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The *Helicobacter pylori* vacuolating cytotoxin: from cellular vacuolation to immunosuppressive activities

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Abstract *Helicobacter pylori* is a highly successful bacterial pathogen of humans, infecting the stomach of more than half of the world's population. The *H. pylori* infection results in chronic gastritis, eventually followed by peptic ulceration and, more rarely, gastric cancer. *H. pylori* has developed a unique set of virulence factors, actively supporting its survival in the special ecological niche of the human stomach. Vacuolating cytotoxin (VacA) and cytotoxin-associated antigen A (CagA) are two major bacterial virulence factors involved in host cell modulation. VacA, so far mainly regarded as a cytotoxin of the gastric epithelial cell layer, now turns out to be a potent immunomodulatory toxin, targeting the adapted immune system. Thus, in addition to the well-known vacuolating activity, VacA has been reported to induce apoptosis in epithelial cells, to affect B lymphocyte antigen presentation, to inhibit the activation and proliferation of T lymphocytes, and to modulate the T cell-mediated cytokine response.

Introduction

Since the discovery of vacuolating cytotoxin (VacA) 16 years ago (Leunk et al. 1988) and the first description of its purification (Cover and Blaser 1992), numerous studies have been performed to elucidate its mechanism(s) of action and its biological role. Particularly the effect of VacA on epithelial cells has been examined in great detail. The biological significance of VacA has been derived from several observations. First of all, large quantities of purified VacA can induce ulcer-like erosions when administered into the mouse stomach (Marchetti et al. 1995). In addition, VacA increases the risk of gastric ulcer formation in experimentally infected gerbils (Ogura et al. 2000) and also enhances the bacterial colonization rate in a mouse model of infection (Salama et al. 2001). Furthermore, certain *vacA* genotypes causing a high vacuolating activity are correlated with more severe disease in humans (Atherton et al. 1995). The *vacA* gene displays a considerable polymorphism, especially in the signal region (where the genotypes s1 and s2 may be dis-

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criminated) and in a mid region (genotypes m1 and m2). Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and absent in s2/m2 genotypes (Atherton et al. 1995). Consequently, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma. One further difference is a higher expression level of the s1/m1 as compared to the s2/m2 *vacA* genes (Forsyth et al. 1998).

Structure of the *vacA* gene and its product

The VacA protein is synthesized as a 140-kDa precursor protein (Cover et al. 1994; Schmitt and Haas 1994; Telford et al. 1994), consisting of an N-terminal signal sequence, the toxin domain, and a C-terminal extension of about 50 kDa. The C-terminal part of this extension is sufficient to accomplish secretion to the extracellular medium via an autotransporter mechanism (Fischer et al. 2001). Extracellularly, the mature 95-kDa toxin may be cleaved into an N-terminal 33-kDa (here p33, sometimes also designated as p37) and a C-terminal 55-kDa protein (here p55, sometimes also designated as p58) (Telford et al. 1994; Nguyen et al. 2001), respectively, that remain non-covalently associated (Telford et al. 1994). Purified VacA was shown to assemble into hexameric, heptameric, or dodecameric structures (Lupetti et al. 1996; Cover et al. 1997). Mutant forms of VacA that are unable to form oligomers also lack a vacuolating activity (Vinion-Dubiel et al. 1999), suggesting that oligomerization is an important feature of purified VacA. Under acid or alkaline treatment, these oligomeric forms become activated by dissociation into monomers (Cover et al. 1997; Molinari et al. 1998a; Yahiro et al. 1999). However, culture supernatants and VacA associated with the bacteria do not seem to require activation (Leunk et al. 1988; Pelicic et al. 1999), suggesting that active VacA may be monomeric in vivo, and oligomerization, which requires acid activation for its function, might be an artefact of protein purification.

Effects of VacA on epithelial cells

The vacuolating activity on epithelial cells, originally described by Leunk et al. (1988), is the most apparent effect of VacA. Despite a very extensive characterization, the mechanism of vacuolation is not completely understood. The vacuolating activity is also observed when the VacA protein is produced in epithelial cells, e.g., after transfection of the *vacA* gene cloned in an appropriate plasmid (de Bernard et al. 1997), suggesting an intracellular target for VacA vacuolating activity. On the other hand, VacA is able to form anion-selective membrane channels both in lipid bilayers (Czajkowsky et al. 1999; Szabo et al. 1999; Iwamoto et al. 1999) and in the plasma membrane of epithelial cells (Szabo et al. 1999). However, the channels seem to be structurally different in these two situations (Adrian et al. 2002). These pores induce a membrane depolarization (Szabo et al. 1999; Iwamoto et al. 1999; Schraw et al. 2002) and seem to be necessary for vacuole formation. In fact, inhibition of channel formation by addition of anion-channel blockers to VacA-treated epithelial cells also prevents vacuolation (Szabo et al. 1999; Tombola et al. 1999). Moreover, mutant forms of VacA lacking an N-terminal hydrophobic region or containing site-specific mutations in this region are defective in membrane channel as well as vacuole formation (Vinion-Dubiel et al. 1999; McClain et al. 2003). The pores are formed by oligomerization of membrane-bound monomers (Vinion-Dubiel et al. 1999), for which the N-terminal hydrophobic region is essential (McClain et al. 2001b, 2003). The N-terminal

sequence of s2-encoded VacA proteins contains a hydrophilic extension in comparison to s1-encoded VacA proteins, which is necessary and sufficient to render the proteins non-toxic (Letley and Atherton 2000; Letley et al. 2003; McClain et al. 2001b).

VacA binding to receptor and/or lipid rafts

As reported recently by Ilver et al. (2004), a significant portion of the toxin remains associated with the bacterial surface, where it seems to be organized into distinct domains. The bacteria-associated toxin was shown to be biologically active and may be transferred from the bacteria to the target cell by a contact-dependent mechanism (Ilver et al. 2004). The mechanism of a direct delivery or the involvement of a potential receptor for the direct toxin delivery to eukaryotic cells is still unknown. In contrast to surface-associated VacA, the interaction of secreted VacA with the target cell was reported to be dependent on specific protein receptors, but VacA inserts into artificial lipid membranes in the absence of protein receptors as well (Molinari et al. 1998a; Moll et al. 1995; Pagliaccia et al. 2000). This indicates that the toxin may not bind specifically to a single receptor but rather interacts with multiple components on the cell surface (Fig. 1). The first protein described functioning as a receptor for VacA was the epidermal growth factor (EGF) receptor (Seto et al. 1998). Later, Yahiro et al. (1999) identified the receptor-like protein tyrosine phosphatase (RPTP)-β, a 250-kDa surface glycoprotein, as a VacA-binding receptor on the surface of the gastric adenocarcinoma cell line AZ-521. Interestingly, this interaction was shown to be relevant for the development of VacA-induced gastric ulcers. Fujikawa et al. (2003) reported that wild-type mice, but not mice deficient in RPTP-β, showed mucosal damage and ulcer induction by VacA. RPTP-β is known to be widely expressed in cells of the nervous system, where it regulates the maturation, development, and differentiation of neuronal and glial cells. But Fujikawa et al. demonstrated its expression in gastric tissue as well (Fujikawa et al. 2003). Studies with primary RPTP-$\beta^{+/+}$ and RPTP-$\beta^{-/-}$ gastric epithelial cells indicated that VacA was taken up and induced vacuolation in both cell types to the same extent (Fujikawa et al. 2003). This suggested the existence of other receptors or an internalization mechanism for uptake of VacA besides RPTP-β. Remarkably, binding of VacA to RPTP-β increased significantly the tyrosine phosphorylation of the G protein-coupled receptor kinase interactor (Git)-1, a substrate of RPTP-β, and subsequently induced the detachment of the cells from a reconstituted basement membrane. The authors concluded that erroneous RPTP-β signaling might be involved in the development of gastric ulcers (Fujikawa et al. 2003). Co-immunoprecipitation experiments using RPTP-β-negative, but VacA-sensitive cells led to the identification of another receptor-like protein tyrosine phosphatase, RPTP-α, as a receptor for VacA (Yahiro et al. 2003). In contrast to RPTP-β, RPTP-α is ubiquitously expressed (Yahiro et al. 2003). Besides the interaction of VacA with these specific receptors, VacA was also shown to bind to detergent-resistant microdomains (lipid rafts) of the cell membrane, which are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-anchored proteins (GPI-APs) (Fig. 1). As reported by several groups, depletion of membrane cholesterol significantly reduces the entry of VacA into target cells (Patel et al. 2002; Schraw et al. 2002; Kuo and Wang 2003). Contrasting results exist concerning the involvement of GPI-APs in internalization of VacA via lipid rafts. Kuo and Wang (2003) demonstrated that removal of GPI-APs from the cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) blocked VacA-induced vacuolation, but did not affect binding of VacA to lipid rafts. Similar data were reported by Ricci et al. (2000). In agreement with these findings,

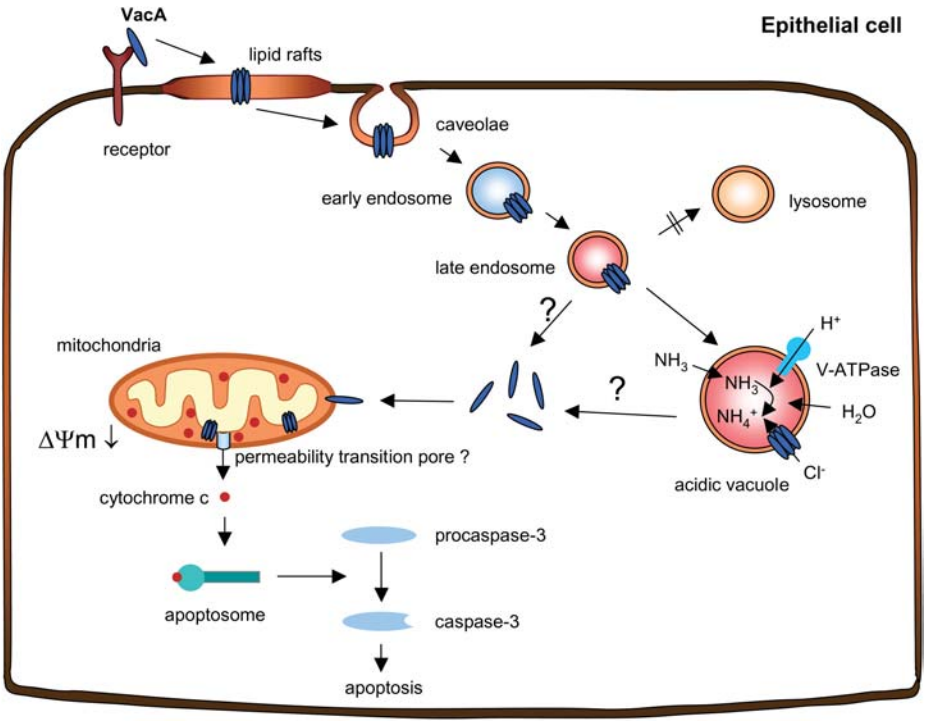

Fig. 1 VacA membrane interaction, intracellular vesicle trafficking, and induction of apoptosis. VacA interacts with a cellular receptor and associates with lipid rafts, where it inserts and oligomerizes to form anion-selective channels (chloride channels). The channels are endocytosed via caveolae and mature from early endosomes via late endosomes into acidic vacuoles. The activation of a vacuolar proton pump (V-ATPase) acidifies the endosomal compartment, which takes up weak bases (NH_3) and H_2O by diffusion. The VacA channel supports the entry of Cl^- ions, finally resulting in swelling and acidic vacuole formation. VacA then enters the cytosol and accumulates in mitochondria by a yet-unknown mechanism. Either direct insertion of VacA into the mitochondrial inner membrane or the activation of endogenous channels results in a reduction of the membrane potential ($\Delta\Psi m$) and cytochrome *c* release, which activates the caspase 3 pathway and finally leads to apoptosis

Gauthier et al. (2004) showed that GPI-APs are required for the formation of active VacA channels in the plasma membrane, but not for binding of the toxin to lipid rafts. This indicated that GPI-APs might be necessary for internalization of VacA, but not for binding to the eukaryotic cell. Kuo and Wang (2003) suggested a common endocytic pathway of GPI-APs and VacA, since they found that internalized VacA co-migrates along with the model lipid raft antigen GPI-AP fasciclin I, which has been constitutively produced in a Chinese hamster ovary (CHO) cell line. In contrast to these results, Schraw et al. (2002) demonstrated VacA cytotoxicity independent of GPI-APs. VacA was taken up into GPI-AP-positive and GPI-AP negative CHO cells to the same extent, but vacuolation was induced in both cell types (Schraw et al. 2002). After insertion into the plasma membrane, VacA channels are internalized. Endocytosis of the VacA channels does not occur via the clathrin-coated pit pathway but rather via an actin-dependent caveolae-like internalization process (Ricci et al. 2000) (Fig. 1). Disruption of the actin cytoskeleton by treatment with cytochalasin D did not inhibit binding of VacA to the lipid rafts, but blocked internalization of VacA and vacuole formation (Gauthier et al. 2004).

Trafficking and formation of acidic vacuoles

VacA-induced vacuoles are hybrid compartments of late endosomal origin with some lysosomal markers (Molinari et al. 1997). The formation of visible vacuoles depends on the presence of weak bases, such as NH_4Cl, that promote osmotic swelling. But even without the addition of weak bases, VacA induces a perinuclear redistribution and clustering of late endosomal compartments, which may be considered as a prerequisite for the development of vacuoles (Li et al. 2004). Vacuole formation depends on the presence and activity of a number of cellular molecules, such as the vacuolar (V-) ATPase (Papini et al. 1993), Rab7 (Papini et al. 1997), Rac1 (Hotchin et al. 2000), or dynamin (Suzuki et al. 2001). The SNARE protein syntaxin 7 also seems to be involved in trafficking or vacuolation, as confirmed by transfection with a dominant-negative syntaxin 7 construct (Suzuki et al. 2003). Another study, however, excluded the involvement of SNARE proteins and suggested that the enlargement of vacuoles might be achieved by fusion of internal vesicles of multivesicular body-like structures (de Bernard et al. 2002). Notably, an involvement of the protein kinase PIKfyve in vacuole formation was demonstrated as well. In this study over-expression of PIKfyve, or microinjection of its substrate, phosphatidylinositol-3,5-bisphosphate, was sufficient to induce vacuole formation (Ikonomov et al. 2002). Apart from these proteins necessary for vacuolation, VacA was shown by a yeast two-hybrid analysis to interact with the intermediate filament protein VIP54 (de Bernard et al. 2000) and with the scaffolding protein RACK1 (Hennig et al. 2001). In both cases, the significance of these interactions is not clear. The generation of late endosome–lysosome hybrid compartments in VacA-intoxicated cells is accompanied by an inhibition of vesicle trafficking, which leads to a defect in procathepsin D maturation, or EGF degradation (Satin et al. 1997).

In order to exert a vacuolating activity, the 33-kDa fragment is not sufficient; at least part of the 55-kDa domain must be present when VacA is produced intracellularly. The minimal intracellular active domain consists of p33 and the N-terminal 100 amino acids of the p55 (de Bernard et al. 1998; Ye et al. 1999). The vacuolating activity also depends on interactions between p33 and p55 (Ye and Blanke 2002; Willhite et al. 2002; Torres et al. 2004) and requires a hydrophobic N-terminal region (Ye and Blanke 2000; McClain et al. 2001a). This hydrophobic region probably contains a transmembrane dimerization sequence, which contains an essential proline residue and a GXXXG motif, which is typical for transmembrane dimerization sequences (Ye and Blanke 2000; McClain et al. 2001a, 2003). Interestingly, an inactivating mutation in the 55-kDa domain can functionally complement another inactivating mutation within this hydrophobic sequence (Ye and Blanke 2002). All mutations that inactivate VacA for vacuolation also lead to a defect in channel formation.

VacA and apoptosis

Several studies correlated *H. pylori* with an increased level of apoptosis in the human gastric mucosa (Moss et al. 1996; Mannick et al. 1996; Rudi et al. 1998). Several *H. pylori* antigens were described to be responsible for apoptosis induction, such as urease (Fan et al. 2000), proteins of the *cag* pathogenicity island (Peek et al. 1999), and lipopolysaccharide (Wagner et al. 1997). Recently, several groups presented conclusive evidence that the VacA toxin is able to induce apoptosis in epithelial cells in the absence of other *H. pylori* factors (Cover et al. 2003; Galmiche et al. 2000; Kuck et al. 2001). Cover et al. (2003)

clearly demonstrated that wild-type bacteria, or their culture supernatants, but not the isogenic *vacA* mutant strains or the corresponding culture supernatant, induced apoptosis. In the same way, purified toxin caused apoptosis in a dose-dependent manner in the presence of ammonium chloride. Furthermore, the induction of programmed cell death was shown to be dependent on the s1m1 form of the VacA protein, since a chimeric s2m1 VacA protein did not initiate apoptosis.

Mitochondria as target for VacA and apoptosis induction

It was reported that intracellularly expressed VacA and the N-terminal p37 VacA fragment, as well as extracellularly applied VacA, are selectively targeted to the mitochondria. VacA is found in the inner mitochondrial membrane, where it induces the release of cytochrome *c*, activating the caspase-3-dependent cell death signaling cascade (Galmiche et al. 2000; Willhite and Blanke 2004) (Fig. 1). Over-expression of the anti-apoptotic Bcl-2 protein blocked apoptosis induction (Galmiche et al. 2000), indicating that VacA-induced apoptosis occurs via a mitochondria-dependent pathway. Later, Willhite et al. (2003) examined the relation between vacuolation, channel forming activity, and induction of apoptosis in more detail. They found that vacuolation and induction of cytochrome *c* release are independent outcomes of VacA intoxication and that both depend on internalization of VacA and formation of anion-selective membrane channels. Interestingly, mutant forms of VacA known to be deficient in channel formation neither caused vacuolation nor cytochrome *c* release, although they localized to the mitochondria as the wild-type toxin does (Willhite and Blanke 2004). Furthermore, the authors investigated the mechanism of VacA-mediated cytochrome *c* release and demonstrated that VacA intoxication of cells leads to a breakdown of the mitochondrial transmembrane potential (Willhite and Blanke 2004), which may be associated with changes in the permeability of the mitochondrial membrane (Fig. 1). Experiments with the well-characterized mutant forms, VacA-P9A and VacA-G14A, which are unable to form membrane channels, and with the specific channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) clearly showed that VacA membrane channel formation is essential for reduction of the mitochondrial membrane potential ($\Delta\psi m$) and subsequent cytochrome *c* release. The authors speculate that VacA might induce changes in the mitochondrial membrane permeability by a mechanism that is independent of cellular caspase activity or the mitochondrial permeability transition.

The simplest model might be that VacA directly permeabilizes the mitochondrial inner membrane by inserting and forming pores. Such pores could result in a reduction in $\Delta\Psi m$, ultimately leading to further changes in the outer membrane and release of cytochrome *c* into the cytosol (Fig. 1). Alternatively, VacA interaction with the mitochondria could activate endogenous channels of the mitochondria, such as the adenine nucleotide translocase within the inner membrane, or the voltage-dependent anion channel (VDAC), an abundant protein located in the outer membrane of mitochondria, which is thought to facilitate cytochrome *c* release (Tsujimoto and Shimizu 2002). Interestingly, the ability of VacA to induce apoptosis seems to be cell type-dependent, since it was found that VacA induces apoptosis in parietal cells (Neu et al. 2002), which might facilitate *H. pylori* colonization of the gastric mucosa, but no apoptosis could be detected in VacA-treated activated T cells (Gebert et al. 2003; Boncristiano et al. 2003).

Novel activities of VacA toward cells of the immune system

The effect of VacA has been studied for years exclusively on epithelial cells. This might be explained by the fact that the epithelial cell layer constitutes the first barrier for VacA in the gastric mucosa. Originally, the vacuolating cytotoxin was described as a secreted molecule (Leunk et al. 1988; Cover and Blaser 1992). The contact-dependent transfer of VacA described by Ilver et al. (2004) might be relevant for delivery of VacA into gastric epithelial cells, but secreted VacA is able to penetrate into deeper tissues, getting possibly into contact with many other cell types, especially cells of the immune system, such as granulocytes, macrophages, dendritic cells, B cells, and T cells. The activity of VacA on these cell types is discussed below.

Opening of tight junctions and cooperation of CagA and VacA

The intact epithelial barrier is usually very tight and the deeper tissue is not accessible for molecules of the gastric surface. It has been observed, however, that colonization of the gastric surface by *H. pylori* results in a partial opening of the tight junctions, which are responsible for the controlled sealing separating the apical side of the gastric surface from the sub-mucosa (Terres et al. 1998; Suzuki et al. 2002). Originally, the transepithelial electrical resistance (TER) of polarized epithelial cell monolayers was found to be lowered by VacA, which was believed to allow the epithelial permeability of Fe^{3+} and Ni^{2+} ions, essential for *H. pylori* survival in vivo (Papini et al. 1998). This effect does not require acid activation and is also observed with m2-type VacA (Pelicic et al. 1999). VacA might also act as a specific urea transporter, as demonstrated by the generation of a transepithelial flux of urea across model epithelia from various polarized and non-polarized epithelial cell lines (Tombola et al. 2001). A more profound effect on the integrity of tight junctions may be caused by cytotoxin-associated antigen (CagA). As demonstrated under in vitro (Odenbreit et al. 2000) and in vivo (Azuma et al. 2003) conditions, CagA might be directly injected into polarized epithelial cells via the type IV secretion system of *H. pylori*. Notably, CagA was reported to disrupt the epithelial barrier function by directly targeting the epithelial tight-junction scaffolding protein ZO-1 and the transmembrane protein junctional adhesion molecule (JAM) (Amieva et al. 2003). It can be assumed that a long-term CagA delivery to polarized epithelia causes a severe disruption of the epithelial barrier function. Thus, it seems that the two major virulence factors of *H. pylori,* VacA and CagA, have a possible synergistic effect within the gastric mucosa, a finding assumed already for a long time because of a frequent association of certain *vacA* and *cagA* genotypes. CagA opens the tight junctions allowing VacA to enter the gastric sub-mucosa and to come into contact with a number of non-epithelial cell types, such as polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells, B cells, and T cells.

Interaction of *H. pylori* with polymorphonuclear granulocytes, monocytes, and macrophages and role of VacA

H. pylori infection induces a specific immune response, as demonstrated by high titers of *H. pylori* antibodies (Blanchard et al. 1999) and the recruitment of PMNs, mononuclear phagocytes, and lymphocytes to the gastric mucosa of infected individuals (Blaser and

Parsonnet 1994; Telford et al. 1997). Despite the presence of such a vigorous immune response, *H. pylori* eradication is not observed, unless specific antibiotic therapy is administered. This finding suggests, that *H. pylori* possesses properties allowing evasion of the host immune response.

Against extracellular bacterial pathogens, activated granulocytes and macrophages play a central role in the inflammatory response. Despite a predominantly extracellular lifestyle in vivo, *H. pylori* has also been described as occasionally entering epithelial cells, especially under in vitro conditions (Kwok et al. 2002), or to be taken up by professional phagocytes (Odenbreit et al. 2001). In epithelial cells, *H. pylori* seems to enter large cytoplasmic vacuoles, where the bacteria are reported to remain viable and motile (Amieva et al. 2002). It has been speculated that such intracellular vacuoles may constitute a reservoir of live *H. pylori*, difficult to attack by antibiotics or phagocytes. Whether or not VacA actually supports survival of *H. pylori* in such vacuoles is still a point of controversy (Petersen et al. 2001; Amieva et al. 2002). Whereas Ramarao et al. (2000) provided evidence that phagocytosis of *H. pylori* is inhibited by the *cag*-PAI type IV secretion system of *H. pylori*, another study found no difference in phagocytosis and intracellular survival between type I strains and isogenic mutants lacking the type IV secretion system (Odenbreit et al. 2001). Delayed phagocytosis, homotypic phagosome fusion, and prolonged survival in macrophages was reported for type I, but not type II, *H. pylori* strains (Allen et al. 2000). A further study found an enhanced survival of *H. pylori* in professional phagocytes, such as the macrophage cell lines THP-1 (human) and RAW 264.7 (murine), which was dependent on the production of VacA (Zheng and Jones 2003). They reported that VacA-producing strains of *H. pylori* reside in a compartment with early endosome properties and avoid fusion with lysosomes (Zheng and Jones 2003). The vacuolating cytotoxin seems to arrest phagosome maturation by recruiting and retaining tryptophane aspartate-containing coat protein (TACO), a mechanism also exploited by *Mycobacterium bovis* to prevent phagosome trafficking and maturation (Ferrari et al. 1999). In another study the presence of *H. pylori*-induced homotypic fusions were confirmed in isolated human monocytes, but neither VacA nor the *cag*-PAI were involved in prolonged intracellular survival (Rittig et al. 2003). Thus, the role of bacterial invasion into epithelial cells and of uptake by phagocytes as a bacterial evasion mechanism has to be clarified more rigorously in future.

Interaction of *H. pylori* with B cells and interference of VacA with antigen presentation

As discussed above, the cytotoxin might enter the gastric sub-mucosa by using the intercellular route. At this time it cannot be excluded, however, that VacA also uses a pathway of transcytosis to enter the sub-epithelial layer. In the sub-mucosa, VacA will come into contact with a number of different cell types. Molinari et al. (1998b) presented the first detailed evidence of how VacA might be able to interfere with the immune response. They demonstrated that VacA can inhibit processing of antigenic peptides in B cells and their presentation to human $CD4^+$ T cells. VacA-treated B cells showed a selective inhibition of antigen processing and surface presentation by the invariant chain (Ii)-dependent pathway. This pathway is associated with peptide loading and T cell presentation of newly synthesized major histocompatibility complex (MHC) class II antigen. The antigen presentation, which is dependent on recycling MHC class II was not affected by VacA.

Immunosuppressive activities of VacA

The reason for the successful chronic persistence of *H. pylori* in its special niche is not well understood. As postulated earlier, *H. pylori* may evade host responses through the inhibition of antigen-specific T cell proliferation (Knipp et al. 1994; Fan et al. 1994). Induction of apoptosis in gastric T cells expressing Fas ligand (FasL) by *H. pylori* was also suggested as a mechanism to explain immune evasion of the bacterial pathogen (Wang et al. 2001). Vaccination data from the *H. pylori* mouse model indicated that $CD4^+$ T cells are crucial for control of the *H. pylori* infection by the host (Ermak et al. 1998; Aebischer et al. 2000). A direct correlation was reported between the level of protection against *H. pylori* infection and the density of T cells recruited to the gastric mucosa. In contrast to MHC class I and B cell knockout mice, MHC class II knockout mice were not protected by vaccination, indicating that control of the *H. pylori* infection is dependent on MHC class II-restricted, cell-mediated mechanisms. Furthermore, the adoptive transfer of UreA-specific $CD4^+$ T cells from vaccinated BALB/c mice into naïve syngeneic recipients also demonstrated the importance of $CD4^+$ T cells in controlling the *H. pylori* infection (Lucas et al. 2001). Whether or not VacA has an immunosuppressive activity in vivo in infected individuals is not known. Clinical data by Fan et al. (1994) show significantly lower peripheral blood lymphocyte proliferative responses to *H. pylori* in *H. pylori*-positive as compared to *H. pylori*-negative patients. Furthermore, the observation that *H. pylori* infection of mice infected with vaccinia virus reduces the vaccinia-specific cytotoxic T cell response and prolongs the viral infection would support such an activity (Shirai et al. 1998). Taken together, these data suggest that T cells might be a putative target of manipulation by *H. pylori* during infection.

Until recently, neither a bacterial effector nor any target for immunosuppression by *H. pylori* were known. Now, T cells have been identified as a major target for *H. pylori* VacA (Gebert et al. 2003; Boncristiano et al. 2003). Two factors of *H. pylori*—an hitherto unknown membrane-associated factor and VacA—were shown to efficiently inhibit the proliferation of T cells activated by polyclonal stimulators, such as phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) and Ca^{2+} ionophores (ionomycin, A23187). A productive T cell receptor ligation, together with a costimulatory signal via the CD28 surface receptor triggers an effective interleukin-2 (IL-2) secretion. In parallel, the high-affinity IL-2 receptor α (IL-2Rα) is up-regulated on T cells. VacA was found to efficiently block both IL-2 secretion and IL-2Rα surface location and thus inhibits the existing autocrine loop (Gebert et al. 2003) (Fig. 2). Notably, a low ratio of one to five bacteria per T cell resulted already in a clear effect on IL-2 secretion of T cells (Gebert et al. 2003). Such low amounts of VacA, that may be present during gastric infection in vivo, support the idea that a high-affinity receptor for VacA is present on T cells (Boncristiano et al. 2003). The production of cyclins D3 and E were efficiently down-regulated in purified human peripheral blood lymphocytes (PBLCs) treated with VacA, resulting in a reduced phosphorylation and activation of the retinoblastoma protein (Rb), a key regulator of the cell cycle G1/S phase progression (Gebert et al. 2003). This implies that VacA might have a direct effect on the proliferative response of T cells by modulating the cell cycle.

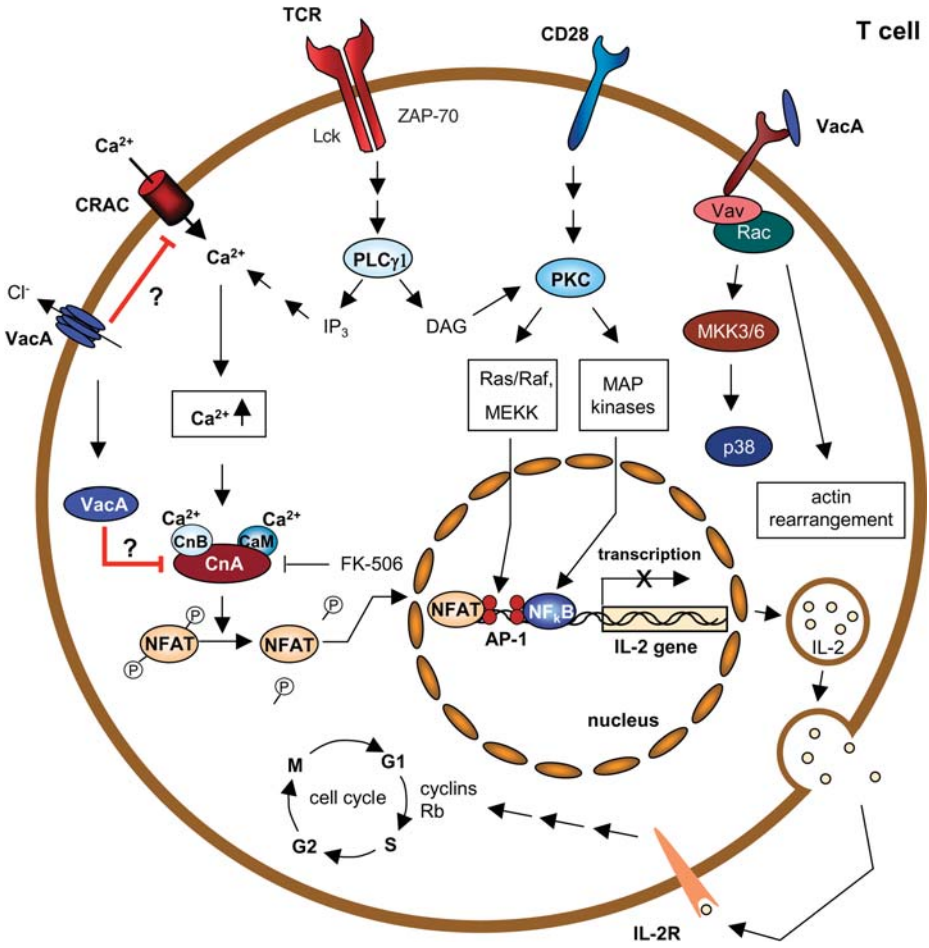

Fig. 2 Model of the interference of VacA with T cell activation. VacA inhibits T cell activation and proliferation by interfering with the T cell receptor (*TCR*) signaling pathway. Upon stimulation of T cells via TCR and CD28, two major pathways are activated [Ca^{2+}-dependent pathway and mitogen-activated protein (*MAP*) kinase pathway]. The anion-selective channel activity of VacA is supposed to depolarize the plasma membrane and to prevent the opening of the Ca^{2+} release-activated Ca^{2+} (*CRAC*) calcium channel (Boncristiano et al. 2003), which is operated by calcium released from intracellular stores. Alternatively, VacA might also block calcineurin activation directly. Inhibition of calcineurin, a prominent target for the immunosuppressive drugs cyclosporin A and FK506, prevents dephosphorylation and translocation of NFAT into the nucleus. As a result, transcription of IL-2 and IL-2Rα genes cannot be initiated (Gebert et al. 2003). The missing IL-2 signaling may arrest the cell cycle via cyclin expression and Rb protein phosphorylation. At low doses, VacA inhibits T cell activation by inducing a cascade of phosphorylation events involving a still-unidentified receptor, Vav, and MKK3/6, resulting in an increase of the active form of p38. Vav induces actin rearrangement through the small GTPase Rac, which leads to inhibition of T cell proliferation (Boncristiano et al. 2003). *AP-1* activator protein 1, *CaM* calmodulin, *CD28* costimulatory molecule, *CnA* calcineurin A subunit, *CnB* calcineurin B subunit, *CRAC* Ca^{2+} release-activated Ca^{2+} channel, *DAG* diacylglycerol, *IL-2* interleukin-2, *IL-2R* interleukin-2-receptor, *IP3* inositol-1,4,5-trisphosphate, *MEKK* MAP/ERK (extracellular regulated kinase) kinase kinase, *MKK* MAP kinase kinase, *NFAT* nuclear factor of activated T cells, *PKC* protein kinase C, *PLCγ1* phospholipase C γ1, *Rb* retinoblastoma protein, *TCR* T cell receptor

Mechanism of T cell inhibition resembles the activity of cyclosporin A and FK506

IL-2 is a major cytokine essential for the proliferation of T cells on antigen stimulation. Transcription of the IL-2 gene is under control of several transcription factors, such as nuclear factor (NF)-κB, activator protein (AP)-1, and nuclear factor of activated T cells (NFAT). In resting T cells, NFAT is phosphorylated at serine–threonine motifs and located in the cytosol. On T cell activation, a massive cytoplasmic Ca^{2+} influx is triggered, first from internal stores and subsequently from external milieu by opening of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. VacA is able to interfere with the Ca^{2+} signaling pathway, which results in blocking of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (PP2B) (Fig. 2). Ca^{2+}-activated calcineurin dephosphorylates NFAT, which subsequently migrates into the nucleus to initiate transcription of a number of genes, including the IL-2 gene. VacA abolishes the translocation of NFAT into the nucleus by specifically blocking the activation of calcineurin, but leaves NF-κB and AP-1 unaffected (Gebert et al. 2003; Boncristiano et al. 2003). This results in a failure of the activated T cell to produce IL-2 and IL2Rα, but also a number of chemokines—such as single C motif-1β (SCM-1β), SCM-1α (lymphotactin, ATAC), macrophage inflammatory protein (MIP)-1α, and MIP-1β—that might be essential for a concerted immune response (Gebert et al. 2003). Thus, VacA displays a similar effect on T cells as the well-known immunosuppressive drugs cyclosporin A and FK506, which block activation of calcineurin. One explanation of calcineurin inhibition might be due to the VacA anion channel activity (Szabo et al. 1999). In the presence of NPPB, VacA had no effect on the A23187-induced cytosolic Ca^{2+} increase, which suggests that the opening of anion channels might influence the CRAC channel activity and inhibit Ca^{2+} influx (Boncristiano et al. 2003). Other possibilities, for example a direct interaction of VacA with calcineurin, cannot be excluded yet, and further investigations are necessary to clarify the underlying mechanisms.

Immune suppression and proinflammatory activities of VacA

In addition to the immune-suppressive activity of the complete VacA protein, which implies the uptake of VacA into cells, Boncristiano et al. (2003) reported on a second activity of VacA mediated from outside by binding of the COOH-terminal p58 domain of VacA to an unknown high-affinity receptor. This binding was supposed to cause activation of Rac and phosphorylation of stress kinases, such as p38, but not Erk1/2, resulting in actin rearrangement, inhibition of T cell proliferation, and an anergic state of T cells (Boncristiano et al. 2003; Montecucco and de Bernard 2003) (Fig. 2). In the gastric cell line AZ-521, VacA treatment induced p38 as well as Erk1/2 phosphorylation and induction of the p38/ATF-2-mediated pathway (Nakayama et al. 2004). In addition to immune suppression, VacA is also postulated to have proinflammatory effects, e.g., by stimulating expression of the proinflammatory enzyme cyclo-oxygenase (COX)-2, not only in T cells, but also in granulocytes and macrophages (Boncristiano et al. 2003). A further study published by Jüttner et al. (2003) showed transcriptional up-regulation of the COX-2 gene in AGS epithelial cells, but the effect was independent of the *cag*-PAI and of VacA (Jüttner et al. 2003). VacA-independent stimulation of COX-2 was also reported for gastric epithelial cells by Busiello et al. (2004). These authors purified the stimulating activity from bacterial culture supernatant and identified it as the secreted protein gamma-glutamyltranspeptidase. Furthermore, VacA was reported to activate mast cells to produce proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-6 (Supajatura et al. 2002).

Conclusions

Over the last 16 years, significant progress has been made in the understanding of the structure and function of VacA. Besides its vacuolating activity, it was surprising to find completely novel effects of VacA, especially on cells of the immune system. The interference of VacA with antigen presentation and the blocking of T cell activation might be a special form of immunosuppression by *H. pylori,* which might help the bacteria establish a chronic persistent infection. The targeting of VacA to mitochondria and the induction of apoptosis in certain cell types has been well documented meanwhile, but the mechanism of action of VacA has to be further established. In future it will be important to analyze the novel activities observed in vitro for their role in vivo during *H. pylori* chronic infection—in animal models or patients—to find out which of the activities are finally relevant.

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