

It is of note that also in another mouse model of Th2-mediated disease, such as *Trichinella spiralis* infection, HP-NAP was able *in vivo* to enhance endogenous IL-12 and IFN-γ response and to exert a powerful anti-Th2 activity, targeting both the IL-5-induced eosinophilia and the IL-4-mediated hyper-IgE responses induced by parasitic infection (Del Prete *et al.*, 2009).

Concluding remarks

Asthma is one of the most common chronic diseases in industrialized countries and consists of airway inflammation, bronchial hyper responsiveness and airway obstruction. Typical pathological features include infiltration of the airways by activated lymphocytes, particularly Th2 cells and eosinophils. 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, such as *H. pylori* infection, can influence the development of allergic disorders. 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. HP-NAP by acting on both neutrophils and monocytes via TLR2 agonistic interaction, significantly contributes to induce an IL-12 and IL-23 enriched milieu, and represent a key bacterial factor able to drive the differentiation of antigen-stimulated T cells towards a polarized Th1 phenotype. HP-NAP has in vitro the potential to redirect allergen-specific T cell response from a predominant Th2 to a Th1 response polarized. Furthermore, HP-NAP in vivo administration resulted in inhibition of the typical Th2-mediated bronchial inflammation of allergic bronchial asthma. Thus, altogether these results support the view that the increased prevalence and severity of asthma and allergy in western countries may be related, at least in part, to the decline of *H. pylori* infection able to elicit a long-lasting Th1 background, and suggest

HP-NAP as an important candidate for novel strategies of prevention and treatment of asthma and allergic diseases.

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Statements

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Th1	Th2
- *H. pylori* infection	- Allergic diseases
- Atherosclerosis	- Vernal conjunctivitis
- Organ-specific autoimmunity	- Parasitic infections
- Acute allograft rejection	- Systemic sclerosis
- Crohn's disease	- Some hypereosinophilic syndromes
- Some recurrent abortions	- Chronic graft versus host disease
- Sarcoidosis	- Some patients with AIDS

Table 1. Human pathological conditions associated to Th1 and Th2 predominant responses.

Figure legends

Figure 1. HP-NAP. A) Ribbon representation of the HP-NAP dodecamer. Each subunit is represented with a different color. Red spheres show the positions of the twelve structural Fe ions. B) Surface of the dodecamer of HP-NAP. Positively and negatively charged residues on the surface are colored: Arg and Lys, blue; His, cyan; Glu and Asp, red. Whilst in the overall the surface presents a prevalence of positively charged residues, the pore in the center of the picture, which is the postulated entrance for the Fe ions, is characterized by negative charges (Glu1145, Asp126 and 127).

Figure 2. Intraperitoneal and intranasal administration of HP-NAP inhibited the development of airway eosinophilia in OVA-sensitized animals. On day 1, mice were sensitized with OVA alone, or with OVA plus systemic HP-NAP (Sy HP-NAP), or with intranasal aerosolized HP-NAP (Mu HP-NAP) on days 7 and 8, and then exposed to aerosoloized OVA, followed by repeated challenge from days 15 to 18. Control animals (saline) were injected with saline alone and then exposed to aerosolized PBS. On day 18, cytocentrifuge preparations from BAL of the different groups of animals were stained to calculate the proportions of eosinophils. Absolute counts of eosinophils ($\pm$ SD) were calculated from the number of total cells in the BAL. ***$P < 0.01$ versus mice treated with OVA alone.

Figure 3. Intraperitoneal and intranasal administration of HP-NAP resulted in reduced Th2 accumulation in the airway lumen. BAL samples were collected from saline-, OVA-, OVA plus i.p. HP-NAP (OVA + Sy HP-NAP), and OVA plus i.n. HP-NAP (OVA + Mu 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-treated mice.

Figure 4. Schematic representation of HP-NAP activities in asthma and allergic diseases. Following mucosal or systemic administration HP-NAP, via production of IL-12, and IL-23, inhibits allergic inflammation and redirect Th2 into Th1 responses.

Figure 1.

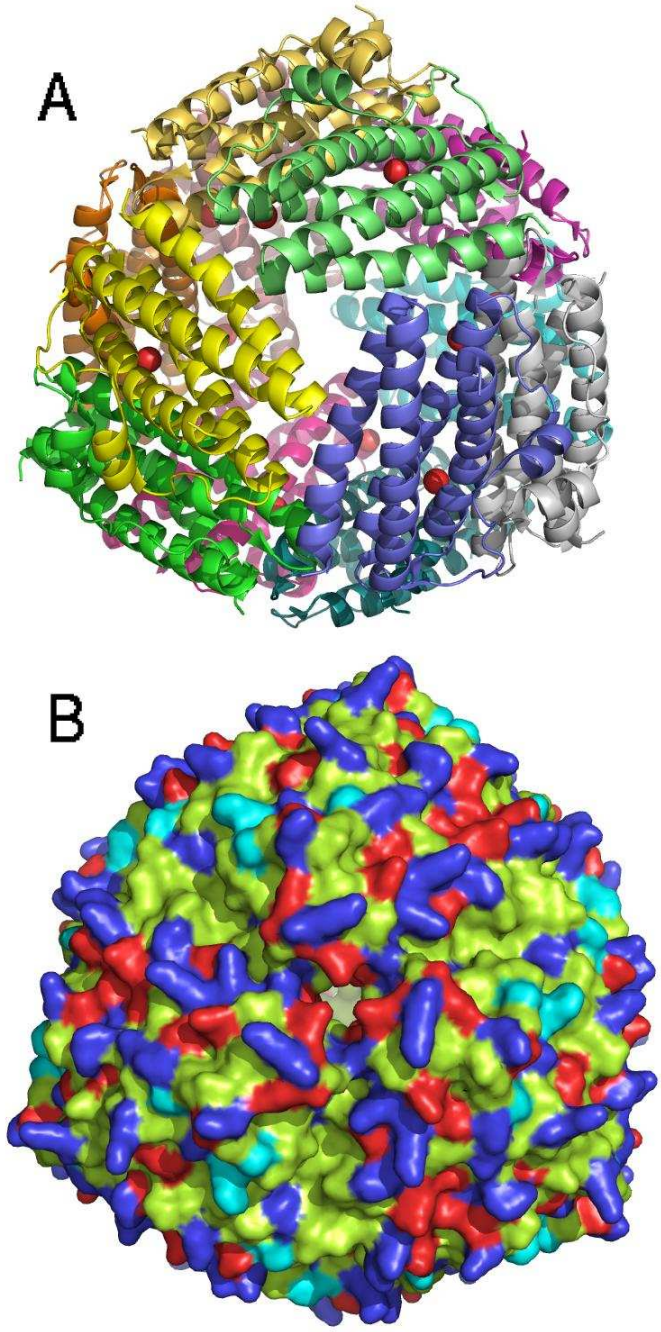

Figure 2.

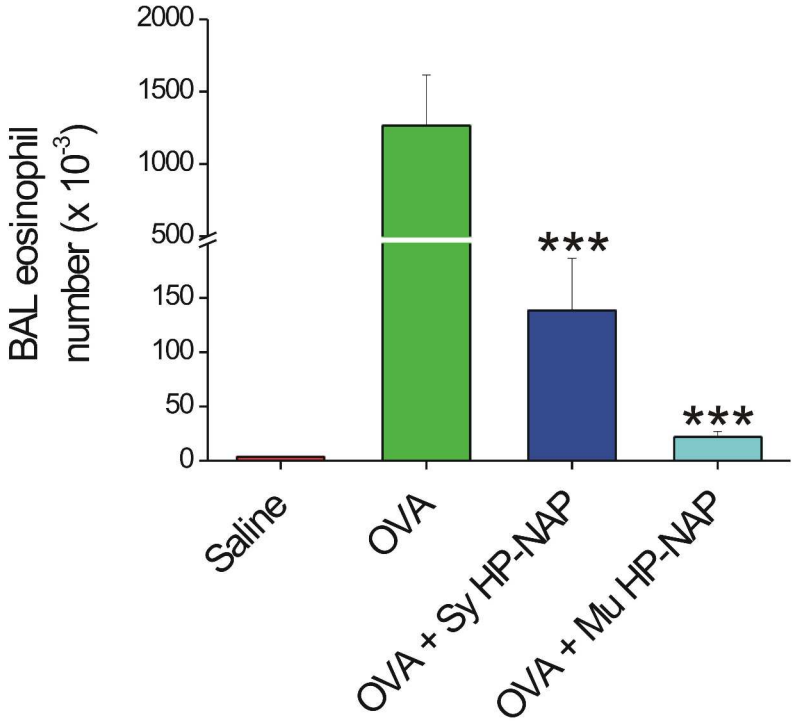

Figure 3.

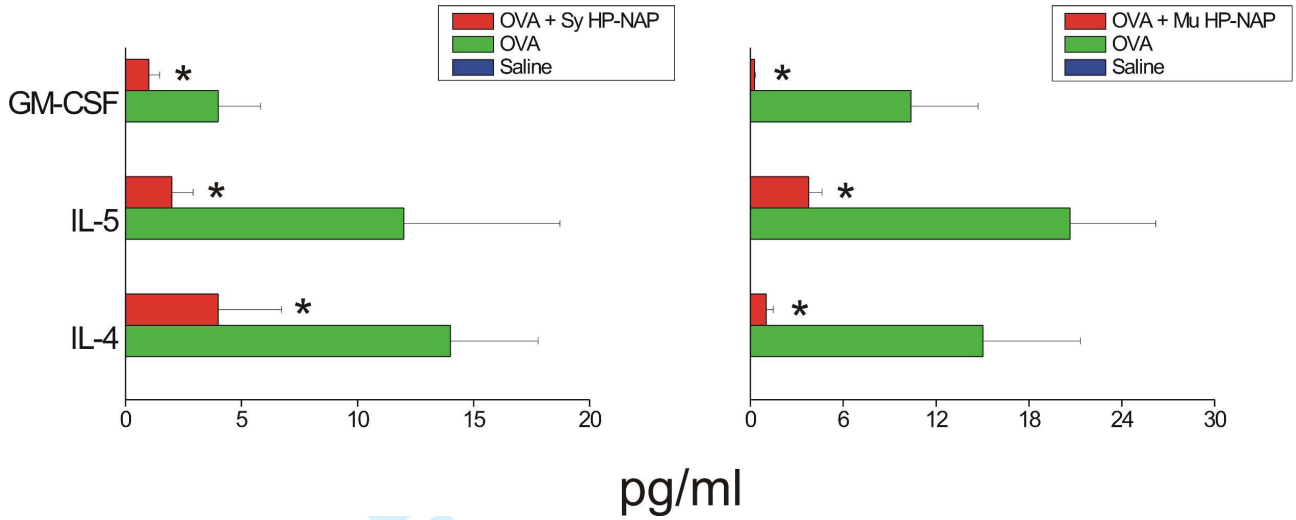

Figure 4.