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Review

The immune modulating activity of the *Helicobacter pylori* HP-NAP: Friend or foe?

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ABSTRACT

The *Helicobacter pylori* HP-NAP is a dodecameric protein with a three-dimensional structure similar to that of bacterioferritins. Originally defined as neutrophil-activating protein, because of its ability to stimulate neutrophils to produce oxygen radicals, HP-NAP is now considered a crucial factor in driving the Th1 inflammation in *H. pylori* infection. This review summarizes recent studies that have provided a deeper understanding of the pro-inflammatory and immune modulatory properties of HP-NAP. We first examine the role of this protein in the *H. pylori*-associated disease, and then we discuss recent findings that support the possibility for HP-NAP to become a new tool for therapeutic strategies aimed at redirecting Th2 into Th1 responses, for example in atopy, vaccinology and cancer immunotherapy.

1. Introduction

Infection of humans with the Gram negative bacterium *Helicobacter pylori* is associated with the development of severe gastroduodenal diseases, including chronic gastritis and stomach cancer (Goodwin, 1997; Montecucco and Rappuoli, 2001; Atherton, 2006). A number of virulence factors produced by the bacterium have been identified. Among them, a relevant role is played by an oligomeric protein, termed neutrophil-activating protein (HP-NAP), because of its ability to induce neutrophils to produce reactive oxygen radicals (Evans et al., 1995).

The *napA* gene is highly conserved among many isolates of *H. pylori*, which may indicate a precise structurally linked function or a lack of immune selection for diversification of HP-NAP. The atomic structure of HP-NAP shows a ball-shaped dodecamer formed by four-helix bundled subunits (17 kDa) with a hollow central part, similar to the *Escherichia coli* DNA-binding protein Dps (Zanotti et al., 2002; Papinutto et al., 2002). Dps proteins are a family of bacterial stress proteins that are induced under nutrient limitations. They protect bacterial DNA from oxidizing radicals generated by the Fenton reaction and also from various other damaging agents. DNA protection has a chemical component based on the highly conserved ferroxidase activity of Dps proteins, and a physical one based on the capacity of Dps proteins containing a positively charged N-terminus to bind and condense DNA (Ceci et al., 2004). HP-NAP does not possess a positively charged N-terminus but, unlike the other members of the family, is characterized by a positively charged protein surface which has been proposed to be

Abbreviations: DC, dendritic cells; HP-NAP, *Helicobacter pylori* neutrophil neutrophil-activating protein; I-CAM, intercellular cell adhesion molecule; IFN, interferon; IL, interleukin; OVA, ovalbumin; PAF, platelet activating factor; PAI-2, plasminogen activator inhibitor-2; PMN, polymorphonuclear cells; ROI, reactive oxygen radicals; TF, tissue factor; Th, T helper cells; TLR-2, toll-like receptor 2; TNF, tumor necrosis factor; V-CAM, vascular cell adhesion molecule.

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responsible for binding and condensing DNA (Ceci et al., 2007). Interestingly, the same report showed that the DNA condensation occurs at pH values that correspond to stress conditions encountered by *H. pylori* during gastric colonization. Besides these results, several reports concerning HP-NAP–DNA interaction and protection have been published: two independent studies reported that the newly purified HP-NAP protein as well as that expressed in *Bacillus subtilis* do not bind DNA (Wang et al., 2006; Tonello et al., 1999); another study demonstrated that HP-NAP, when expressed in *E. coli* cells, colocalizes with the nuclear material, suggesting that it can interact with DNA *in vivo* (Cooksley et al., 2003). Moreover, Wang et al. (2006) demonstrated, in a mouse model, that HP-NAP, although able to induce oxygen radicals' release, protects *H. pylori* from iron-mediated oxidative DNA damage.

HP-NAP can bind up to 500 atoms of iron per dodecamer and probably iron plays an important role in generation of the quaternary structure of HP-NAP by promoting stable dimers that are crucial for the ensuing dodecamer structure (Kottakis et al., 2008). HP-NAP was originally thought to be a bacterial ferritin, based on the nucleotide sequence homology (Evans et al., 1995), with a role in iron binding; however, other findings revealed that the bacterial protein is constitutively expressed under iron-depletion, that its expression is not regulated by the presence or absence of iron and that it has no part in the metal resistance of *H. pylori* (Dundon et al., 2002). Therefore, the role of HP-NAP for the bacterium remains an elusive issue: one possibility is that HP-NAP has evolved as a pro-inflammatory molecule to induce a moderate state of inflammation, which would promote *H. pylori* growth by the release of nutrients from the inflamed tissue.

HP-NAP is released most likely after cell lysis, and is capable of crossing the stomach epithelial layer reaching the underlying tissue and of inducing the activation of the resident mast cells (Montemurro et al., 2002). These cells are likely to be the first that encounter HP-NAP and their activation by the bacterial protein, with granule exocytosis and release of pro-inflammatory cytokines, is expected to trigger the inflammatory response. Once accumulated within the tissue, HP-NAP alone or together with other bacterial factors would recruit neutrophils and monocytes from the blood, thus amplifying the flogistic process. The molecular mechanisms by which HP-NAP exerts such effects will be discussed in detail in the following two paragraphs (Fig. 1).

Notably, HP-NAP is a major antigen in the human immune response; in fact, the majority of the infected patients have antibodies against this antigen (Satin et al., 2000). Antibodies against HP-NAP have been documented in patients with severe gastric disease, such as peptic ulcer and gastric cancer, suggesting that the presence of anti-HP-NAP antibodies can be considered as an indicator of severity of the infection (Long et al., 2009). Considering the protection conferred by the protein against the subsequent challenge with *H. pylori*, HP-NAP has been included in a very promising vaccine which is now in clinical trial (Satin et al., 2000; Del Giudice et al., 2001; Malfertheiner et al., 2008). Moreover, interesting results have been recently reported on an oral recombinant DNA vaccine based on a live attenuated *Salmonella typhimurium* strain harbouring the HP-NAP gene (Sun et al., 2006).

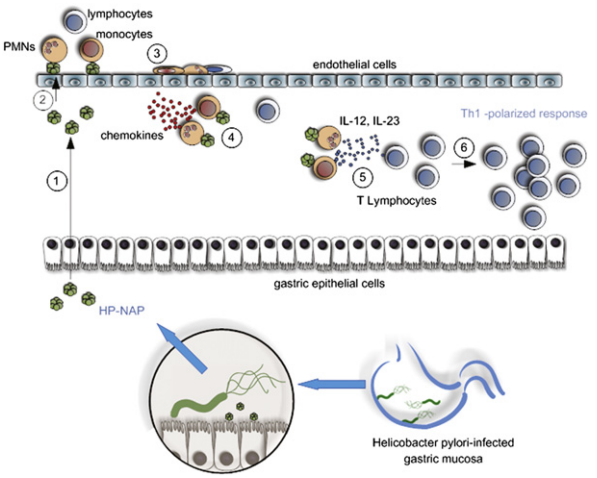

Fig. 1. Model illustrating the pivotal role of HP-NAP in triggering the flogistic process associated to *H. pylori* infection and its immunomodulating activity. HP-NAP, once released from the bacterium in the stomach lumen, crosses the gastric epithelial lining (1) and the endothelium (2), and stimulates directly leukocytes to adhere and to extravasate (3). HP-NAP also activates neutrophils and monocytes to secrete chemokines (4). In this way the protein, by acting on the recruited cells, could contribute to the maintenance of the flogistic status. Furthermore, the protein is able to create an IL-12/IL-23-enriched milieu (5) which is responsible for driving the differentiation of T helper cells towards the Th1 phenotype (6).

Interestingly, the effects on lymphocytes exerted by HP-NAP are not limited to the activation of T and B cells, a typical scenario which follows the presentation of an antigen by the professional antigen presenting cells; HP-NAP is also able to modulate the effector functions of T lymphocytes, which are stimulated to differentiate towards the Th1 phenotype. Such an ability of the protein, that will be examined in the third part of the review, is probably responsible for the predominant activation of Th1 cells occurring in the antrum of *H. pylori*-infected patients, as it will be discussed in detail in the fourth part.

The review concludes focusing on the therapeutic potential of HP-NAP in all the conditions in which an effective Th1 response is desired, i.e. vaccinology and cancer immunotherapy.

2. Effects of HP-NAP on neutrophils: recruitment and activation

H. pylori colonization is typically followed by infiltration of the gastric mucosa by polymorphonuclear leucocytes, macrophages and lymphocytes (Dixon et al., 1996; D'Elios et al., 1997). A strong correlation exists between gastric infiltration by neutrophils (polymorphonuclear cells; PMN), mucosal damage and development of duodenal ulcer disease in *H. pylori* infections (Davies et al., 1994; Hamlet et al., 1999). The mechanism underlying the sustained recruitment of PMN to the *H. pylori*-infected tissue *in vivo* is not completely understood; however, compelling evidence indicates that the neutrophil-activating protein of *H. pylori* (HP-NAP) exerts a pivotal role. Until recently, all

the experiments illustrating the capacity of HP-NAP of inducing leukocytes to adhere to the endothelium were conducted *in vitro* putting PMN and HP-NAP directly in contact, without considering that *in vivo* the protein has to cross the endothelium in order to activate neutrophils (Evans et al., 1995). A recent report, however, showed that HP-NAP promoted transendothelial neutrophil migration also when added into a lower chamber of a transwell system consisting of a cultured monolayer of human endothelial cells as barrier between two chambers (Brisslert et al., 2005). These findings suggested that HP-NAP was able to cross the endothelium to contact leukocytes, but failed to provide direct evidence for the transendothelial passage of HP-NAP. Moreover, it was not considered that *in vivo* the under-flow conditions may bias the effects observed *in vitro*. The first *in vivo* evidence that HP-NAP promotes leukocyte adhesion to the endothelium and the subsequent extravasation was obtained by applying intravital microscopy analysis to rat mesenteric venules topically exposed to HP-NAP (Polenghi et al., 2007). The same study showed that HP-NAP is effectively transported across the endothelium via intracellular route and a proportion of the protein remains bound to the endothelium after transcytosis, similarly to what was reported for the chemokine CXCL8 (Middleton et al., 1997). It is conceivable that in this form HP-NAP comes in contact with rolling neutrophils and monocytes and promotes the up-regulation of β2 integrin expression on their surface (Satin et al., 2000). β2 Integrins, which are crucial for the tight adhesion of leukocytes to the endothelium before extravasation, need also to be activated to acquire their ligand-binding capacity. Again HP-NAP shows a role since it induces a conformational change of β2 integrins, resulting in an increased affinity for the endothelial partner (Polenghi et al., 2007). Collectively these data suggest that HP-NAP, via its direct intervention, has a crucial role in recruiting leukocytes towards the infected area and therefore, in triggering the inflammation process. Furthermore, HP-NAP stimulates mast cells and macrophages to release TNF-α (Montemurro et al., 2001; Amedei et al., 2006). TNF-α is a pleiotropic cytokine which increases the adhesiveness of endothelial cells by up-regulating adhesion molecules, such as V-CAM and I-CAM (Silverstein et al., 2000). Moreover TNF-α can induce activation of integrins on PMN, directly or via CXCL8 whose endothelial secretion is stimulated by TNF-α (Laudanna et al., 2002; Gamble et al., 1985). Thus, HP-NAP, both alone and together with other host-derived factors, triggers *in vivo* the PMN accumulation within the tissue, evoking the adhesive properties of PMN and endothelium. In addition, considering that recruited PMN release cytokines and chemokines and that, under HP-NAP stimulation, they release TNF-α, CXCL8, CCL3 and CCL4, PMN may contribute to the generation of the condition required for the recruitment of additional neutrophils, monocytes and lymphocytes (Polenghi et al., 2007). Although it is undoubted that HP-NAP plays a major role in PMN recruitment and activation, other bacterial factors are also important (Unemo et al., 2005). The acronym HP-NAP was originally attributed to the *H. pylori* dodecameric bacterioferritin because of its ability to induce neutrophils to produce reactive oxygen radicals (ROI) (Evans et al., 1995). Such a property has been further documented by Satin et al. (2000) and Wang et al. (2008), the latter also showing that HP-NAP promotes the release of myeloperoxidase from human neutrophils. It has been reported that the ability of HP-NAP in inducing the release of oxygen radicals is exerted also on monocytes and depends on the activation of the NADPH oxidase. HP-NAP acts through a cascade of intracellular activation events, which is completely prevented by pertussis toxin: it includes the increment of cytosolic Ca^{2+} and the phosphorylation of proteins, leading to the assembly of active NADPH oxidase on the neutrophil plasma membrane (Satin et al., 2000; Montecucco and Rappuoli, 2001). The pattern of events triggered by HP-NAP is closely similar to that triggered by heptahelical receptors specific for the chemotactic agonist fMLP, C5a, platelet activating factor (PAF), and CXCL8 (Rossi et al., 1985; Thelen et al., 1993). Such similarity strongly suggests that one possible HP-NAP receptor is a G-coupled heptahelical protein receptor, still to be identified (Nishioka et al., 2003).

3. Effects of HP-NAP on monocytes

HP-NAP efficiently stimulates human monocytes to synthesize tissue factor (TF) and plasminogen activator inhibitor-2 (PAI-2) (Montemurro et al., 2001). Consequently, the procoagulant potential of monocytes increases while their fibrinolytic capacity decreases, tilting therefore the cell coagulation–fibrinolysis balance towards fibrin formation and pro-thrombotic events. This action of HP-NAP is expected to favor chronic development of gastritis and tissue disruption by hampering tissue healing, which requires degradation and removal of fibrin and tissue debris. At the same time, fibrin deposition might hinder movement of phagocytes towards *H. pylori* cells and protect them from phagocytosis (Montemurro et al., 2001).

Stimulation of monocytes and dendritic cells (DC) by HP-NAP results also in a prompt and remarkable up-regulation of cytokines including IL-12p35 and IL-12p40, which pair to form the active IL-12 molecule, and IL-23p19 which assembles with the IL-12p40 chain to form the IL-23 heterodimer (Amedei et al., 2006). In other words HP-NAP induces an IL-12- and IL-23-enriched milieu, which has the potential to drive the differentiation of antigen-stimulated T cells towards the Th1 phenotype (Oppmann et al., 2000; Trinchieri, 2003) (Fig. 1).

The unique role of HP-NAP with regard to the whole bacterium was better specified by comparing HP-NAP-null *H. pylori* mutant with the wild-type bacterium for the efficiency in inducing monocyte cytokine synthesis. Both wild-type and HP-NAP-null mutant bacteria were able to induce the production of comparable amounts of TNF-α, IL-6, and IL-8 by monocytes. In contrast, stimulation with the highest dose of HP-NAP-null mutant *H. pylori* (5×10^5 CFUs/ml) resulted in very poor secretion of IL-12, which was lower than that induced by a 25 times lower concentration of wild-type *H. pylori* (0.2×10^5 CFUs/ml). These data suggest that a number of *H. pylori* components can activate monocytes to cytokine synthesis, but HP-NAP represents the critical molecule for the induction of substantial IL-12 secretion (Amedei et al., 2006).

HP-NAP activity on monocytes results not only in the strong up-regulation of Th1-polarizing cytokines, TF and PAI-2 production, but also in the induction of a progressive maturation of monocytes into mature dendritic cells showing high expression of HLA-DR, CD80 and CD86, longer survival, and a tendency to cluster and to detach from the substrate (Amedei et al., 2006). A previous study showed that *H. pylori* induces dendritic cells to release IL-6, IL-8 and IL-12, and represents a maturation stimulus for human DC (Kranzer et al., 2004). However, the bacterial factor responsible for such effects was not identified: HP-NAP might represent that factor, due to its ability to mimic all the effects induced by *H. pylori*. Interestingly, the immune modulating activity of HP-NAP does not result from the engagement of a G-coupled heptahelical receptor, rather it is triggered by the activation of the toll-like receptor 2 (TLR-2) (Nishioka et al., 2003; Amedei et al., 2006).

4. HP-NAP and gastric Th1-polarized response

In infectious diseases, T helper cells (Th) orchestrate the host defense against pathogens via various types of cytokines and effector functions. Th1 cells produce IFN-γ and TNF-β, and elicit macrophage activation and TF production, whereas Th2 cells produce IL-4, IL-5 and IL-13, and inhibit several macrophage functions, including TF synthesis (D'Elios and Del Prete, 1998). Th0 cells do not express a polarized Th1 or Th2 profile, and represent a population of effector cells secreting different combinations of Th1 and Th2 cytokines. In *H. pylori* infection, a predominant activation of Th1 cells with production of IFN-γ and increased expression of IL-12, IL-18, IL-17 and TNF-α occurs *in vivo* in the antrum (D'Elios et al., 1997; Bamford et al., 1998; Luzza et al., 2000; Tomita et al., 2001; Lehmann et al., 2002). In the gastric mucosa of *H. pylori*-infected patients a remarkable proportion of Th cells showed significant proliferation in response to different *H. pylori* antigens, including HP-NAP (D'Elios et al., 1997; Amedei et al., 2006). Upon HP-NAP stimulation, antigen-specific gastric Th cells produced large amounts of IFN-γ and TNF-α and displayed a powerful cytotoxic activity, thus showing a polarized Th1 effector phenotype (Amedei et al., 2006). Collectively, these results demonstrate that there is a strict correlation between *in vitro* and *in vivo* effects of HP-NAP and they define the bacterial protein as responsible for driving the Th1 response in the gastric antrum of patients affected by *H. pylori* (Fig. 1).

5. Effect of HP-NAP on lymphocytes: Th1 induction and Th2 down-modulation

Consistent with the ability to trigger the secretion of important Th1 inducers, such as IL-12 and IL-23, HP-NAP was found able to modulate *in vitro* the cytokine profile of human T cell response, towards the Th1 profile. Indeed, by conditioning T cell cultures with HP-NAP a remarkable increase of IFN-γ-producing cells and a decrease of IL-4-secreting cells were observed. This cytokine profile of antigen-specific Th cells, generated in the presence or absence of HP-NAP, was evaluated at clonal level, using tetanus toxoid (TT) and mite allergens as antigen. The results obtained in the case of mite allergen were particularly interesting because Th cell responses to allergens are usually definitely oriented to the Th2 pattern. In the series of allergen-specific Th clones, not conditioned with HP-NAP, 1% of clones were Th1, 10% were Th0 (cells producing either IFN-γ and IL-4), and 89% were Th2. In contrast, in the series of allergen-specific Th clones conditioned with HP-NAP, Th1 and Th0 clones accounted for 38% and 33%, respectively, and Th2 clones accounted for only 29%. In other words, addition in culture of HP-NAP resulted in a shift from preferential Th2 to predominant Th1 T cell responses, with a remarkable increase of IFN-γ producing T cell clones and a strong reduction of allergen-specific clones with the Th2 profile. Interestingly, the majority of allergen-specific Th cell clones obtained upon HP-NAP conditioning displayed strong cytolytic activity, suggesting that the Th1-immune modulatory effect of HP-NAP not only affects the T cell cytokine production, but also triggers the expression of the cytotoxic program, a property of the fully Th1-polarized effector T cells (D'Elios and Del Prete, 1998; Amedei et al., 2006). Of note, HP-NAP had a strong Th1-polarizing effect also on non-allergen-specific (bystander) Th clones that were grown in the context of allergen-induced T cell lines (Amedei et al., 2006; D'Elios et al., 2009).

6. HP-NAP exerts a powerful anti-Th2 activity in vivo

Although *in vitro* experiments carried on allergen-induced T cell lines generated from mononuclear cells of house dust mite allergen-sensitive donors represented a strong evidence for the Th1-promoting and Th2-inhibiting activity of HP-NAP, the first evidence that the bacterial protein was capable of redirecting Th2 responses towards the Th1 phenotype came from two recent reports (Codolo et al., 2008a; Del Prete et al., 2008). In both studies HP-NAP was applied *in vivo* to a Th2-dominated animal model, such as mice affected by allergic asthma in the former study and mice infected with *Trichinella spiralis* (Ts) in the latter.

The study carried out by Codolo et al. (2008a) was based on a mouse model of allergic asthma induced by inhaled ovalbumin (OVA) (Gonzalo et al., 1996). After intra-peritoneal priming followed by repeated aerosol challenge with OVA, Th2 responses were induced in the mouse lung, resulting in eosinophils recruitment in bronchial airways and serum IgE levels increment. In this study, HP-NAP was administered simultaneously with OVA priming, by intraperitoneal injection, or via intranasal instillation 7 day after the phase of sensitization. Both systemic and mucosal administration of HP-NAP strongly inhibited the development of airway eosinophilia and bronchial inflammation and led to reduction of total serum IgE and to increase of IL-12 plasma levels (Codolo et al., 2008a).

IgE hyper-production and eosinophilia are typical immune responses evoked by Ts. Eosinophilia is due to the selective induction and expansion of Th2 cells that produce IL-5 and the high IgE response is due to the concomitant production of IL-4 and IL-13, which are key molecules for differentiation of B cell to IgE-producing cells (Mosmann

and Coffman, 1989; D'Elios and Del Prete, 1998; Romagnani, 2000). Treatment with HP-NAP of mice with established Ts infection resulted in a strong anti-Th2 effect, as demonstrated by reduced eosinophilia and lower levels of total IgE in comparison with control animals. In addition, evidence was provided that HP-NAP *in vivo* resulted in ongoing production of endogenous IL-12 and IFN-γ even days after its delivery, as well as in persistent inhibition of the Ts-induced expression of IL-4 and IL-5 (Del Prete et al., 2008).

Interestingly, both *in vivo* studies demonstrate that the binding of HP-NAP to the TLR-2 is crucial for the bacterial protein to exert its anti-Th2 activity.

7. Concluding remarks

In *H. pylori* infection the bacterium induces an inflammatory response in the gastric mucosa, characterized by polymorphonuclear and mononuclear cell infiltration. A key factor in orchestrating the recruitment and activation of these cells is HP-NAP, which not only directly promotes the accumulation of inflammatory cells within the infected gastric tissue but also activates them to release pro-inflammatory cytokines and chemokines, that contribute to the maintenance of the flogistic process. Notably, HP-NAP stimulates neutrophils and monocytes to release IL-12 and IL-23, the two pivotal cytokines responsible for driving the Th response towards the Th1 phenotype. This T cell subset is the most represented in the stomach of *H. pylori*-infected individuals and is associated with more serious diseases. Indeed, Th1 cells, by producing large amounts of IFN-γ and by activating a cytolytic cascade strongly contribute to gastric damage. Moreover, high levels of TF, which is also induced by HP-NAP, IFN-γ and TNF-α, might result in procoagulant activity and in an increment of gastric secretion and of pepsinogen release. The ability of HP-NAP to shift a Th response towards the Th1 phenotype has been clearly documented *in vivo*: in the gastric mucosa of *H. pylori*-infected patients a remarkable proportion of Th cells show significant proliferation to different *H. pylori* antigens, including HP-NAP. Upon HP-NAP stimulation, antigen-specific gastric Th cells produce high amounts of IFN-γ and TNF-α.

Epidemiological studies and experimental data provided evidence of an inverse association between *H. pylori* infection and the frequency of allergic asthma (Blaser et al., 2008); however, the absence of a convincing molecular mechanism represented a limitation of this study and raised several criticisms. In virtue of its striking immune modulating activity, HP-NAP might be part of the molecular mechanism underlying such a negative association. Most importantly, the capacity of HP-NAP in inhibiting Th2 responses *in vitro* and *in vivo* in allergic bronchial asthma, in humans and mice, makes this bacterial protein an important candidate for novel strategies of prevention and treatment of asthma and allergic diseases. Furthermore, considering that HP-NAP is a very powerful inducer of IL-12 and that IL-12 represents the most effective cytokine in terms of tumor eradication, anti-metastatic activity and long-term anti-tumor immunity, it is tempting to speculate that HP-NAP might be beneficial not only against allergic disease but also to fight cancer, e.g. as adjuvant for local immunotherapy of some neoplasias (Colombo and Trinchieri, 2002; Trinchieri, 2003; D'Elios et al., 2007).

Noteworthy, HP-NAP shares significant homology with other Dps-like proteins, produced by bacteria associated with chronic inflammation, such as NapA of *Borrelia burgdorferi* (Li et al., 2007; Codolo et al., 2008b): also this protein is endowed of specific immune modulatory properties, although different from those ascribed to HP-NAP. In fact, NapA is able to drive the expression of IL-6, IL-1, IL-23, and TGF-β by cells of the innate immune system and to elicit a synovial fluid Th17 cell response that might play a crucial role in the pathogenesis of Lyme arthritis (Codolo et al., 2008b).

In conclusion, HP-NAP emerges as the prototype of a novel class of Dps-like proteins, characterized by the ability of driving and orchestrating the inflammation associated with bacterial infections. From this point of view it could be considered as "foe" because of its contribution to the tissue damage associated with the flogistic process. However, in virtue of its immune modulatory properties it should also be seen as a promising and "friendly" therapeutical tool.

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Conflict of interest

M.d.B. and M.M.D.E. are inventors and applicants of patent EU05425666.4, for potential use of HP-NAP as therapy of cancer, infectious and allergic diseases.

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