The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* as immune modulating agent

running title: HP-NAP, a novel immune modulating agent

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Abstract

Microorganisms during evolution have developed several immune modulating strategies. The *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is a virulence factor that attracts and activates neutrophils, promotes their endothelial adhesion, production of oxygen radicals, and chemokines, including CXCL8, CCL3 and CCL4. HP-NAP, a TLR2 agonist, is an immune modulator able to induce the expression of IL-12 and IL-23 by human neutrophils and monocytes. In fact, HP-NAP has the potential of shifting antigen-specific T-cell responses from a predominant Th2 to a polarized Th1 cytotoxic phenotype, characterized by high level of IFN-γ and TNF-α production. Thus, HP-NAP is both a key factor driving Th1 inflammation in *H. pylori* infection and a new tool for future therapeutic strategies aimed to redirect Th2 into Th1 responses, e.g. in atopy, vaccinology and cancer immunotherapy.

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 (Marshall, 1994; Parsonnet *et al.*, 1991; Wotherspoon *et al.*, 1991; D'Elios *et al.*, 2004). *H. pylori* gastric colonization is typically followed by mucosa infiltration of polymorphonuclear leukocytes (PMNs), macrophages and lymphocytes, and the degree of mucosal damage is correlated with neutrophil infiltration.

The *H. pylori* neutrophil-activating protein (HP-NAP) is a dodecameric protein of 150 kDa, with a structure similar to bacteriferritin, including a central cavity for iron accumulation (Tonello *et al.*, 1999). It was initially identified as a promoter of endothelial adhesion of neutrophils and was defined as neutrophil activating protein, because it stimulates high production of oxygen radicals from PMNs (Evans *et al.*, 1995). In addition, HP-NAP increases in monocytes the synthesis of tissue factor and the secretion of type-2 plasminogen activator inhibitor (Montecucco & de Bernard 2003; Montemurro *et al.*, 2001). HP-NAP is chemotactic for neutrophils, and represents a major *H. pylori* virulence factor participating *in vivo* in creating a peculiar cytokine *milieu* at the site of infection (Montecucco & de Bernard 2003; Amedei *et al.*, 2006; Polenghi *et al.*, 2007).

Despite a high rate of infection worldwide only a minority (10-20%) of *H. pylori*-infected patients develop severe diseases, such as peptic ulcer, gastric cancer and lymphoma, and autoimmune gastritis, throughout their life and this evidence suggests that the type of host response to *H. pylori* may represent an important factor able to influence the outcome of the infection. Many reports, in humans and animal models, indicated that T helper (Th) 1 polarization of immune response in *H. pylori*-infected stomach is associated with severity of inflammation and gastric diseases (reviewed in D'Elios *et al.*, 2005). This article will focus on the *H. pylori*-related factors involved in eliciting host Th1 response, and particularly on the Th1 immune-modulating activities exerted and promoted by HP-NAP on neutrophils, macrophages, and T lymphocytes.

H. pylori neutrophil-activating protein

The infiltration of neutrophils and mononuclear inflammatory cells within the gastric mucosa is a common finding in *H. pylori* infection, and the degree of mucosal damage correlates with neutrophil infiltration. Different components present in *H. pylori* extracts directly attract and activate neutrophils and other inflammatory cells (Karttunen *et al.*, 1990; Mai *et al.*, 1991; Craig *et al.*, 1992; Nielsen & Andersen 1992; Kozol *et al.*, 1993; Reymunde *et al.*, 1993; Marchetti *et al.*, 1995; D'Elios *et al.*, 1997; Betten *et al.*, 200; Polenghi *et al.*, 2007). A 150-kDa oligomeric protein isolated from *H. pylori* was found to promote neutrophil adhesion to endothelial cells (Yoshida *et al.*, 1993; Evans *et al.*, 1995). This protein was designated as *H. pylori* neutrophil-activating protein (HP-NAP) because of its ability to induce neutrophils to produce reactive oxygen radicals (Evans *et al.*, 1995; Satin *et al.*, 2000). HP-NAP is released in the medium, most likely after cell lysis, and binds to the bacterial surface, where it can act as an adhesin, mediating binding to mucin or to polymorphonuclear leukocyte sphingomyelin (Namavar *et al.*, 1998; Teneberg *et al.*, 1997). Purified recombinant HP-NAP has been produced in *Bacillus subtilis* to avoid contamination by *Escherichia coli* LPS. This purified material was found to be chemotactic for human neutrophils and monocytes in vitro (Satin *et al.*, 2000). Moreover, using intravital microscopy, it has recently been demonstrated in rats that HP-NAP is able to efficiently cross the endothelia and to promote rapid neutrophil adhesion in vivo (Polenghi *et al.*, 2007). HP-NAP-induced adhesivity depends on the induction of expression and on the acquisition of a high affinity state of β2-integrin on the plasma membrane of PMNs (Satin *et al.*, 2000; Polenghi *et al.*, 2007). This conformational change requires a functional p38 mitogen-activated protein kinase (MAPK). Collectively, these observations suggest that HP-NAP play a central role in the accumulation of leukocytes at the site of infection (Evans *et al.*, 1995; Satin *et al.*, 2000; Polenghi *et al.* 2007). HP-NAP stimulates PMNs to synthesize and release several chemokines, including CXCL8 (interleukin 8), CCL3 (MIP-1α) and CCL4 (MIP-1β) (Polenghi *et al.*, 2007). Because neutrophils rapidly migrate in large numbers at the infection sites, the fact that they serve also as a chemokine source may contribute to the generation of the

conditions necessary for both the recruitment and activation not only of additional neutrophils, via CXCL8, but also of monocytes, dendritic cells, and lymphocytes through CCL3 and CCL4. After crossing epithelial monolayers, HP-NAP is also able to activate the underlying mast cells to release TNF-α and other pro-inflammatory molecules (Montemurro *et al.*, 2002).

The *napA* gene is highly conserved within different isolates of *H. pylori*. The atomic structure of HP-NAP consists of a dodecamer formed by four-helix bundled subunits with a hollow central part, similar to the *E. coli* DNA-binding protein Dps (Zanotti *et al.*, 2002). Dps proteins are a heterogenic family of bacterial stress proteins that are induced during periods of nutrient limitation. However, unlike Dps, which binds DNA, HP-NAP can bind up to 500 atoms of iron per dodecamer. HP-NAP was originally thought to be an efficient bacterial ferritin, based on the nucleotide sequence homologies (Evans *et al.*, 1995). The *nap* gene has several A+T-rich regions of dyad symmetry immediately upstream of its start codon, which could function as Fur-regulated promoters for iron regulation. Similar A+T-rich regions exist upstream of the *H. pylori* ferritin *pfr* gene. Iron is an essential nutrient for the growth of most bacteria, including *H. pylori* and the unique niche inhabited by *H. pylori* is probably very poor in iron: HP-NAP may have a role in iron binding although its expression would not be regulated by the presence or absence of iron and therefore it would not play a major role in the metal resistance of *H. pylori* (Dundon *et al.*, 2002). HP-NAP might have evolved as a peculiar pro-inflammatory molecule able to induce a moderate state of inflammation, which would promote *H. pylori* growth by nutrient factors released from the inflammed tissue.

HP-NAP and innate immunity

HP-NAP and IL-12, IL-23 production in neutrophils, monocytes and dendritic cells

To address whether HP-NAP had any impact on the host innate immune response the ability of HP-NAP in stimulating the IL-12, IL-23, and TNF-α production by different cell types, as neutrophils, monocytes and dendritic cells (DC) has been evaluated. Stimulation of neutrophils, monocytes and dendritic cells with HP-NAP resulted in prompt and remarkable up-regulation of cytokines mRNA

expression and protein secretion, including IL-12p35 and IL-12p40, that pair to form the active IL-12 molecule, and IL-23p19, which assembles with the IL-12p40 chain to form the IL-23 heterodimer. The kinetics and cytokine levels were rather different between monocytes, and neutrophils, the former cells being in general more efficient in their expression of IL-12 p40 and IL-23 p19 mRNAs (Fig. 1) (Amedei *et al.*, 2006). Dose-response experiments demonstrated that HP-NAP, as many bacterial products, acts via Toll-Like Receptor (TLR), being able to activate NF-kB after TLR2 activation, whereas it was inactive on other TLRs (Amedei *et al.*, 2006; Takeda *et al.*, 2003; Napolitani *et al.*, 2005). These findings suggest that HP-NAP, by acting on both neutrophils and monocytes, significantly contributes to induce a IL-12 and IL-23 enriched *milieu*, which has the potential of driving the differentiation of antigen-stimulated T cells toward a polarized Th1 phenotype (Trinchieri *et al.*, 2003; Oppmann *et al.*, 2000). Besides HP-NAP other *H. pylori* factors may contribute to the generation of the Th1-type milieu found in the gastric mucosa of *H. pylori* infected subjects (D'Elios *et al.*, 1997). The products of the *cag* pathogenicity island might be involved in activation of IL-12 expression considering that IL-12 expression is reduced by 50% when an isogenic *cagE* mutant is used (Guiney *et al.*, 2003). Other factors, which might be involved in IL-12 induction and Th1 polarization, include different *H. pylori* gene products, such as those of the plasticity region, frequently isolated from patients affected with gastric adenocarcinoma or peptic ulcer, the outer membrane protein 18, and the cysteine-rich protein A (de Jonge *et al.*, 2004, Rathinavelu *et al.* 2005, Deml *et al.*, 2005; Kranzer *et al.* 2005).

HP-NAP and Tissue Factor production, MHC class II up-regulation in monocytes

HP-NAP has been shown to increase the synthesis of tissue factor (TF) and plasminogen activator inhibitor-2 in mononuclear cells. Macrophages TF production and procoagulant activity are cross-regulated by Th1 and Th2 cells: in particular IFN-γ and other Th1 cytokines are required for optimal TF synthesis whereas Th2 cytokines, as IL-4, IL-10 and IL-13, are inhibitory (Del Prete *et al.*, 1995; D'Elios & Del Prete 1999). By inducing the coordinate expression of procoagulant and

antifibrinolytic activities, HP-NAP might favour fibrin deposition and contribute to the Th1 inflammatory response of gastric mucosa elicited by *H. pylori* (Montemurro *et al.*, 2001; D'Elios *et al.*, 1997).

HP-NAP activity on monocytes resulted not only in strong upregulation of Th1-polarizing cytokines and TF production, but also in the induction of a progressive and consistent maturation process of monocytes into mature dendritic cells showing high expression of HLA-DR, CD80, and CD86, longer survival, tendency to cluster and to detach from the substrate (Amedei *et al.*, 2006). Furthermore HP-NAP is able to significantly up-regulate the MHC class II expression of human macrophages *in vitro* (de Bernard *et al.*, unpublished) (Fig. 2). A previous study showed that *H. pylori* induced dendritic cells to release IL-6, IL-8, IL-10 and IL-12, and represented a maturation stimulus for human DCs (Kranzer *et al.*, 2004). However, the bacterial factor responsible for such effects was at that time not identified: HP-NAP can represent that factor, given its ability to mimic all the effects induced by *H. pylori*.

HP-NAP and adaptive immunity

HP-NAP and gastric Th1 polarized response

In infectious diseases T helper cells (Th) orchestrate host defence against pathogens via different types of cytokine secretion patterns 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 (Del Prete *et al.*, 1991a; D'Elios & Del Prete 1999). Th0 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, mRNAs occur *in vivo* in the antrum (D'Elios *et al.*, 1997; Bamford *et al.*, 1998; Luzza *et al.* 2000 Tomita *et al.*, 2001; Lehman *et al.*, 2002). In the gastric mucosa of *H. pylori*-infected patients a remarkable proportion of Th cells showed significant

proliferation to different *H. pylori* antigens, including HP-NAP (D'Elios *et al.* 1997; Amedei *et al.*, 2006). Upon HP-NAP stimulation, Ag-specific gastric Th cells produced high amounts of IFN-γ and TNF-α, and displayed a powerful cytotoxic activity, thus showing a polarizing Th1 effector phenotype (Amedei *et al.*, 2006).

HP-NAP as Th1 inducer and Th2 down-modulator

In view of its ability to activate the secretion of important Th1-inducers, such as IL-12 and IL-23, it was investigated whether HP-NAP was able to modulate the cytokine profile and effector functions of human T-cell responses. After antigen (Ag) stimulation in the presence or absence of HP-NAP the cytokine profile of Ag-specific T cells was evaluated by ELISPOT. Conditioning of cell cultures with HP-NAP resulted in remarkable increase of IFN-γ-producing T cells and decrease of IL-4 secreting cells. The cytokine profile of Ag-specific Th cells generated in presence or absence of HP-NAP was evaluated at clonal level, using tetanus toxoid (TT) and mite allergen as antigens (Fig. 3). In the series of Th clones from the TT cell lines not conditioned with HP-NAP, 30% clones expressed a Th1 profile, 42% were Th0 producing both IFN-γ and IL-4, whereas 28% were Th2 clones. In contrast, in the series of TT-specific clones from the TT-induced lines conditioned with HP-NAP, 68% were Th1, 29% were Th0, whereas Th2 clones were 3% only (Fig. 3A-3B). In order to assess whether HP-NAP substantially influenced the in vitro development of Th cell responses to allergens usually orienting to the Th2 pattern, allergen-induced T-cell lines were generated from PBMC of house dust mite allergen-sensitive donors and HP-NAP was added or not at the time of allergen exposure. In the series of allergen-specific Th clones not conditioned with HP-NAP, 1% of clone expressed a Th1 profile, 10% were Th0 producing both IFN-γ and IL-4, whereas 89% were Th2 clones. In contrast, in the series of allergen-specific Th clones conditioned with HP-NAP, Th1 and Th0 clones were 38% and 33%, respectively, whereas Th2 clones were 29% only. Addition in culture of HP-NAP, resulted in a shift from preferential Th2 to predominant Th1 T cell responses,

with remarkable expansion of IFN-γ-producing T-cell clones and strong reduction of allergen-specific clones with Th2 profile (Fig. 3C-3D).

Of note, HP-NAP did have 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. To assess the cytokine profile of these bystander clones, generated from T-cell lines induced with or without HP-NAP, they were stimulated with PMA plus anti-CD3 mAb. In the series of bystander clones not conditioned with HP-NAP none expressed a Th1 profile, whereas 20% and 80% were Th0 and Th2, respectively. In contrast, bystander Th clones, generated from the T-cell lines conditioned with HP-NAP, Th1 and Th0 clones were 47% and 37%, respectively, whereas 16% only belong to the Th2 phenotype. Therefore, conditioning with HP-NAP resulted in significant reduction of Th2 clones and shifting to Th1 and Th0 of the development of the cytokine profile of bystander clones grown in the context of allergen-induced T-cell lines. Since cytolytic activity is a property of some activated Th, usually missing in Th2 clones (D'Elios & Del Prete, 1999), the cytolytic potential of Th clones generated in the presence or absence of HP-NAP was investigated (Amedei *et al.*, 2006). At an effector to target ratio of 5:1, the majority of CD4 clones generated from allergen-induced T-cell lines not conditioned with HP-NAP were non-cytolytic whereas few clones only (of the Th0 phenotype) were cytolytic. In the series of Th clones derived from T cell lines conditioned with HP-NAP, a significant proportion of Th clones expressed cytolytic activity and TNF-α production, whereas the non-cytolytic Th2 clones were significantly reduced in comparison to clones from lines not conditioned with HP-NAP. Th clones modulated by HP-NAP had higher killing-potential, and the specific ^{51}Cr-release was still detectable at an effector to target ratio of 1:1, implying that the cytolytic "killing license" of those clones was really impressive. Thus, conditioning with HP-NAP resulted in the outgrowth of allergen-specific clones, the majority of which showed cytolytic activity, suggesting that the Th1-immune modulatory effect of HP-NAP does not merely affect the T-cell cytokine production, but also triggers the expression of the cytotoxic program, a property of fully Th1-polarized effector T cells (D'Elios & Del Prete 1999; Amedei et al., 2006,).

Concluding remarks: HP-NAP, the "Th1-activating protein of *H. pylori*"

In *H. pylori* infection the bug induces an inflammatory response in the gastric mucosa characterized by polymorphonuclear and mononuclear cell infiltration. A polarized Th1 response occurs in the stomach of infected individuals and is associated with a more severe disease. Different factors (related to bacterium, host, environment, age, sex, other concomitant or previous infections) influence the type of host gastric immune responses. HP-NAP is a key factor in gastric inflammation induced by *H. pylori* (Fig. 4). HP-NAP could first attract and activate neutrophils to produce IL-12 and IL-23. Then those two Th1-polarizing cytokines together with HP-NAP and other *H. pylori* factors stimulate IL-12 and IL-23 production and secretion by macrophages and dendritic cells and drive gastric T-cell response towards a Th1 type of immune response, characterized by huge IFN-γ production and activation of cytolytic program with consequent gastric damage. Predominant activation of Th1 cells, with high levels of TF, IFN-γ, and TNF-α might result respectively in procoagulant activity and increase of gastrin secretion and pepsinogen release. A strong long-lasting Th1 polarized response would lead to gastric immunopathology if not fine tuned by appropriate regulatory mechanisms. On the other hand, in other infectious and non-infectious immunopathological conditions, extreme polarization of Th2 cell responses are responsible for disease. These Th2-dominated diseases, such as helminth infection or atopy, or conditions in which an effective Th1 response is desired (vaccinology and cancer immunotherapy), could benefit from a powerful Th1-polarizing signal, like HP-NAP. This signal could boost the Th1-polarizing capacity of DCs and re-direct the predominant Th2 cell response into a balanced, less pathogenic mixed Th1/Th2 response. Given its Th1-promoting activity, HP-NAP might represent a new tool for immunotherapeutic protocols.

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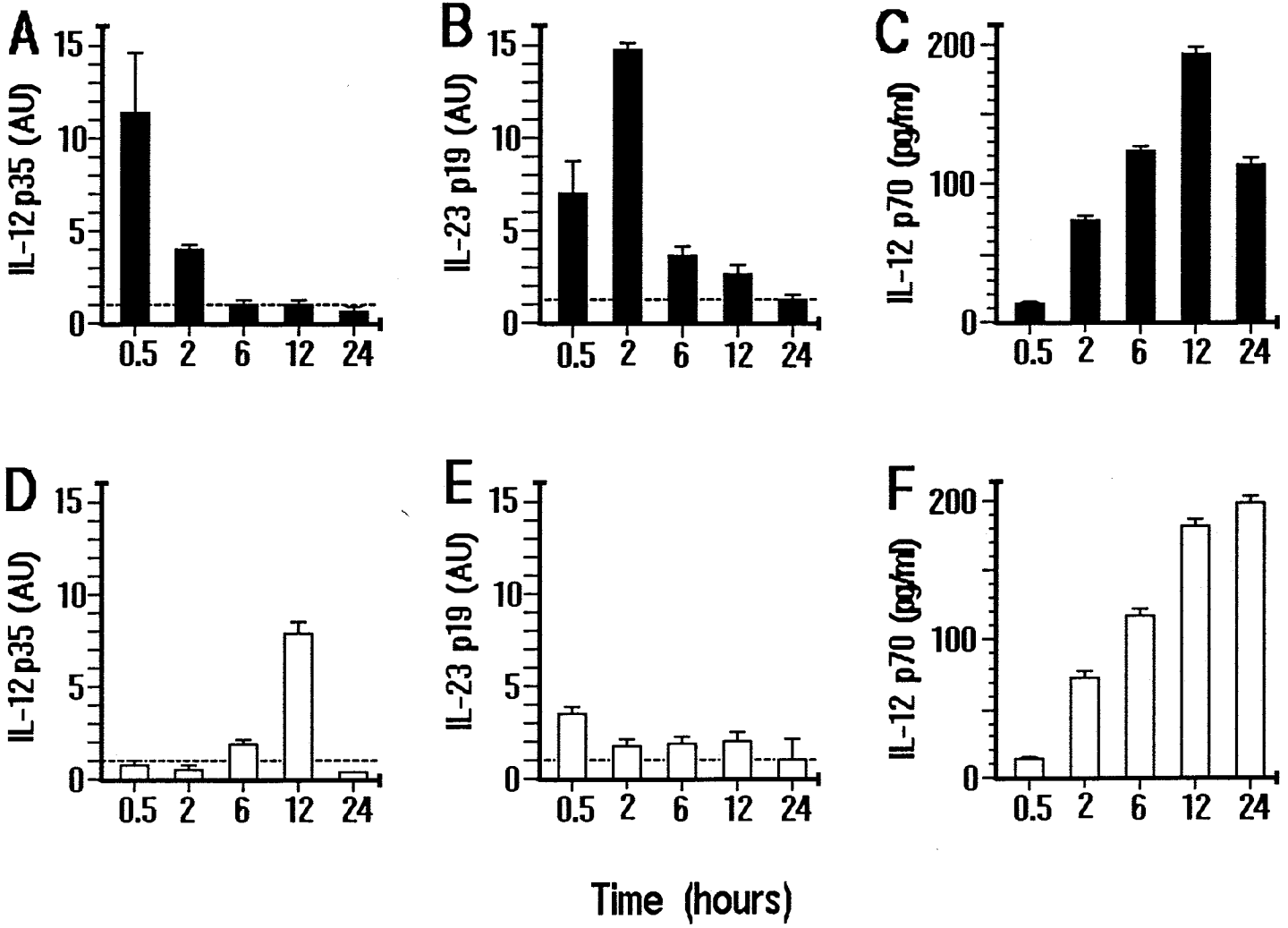
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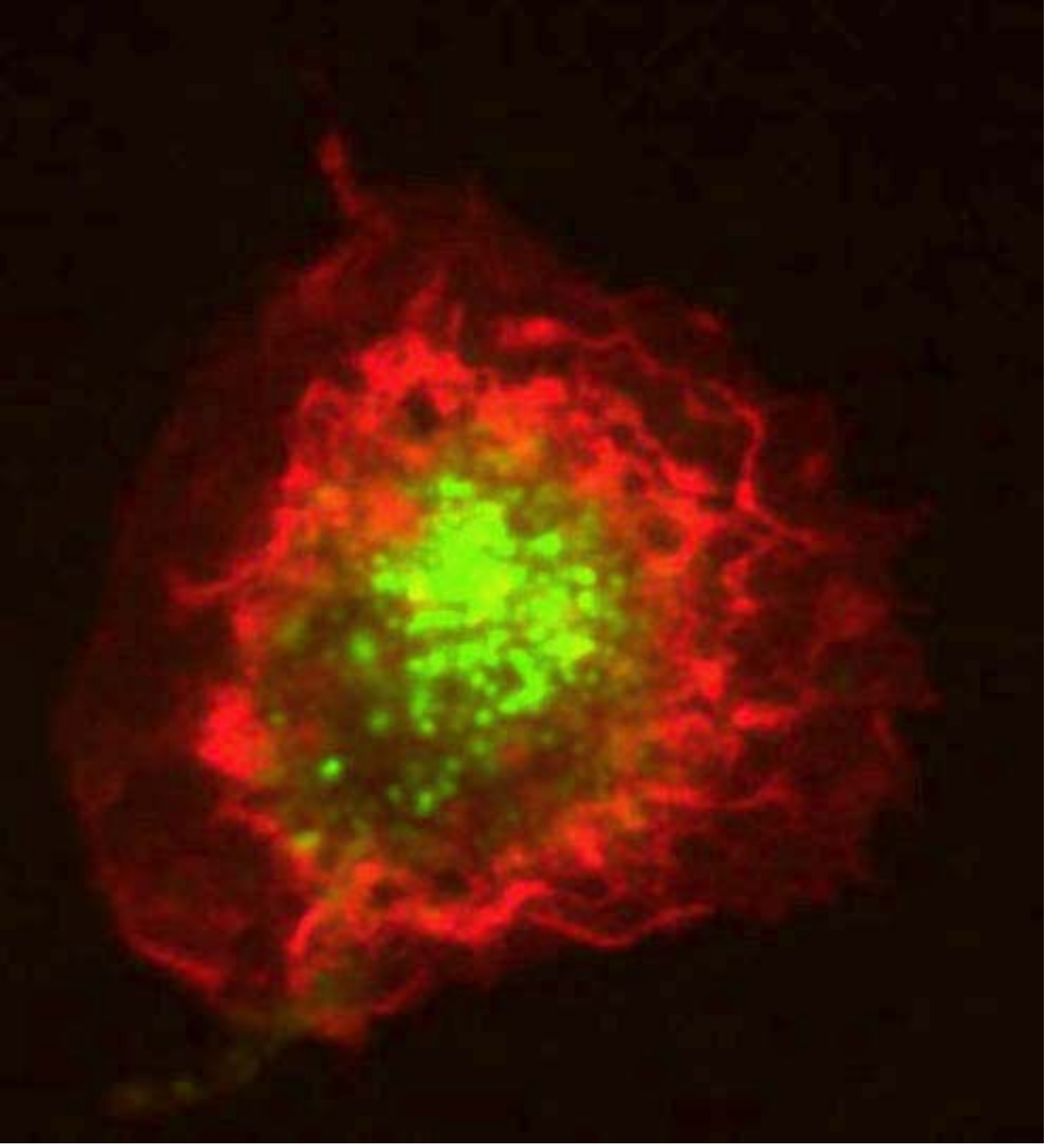
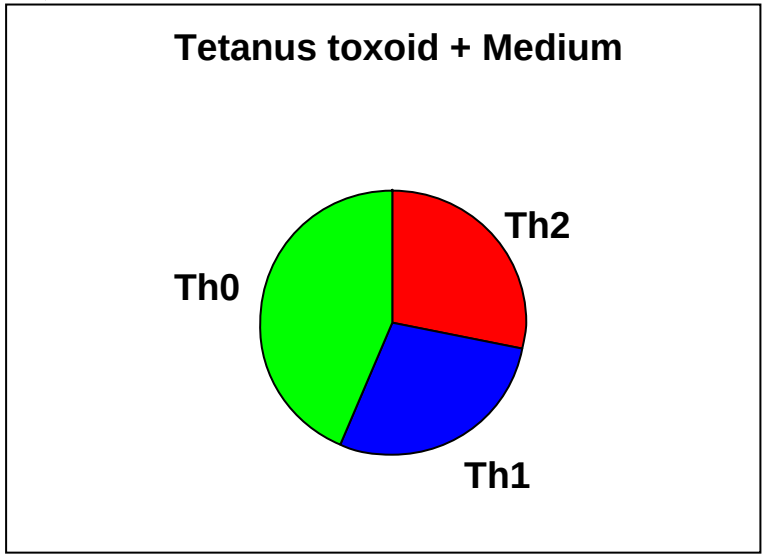
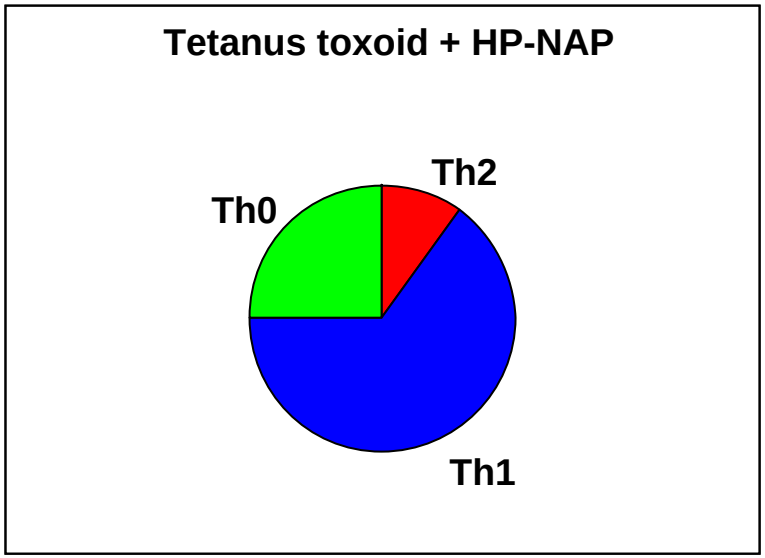
24
Time (hours)



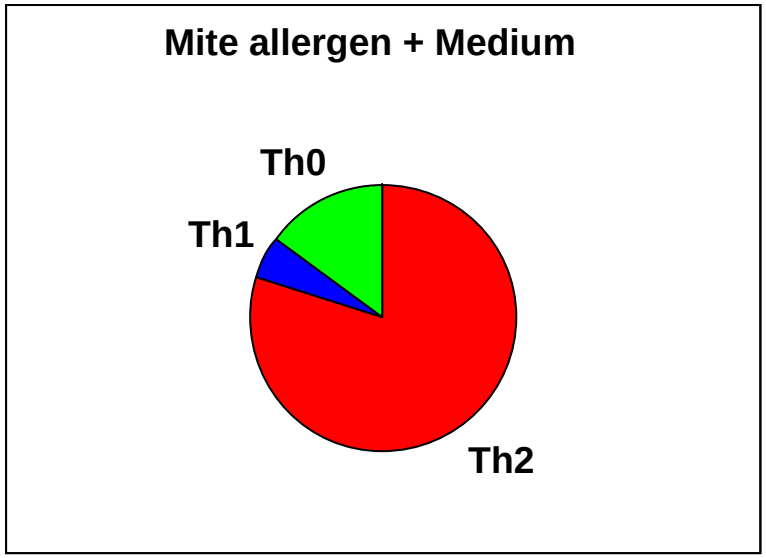
A)


Tetanus toxoid + Medium
Th2
Th0
Th1

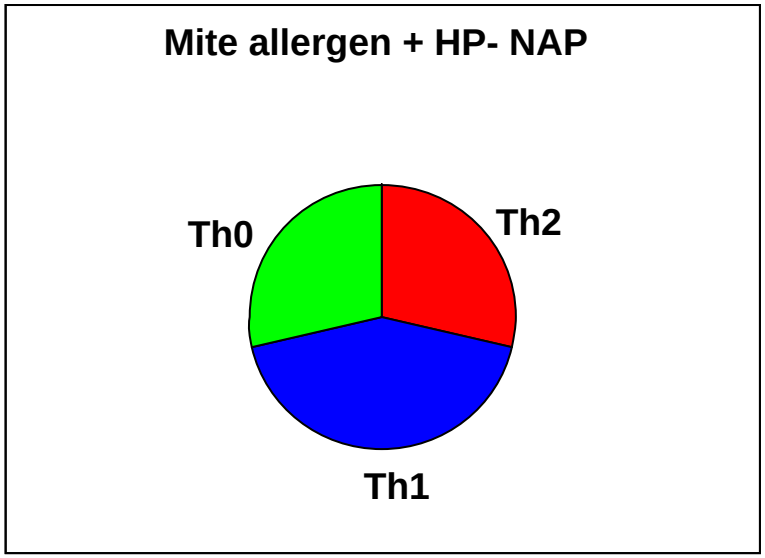
B)


Tetanus toxoid + HP-NAP
Th2
Th0
Th1

C)


Mite allergen + Medium
Th0
Th1
Th2

D)


Mite allergen + HP- NAP
Th0
Th2
Th1

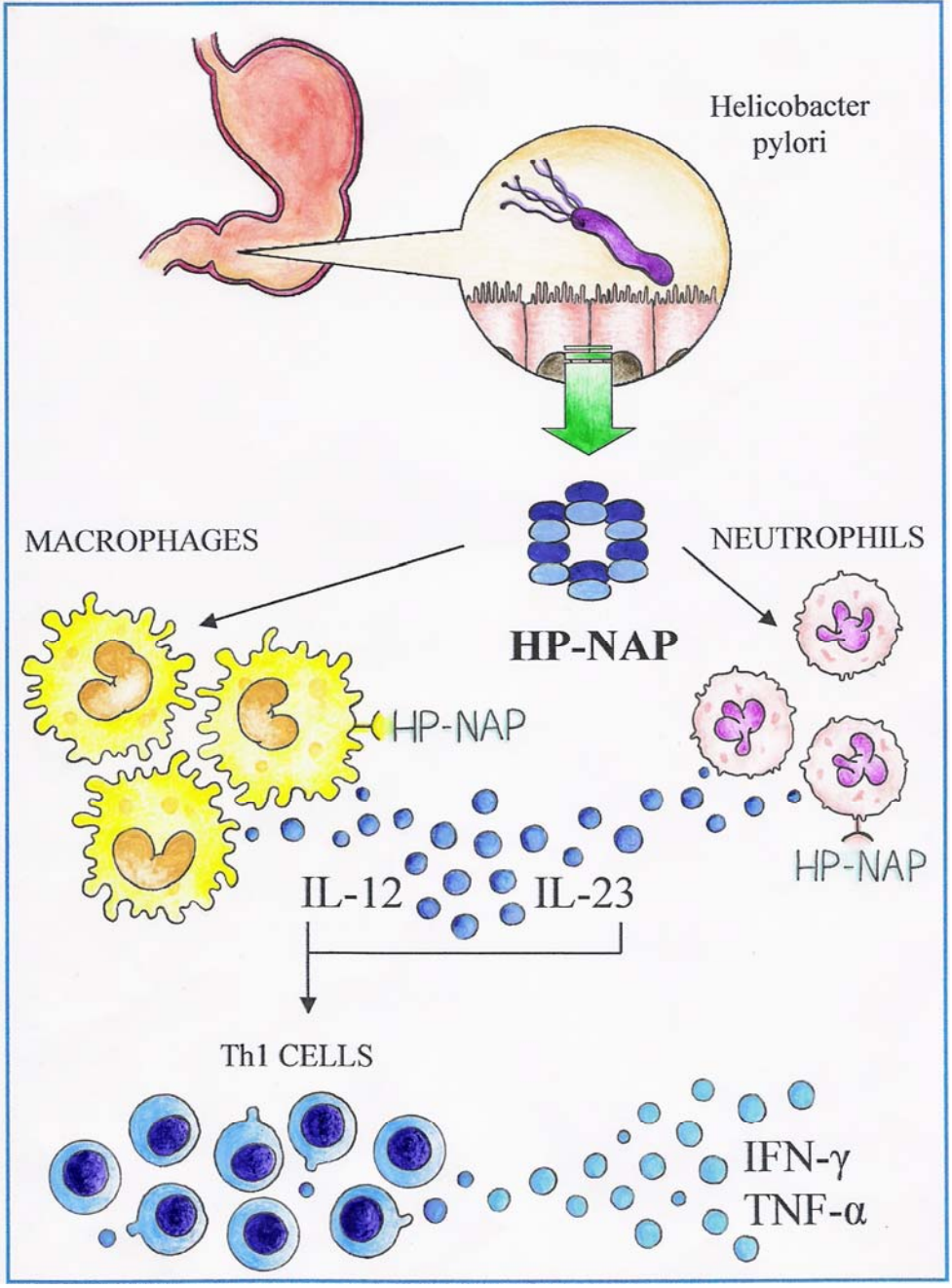

Helicobacter
pylori
MACROPHAGES
NEUTROPHILS
HP-NAP
HP-NAP
HP-NAP
IL-12
IL-23
Th1 CELLS
IFN-γ
TNF-α

Figure legends

Figure 1

Kinetics of cytokine mRNA levels and IL-12p70 production in neutrophils and monocytes stimulated with HP-NAP. Cytokine mRNAs in monocytes (A, B) and neutrophils (D, E) were determined by quantitative real time PCR. The experiment shown is one representative out of seven experiments conducted with different cell preparations. (AU, arbitrary units). The dotted line represents the level of cytokine mRNA produced by mock cells. IL-12p70 protein levels were measured in the culture supernatants of the same monocytes (C) and neutrophils (F) harvested for messengers evaluation. Levels were assessed by a specific ELISA method. IL12p70 protein levels at time 0 were under the lower limit of sensitivity of the assay (7.8 pg/ml). The kinetics of production were comparable among different experiments whereas the amounts varied among different donors.

Figure 2

HP-NAP promotes MHC class II expression by activated macrophages. Confocal microscopy of MHC class II expression (red fluorescence) by HP-NAP (green fluorescence) activation in macrophage.

Figure 3

Conditioning with HP-NAP promotes the Th1 polarization of antigen-specific T cells.

Tetanus toxoid (TT)- or mite allergen-induced T-cell lines were generated in the presence (B, D) or absence of HP-NAP (A, C). T-cell blasts of each line were then cloned and antigen-specific T cell clones were stimulated for 48 hours with medium or the appropriate antigen in the presence of irradiated autologous APC. Culture supernatants were then collected and assayed for their IFN-γ and IL-4 content. Clones able to produce IFN-γ, but not IL-4, were categorized as Th1, clones

producing IL-4, but not IFN-γ, were coded as Th2, whereas clones producing both IFN-γ and IL-4 were categorized as Th0. Results represent mean percent proportions of CD4 clones with the indicated cytokine profile obtained from series of three T-cell lines for each condition.

Figure 4

Schematic representation of HP-NAP Th1-polarizing activities in *H. pylori* infection.

Following *H. pylori* infection of the gastric antrum HP-NAP firstly attracts and activates neutrophils to produce IL-12 and IL-23 together with CXCL8, CCL3, and CCL4. HP-NAP also triggers macrophages to produce IL-12, IL-23, and other Th1-polarizing cytokines. This cytokine "milieu" together with HP-NAP and other *H. pylori* factors promote the preferential development of *H. pylori*-specific Th1 cells secreting IFN-γ and TNF-*a*.