

Pleiotropic cytotoxicity of VacA toxin in host cells and its impact on immunotherapy

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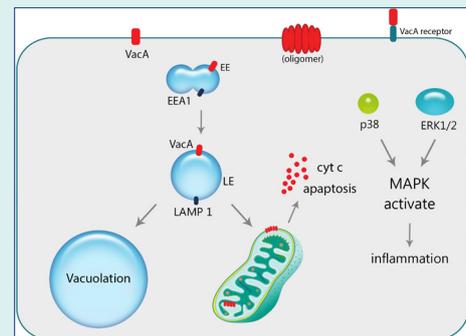
Abstract

Introduction: In the recent decades, a number of studies have highlighted the importance of *Helicobacter pylori* in the initiation and development of peptic ulcer and gastric cancer. Some potential virulence factors (e.g., urease, CagA, VacA, BabA) are exploited by this microorganism, facilitating its persistence through evading human defense mechanisms. Among these toxins and enzymes, vacuolating toxin A (VacA) is of a great importance in the pathogenesis of *H. pylori*. VacA toxin shows different pattern of cytotoxicity through binding to different cell surface receptors in various cells.

Methods: To highlight attempts in treatment for *H. pylori* infection, here, we discussed the VacA potential as a candidate for development of vaccine and targeted immunotherapy. Furthermore, we reviewed the related literature to provide key insights on association of the genetic variants of VacA with the toxicity of the toxin in cells.

Results: A number of investigations on the receptor(s) binding of VacA toxin confirmed the pleiotropic nature of VacA that uses a unique mechanism for internalization through some membrane components such as lipid rafts and glycoposphatidylinositol (GPI)-anchored proteins (GPI-AP). Considering the high potency of VacA toxin in the clinical presentations in infection and assisting persistence and colonization of *H. pylori*, it is considered as one of the pivotal components in production vaccines and monoclonal antibodies (mAbs).

Conclusion: It is possible to generate mAbs with a considerable potential to convert into secretory immunoglobulins that could penetrate into the niche of *H. pylori* and inhibit its normal functionalities. Further, conjugation of *H. pylori* targeting Ab fragments with the toxic agents or drug delivery systems (DDSs) offers new generation of *H. pylori* treatments.



Introduction

Long-term colonization of *Helicobacter pylori* in the human stomach is often associated with the high incidence of development of peptic ulcers, gastric lymphoma, and gastric adenocarcinoma. Gastric cancer is considered as one of the most prevalent malignancies worldwide, especially in the developing countries. It is largely related to the high colonization of *H. pylori*, which shows a high rate of mortality, in large part because of diagnosis at the late stages of the disease.¹⁻³ *H. pylori* is classified as type I

carcinogenic bacteria because more than 60% of the non-cardiac gastric cancers are related to *H. pylori* infection.⁴⁻⁶ Although the presence of *H. pylori* is not enough for the initiation of cancer, it is necessary for such phenomenon. *H. pylori*-associated gastric cancer seems to initiate by the loss of acidic juice secretion due to the persistent atrophic gastritis.⁷

H. pylori can survive and colonize in the human stomach through the functional expression of VacA that is produced by all strains, even though with different levels

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of expression. VacA toxin creates a selective ion channel in order to utilize the electrolyte and metabolites of the host cells for the survival of *H. pylori* in the mucosal layer.⁸ The effects of VacA toxin on the epithelial cells are mediated by diverse mechanisms. Endocytosis of VacA associates with the pattern alteration of intracellular endocytic pathways, resulting in formation of vacuoles, and the impairment of the mitochondrial membrane potential and hence induction of apoptosis.^{9,10} Besides, VacA binds to variable cell surface receptors and triggers several intracellular signaling pathways, in particular, mitogen-activated protein kinase (MAPK)/p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2), which lead to recruitment of cytokines initiating inflammatory response.¹¹⁻¹³

The active VacA toxin is made up of two functional (p33) and binding (p55) domains. Based on sequence diversities in these domains, *H. pylori* is classified into 4 strains, including: s1m1, s1m2, s2m2 and s2m1. These strains were shown to pose different extent of vacuolation in various cells.^{13,14} Depending on the cell type and *H. pylori* strain, the VacA interaction with cells occur through various receptors such as receptor protein tyrosine phosphatase beta (RPTP β), sphingomyelin, epidermal growth factor receptor (EGFR) and heparin/heparan sulfate. Of these, the RPTP β is the main receptor for VacA binding, which guaranties survival of *H. pylori* within the host cells.^{15,16} The influence of cell surface receptors, in particular RPTP β , on the VacA-induced cell vacuolation *in vitro* is still controversial. Collectively, the VacA toxin is considered as a multi-receptor toxin that imposes cellular vacuolization.¹⁷ In addition to the effect of VacA on long-term persistence of *H. pylori*, VacA is deemed to be a marker for gastric cancer diagnosis, which is also a determinant for the initiation of metastasis in infectious patients.^{18,19}

Former reviews on this subject have not covered the exact roles and effects of multiple receptors on binding and *in vitro* cytotoxicity of VacA and its influence on immunotherapeutical approaches. Hence, the foremost goal of the current review is to discuss the genotype of VacA toxin and articulate the cell specific toxicity on epithelial cells elicited by VacA and its potential as a candidate for development of vaccine against *H. pylori*.

Structure and expression of VacA

The *VacA* gene is a monostrophic gene with the length of 3860-3940 base pairs. All strains of *H. pylori* possess the *VacA* gene, but with different levels of expression leading to different degrees of cytotoxicity. Expression level of the VacA toxin can be affected by several factors, including: (a) promoter strength, (b) stem-loop structures at the 3' end of mRNA resulting in mRNA endurance, (c) presence of activators or inhibitor sequences, and (d) amino acid sequence differences found between *H. pylori* strains leading to distinct secretion pattern.^{20,21}

The expression of *VacA* gene produces a 1287 amino acids (aa), a 140 kDa length pre-prototoxin, that is

processed to a mature toxin through two cleavages at the N- and C-terminal ends of the toxin by membrane-associated proteases. Similar to autotransporter proteins (ATs), the first proteolysis is occurred by LepB peptidase at the N-terminal signal sequence (33 aa) responsible for transporting toxin to the bacterial periplasm.²²⁻²⁴ The second proteolysis at the C-terminal domain, which happens simultaneously with the secretion of toxin to the outer membrane, produces a 88 kDa mature toxin, a 12 kDa secreted peptide (linker peptide), and a 33 kDa C-Ter autotransporter domain remaining associated with the bacterial membrane.^{22,25,26}

The complete processing of VacA produces the mature VacA toxin that is considered as an A-B type toxin (Fig. 1).^{27,28} The mature VacA toxin is consisted of p33 and p55 domains, which are essential for its activity and binding to the host cells and is performed by various residues confirmed by several studies (Table 1).

P55 domain

The P55 domain shows different binding specificity to various cells, which is related to a divergent region known as "m region" with the least recombination between strains. Based on the m region, VacA toxin is classified into two types of m1 and m2, which are different in receptor binding and interaction with the host cells as well as the expression level.^{33,34} Unlike the m1 VacA, the m2 VacA has an additional 23 aa insert located at residues of 460-496, a region that has no effect on the toxin cytotoxicity and/or cell specificity.^{5,22}

Differences in the m region contributes to both different degrees of cytotoxicity in various cells and discrete incidence of gastritis and gastric cancer, even though no relation has been observed between the m region and incidence of peptic ulcer in patients.^{5,35,36}

P33 domain

The p33 domain (i.e., residues 1 to 311) is a functional domain of the VacA toxin that causes cell vacuolation through formation of anion selective channels.^{37,38} A hydrophobic 32 aa residual region with α -helix structure is located at the vicinity of the N-terminus of the p33 domain which assumes to be critical for the toxicity of VacA and is designated as s region.³⁹ Further, some researches have confirmed the importance of the transmembrane dimerization sequence consisted of three tandem repeats of GXXXG motifs for the channel and vacuole formation.⁴⁰

Based upon the sequence diversity in the "s region", VacA toxin has been classified into two types of s1 and s2. These two types -like m types- impose the toxicity potential of the toxin via (a) increased expression of the BAbA2 and cagPAI and VacA, (b) enhanced efficiency of signal sequence that facilitates toxin transformation, and (c) elevated capacity of anion selective channels. All these result in phenotypic manifestation of the toxin like peptic ulcer and gastric inflammation.⁴¹ The s1 region shows hydrophobic characteristics that mediates dimerization of toxin leading to channel formation. The s2 region

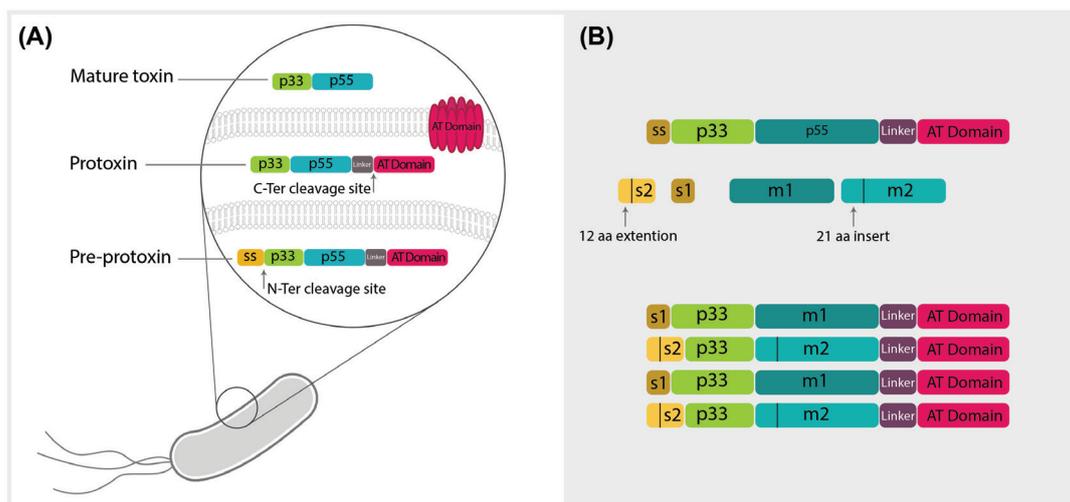


Fig. 1. The molecular structure of vacuolating toxin of *Helicobacter pylori*. A) The vacuolating toxin of *Helicobacter pylori* is expressed as pre-prototoxin inside bacteria containing p33 and p55 domains, a linker domain and autotransporter (AT) domain responsible for toxin secretion from bacteria. Maturation of the toxin occurs through two enzymatic cleavages. First, spontaneous N-ter cleavage site leads to breakage of signal sequence (ss), which is a determinant of directing of toxin to periplasmic space. Next, cleavage of the AT domain occurs simultaneously with the secretion of mature toxin to external environment of bacteria. The linker domain degrades by exposure to the environment and mature toxin is produced including two major regions known as p33 and p55. B) There are two diverse regions inside the mature toxin called s in p33 domain and m in p55 domain. The s region is located in the signal sequence of toxin in p33 with two types of s1 and s2 and an extra 12 amino acid extension sequence. The other divergent portion in p55 is also classified in two types, m1 and m2, with 21 amino acid insertion and shows different cell binding capacity to various receptors. Based on these two portions, VacA is classified into four groups of s1m1, s1m2, s2m2 and s2m1, all of which determine various pathogenicity based on their genotype.

possesses a hydrophilic sequence extension of 12 aa providing a distinct cleavage site from s1, which results in inhibition of cell vacuolation.^{42, 43}

Other polymorphic regions inside VacA

Besides two mentioned divergent regions inside VacA toxin, two additional regions are identified known as intermediate (i) and deletion (d). The i region, which is located between s and m is classified in 3 diverse types known as i1, i2 and i3. This region is considered to be responsible for increased potential of carcinogenesis and expression of CagA,⁴⁴ though one study confirmed that the presence of i is not as efficient as s and m regions on the incidence of disease.⁴⁴⁻⁴⁶ The type of i region is

somehow dependent on the presence of types of s and m, so that presence of s1m1 increases the incidence of i1 to be expressed and result in an increased outcome in severity of the disease.⁴⁷

The deletion of 81 residues between i and m regions is another polymorphic region inside VacA toxin, which is located at the N-terminus of p55 and is considered to be related to the vacuolating activity and binding to host cells, gastric mucosal atrophy and neutrophil infiltration. Despite the influence of s, m and i regions diversity on toxicity of VacA, it seems that different d regions provide the same function(s).⁴⁵

In general, it is believed that various types of VacA toxin are classified based on their polymorphic regions and distributed around the world. They are responsible for various types of diseases with different severity due to their phenotypes expressed and also the effect of host genetic background and environmental effects.⁴⁵

Table 1. Function of various domains of the VacA toxin

Domain	Amino acid sequence	Role of sequence in toxin activity	Ref.
P33	6-27	Vacuolation-apoptosis-oligomerization	27, 29
	1-32	Ion channel formations	30
	49-57	Oligomerization	30
P55	346-347	Vacuolating-depolarization-oligomerization	29
	599-628	Folding and secretion of protein	31
	312-478	Interaction with p33	29
	342-361	Cleavage site	32
	313-478	P33/p55 interaction	32

The function of VacA is not fully restricted to the domains introduced for, and each domain- p33 and p55- is participating in the role of the other domain.

Cell surface receptors for VacA toxin

Receptor-like protein tyrosine phosphatase β (RPTP β)

RPTP β is considered as the major cell receptor for binding of VacA toxin. Similar to inhibitory ligand of RPTP β , pleiotrophin, the VacA prohibits the phosphatase activity of RPTP β via interacting with QTTQP motif located at 747-751 containing two O-glycosylation sites that is distinct from the binding site of pleiotrophin.⁴⁸

Inhibitory effect of VacA on RPTP β leads to vacuolation-independent cytotoxicity through phosphorylation of Git-1 that triggers several intracellular signal transductions

related to pro-inflammation and ulcerogenesis.⁴⁸ Phosphorylation of Git-1 initiates signaling cascades, leading to actin cytoskeleton impairment and focal adhesion segregation known as the first steps in ulcerogenesis.^{49,50} Further, VacA induces the activation of MAPK pathways through the activation of p38 and ERK1/2 messengers, resulting in expression of various chemokines and inflammatory agents specially interleukins.¹²

Several studies have been carried out to investigate whether RPTP β is related to the VacA induced cellular vacuolation. There is somewhat controversy about relation between the presence of RPTP β on the cell surface and the toxicity of VacA. It has been shown that the VacA-induced vacuolation in the RPTP β -positive human promyelocytic leukemia HL-60 cells can be prohibited by antisense oligonucleotide against RPTP β receptor.^{51, 52} Adversely, the vacuolation, induced by VacA on RPTP β expressing rabbit kidney epithelial RK13 cells, was not inhibited by phorbol 12-myristate 13-acetate (PMA). In addition, *in vivo* study with RPTP β -deficient mice (RPTP β -/- strains) indicated that the vacuolation is not dependent on the presence of RPTP β .⁵³⁻⁵⁵ Consequently, RPTP β is not necessary for the vacuolating activity of VacA, but rather for signal induction. Dependency of the vacuolation to RPTP receptors in various cells is shown in Table 2. These findings highlight the necessity of further justification of a pattern for the receptor-dependent vacuolation in different cells.

Receptor-like protein tyrosine phosphatase α (RPTP α)

RPTP α is a 140-KDa protein receptor (p140) recognized on Wilm's tumor G-401 cell line for the first time.⁵⁶ RPTP α , unlike RPTP β , has shorter glycosylated extracellular domain - necessary for the binding to VacA. In fact,

attenuating VacA-induced vacuolation on G401 cells through neuraminidase- α glycosidase treatment indicated the necessity of sialic acid and glycosylation for the binding of VacA.⁴⁹ Early studies showed that the function of RPTP α in signaling cascades related to pathogenesis of VacA is still unclear.⁴⁸

Epidermal Growth Factor Receptor (EGFR)

VacA was shown to be internalized by HeLa cells through EGFR receptor inducing vacuolation. This finding was confirmed through inhibiting the vacuolation by anti-EGFR antibody as well as immunoprecipitation of two protein fragments (p33 and p55) equivalent to the VacA domains with anti-EGFR antibody.⁵⁷ Furthermore, interfering VacA with EGFR-mediated signaling pathways has been shown by ERK1/2 signaling path, actin stress fiber formation, and wound healing. These findings suggest that VacA imposes its impact(s) on HeLa cell through EGFR-mediated signaling pathways leading to inhibition of wound re-epithelialization, renewal of the gastric mucosa and cell proliferation.^{58,59}

Lipid raft/glycosylphosphatidylinositol-anchored proteins (GPI-AP)

Lipid raft as a cholesterol-enriched area acts as an accumulation center in the plasma membrane. It appears to be the main pathway, which enables VacA toxin to form oligomerized structure necessary for the flow of electrolytes and ions.^{39,60} Experiments about low-affinity binding of VacA toxin to the membranes containing low-cholesterol has confirmed the cholesterol-dependent nature of the VacA association to lipid rafts.⁶¹ Furthermore, GPI-AP was shown to be possibly involved with the VacA intoxication through recruitment of GPI-AP-associated VacA by lipid rafts.⁶² Incubation of Hep-2 cells with phosphatidylinositol-specific phospholipase C (PI-PLC) was shown to diminish the sensitivity to VacA, supporting the GPI-AP involvement. Also removal of GPI-AP from HeLa cells has confirmed that the localization of VacA into lipid rafts is not a GPI dependent process, but the flux of chloride ions through the membrane is affected.^{63,64}

There are evidences that the translocation of VacA to lipid raft is important for the vacuolation-dependent cytotoxicity. Further, addition of methyl- β -cyclodextrin (MCD), a -cholesterol depleting drug, to HeLa cells and human gastric carcinoma AZ-521 cells was shown to interfere with the VacA internalization and vacuolation, but not with its binding to cells. Thus, it can be conquered that lipid rafts are necessary for the intoxication process. Also, treatment of HeLa and AZ-521 cells by PI-PLC was shown to inhibit the internalization and vacuolation supporting the importance of GPI-AP in the VacA-induced cytotoxicity.⁶⁵ Conversely, another study using GPI-AP-deficient mutant Chinese hamster ovarian CHO-LA1 cells has revealed that lipid rafts might play an alternative role of GPI-AP for the VacA toxin.⁶⁶

In addition to intoxication, lipid raft influences signaling cascades induced through the binding of VacA toxin to

Table 2. Phenotype specificity of VacA in relation with RPTPs

Cell line	Type of receptor	Toxic strain of VacA
RK13	RPTP β	m1 and m2
Az-521	RPTP β and RPTP α	m1 and m2
AGS	RPTP β and RPTP α	m1
HeLa	RPTP α	m1
G401	RPTP α	m1 and m2
HL60	-	No toxicity

Phenotype specificity caused by the m region of VacA toxin is related to the functional expression of RPTPs.⁵⁴ HeLa and G401 cell lines expressing one type of receptor (RPTP α) demonstrate different toxicity responses. Difference in the post-transcriptional processing of RPTP α causes formation of smaller RPTP α in HeLa cell line and reacting solely to the m1, while RPTP α in G401 responds to both m1 and m2.⁵⁵ In some cell lines, lack of RPTP is the reason of VacA untoxic behavior. HL-60 cell line with no RPTP receptor and no impression by VacA intoxication responded to VacA m1 when treated with PMA, but still unaffected by m2.^{51,55} Also two AGS and AZ521 cell lines expressing both RPTP receptors show various reaction to VacA treatment. Additionally, in spite of expressing just RPTP β in RK13 cell line both m1 and m2 types are considered as toxic strains. As a result, although m1 and m2 both respond to RPTP β , it seems that in all cell lines presence of RPTP β does not guarantee the vacuolation potential of VacA.

the host surface receptors as well as GPI-APs. Treatment of AZ-521 cells with PI-PLC and MCD was shown to inhibit the p38/MAPK signaling pathway, in large part due to the inhibition of RPTP β /VacA translocation to lipid raft assisting GPI-AP. As a result, VacA and RPTP β complex can be translocated to lipid rafts, which is the most important step in triggering the RPTP β -mediated signaling cascade while the presence of GPI-AP is contributory for the accumulation of VacA in lipid rafts.⁶⁵

Sphingomyelin (SM)

SM serves as another receptor of the VacA-mediating interaction of toxin with the plasma membrane. The importance of SM in vacuolation was shown by the inhibition of the vacuolation in multiple cell lines (e.g., HeLa, human Caucasian gastric adenocarcinoma AGS cells as well as AZ-521 cells) treated with sphingomyelinase C. Enhanced VacA-induced vacuolation has been shown through exogenously overexpressed SM onto the plasma membrane of HeLa cells, which further indicates the role of the SM in cell vacuolation. In addition, the fate of intracellular pathway in which VacA is involved could be strictly affected by the length of SM carbon chain. In cells with long chain SM (C16 and C18), the VacA intoxication occurs via a cdc42-dependent pinocytotic pathway/pinocytosis trafficking to Rab7-containing late endosomal compartment essential for the vacuolating activity of the VacA toxin. However, in the short acyl chains (C2 and C4), the VacA is internalized through a cdc42-independent pathway directing to Rab11-containing compartments of the eukaryotic cells. Interestingly, the length of acyl chain affects the distribution of VacA into lipid or non-lipid raft and also its uptake by GPI-AP in the plasma membrane.^{67,68}

Fibronectin (FN)

FN, a 440 kDa extracellular matrix (ECM) glycoprotein, functions as a receptor for the VacA toxin that binds through its RGD (Arg-Gly-Asp) motif in a dose-dependent manner.⁶⁹ Adhesion of the VacA to FN or any other ECM proteins can initiate several mechanisms in favor of bacteria survival, in large part through retarding the clearance of *H. pylori* and preserving the infection via invasion and penetration into the intracellular junction.^{70,71}

In addition, the VacA binding to FN was shown to impose cell disruption and changes in cell morphology through cytoskeletal reorganization.⁷²

Heparin (H) and heparan sulfate (HS)

The H and HS as two types of receptors are cellular surface and extra cellular matrix-associated proteoglycans and present different binding sites for m1 and m2 strain of VacA in favor of *H. pylori* survival.⁷³ It should be also stated that *H. pylori* evades immune response of the host by binding of VacA to H/HS that, in return, recruits and inactivates complement components, C3b and C4b, known as one of the first barriers of the native immune system.⁷⁴ Further, attachment of H/HS onto the surface of

microorganism enables *H. pylori* to avoid phagocytosis.⁷⁵

Low-density Lipoprotein Receptor-related Protein-1 (LRP1)

LRP1, an endocytic receptor from LDL receptor family, is thought to be the receptor mediating VacA-dependent autophagy. The study investigating the influence of LRP1 was carried out on AZ-521 cell. LRP1 gene silencing by siRNA and confocal microscopy results in the AZ-521 cells determined dependency of binding and internalization of toxin to LRP1. Furthermore, VacA-induced apoptosis occurs through autophagy and caspase independent via binding to LRP1. Interestingly, formation of small vacuoles via binding to LRP1 is exhibited by formation of phagosomes, which is different from the RPTP β -mediated vacuolation. Overall, LRP1 is considered as one of the main receptors for binding and internalization leading to autophagy dependent apoptosis and vacuolation.⁷⁶

CD18

In addition of various receptors on epithelial cells for binding of VacA, one receptor has been identified as a ligand for VacA on T cells named CD18 and known as PKC-associated internalization. Similar to the epithelial cells internalization, the mechanism of intoxication after concentrating within the lipid raft is clathrin-independent due to phosphorylation of cytoplasmic domain of CD18, PKC, by the regulation of Rac1 and cdc-42.⁷⁷ As a result, T-cell proliferation and IL-2 signaling pathway is prohibited by inhibition of nuclear factor of activated T-cell (NFAT) making it an immunomodulator toxin.⁷⁸ Another immunosuppressive effect of VacA has been observed on B cells, causing interference with antigens (Ags) presenting cells (APCs).⁷⁹

Internalization and trafficking of VacA toxin

Generally, VacA is released from bacteria in two types, including: (a) VacA associated with vesicles known as outer membrane vesicles (OMV), and (b) free VacA. The biological activity of free VacA is triggered immediately after the internalization (known as the early internalization), while the VacA-containing OMVs remain intact for about 72 hours after the internalization (known as the late internalization).⁸⁰ Both types of VacA are internalized through clathrin-independent pathway that is believed to be regulated by the small GTPase proteins, Rack1 and Cdc42. Upon internalization, the VacA toxin is located beneath the membrane in GPI-AP early endosome compartment (GEECs). The tubulovesicular compartments containing GPI-AP are part of the GPI recycling system. Once in the early and late endosomes, the toxin either is directed to mitochondria and induces apoptosis or is oligomerized on the vacuole membranes and causes vacuolation.^{65,81,82}

Similar to the diphtheria and tetanus toxins, VacA intoxication through lipid rafts is a pH- dependent process. An increased interaction of VacA with lipid raft at an acidic pH is attributed to the structural alteration of p55 domain. Although the effect of low pH on the p33 domain

has been shown, its impact(s) on the p55 domain appears to be higher on the toxin binding because the interaction of p33 with lipid raft is weaker/lower than that of the p55. The low pH-induced conformational changes were shown to increase the surface exposure of the hydrophobic domain of toxin in favor of its binding to the lipid rafts, which facilitates the membrane internalization.⁸⁰

Pathogenesis of VacA and signaling pathways

VacA can trigger intracellular signal transductions related to pro-inflammation, vacuolation and apoptosis. In general, the behaviour of the host cells in relation with VacA toxin is relevant to the type of toxin-cell interaction. Binding of the VacA toxin to the host cell membrane through its receptors can activate several signaling pathways leading to pro-inflammatory responses. On the other hand, the uptake of VacA toxin by cells can direct the toxin to the mitochondria and induce apoptosis or vacuolation (Fig. 2).²³

A- Vacuolation

After pinocytic-dependent and clathrin-independent endocytosis of the VacA toxin through GEECs, they are directed to the early endosomal (EE) compartments containing EEA1 as a marker with assistance of the F-actin and CD2AP as VacA-F-actin interaction mediator.¹⁵ Vesicular trafficking of early endosomes to the LAMP1-containing late endosomes (LE) is the next step that initiates the oligomerization of VacA on the late endosomal compartment membrane. The oligomerized

VacA acts as an ion selective channel altering chloride concentration and enhancing proton pump activity that results in LE swelling and vacuole formation.^{24, 83}

B- Programmed Cell Death (apoptosis)

In addition to the vacuolation of the host cells caused by the VacA, the internalization through endocytosis can elicit apoptosis via mitochondrial-dependent cell death mechanism. Internalization to the mitochondria occurs via TOM complex through the recognition of N-terminus of VacA, which is believed to be futile for the channel formation but important for directing toxin to the mitochondrial membrane. VacA, containing LE compartment and mitochondrial membrane interaction, is also able to initiate the programmed cell death through membrane potential fluctuations.^{84,85} The release of cytochrome C through translocation of the pro-apoptotic compounds- Bax and Bak caused by the alterations of membrane potential is the basic mechanism for leading cells towards apoptosis.^{86,87}

C- MAPK signaling pathways

Furthermore, VacA can impose its effects on the host cell through binding to certain membrane receptors and inducing p38 and ERK1/2 signaling cascades.⁸⁴ Activation of p38/MAPK pathway through binding of the VacA initiates the activating transcription factor-2 (ATF-2), which functions as a regulator on the Cox-2 by binding to its promoter via TLR2/9. Induction of Cox-2 expression was shown to enhance the expression of PGE2 - known

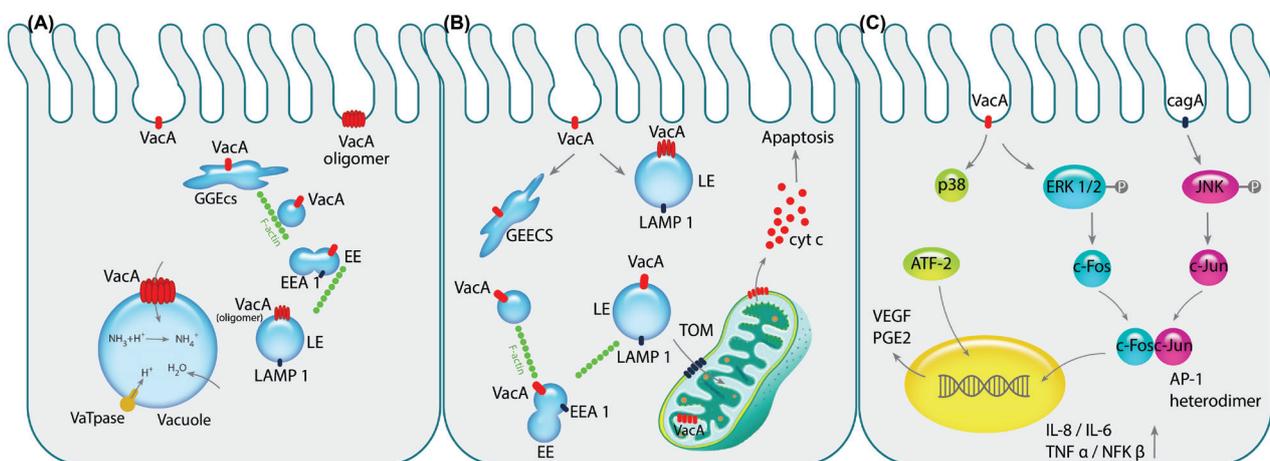


Fig. 2. Pathogenicity of VacA toxin is exhibited on epithelial cells. A) After the pinocytosis of VacA into intracellular environment, it is directed towards early endosomal compartment GEEC, EE and LE respectively by the aid of F-actin filaments. Next, VacA is oligomerized on the LE membrane acting as the channel discharging chloride ions that results in LE swelling since water molecules are imported due to overpopulation of ammonium resulted from the change in chloride ions. Consequently, over-hydration results in vacuolation of the cell. B) Following aforementioned trafficking of the vacA to the endosomal compartments, its transportation to the mitochondria by N-ter signal sequence of the toxin can commence the programmed cell death (apoptosis). Importing of VacA through TOM complex and oligomerizing alters membrane potential of the mitochondria which recruit pro-apoptotic Bax and Bak complexes indirectly leading to release of cytochrome c. C) Binding of the VacA to cell surface receptor RPTPβ activates the MAPK signaling cascades independently or in cooperation with CagA. The activation of MAPK/p38 pathway induces stimulation of transcription factor ATF-2 that increases the expression of PGE2, known as the angiogenic factor and mitotic signal, via binding to COX-2 promoter. Additionally, VacA phosphorylates ERK1/2 that activates the downstream component c-fos, while CagA activates the other components of AP-1 heterodimer, c-jun, by phosphorylation of JNK. Interaction of c-fos and c-jun together would produce the AP-1 heterodimer which is counted as the transcription factor for several cytokines such as IL-1, IL-6 and TNFα leading to inflammation of the gastric mucosa.

as a mitogenic signal - and contributes to angiogenesis through up-regulation of the vascular endothelial growth factor (VEGF).^{16,88}

VacA also contributes to the activation of ERK1/2 via its phosphorylation and formation of AP-1 that contains heterodimer of c-fos and c-jun. While the c-fos expression is regulated by the phosphorylated ERK1/2, the other part of the AP-1 complex is activated via JNK phosphorylation through binding of cagA that is another toxin of *H. pylori*. As a result, AP-1 regulates the expression and recruitment of cytokines such as IL-8, IL-6, TNF- α and NF- κ B through binding to their promoters.^{12,89}

D- Toxin oligomerization on plasma membrane

In the host cells, the VacA toxin is functionalized in two forms of monomer and oligomer. The latter can reshape as “rosettes” in the solution and form low-conductance, anion-selective and voltage-dependent bio-structures that are water-soluble 30 nm-diameter channels as two single-layered hexameric and heptameric flowers and

bilayered shapes consisting of 6-9 and 12-14 monomers, respectively.^{40,90,91}

A study predicting structure of oligomerized VacA by deep-etch electron microscopy (EM) viewed 3 possible forms for hexameric structure, including: (i) the flower shape, a dodecamer constructed of two hexameric flat forms with two symmetrical six-subunit arrays, (ii) the flat form composed of an individual six-petal structure known as half of a dodecamer with central ring and counterclockwise chirality, and (iii) the other flat form with no central ring and clockwise chirality with a more abundance than the former and limited accessibility in the environment (Fig. 3).⁹¹

Affluence of the heptameric structure seems to be dependent on the length of the loop region located between p33 and p55. Since the m1 type possesses longer residue than the m2 type, the heptameric structure is more abundant. These multi-shaped structures are generated in the presence of two interacting sites and environmental effect of pH, which aids the assembly of monomeric structures in the neutral pH.^{92,93} The oligomerized VacA on the host cells are able to permeabilize the membrane through rosette form channels that can efflux/transfer the metabolites and ions of the host cell to the extracellular environment in favor of survival of bacteria.⁴⁰

VacA and CagA interaction

Besides VacA-induced pathogenesis on the host cell in gastric mucosa, it is proved that the VacA association with CagA is a key regulator for the disease severity. Phosphorylated and unphosphorylated CagAs are able to function as the inhibitor of intracellular trafficking of VacA and the simulator of anti-apoptotic BCL2 in order to prevent initiation of apoptosis induced by VacA, respectively.^{94, 95} Besides this manifestation of CagA, it affects the internalization of the VacA to the host cells to which bacteria is attached in order to assist the survival of the bacteria. It is determined that the binding of VacA to distant cells out of access of *H. pylori* induces vacuolation and apoptosis, thus the intracellular nutrients become accessible.^{96,97}

VacA as a candidate for molecular therapy

For decades, the first line treatment of *H. pylori* infection is a triple drug combination, including: proton pump inhibitor (PPI), clarithromycin and amoxicillin. However, this traditional regimen often associate with some drawbacks, including: an increased resistance to antibiotic, low patients compliance against the long course of treatment with significant side effects, and relapse of the infection.^{98,99} Thus, new generation molecular therapies need to be alternatively implemented since they impose less detrimental impacts on the healthy tissues with lower side effects, thereby improving the efficacy of treatment.

In several experiments, few vaccines and immunotherapies have successfully been developed against various *H. pylori* Ags related to infection and colonization of the bacteria and incidence of peptic ulcer

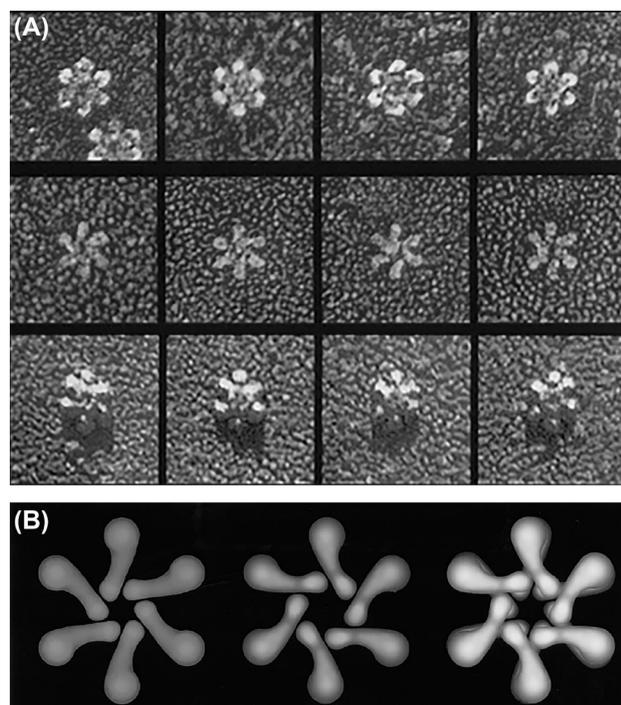


Fig. 3. Freeze-dried transmission electron microscopy micrographs of *H. pylori* VacA toxin. A) Rotary replicas of oligomerized structures of VacA toxin. The first, second and third rows respectively show the flower shaped dodecamer form of VacA toxin, the flat form of VacA in oligomerized form, and the seven-membered structure of toxin. B) Three structures predicted based on the studies on the oligomerized forms of VacA with deep-etch electron microscopy analysis. The right, middle and left images respectively show the symmetrical pair of hexameric VacA flower placed face-to-face that is a complete dodecamer constructed, the minor flat form included half of a dodecamer with the central ring that resembles the complete VacA structure, and the more common flat form of oligomerized VacA with no central ring that is sandwiched in the center of dodecamer. The data were adopted with permission from a published work conducted by Cover et al.⁹¹

and gastric cancer. The candidate Ags for molecular therapy include flagella, hsp60, urease, adhesions, cagA and VacA.¹⁰⁰

Of these, VacA plays a pivotal role in pathogenesis of *H. pylori*. It contributes to the persistent colonization of bacteria, leading to the longevity of infection through escaping from the autophagy and immunosurveillance of host.¹⁰¹ VacA is considered as one of the main virulence factors involving in ulcerogenesis and gastric cancer. Further, the severity of disease symptoms among patients suffering from the gastritis and peptic ulcer disease appears to be VacA strain-dependent.¹⁰²

Vaccine development

Generally, based on multiple studies on developing an effective vaccine against *H. pylori*, there are two types of vaccines, including: (a) the prophylactic vaccine that prevents the infection by reducing or clearing bacteria in the healthy or previously infected individuals, and (b) the therapeutic vaccine that treats the infection in patients via stimulating immune system different from the infection-induced immunity.^{103,104} To control this formidable diseases, however, multi-component vaccines need to be developed. To achieve an effective vaccine against *H. pylori*, multiple virulence factors mediating various clinical manifestations should be targeted. Although multivalent/polyvalent vaccines would be helpful, development of multi-antigen vaccines against *H. pylori* seems to be inevitable. For this purpose, the vaccine could be designed based on the *H. pylori* whole cell (HWC) or antigen cocktail which is composite of two or more major virulence factors of bacterium.¹⁰⁵ Although approaches exploiting HWC in mouse models or different clinical trials confirmed the efficacy of these vaccines, in some cases, these types of vaccines show low quality of Ags and possible cross-reactivity and immunologic reactions.^{106,107} Therefore, an efficient vaccine against *H. pylori* should contain a series of virulent Ags presented in all strains that are involved in bacterial adhesion and intoxication. Owing to its function in pathogenicity of *H. pylori*, VacA has a potential ability of being a suitable candidate for vaccine development. This notion has further been verified because of the high amount of IgGs detected in patient's serum, and the high expression rate in most *H. pylori* strains specially those causing the peptic ulcer. Furthermore, establishing protection with no need to native conformation by VacA toxin makes it an appropriate Ag for development of vaccine and immunotherapy.^{104,108}

A couple of studies have reported the efficacy of the VacA-containing vaccine *in vitro* and animal models. For example, formaldehyde-inactivated VacA vaccine was shown to elicit immune response(s) in mice as compared to that of the native toxin.¹⁰⁹ It should be noted that formaldehyde is a detoxifying agent via interacting with L-lysine residues of the toxin, and hence can be exploited as an adjuvant in vaccination. Since toxin inactivation by formaldehyde also causes distortion of some epitopes that do not present in the native Ags, the mutant type of

heat-labile enterotoxin of *E. coli*, LTK63, was utilized as a suitable adjuvant for the vaccine development against *H. pylori*.¹¹⁰ In addition, VacA has been exploited for the production of multi-component vaccines. Intra-gastric immunization of mice infected with *H. pylori* SPM326 by the therapeutic vaccine consisting of recombinant VacA and CagA along with LTK63 adjuvant was shown to eradicate *H. pylori* for 3 months and also provide tenacity of eradication for 2 months after infection rehearsal.¹¹¹ Immunization of Beagle dog suffering from gastritis with multi-component vaccine, VacA-CagA- neutrophil-activating protein (NAP), supplemented with adjuvant Al(OH)₃, resulted in an increased level of antibody (Ab) and hence protection for 4 months post-vaccination. Despite such therapeutic impacts, the gastritis was recurred after 29 weeks indicating the partial periodic effect of vaccine.¹¹² In contrary to mice, Beagle dogs, as animal model for the human *H. pylori* infection, provide researchers with high ability of tandem biopsy with no need to sacrifice the animal, which helps to gain a better understanding of the overall process of infection and immunity.

Further, a prophylactic vaccine containing three Ags (VacA, CagA and NAP) with adjuvant Al(OH)₃ has been developed and examined in the non-infected individuals through a phase 1 clinical trial. This intramuscular booster vaccination demonstrated satisfactory safety and immunogenicity with anamnestic antibody and cellular responses 18–24 months post-vaccination. Long memory immunity was originated from VacA- and CagA-driven interferon γ production, which lasted 4.5 months post-immunization. After the third immunization, all volunteers responded to one or two Ags (mostly VacA and NAP), while 86% responded to all three Ags. VacA induced Ab response after two immunizations, whereas CagA showed cell-immune response after three immunizations.¹¹³

Yet, there exist some debates to figure out what portion of the VacA toxin is more efficient for the vaccine development. A study using different recombinant fragments of VacA confirmed that the highest titer of Ab was produced against 297-317 residues including both p33 and p55 regions of the toxin.¹¹⁴

Nonetheless, another effort was carried out to study the VacA neutralizing activity of 10 monoclonal antibodies (mAbs) produced in mice via epitope mapping using a panel of VacA deletion mutants and VacA chimera. Among these, two mAbs were able to neutralize the cytotoxic activity of VacA through recognition of amino acids 685 to 821 located at the receptor binding region.¹¹⁵ In the following context, mAbs against *H. pylori* infection will be discussed.

Monoclonal antibody

Development of mAbs has provided new insights in the diagnosis and therapy of various diseases, in particular infections and malignancies. As a result, commercial therapeutic mAbs have made their way to the therapeutic markets in the early 1980s.¹¹⁶

Passive administration of mAb directed at protective Ags such as VacA and urease might be particularly relevant as a substitute to the current therapies and diagnosis methods. Thus, VacA toxin has become a target for the production of therapeutic mAbs to neutralize the cytotoxin activity. Accordingly, various studies reported that some variants of VacA are highly associated with an increased risk of symptomatic gastroduodenal disease. Two anti-VacA mAbs, known as V36E and V41, were shown to be able to neutralize the vacuolation of rabbit kidney cell line RK13 through binding to the native structure of VacA.¹¹⁷ Another experiment was conducted to produce mAbs against small and large subunits of urease. As a result, two Abs designated as S2 and L2 were determined based on their strong interactions respectively with small and large subunits, and showed significant blockage of the enzyme activity.¹¹⁸

Besides application of mAbs in the field of therapy, it seems that they are promising tools for increasing accuracy of diagnosis. Common diagnostic methods of *H. pylori* infection have been developed on the basis of biopsy, which is painful. Further, various diagnostic methods such as Urea Breath Test (UBT) and serological techniques may provide false negative and positive results specially in asymptomatic patients, thus leading to failure of accurate diagnosis and efficient treatments.¹¹⁹ Gamma-glutamyl transpeptidase (GGT) antigen is a virulence factor, necessary for colonization and cell apoptosis of *H. pylori*, which appears to be a promising Ag for development of mAb. In 2014, a phase 1 clinical trial (ID: NCT02123771) was successfully carried out to detect the *H. pylori* infection in the stool of patients using ELISA and anti-GGT mAb. Given that several toxins of *H. pylori* participate in the pathogenesis and survival of bacteria, a multi-component Ab cocktail may provide the next generation immunotherapy of *H. pylori* infection, similar to Ab cocktail used for treatment of Antrax.¹²⁰ Ab cocktail is a combination of several Abs that can recognize different virulence factors, through which the treatment outcome can be maximized by expanding the spectrum of protection and enhancing the protective efficacy.

Production of Ab cocktail to recognize the major virulence factors *H. pylori* (i.e., VacA, urease, CagA, GGT and flagellin) appears to be very desirable for the treatment of *H. pylori* infection. Use of class immunoglobulin A (IgA) in Ab cocktail against *H. pylori* seems to provide an improved immunotherapy modality, in large part because IgA secreting cells often react with *H. pylori* membrane proteins (e.g., flagellin and urease) in *H. pylori*-infected patients.¹²¹ Given the high stability and avidity of IgA in the gastric environment and habituating *H. pylori* as a noninvasive organism in gastric mucosa, passive immunotherapeutic approaches using secretory IgA (sIgA) might provide a robust treatment modality. For example, in a study, a scFv polypeptide specific to *H. pylori* urease subunit A was compared with its reformatted polymeric IgA (IgAp/d) and sIgA for the degree of enzyme activity inhibition. Due to increased avidity, the sIgA and IgAp/d

Review Highlights

What is current knowledge?

- ✓ Application of VacA in production of a vaccine for *H. pylori* treatment has been confirmed, and used as a part of multi-component vaccine including several virulence factors of the bacterium.
- ✓ Efficiency of therapeutic vaccines has been investigated in various *in vivo* and *in vitro* experiments, as well as clinical trials.
- ✓ Despite success in the treatment of infection in various studies, relapse of infection after several months appears as a challenging issue.

What is new here?

- ✓ Besides production of systemic immunoglobulins confronting *H. pylori* infection, local antibody secreting cells (ASCs) appear to be the major class of Abs representing the state of infection in patients.
- ✓ New multivalent vaccines may provide novel treatment modalities against *H. pylori*.
- ✓ New immunotherapies could be designed based on the IgG and IgA Ab cocktail.
- ✓ Novel methods of producing recombinant Ab fragments (scFv and Fab) such as display technologies are able to produce mAbs with higher abilities of multimerization into secretory forms in comparison to traditional full length Abs.

were found to be able to efficiently block the enzymatic activity of free and membrane-associated ureases.^{122,123}

Although several antibody fragments have been designed against the main virulence factor of *H. pylori*, urease,¹²⁴ no antibody fragment has been developed against domain of VacA by phage display ever. New approaches for production of antibodies, such as phage display technique,^{125,126} have been developed in the recent decades that provide a convenient and economic procedure for production of monoclonal antibodies. To the best of our knowledge, we are among the first teams who work on the development of mAb fragments by phage display against VacA toxin.^{127,128}

Concluding remarks

VacA is considered as one of the main virulence factors in pathogenesis of *H. pylori* in the stomach with variety of cytotoxicity, in which the toxicity is assumed to be dependent on different variants of the toxin and various types of receptors and cells. Despite some attempts to justify the binding pattern of VacA, the exact receptor(s) of host cells responsible for the binding of VacA toxin is yet to be fully addressed. In general, it is believed that, regardless of the receptor involved (e.g., RPTP β or EGFR), the process of VacA internalization occurs in the regions enriched with lipid rafts with the aid of cholesterol, GPI-AP and sphingomyelin. Notwithstanding unprecedented volume of investigations on the *H. pylori* infection worldwide, its effective eradication remains

as a dilemma. Failure of the currently applied therapies, consisting of antibiotic regimens, is largely attributed to inaccurate treatment causing point mutations in *H. pylori*. New molecular therapies such as vaccines and mAbs seem to offer much more effective treatment modalities with much higher patient compliance and minimal side effects. Although successful vaccination in animal models has been reported, attempts for translation of such therapies to human cases are still under investigation. Among various types of mAbs produced against *H. pylori*, IgA class of Abs may be considered as one of the best candidates for passive immunotherapies since the infection of gastric mucosa by *H. pylori* recruit cells that are responsible for the production of IgA specific for a variety of virulence factors. Besides, sIgA was shown to prevent the cellular attachment of *H. pylori* and its infection. In this review, we discussed genetic diversity and receptors of VacA toxin on epithelial cells and tried to justify whether VacA cytotoxicity is dependent on the type of receptor and genetic diversity for the first time. It is obvious that receptor and genetic diversity are regulators of *H. pylori* pathogenesis regardless of the genetic background and environmental issues affecting the severity of the disease worldwide and also the effect of cagA on the potential of VacA. Overall, the cytotoxicity of the VacA *in vivo* is exhibited based on the position/condition of the host cells. Non vacuolating toxicity (e.g., peptic ulcer and gastritis) is resultant from the pathogenicity for adjacent cells with attached *H. pylori* and vacuolation is observed in distant cells in order to feed on cellular nutrients. Collectively, due to growing trend of mAb applications, recombinant Ab fragments (e.g., scFv, Fab, single domain Abs and bispecific Abs), multivalent Abs and Ab-conjugates with higher avidity as a cocktail of IgG and sIgA Abs as well as multivalent vaccines may be our best shot to tackle this formidable disease.

Ethical approval

Not applicable.

Competing interests

The authors declare no conflict of interests.

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