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VacA and HP-NAP, *Ying and Yang* of *Helicobacter pylori*-associated gastric inflammation

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Abstract

Background: *Helicobacter pylori* is a Gram negative bacterium which infects almost half of the population worldwide and represents the major cause of gastroduodenal pathologies, such as duodenal and gastric ulcer, gastric cancer, B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) and autoimmune gastritis. **Methods:** *H. pylori* colonization is followed by infiltration of the gastric mucosa by polymorphonuclear cells, macrophages and lymphocytes. Two of the major *H. pylori* virulence factors are the vacuolating cytotoxin (VacA) and the *Helicobacter pylori* neutrophil-activating protein (HP-NAP). VacA has been proposed as a modulator of immune cell function because of its capacity to interfere with antigen presentation and to inhibit T cell activation. HP-NAP was designated as neutrophil activating protein because it stimulates high production of oxygen radicals from neutrophils. We have recently demonstrated that HP-NAP is able to recruit leukocytes *in vivo* and to stimulate either neutrophils or monocytes to release IL-12, a key cytokine for the differentiation of naïve Th cells into the Th1 phenotype. **Conclusion:** Altogether these evidences indicate that both VacA and HP-NAP play a major role in generating and maintaining the gastric inflammatory response associated with the *H. pylori* infection.

Keywords: *Helicobacter pylori*, inflammation, VacA, HP-NAP, mucosal immunity

1. Introduction

More than 50% of the entire human population is infected with *Helicobacter pylori*, and the prevalence of infection peaks >90% in some African and Asian regions [1]. This microaerophilic Gram-negative bacterium is a strictly human pathogen which localizes in the stomach mucosa. *H. pylori* has been associated with chronic superficial gastritis (inflammation of the gastric mucosa), chronic active gastritis (gastric mucosa inflammation with polymorphonuclear cell infiltration), peptic ulcers and gastric cancers [2,3].

H. pylori colonization is typically followed by infiltration of the gastric mucosa by polymorphonuclear leukocytes, macrophages and lymphocytes [4,5]. A strong correlation exists between gastric infiltration by neutrophils (PMNs), mucosal damage, and development of duodenal ulcer disease in *H. pylori* infections [6,7].

A number of *H. pylori* virulence factors have been characterised so far. The molecular mechanisms of action of a vacuolating cytotoxin, termed VacA, and its possible roles in the *H. pylori*-associated gastro-duodenal diseases will be discussed in the first part of this paper. The role of the Neutrophil-Activating Protein HP-NAP in inducing and maintaining the inflammatory status associated with the *H. pylori* infection will be dealt with in the second part of the paper.

2. The Vacuolating Cytotoxin A (VacA)

2.1 Structure and diversity

Soon after the discovery of *H. pylori* it was reported that a protein in *H. pylori* broth culture filtrates could cause the formation of large vacuoles in the cytoplasm of cultured mammalian cells [8]. The bacterial protein responsible for this effect (named vacuolating cytotoxin VacA) is encoded by a chromosomal gene known as *vacA*. The *vacA* gene encodes a pro-toxin of 140 kDa; both an amino-terminal signal sequence (s) and a carboxy-terminal fragment are proteolitically cleaved during the process of VacA secretion and an 88 kDa mature toxin is exported [9,10]. Mature toxin molecules are released into the extracellular space, or they may be retained on the surface of the

bacterium [11]. The single polypeptide forming the mature VacA tends to be nicked with the generation of a N-terminal 34 kDa (p37) and a C-terminal 54 kDa (p58) fragment [12], which remain non-covalently associated (Fig. 1). VacA is purified from the culture supernatant as an oligomer with a mass >900 kDa [9]. Electron microscopy shows individual VacA oligomers as flower-shaped complexes about 30 nm in diameter (Fig. 2), thought to be composed of one or two rings, each comprised of 6-7 VacA monomers [12,13]. This oligomeric form of VacA is poorly active, but it is strongly activated upon short exposure to acidic or alkaline pH *milieu* [14,15]. Such a treatment leads to the disassembly of the oligomer into monomers (Fig. 2) and to the exposure of surface hydrophobic patches, capable of mediating the insertion of the toxin into biological membranes with the formation of anion-specific channel [16-18].

There is considerable, either quantitative and qualitative, variation in the VacA released by various *H. pylori* strains [19,20]. Signal sequences can be grouped into at least three different types (s1a, s1b and s2) and another highly divergent segment, referred to as the m-region, is present within p58 (Fig. 1). Atherton and colleagues [19] have divided VacA isoforms into two main groups: m1 and m2. s1/m1-positive strains generally produce high levels of VacA protein and are highly toxigenic in the standard HeLa cell vacuolation assay [21]. The s2 type signal sequence appears to be poorly efficient since most strains containing it fail to release the toxin [19]. Strains which express an s1/m2 toxin produce large quantities of toxin, which assembles into the correct structure, but which has no activity on HeLa cells. These VacA isoforms do vacuolate other cell lines in culture, and this differential activity is linked to differences in the nature and/or availability of cell surface receptors [22].

2.2 VacA modulates epithelial cell function

Vacuolization and intracellular trafficking alteration

The first documented effect of VacA was its ability to induce cell vacuolization (Fig. 2). Water-soluble VacA oligomers are relatively inactive in inducing vacuolization, but acid or alkaline

activation of these complexes markedly increases VacA activity [14,15]. VacA-induced vacuoles are acidic and can accumulate membrane permeable weak bases, including dyes such as neutral red or acridine orange [23,24]. Vacuoles are acidic and derive from late endocytic compartments [25,26]; their biogenesis is strictly dependent on the presence of an active small GTPase rab7 [27]. Electron microscopy reveals that vacuoles in biopsies of stomach mucosa of *H. pylori*-infected patients and of HeLa cells in culture are largely devoid of the large array of multivesicular bodies, characteristic of late endosomes (LE) and lysosomes (LY) [8,23,24]. It appears that VacA induces a large rearrangement of the organization of LE and LY, with extensive SNARE-independent membrane fusion and swelling [28]. Vacuolization is strictly dependent on the channel forming ability of the toxin: indeed it is completely abrogated by the chloride channel inhibitor NPPB [18]. In the absence of weak bases, VacA does not cause vacuole formation but still causes detectable alteration in late endocytic compartment. These alterations include inhibition of the intracellular degradation of epidermal growth factor, inhibition of pro-cathepsin D maturation, associated with mis-targeting of pro-cathepsin D outside the cell [29], and inhibition of antigen presentation [30].

Transcellular and paracellular permeability increase

Addition of VacA to various polarized epithelial monolayers *in vitro* results in a rapid increase of ion conductivity, which can be measured as a drop in trans-epithelial resistance (TER). This appears to be due to modulation of the resistance of cell-cell junctions (the paracellular pathway) [31]. This effect of VacA is not accompanied by either vacuolation or inhibition of EGF degradation. TER decrease is not inhibited by bafilomycin A1 and it is not influenced by weak bases, two factors which affect vacuolation, and it is only partially affected by inhibitors of the VacA anion channel [18]. Not only ions but also small neutral molecules with a molecular weight <450 kDa (for example, mannitol and sucrose) exhibit increased transepithelial diffusion upon VacA treatment. It has been suggested that in this way VacA increases the supply of essential nutrients that are necessary for the growth of *H. pylori* on the gastric mucosa [31]. In addition to

altering paracellular permeability, vacA also increases the transepithelial flux of certain molecules, including urea and bicarbonate (Fig. 2). The release of the latter factors from cells is attributed to the formation of VacA channels in the plasma membrane [32].

Alterations in mitochondria and apoptosis

VacA causes alteration in mitochondria, such as reduction of the mitochondrial transmembrane potential and release of cytochrome C [33-36]. Release of cytochrome C from mitochondria and activation of the caspase-3 are pro-apoptotic phenomena and, accordingly, prolonged exposure of AGS gastric cells to VacA induces apoptosis [37] (Fig. 2). Although it has been reported that VacA localized to the mitochondria [34,35], it is not clear whether cytochrome C release results from a direct action of VacA on mitochondria. A recent study demonstrated that VacA is mostly associated with vacuoles rather than mitochondria even in cells in which cytochrome C release is induced, and VacA did not induce cytochrome C release from isolated mitochondria. It appears that VacA-induced cytochrome C release does not necessarily require direct interaction of VacA with mitochondria; rather, an alternative signalling pathway resulting in Bax and Bak activation seems to be important in VacA action [38]. Moreover, VacA-induced apoptosis depends on the channel forming ability of the toxin [35], but is rather independent from vacuolization [38].

2.3 VacA modulates immune cell function

Immune suppressive activity

The antigen processing compartment of antigen presenting cells is highly homologous to late endosomes [39], and VacA was found to profoundly affect the processing of the model antigen tetanus toxin by human antigen presenting cells in such a way that the proliferation of tetanus toxin specific T cells was strongly reduced [30]. This led to the proposal that VacA could exert a local immunodepressive activity within the stomach mucosa. In support to this hypothesis there is the

evidence that VacA inhibits the production of IL-2, required for T cell viability and proliferation, and down-regulates surface expression of IL-2 receptor- α [40,41]. T cell activation and production of cytokines, including IL-2, is mediated by an increase in cytosolic calcium concentration and it is under the control of different transcription factors among which NFAT plays a major role [42]. In resting lymphocytes, NFAT is cytosolic and its translocation into the nucleus follows the dephosphorylation of its nuclear localization signal by calcineurin, a phosphatase activated by calcium [42]. VacA was found to inhibit the activation of NFAT, leaving unaffected the other nuclear factors, and prevented its migration into the nucleus [40,41]. This is due to the plasma membrane formation of anion-specific channels by VacA which prevent calcium influx from the extracellular *milieu* [40].

Pro-inflammatory activity

The effects of VacA on T lymphocytes are expected to result in localized immune suppression, however VacA is also reported to induce pro-inflammatory effects. This is suggested by the finding that VacA activates lymphocytes and mast cells to produce pro-inflammatory cytokines such as TNF and IL-6 [5,43,44]. Moreover it has been reported that VacA stimulated the expression of cyclooxygenase-2, a pro-inflammatory enzyme, in neutrophils and macrophages [40].

Pro-inflammatory activities and immunosuppressive effects are only apparently contradictory. *H. pylori* indeed has evolved a very efficient and fascinating strategy to reside chronically in a particular ecological niche such as gastric antrum. The localised and moderate inflammation of the stomach mucosa caused by the presence of *H. pylori* promotes the release from the tissue of nutrients needed to its growth and this necessity, apparently, cannot be dealt with in other ways, given the very limited permeability of the stomach mucus layer and of the epithelial lining. On the other hand, it can be speculated that inhibition of the local immune response is required for chronic persistence of the bacterium in order not to get killed by the host.

3. The Neutrophil Activating Protein HP-NAP

H. pylori colonization is typically followed by infiltration of the gastric mucosa by polymorphonuclear leukocytes, macrophages and lymphocytes [5,45]. A strong correlation exists between gastric infiltration by neutrophils (PMNs), mucosal damage, and development of duodenal ulcer disease in *H. pylori* infections [7,46]. A major pro-inflammatory factor produced by *Helicobacter pylori* is HP-NAP [47]. It is a dodecameric protein of 150 kDa with a structure similar to bacterioferritins, including a central cavity for iron accumulation [48,49]. It was originally defined as PMNs-activating protein because it stimulates PMNs to produce reactive oxygen radicals [50]. In addition, HP-NAP crosses the epithelia and becomes in close contact with inflammatory cells resident in the tissue following inflammation [51]. HP-NAP stimulates the synthesis of tissue factor and the secretion of type 2 plasminogen activator inhibitor by monocytes and macrophages [47,52].

3.1 HP-NAP crosses endothelia to promote neutrophil adhesion in vivo

The possibility that HP-NAP directly stimulates PMNs to adhere derived from several studies [50,53]; in the former study, however, protein and PMNs were assayed together without evaluating whether HP-NAP was actually capable of crossing the endothelium to come in contact with polymorphonuclear cells. The latter study demonstrated that HP-NAP activates PMNs even when they are separated by an endothelium grown on a transwell support, but did not consider the under-flow conditions which are of great relevance *in vivo*. Recently, we have reported that HP-NAP promotes leukocyte adhesion to the endothelium *in vivo*. Intravital microscopy, used in our study to analyze the mobilization of leukocytes in response to HP-NAP, allowed us to show that leukocytes circulating in the rat mesentery begin to adhere rather rapidly to the endothelium of the postcapillary venules after the topical application of HP-NAP (Fig. 3). This effect is directly mediated by HP-NAP, which induces a high affinity state of integrins on PMNs, by recruiting a signalling cascade that involves p38-MAPK. Moreover, we demonstrated that human PMNs

respond to stimulation with HP-NAP by expressing and producing several chemokines, including CXCL8, CCL3 and CCL4. Because neutrophils rapidly migrate in large numbers at the infection sites, the fact that they also serve 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 [54] (Fig. 3).

3.2 HP-NAP promotes Th1 immune responses

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 [5,55-58]. 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 [5,59]. We have recently demonstrated that upon HP-NAP stimulation, Ag-specific gastric Th cells produces high amounts of IFN- γ and TNF- α , and displays a powerful cytotoxic activity, thus showing a striking Th1 phenotype. We have also found that HP-NAP itself is responsible for the modulation of the cytokine profile and effector functions of human T-cell responses, being able to activate the secretion of powerful Th1-inducers, such as IL-12 and IL-23. Indeed, when allergen-induced T cell lines, typically Th2 oriented, are generated in the presence of HP-NAP a significant Th2 to Th1 redirection is observed [59] (Fig. 4).

4. Conclusions

H. pylori lives within the human host from long time ago, approximately 100000 y. It can be speculated that the bacterium has succeed in creating a comfortable habitat in the antral niche, trying to be as less dangerous as possible for its human host. Since the first encounter in Africa with the *Homo sapiens*, *H. pylori* has evolved several mechanisms to get nutrients from the host, e.g. by inducing moderate inflammation, and at the same time to escape the immune surveillance. VacA

and HP-NAP prototypically represent very well *H. pylori* in their ability of displaying either pro- and anti-inflammatory actions. HP-NAP strongly contributes to the onset and maintenance of gastric inflammation. Soon after *H. pylori* reaches the stomach, HP-NAP induces extravasation and migration to the lamina propria of leukocytes. HP-NAP activates high production of oxygen radicals from neutrophils and stimulates the synthesis of tissue factor and the secretion of type 2 plasminogen activator inhibitor by monocytes and macrophages. Moreover HP-NAP promotes in neutrophils and monocytes the production of IL-12 and IL-23, two key cytokines that either stimulates the development of T helper 1 cells and inhibits the T helper 2 type of inflammation. VacA, one of the major virulence factors of *H. pylori*, induces vacuolization of gastric epithelial cells and is able to exert either pro-inflammatory and immune suppressive effects. On one hand VacA stimulates the expression of cyclooxygenase-2 in neutrophils and macrophages and activates lymphocytes and mast cells to produce pro-inflammatory cytokines. On the other hand VacA is able to profoundly interfere with antigen presenting cells, by inhibiting the II-type of antigen presentation. Moreover VacA inhibits the activation of NFAT, and consequently the production of IL-2, required for T cell proliferation, and down-regulates surface expression of IL-2 receptor- α . All together these evidences indicate that VacA and HP-NAP represent two crucial components of the *Ying & Yang* strategy that *H. pylori* has evolved in the course of millennia to reside chronically in the human gastric antrum

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Figure legends:

Figure 1: vacA gene structure and allelic diversity. A) The amino-terminal signal sequence and carboxy-terminal domain are cleaved from the 140 kDa VacA protoxin to yield a 88-kDa mature toxin that is secreted into the extracellular space via an autotransporter mechanism. The mature protein can undergo limited proteolytic cleavage to yield 2 fragments-p37 and p58-which remain linked through non covalent forces. B) There is a high level of diversity among vacA alleles from different *H. pylori* strains. This diversity is particularly striking near the 5' terminus of VacA (the s-region) and in the mid-region of the gene (the m-region). Two main families of s-region sequences (s1 and s2) and 2 main families of m-region sequences (m1 and m2) have been described.

Figure 2: VacA modulates epithelial cell function. After exposure to acidic or alkaline milieu VacA oligomers disassembly in monomers which are able to insert into biological membranes, and to form anionic channels. Such an activity is essential for epithelial cell vacuolization (A), for transepithelial flux of urea and bicarbonate in polarized monolayers (B) and for apoptosis (C). VacA induces also an increase in paracellular permeability (B) which is responsible for the diffusion of ions and small molecules (e.g. sucrose and mannitol). This effect is only partially dependent on the VacA channel activity.

Figure 3: HP-NAP promotes leukocytes recruitment and contributes to sustain inflammation. HP-NAP, after crossing the gastric epithelial lining (1) and the endothelium (2), 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 flogosi status.

Figure 4: HP-NAP promotes Th1 immune responses. HP-NAP following the engagement of the TLR2, stimulate monocytes and dendritic cells to release IL-12 and IL-23. These cytokines, which are potent Th1 inducers, are responsible for the ability of HP-NAP in promoting the preferential development of *H. pylori*-specific Th1 cells secreting IFN- γ .

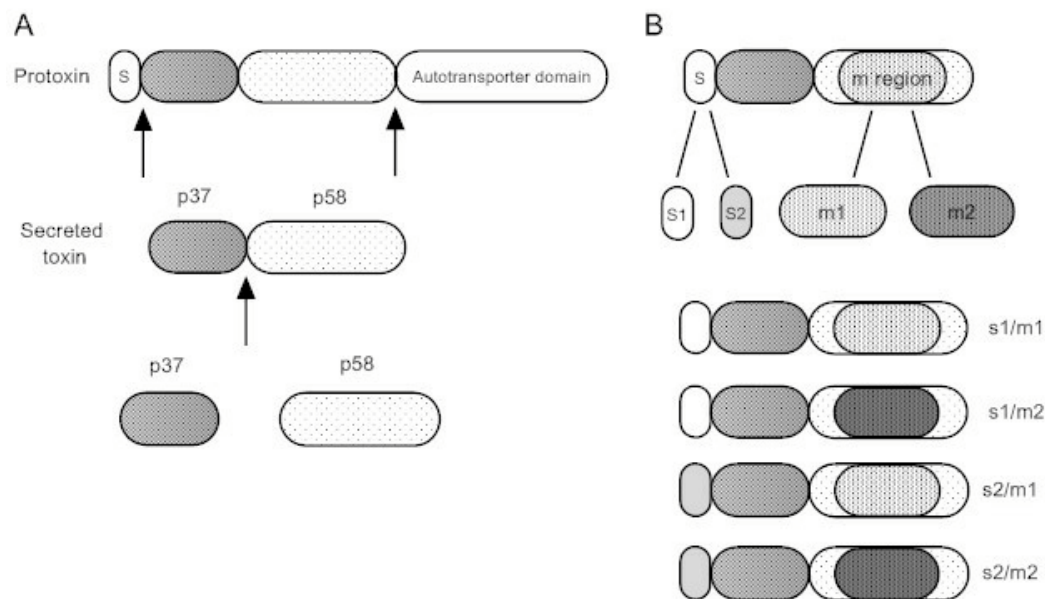


Figure 1

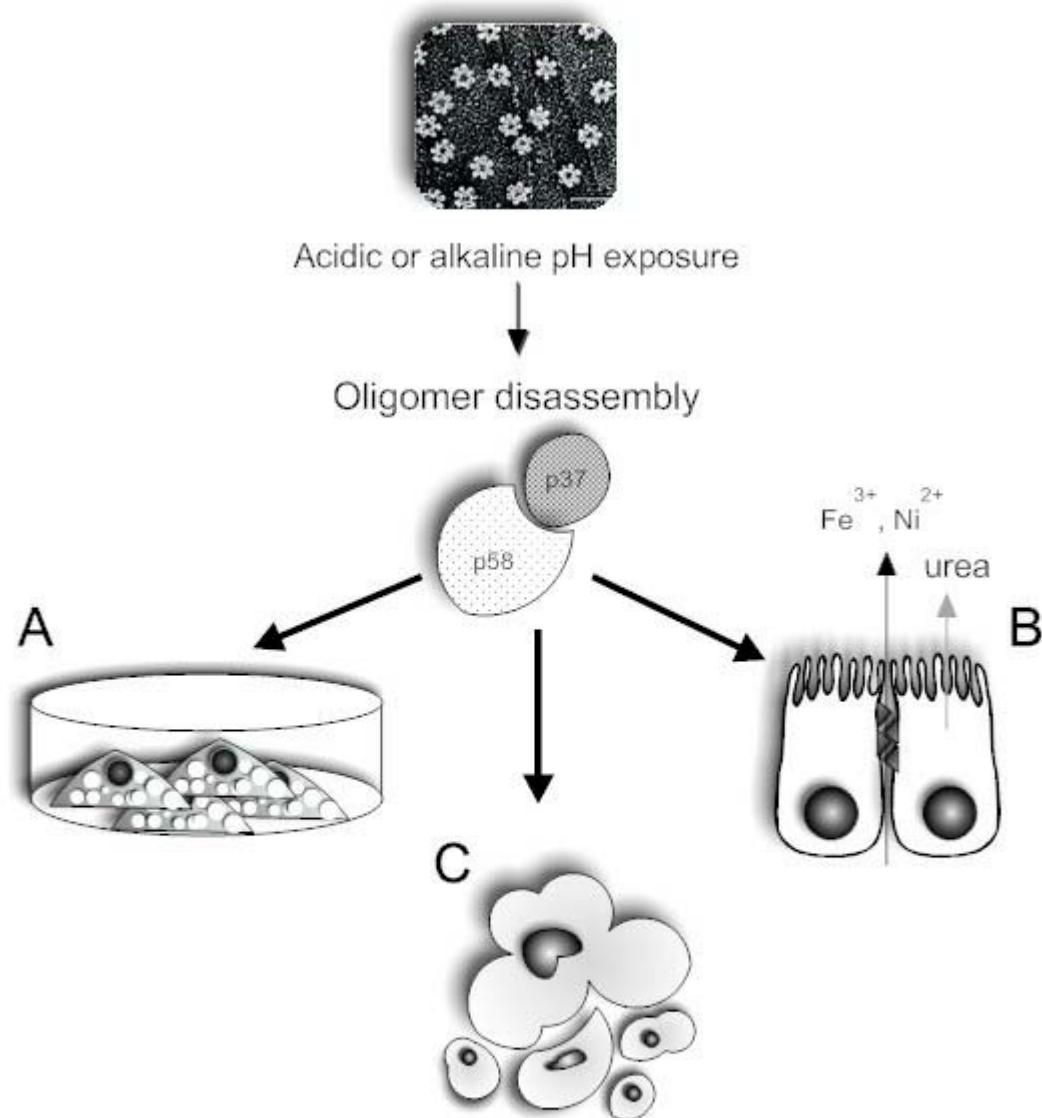


Figure 2

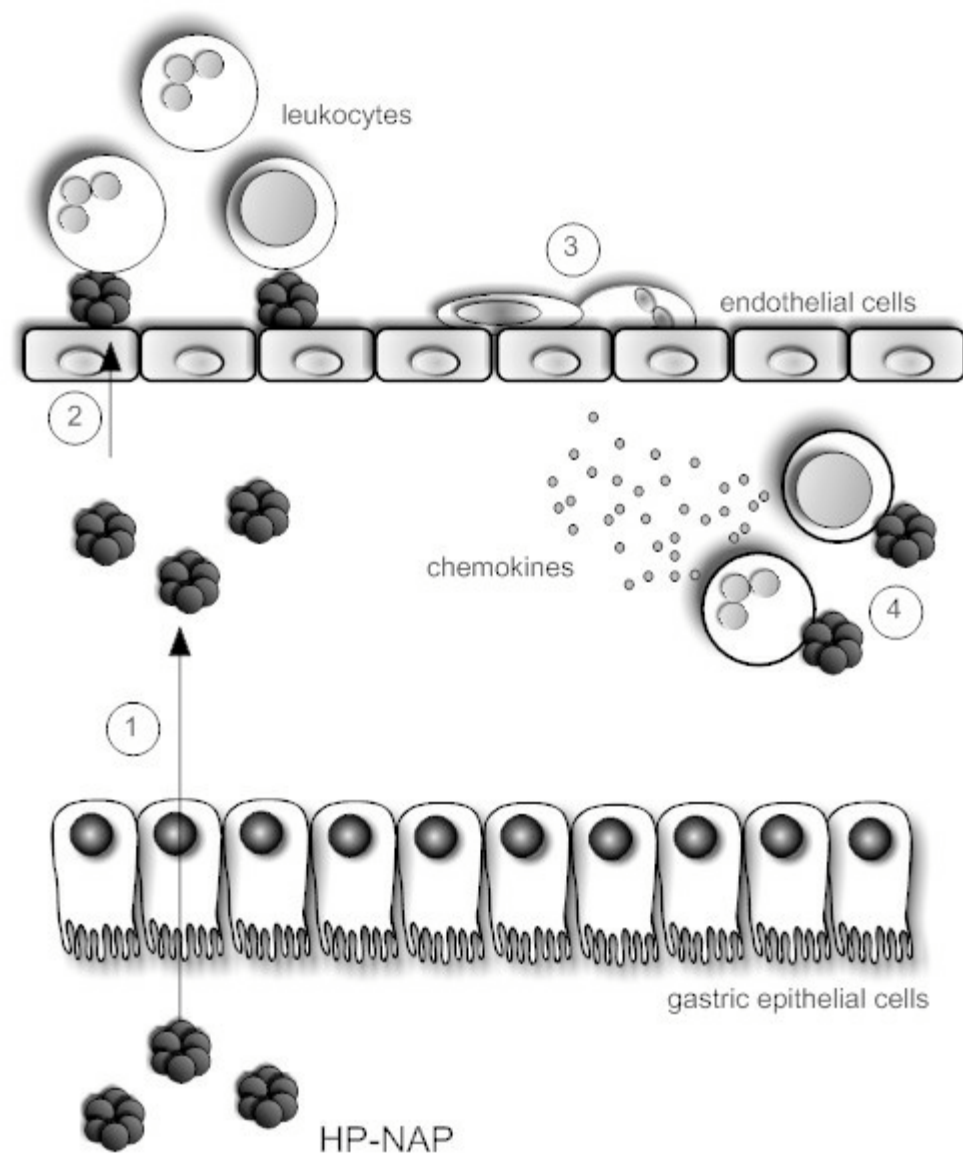


Figure 3

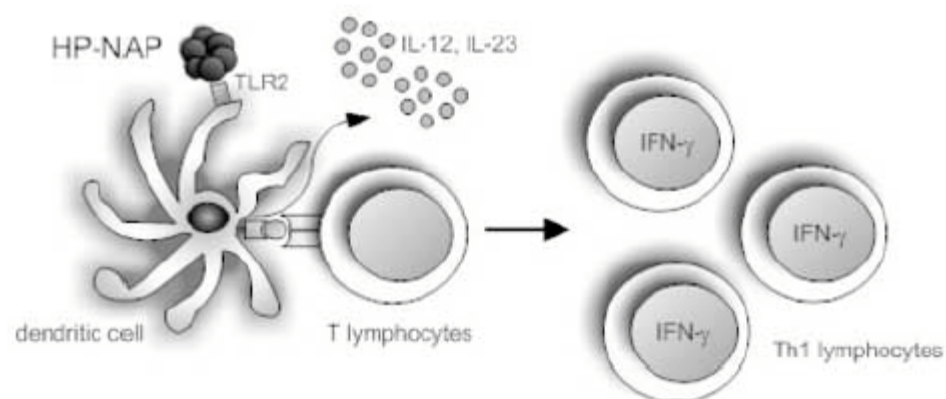


Figure 4