Natural history of *Helicobacter pylori* VacA toxin in human gastric epithelium *in vivo*: vacuoles and beyond

Vittorio Necchi1,2, Patrizia Sommi1, Alessandro Vanoli1, Roberto Fiocca3, Vittorio Ricci1, & Enrico Solcia1,4

Uptake, intracellular trafficking and pathologic effects of VacA toxin from *Helicobacter pylori* have been widely investigated *in vitro*. However, no systematic analysis investigated VacA intracellular distribution and fate in *H. pylori*-infected human gastric epithelium *in vivo*, using ultrastructural immunocytochemistry that combines precise toxin localization with analysis of the overall cell ultrastructure and intercompartmental/interorganellar relationships. By immunogold procedure, in this study we investigated gastric biopsies taken from dyspeptic patients to characterize the overall toxin’s journey inside human gastric epithelial cells *in vivo*. Endocytic pits were found to take up VacA at sites of bacterial adhesion, leading to a population of peripheral endosomes, which in deeper (juxtanuclear) cytoplasm enlarged and fused each other to form large VacA-containing vacuoles (VCVs). These directly opened into endoplasmic reticulum (ER) cisternae, which in turn enveloped mitochondria and contacted the Golgi apparatus. In all such organelles we found toxin molecules, often coupled with structural damage. These findings suggest direct toxin transfer from VCVs to other target organelles such as ER/Golgi and mitochondria. VacA-induced cytotoxic changes were associated with the appearance of autophagolysosomes containing VacA, polyubiquitinated proteins, p62/SQSTM1 protein, cathepsin D, damaged mitochondria and bacterial remnants, thus leading to persistent cell accumulation of degradative products.

*Helicobacter pylori* is a Gram-negative bacterium that colonizes the stomach of about half the global population, thus being one of the most common bacterial infections worldwide1–5. *H. pylori*-dependent gastric pathology ranges from gastritis to mucosal gland atrophy or metaplasia and from peptic ulcer to neoplasia3,4. One of the most important virulence factors of this bacterium is a protein toxin named vacuolating toxin, VacA, because causing massive cytoplasmic vacuolation in cultured cells1–3.

An increasing body of evidence indicates that a functional crosstalk exists between VacA and another key virulence factor of *H. pylori*, the oncoprotein CagA (which is directly injected into host cells by the bacterium through a type IV secretion system) (reviewed in2,6,7). In some instances, these two virulence factors would antagonize each other (e.g., while CagA downregulates both vacuolating and proapoptotic effects induced by VacA, the toxin counteracts the effects of CagA on cell elongation and activation of the transcription factor NFAT and epidermal growth factor receptor). However, VacA and CagA would also act synergistically, for instance in providing *H. pylori* with specific nutrients (e.g., iron) required for its growth. This functional crosstalk would be finalized to achieve an optimal fitness of *H. pylori* with its ecological niche (i.e., the hostile gastric environment), limiting the overall cell damage caused and improving the infection efficiency of the bacterium2,6,7.

To intoxicate its main target host cells (i.e, gastric epithelial cells and T-lymphocytes) VacA exploits a peculiar intracellular trafficking pathway4,8. *In vitro* experimental data show that, after membrane binding, the toxin oligomerizes in lipid rafts and is then internalized through a clathrin-independent endocytic pathway devoted to

---

1Department of Molecular Medicine, Pathologic Anatomy and Human Physiology Units, University of Pavia, Pavia, Italy. 2Centro Grandi Strumenti, University of Pavia, Pavia, Italy. 3Department of Surgical and Diagnostic Sciences, Pathology Unit, University of Genova and IRCCS S. Martino, Genova, Italy. 4Pathologic Anatomy Service, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. Vittorio Ricci and Enrico Solcia jointly supervised this work. Correspondence and requests for materials should be addressed to V.R. (email: vricci@unipv.it)
internalization of glycosylphosphatidylinositol (GPI)-anchored proteins. VacA enters a tubulo-vesicular compartment, named GPI-anchored-protein-enriched early endosomal compartment (GEEC) or clathrin-independent carriers (CLICs), located close to the cell surface. Delivered by a specific subset of early endosomes (motile because the formation of actin comet tails at their surface), most VacA then reaches late endosomes. Here the pore-forming activity of VacA favors the accumulation of osmotically active ions (e.g., NH_4^+) followed by swelling. This leads to the namesake effect of VacA, the development of a massive cytoplasmic vacuolation.

In addition, actin-driven motility of VacA-containing endosomes favors toxin delivery to mitochondria, where VacA translocates and causes apoptosis. However, the exact mechanisms through which VacA reaches its mitochondrial target is still largely unknown.

Kern et al. recently found that VacA reaches also the endoplasmic reticulum (ER) and the Golgi complex, thus identifying these organelles as novel target structures of the toxin. It remains however to be established how the toxin reaches ER and Golgi. Proteomic analysis of VacA-containing vacuoles (VCVs) purified from a T-cell line detected 122 VCV-specific proteins represented, in addition to typical endosomal/lysosomal proteins, by defined proteins from other organelles such as mitochondria, ER, and Golgi. The possibility thus arises that VCVs may exert a specific functional role in the intoxication process of the toxin, acting as a platform to trigger specific trafficking and signaling pathways exploited or influenced by VacA.

VacA impairs immune responses, in particular by modulating the activity of immune effector cells like T lymphocytes, thus favoring the persistence of bacterial infection. In addition, VacA has been found to inhibit antigen processing at endosomal level inside antigen-presenting cells (APCs). Cathepsin E has been identified as a crucial protease for antigen processing in APCs and has also been detected in gastric epithelium where its expression was markedly increased and expanded during H. pylori infection. Thus, a possible VacA-sensitive role for cathepsin E in antigen processing inside gastric epithelial cells should be considered. Furthermore, VacA has been reported to specifically affect the host autophagic and lysosomal machinery, being also associated with autophagosomes, thus suggesting an additional role in the genesis of gastric epithelium pathologic changes.

It must be underlined that most of the data reported derives from in vitro cellular models and that studies indicative of VacA interaction with, trafficking in, and action on the human stomach in vivo are still largely lacking. Thus, at least some of the results obtained so far should be interpreted with caution because, while they may make sense in terms of cell biology, it remains to be established whether, how and to what extent they have actual relevance in H. pylori-infected patients, especially considering the high cell, organ and species specificity of this bacterial infection.

The present study was specifically aimed to investigate the natural history of VacA in H. pylori-colonized human gastric epithelium in vivo by means of transmission electron microscopy (TEM) and ultrastructural immunocytochemistry, the optimal technology to investigate membrane-limited compartments like those involved in VacA intracellular trafficking and action. In particular, we analyzed: a) H. pylori interaction with the gastric epithelium in delivering VacA toxin to host cells, b) VacA uptake at plasma membrane level, c) VacA intracellular trafficking and vacuole development, d) interorganelar interactions and presence of VacA in structurally normal or pathologically altered organelles other than endosomes/vacuoles, such as mitochondria, ER, Golgi, and cellular degradative structures like those of the autophagic-lysosomal pathway or the ubiquitin-proteasome system (UPS).

Results

VacA uptake and accumulation in endocytic-endosomal vesicles of H. pylori-infected human gastric epithelium. In infecting H. pylori, VacA immunoreactivity was mostly concentrated on bacterial outer membrane and its inner and outer leaflets (Fig. 1A,C–F). In addition, in agreement with previous findings on bacterial liquid culture, VacA was found in some outer membrane vesicles (OMVs), ranging from 50 to 300 nm in size, detaching from bacteria and sometimes adhering to the epithelial membrane (Fig. 1A,G,H). Inside H. pylori-infected gastric epithelium, some clear vesicles filling subluminal cytoplasm and infiltrating in between mucin-filled secretory granules also showed VacA reactivity (Fig. 1A,D–F). VacA-reactive bacteria found in the same preparation served as positive controls for the reactivity of such clear vesicles (Fig. 1A,E). Neither such clear vesicles nor VacA reactivity were observed in the gastric epithelium from H. pylori-negative patients taken as a control (Fig. 1B). Bacterial adhesion to the epithelium was characterized either by direct contact, sometimes with apparent fusion, of the respective membranes (Fig. 1A,F), or by connecting thin fibmria-type filaments immunoreactive for LPS O-antigen and VacA (Fig. 1C,D). Both H. pylori products were seen to accumulate on the confronting luminal epithelial membrane (Fig. 1C,D) and to be taken up by subluminal clear vesicles (Fig. 1D,F).

VacA-containing tubular invaginations of the luminal epithelial membrane of H. pylori-infected epithelium were found to penetrate cell cytoplasm and to interact with VacA-positive subluminal clear vesicles (Fig. 1E,F). These tubular invaginations were apparently lacking clathrin coat. They are likely to represent the human in vivo equivalents of the non-coated endocytic pits taking up VacA and generating VacA-positive superficial (early) endosomes (i.e., the aforementioned GEEC/CLICs) in experiments on cell lines in vitro. In addition, clear endosomal vesicles were frequently found to directly contact each other and with larger vacuoles, showing focal loss of their limiting membrane at contact site, a pattern highly suggestive for vesicle fusion (Figs 1A and 2A,B). This may provide a pathway for VacA trafficking to juxtanuclear (late) endosomes and their VCVs derivatives.

VCV role as VacA-distributing platform. VacA-accumulating VCVs were frequently found to directly contact ER, whose cisternae were also observed to open into VCVs, thus allowing free communication between respective lumina and possible transfer of VacA molecules from VCVs to ER (Figs 1I and 2A,B). As in turn ER cisternae frequently enveloped mitochondria (Figs 1I and 2A), this may well generate a pathway for VacA...
Figure 1. VacA uptake in endocytic-endosomal structures of *H. pylori*-infected human gastric epithelium. (A) VacA immunogold particles on bacterial outer membrane (see also C, D, and E) and a few OMVs (arrows). Inside the epithelium, note small clear vesicles with VacA reactivity (enlarged in the inset), subluminal or interspersed among mucin secretory granules. No such vesicles are seen in non-infected, VacA-negative, control epithelium (B). (C) An infected epithelium immunostained for VacA (20-nm gold particles) and *H. pylori* LPS O-antigen (10-nm particles) shows two *H. pylori* lying in the narrow space between luminal mucus (muc) and the epithelial surface (ep). In addition to VacA, mainly on bacterial outer membrane, LPS reactivity (arrows) is shown by filamentous material (fimbriae) connecting the external leaflet of bacterial outer membrane to the apical pole of epithelial plasma membrane. (D) VacA seems to slide along connecting filaments up to the epithelial membrane, where it is apparently taken up by immediately underlying endocytic vesicles. (E) Selective VacA uptake by a subluminal vesicle underlying an adhering bacterium. (F) VacA and LPS reactivities around a bacterium, which adheres to the epithelial membrane in its upper part and to two microvilli (arrowheads) forming two luminal pouches in between bacterial and epithelial plasma membrane. Both pouches and intraepithelial clear vesicles show VacA (black arrows) and/or LPS (white arrows). (G) An epithelial membrane invagination, enlarged in the inset to better recognize its VacA content and non-coated structure, exemplifies the endocytic process of toxin uptake. Note also VacA (arrowhead) in the cytoplasm underlying a bacterium, and focal dilations of bacterial periplasmic space, a likely source of the OMV adhering to the epithelial membrane (arrow). (H) Two enlarged VacA-reactive OMVs interact with luminal epithelial membrane, either with (arrow) or without (lower vesicle) loss of bacterial outer membrane at site of adhesion. (I) Small subluminal endocytic/endosomal vesicles enlarge and fuse into VCVs in the deep supranuclear cytoplasm of an heavily infected cell. n, nucleus. (J) VCVs in the deep supranuclear cytoplasm open directly into ER cisternae (arrows), some of which envelop mitochondria (m). Direct VCV-mitochondria communication is also seen (white arrowhead). n, nucleus.
Figure 2. Intracellular distribution of VacA and damaged organelles. (A) and (B) VCVs imping on RER cisternae, several of which directly open into them (arrows). Note in (A) many well-preserved, VacA-unreactive mitochondria enveloped by RER cisternae and in (B) several endosomal vesicles fusing each other and with VCVs, in addition to lysosomal-type cellular debris (arrowheads) inside the lower VCV. (C) Juxtanuclear Golgi shows focal VacA reactivity and cisternae dilation. Also note VacA positivity of an adjacent ER cisterna. n, nucleus. Unreactive Golgi (D) and ER (E) are shown from uninfected control epithelium incubated with anti-VacA antibodies. (F to H) Cathepsin E reactivity, normally restricted to RER in H. pylori–negative epithelium (F, note unreactive secretory granules in the uppermost cytoplasm), extended to peripheral (G) and juxtanuclear (H) endosomes of a H. pylori–infected epithelium. n, nucleus. Note in (G) the unreactivity of the luminal bacterium and of a forming endocytic vesicle (arrows) for such a host protein. (I) Several mitochondria, partly enveloped by ER cisternae and focally VacA-reactive, are shown; a large one (center of the micrograph) is both VacA-positive (arrows) and heavily damaged (swelled and vacuolated). (J) Several heavily damaged mitochondria (arrows, one of which still VacA-positive) are enclosed in a cytoplasmic vesicle, likely of autophagosomal origin, followed by lysosomal fusion.
transfer also to mitochondria, in addition to the direct VCV-mitochondria communications we occasionally found (Fig. 1J). Of interest was also the finding of VacA reactivity in some Golgi cisternae and adjacent ER cisternae (Fig. 2C). In parallel tests, both the Golgi complex (Fig. 2D) and ER (Fig. 2E) of control gastric epithelium from uninfected human biopsies failed to show any VacA reactivity.

**Cathepsin E in endosomal vesicles and vacuoles.** In normal, non-infected gastric epithelium, cathepsin E was found to be closely restricted to the rough ER (RER) (Fig. 2F). However, in *H. pylori*-infected gastric foveolar epithelium, cathepsin E was also detected in endosomes, both peripheral and juxtanuclear, and related VCVs (Fig. 2G,H).

**VacA, intracellular pathologic changes and cellular degradative structures.** In addition to cytoplasmic vacuoles, several other pathologic changes were found in *H. pylori*-infected gastric epithelium, among which mitochondrial lesions, with loss of cristae or matrix lysis, and increased autophagy of damaged mitochondria (Fig. 2I,J). The close direct or ER-mediated interaction found between VCVs and mitochondria is likely to account for the focal VacA reactivity found in the latter organelles, which sometimes was directly coupled with pathologic changes (Fig. 2I,J).

A frequent finding, especially in cells with consistent cytotoxic changes (larger endosomal vacuoles, damaged mitochondria, autophagic vesicles, lysis of mucin granules or penetrating intercellular clefts), were supranuclear clear bodies (Fig. 3A) of vesicular to solid structure, often containing dense cathepsin D-positive lysosome-type deposits. Such single-membrane-limited bodies, which occasionally stored bacterial or mitochondrial remnants, showed reactivity for p62/SQSTM1 protein, LC3, VacA, cathepsin D, LPS, and K63-linked polyubiquitin (pUb) chains (Fig. 3A–E). Thus, they were interpreted as auto(phago)lysosomes. Notably, no such bodies were found in control uninfected epithelium (Fig. 3F).

Particle-rich cytoplasmic structures (PaCSs)24, characterized by a collection of barrel-like particles heavily reactive for 19S and 20S proteasome, were detected in the cytoplasm below the nucleus (Fig. 3G), especially in cells showing less prominent cytotoxic changes and scarce or no auto(phago)lysosomes. Such structures were also intensely positive for FK1 antibody-reactive polyubiquitinated proteins (Fig. 3G) and, moderately/focally, for VacA (Fig. 3H), while they were unreactive for K63-linked pUb chains (Fig. 3I) and p62/SQSTM1 protein (not shown). They thus reflected structural and cytochemical patterns of the PaCSs previously seen in *H. pylori* gastritis, gastric cancer and several cell lines24–26, including their close topographic relationship with surrounding ribosomes and RER cisternae (Fig. 3G–I). Unfortunately, despite testing different antibodies raised against the K48-linked pUb chains (i.e., the type of pUb chains known to be selectively associated with proteasomal degradation27, we did not find any antibody which worked in our TEM experimental conditions (i.e., aldehyde-osmium fixed resin-embedded specimens). This prevented a direct proof of K48-linked pUb nature of the FK1-positive polyubiquitinated proteins stored by PaCSs.

**Discussion**

A prominent finding of this investigation of *H. pylori*-infected human gastric epithelium *in vivo* was the detection of a population of small subluminal vesicles (and/or tubulovesicles), interposed with or overlying mucin granules, which were essentially lacking in non-infected epithelium. The actual presence of VacA immunoreactivity in a substantial fraction of such vesicles as well as in endocytic pits of the luminal plasma membrane strongly supports the endocytic-endosomal nature of such vesicles and their likely induction by bacterial infection, as previously suggested by *in vitro* experiments22,28–30. The non-coated tubular nature of these endocytic pits suggests that the clathrin-independent non-caveolar pinocytic mechanism9 of VacA internalization documented in experimental models *in vitro*9,32 may have an *in vivo* counterpart in *H. pylori*-infected patients. Given their abundance in the infected epithelium, especially in association with *H. pylori* intimately adhering to surface epithelial cells, it seems likely that the endocytic–endosomal vesicles represent the main route of VacA cellular uptake *in vivo*. Although VacA-containing OMVs as well as whole *H. pylori* bodies were also found to intimately adhere with and enter epithelial cell lines *in vitro* and the gastric epithelium *in vivo*,22,23,33,34 (the present study), these remained less common findings than VacA-containing endocytic-endosomal vesicles inside the cells of *H. pylori*-infected gastric biopsy samples here studied. Our immunocytochemical findings also suggest that endosomal vesicles represent the main route of VacA intracellular trafficking, from subluminal early to juxtanuclear late endosomes, where most VCVs accumulate *in vivo*

Of special interest is our observation of VCVs frequently contacting and also opening into adjacent ER cisternae. Of high interest is also the observation of close simultaneous interactions of VCVs with ER cisternae and mitochondria, of which here we provide the first *in vivo* demonstration as VacA targets. Our findings thus provide the *in vivo* counterpart in *H. pylori*-infected human epithelium of the *in vitro* observations by Kern et al.13 in non-gastric cell lines (i.e., Jurkat T-cell and epithelial HeLa cell lines) showing that VacA also targets ER and Golgi and suggesting that VCVs may have a key role in VacA intoxication processes beyond the vacuoles. Indeed, VCVs seem to act as a platform to trigger specific trafficking pathways exploited by the toxin. Our direct immunocytochemical detection of VacA inside Golgi cisternae further confirms Kern et al.’s data12, although the exact route of VacA transport to the Golgi (from ER to Golgi?) remains unknown. Our *in vivo* findings also support Calore et al.’s *in vitro* observations15 suggesting that VacA might be transferred to mitochondria by endosomal–mitochondrial juxtapositional exchange.

Intriguingly, the existence of distinct interaction domains between the ER and other organelles (such as mitochondria and endosomes), known as membrane contact sites (MCSs), has been recently demonstrated (reviewed in36). At MCSs, organelle membranes are closely apposed and tethered (but apparently do not fuse), and here various protein complexes might work in concert to perform specialized functions as binding, sensing and transferring molecules, as well as engaging in organelle biogenesis and dynamics36. Through the establishment of such
Figure 3. VacA-associated cellular degradative structures. (A) Well-developed, p62/SQSTM1 protein-reactive, ALIS-type auto(phago)lysosomal body (arrows) in the deep supranuclear cytoplasm of a *H. pylori*-infected cell also showing a penetrating luminal cleft (lc), a subluminal collection of mucin granules (asterisk) with interspersed small clear endosomal vesicles and deep VCVs directly interacting (arrowhead) with such a body. This is partly (see box in A) enlarged in (A1) to show p62/SQSTM1-reactivity and heterogeneous lysosomal-type ultrastructure. n, nucleus of a cell adjacent to the body-bearing one. (B) Selective immunoreactivity for LC3 protein of another ALIS-type body further supports its auto(phago)lysosomal nature. (C to E) High enlargements of part of three auto(phago)lysosomal bodies to show their cathepsin D (C), VacA (D) and K63-linked pUb chains (E) reactivity. Also note in (D) the presence inside the body of a VacA-reactive phagocytosed *H. pylori* (arrows). (F) Neither auto(phago)lysosomes nor VCVs were found in the supranuclear cytoplasm (mainly filled by VacA-unreactive RER) of control, uninfected gastric epithelium. (G) A PaCS, formed by a collection of barrel-like, proteasome-reactive particles (see positivity for 19S proteasome subunit by 10-nm immunogold), is found in the cytoplasm below the nucleus of a *H. pylori*-infected foveolar cell, surrounded...
by free ribosomes (asterisk) and RER cisternae (arrow). Also note in (G) selective PaCS reactivity for the polyubiquitinated protein-specific FK1 antibody (20-nm gold). (H) Focal PaCS reactivity for VacA (enlarged in H1). Note VacA reactivity (white arrow) also at the level of a mitochondrion (white asterisk) enveloped by an ER cisterna (black asterisk). (I) The unreactivity of PaCS for K63-linked pUb chains is shown; also note direct merging of several RER cisternae with the PaCS (arrows).

physical contacts with different cell organelles, ER seems thus emerging as a key player in spatiotemporal control of organelar dynamics inside the cell. It has been speculated that these interorganelar "synapses" might serve as a direct delivery route between compartments, bypassing usual trafficking pathways known so far\textsuperscript{27}. Whether canonical MCSs may have a role in VacA trafficking it remains to be investigated.

The endosomal accumulation of VacA toxin and other $H.~pylori$ antigens, like LPS O-antigen, is especially interesting considering 1) the well-known role of endosomes in taking up, storing and processing antigens to be membrane-presented in HLA molecules background\textsuperscript{38,39}, 2) the high de novo expression of HLA-DR by $H.~pylori$-infected gastric epithelium\textsuperscript{19}, 3) the capacity of VacA to interfere with antigen processing by professional APCs at endosomal level\textsuperscript{44}, as well as 4) our present finding of endosomal localization of cathepsin E, an aspartic protease crucial for antigen processing in several APCs\textsuperscript{15–17,40,41}. A VacA-sensitive role for cathepsin E in proteins, and K63-linked pUb chains is highly suggestive for an autophagic component\textsuperscript{53,58–60}. Furthermore, we also obtained ultrastructural and cytochemical (e.g., cathepsin D reactivity) evidence for a lysosomal contribution of misfolded, mutated or anyway damaged cytosolic proteins, known to be produced in excess in neoplastic or fetal cells as well as in hematopoietic cells specifically stimulated by trophic factors and interleukins\textsuperscript{25,26,54}. Our present observations are in keeping with previous in vitro studies concerning the fate of VacA-induced vacuoles\textsuperscript{28,50}. Supranuclear auto(phago)lysosomal bodies in $H.~pylori$-infected gastric epithelium, unlike auto(phago)lysosomal bodies, PaCSs were usually found in the cytoplasm below the nucleus and, preferentially, in cells showing limited cytotoxic changes. Concerning the polyubiquitinated proteins they store, PaCSs differed from autophagosomes/autolysosomes in being unreactive for K63-linked pUb-directed antibodies, while being reactive for the FK1 antibody, known.
to recognize in vitro polyubiquitinated proteins exhibiting either K63- or K48-linked pUb chains. As only K48-linked polyubiquitinated proteins are known to selectively associate with proteasome, it seems likely that the FK1-reactive and K63-linkage- reactive polyubiquitinated proteins associated with proteasome particles inside PaCSs are to be interpreted as K48-linked proteins. Interestingly, the direct opening we found of some ER cisternae into PaCSs may indicate a pathway through which VacA reaches this essentially cytosolic UPS-rich structure and, more in general, might also suggest a way for endocytosed exogenous antigens to reach cytosolic proteasome for class I cross-presentation.

Our present findings show that, notwithstanding their common association with H. pylori infection and common storage of polyubiquitinated proteins, ALIS-type auto(phago)lysosomal bodies and PaCSs are cytochemically linked to two different protein-degradative pathways, namely: 1) the K63-linked pUb, p62/SQSTM1 and LC3 positive autophagic-endolysosomal system, and 2) the proteasome and K48-linked pUb chain positive UPS, respectively. In keeping with our recent findings in human DCs in vitro, PaCSs may represent an early, chaperon protein-promoted and ubiquitin/proteasome-mediated cellular attempt to repair or degrade H. pylori-induced misfolded proteins, whereas the ALIS bodies may result from cellular activation of the autophagic/lysosomal pathway by severe cytotoxic lesions affecting cytoplasmic organelles.

In conclusion, our in vivo study shows that VacA mainly enters H. pylori-infected human gastric epithelium by endocytosis and accumulates into endosomes and endosome-derived VCVs, which directly communicate with ER cisternae and ER-enveloped mitochondria. This latter finding supports toxin trafficking from VCVs to other organelles such as ER/Golgi and mitochondria, as previously suggested by in vitro experiments. De novo endosomal expression by infected gastric epithelium of the antigen-processing proteinase cathepsin E may have a role in the complex VacA-associated host immune-inflammatory response which characterizes H. pylori infection. VacA-induced cytotoxic effects on cell organelles and protein turnover is associated with activation of main cellular degradative systems with persistent accumulation of degradative products inside auto(phago)lysosomes.

Materials and Methods

Human biopsy samples. We reinvestigated biopsy samples of gastric mucosa taken in the period 1981–95 from 26 patients (15 males and 11 females, aged between 26 and 79 years) undergoing routine endoscopic and histologic examination for dyspepsia as requested by the physician in charge of the patient and with the written consent of the patient. The study has been approved by the Ethics Committee of Fondazione IRCCS Policlinico San Matteo (Pavia, Italy) as a re-investigation of archival material along the same line (i.e., diagnosis of H. pylori-dependent gastritis) as for the original written consensus. All the methods were performed in accordance with the relevant guidelines and regulations.

Six biopsy specimens (from the antrum and from the corpus of the stomach) were taken from each patient. From each biopsy site, 2 samples were processed for light microscopy and 1 sample for TEM. Fifteen patients resulted H. pylori-positive in all biopsies at both light microscopy (Giemsa staining and histochemistry for H. pylori LPS) and TEM (detection of characteristic bacterial ultrastructure coupled with VacA and H. pylori LPS cytochemistry) investigation. Their biopsy specimens were thus judged as suitable for present investigation on VacA interaction with human gastric mucosa. Four patients resulted H. pylori-negative in all biopsies from the antrum and corpus, extensively investigated at both light microscopy and TEM as above. The eight TEM-processed biopsy specimens from these 4 patients were thus taken as negative controls in the present study. Biopsy specimens from the remaining 7 patients showed more limited H. pylori colonization, often unequally distributed among different specimens. These cases were not further investigated in the present study.

TEM and ultrastructural immunocytochemistry. For TEM investigation, biopsy samples were fixed for 4 hours with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), followed by 1% osmium tetroxide for 1 hour, and then embedded in Epon-Araldite resin. Thin (~70 nm) sections were stained with uranyl-lead or underwent the immunogold procedure followed by uranyl-lead counterstaining, as previously detailed. Specimens were analyzed by a Jeol JEM-1200 EX II transmission electron microscope equipped with an Olympus CCD camera (Mega View III). Images were processed and assembled by using the Adobe Photoshop CSS software.

Antibodies. The following primary antibodies (Abs) were used: 1) Anti-VacA: a) rabbit polyclonal 123 anti-serum raised against purified VacA (s1/i1/m1 vacA genotype) provided by T.L. Cover, Nashville, TN) and b) rabbit polyclonal HPP-5013-9 Ab (Austral Biologicals, San Ramon, CA) raised against a recombinant VacA fragment (aa 311-819); 2) Anti-LPS O-antigen: rabbit polyclonal V4074 Ab (Biømeda, Foster City, CA) (Andersen 1988 APMIS); 3) Anti-cathepsin E: a) rabbit polyclonal PA3-16821 Ab (Thermo Fisher Scientific, Rockford, IL), and b) mouse monoclonal sc-166343 Ab (clone E-8; Santa Cruz Biotechnology, Santa Cruz, CA); 4) Anti-cathepsin D: a) mouse monoclonal NBPI-04278 (clone 4G2; Novus Biologicals, Littleton, CO), and b) rabbit polyclonal sc-10725 Ab (Santa Cruz Biotechnology); 5) Anti-p62/SQSTM1: a) rabbit polyclonal PM045 Ab (MBL, Nagoya, Japan), and b) mouse monoclonal sc-28359 (clone D-3; Santa Cruz Biotechnology); 6) Anti-LC3: rabbit polyclonal NB100-2220 Ab (Novus Biologicals); 7) Anti-polyubiquitinated proteins: mouse monoclonal FK1 Ab (BML-PW8805; Enzo Life Sciences, Farmingdale, NY); 8) Anti-K63-linked pUb chains: mouse monoclonal BML-PW0600 Ab (clone HWA4C4; Enzo Life Sciences); 9) Anti-K48-linked pUb chains: a) rabbit monoclonal 05-1307 Ab (clone A2; Millipore, Billerica, MA), and b) rabbit monoclonal 140601 Ab (clone EP8589; Abcam, Cambridge, UK); c) rabbit monoclonal A-101 Ab (clone 1001c; BostonBiochem, Cambridge, MA); 10) Anti-20S proteasome α/3 subunits: rabbit polyclonal BML-PW8155 (Enzo Life Sciences); 11) Anti-19S proteasome S2 subunit: rabbit polyclonal 539166 Ab (Calbiochem, La Jolla, CA).

As secondary Abs, anti-rabbit or anti-mouse immunoglobulins labeled with 10, 15 or 20 nm gold particles (British Bio Cell, Cardiff, UK, and Aurion, Wageningen, The Netherlands) were used.
Tests to evaluate the specificity of immunogold labeling were carried out using antibodies absorbed with excess antigen and omitting the specific antibodies in the first layer of the immunogold procedure. Positive and negative controls were obtained by parallel investigation of *H. pylori* cultures, epithelial cell cultures, and *H. pylori*-positive or -negative gastric mucosa specimens as in previous studies. In particular, both anti-VacA antibodies used were tested using parallel TEM investigation on well-characterized bacterial cultures either VacA-producing (*H. pylori* strains 60190, ATCC 49503, and CCUG 17874, from Culture Collection University of Göteborg, Sweden) or not producing the toxin (*H. pylori* strain 60190v1, the isogenic mutant of the 60190 strain in which the vacA gene was disrupted by insertional mutagenesis, kindly provided by T.L. Cover, Nashville, TN). We also assessed the specificity of these antibodies by means of SDS-PAGE, followed by Western blotting, on bacterial lysates and broth culture filtrates of the aforementioned *H. pylori* strains.

**Data availability.** No datasets were generated or analysed during the current study.

**References**

1. Cover, T. L. & Blanke, S. R. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nat. Rev. Microbiol.* 3, 320–322 (2005).

2. Boquet, P. & Ricci, V. Intoxication strategy of *Helicobacter pylori* VacA toxin. *Trends Microbiol.* 20, 165–174 (2012).

3. Cover, T. L. & Peek, R. M. Diet, microbial virulence and *Helicobacter pylori*-induced gastric cancer. *Gut Microbes* 4, 482–493 (2013).

4. Solcia, E. et al. Intestinal and diffuse gastric cancer arise in a different background of *Helicobacter pylori* gastritis through different gene involvement. *Am. J. Surg. Pathol.* 20(Suppl. 1), S8–S22 (1996).

5. Ricci, V., Sommi, P. & Boquet, P. *Helicobacter pylori* vacuolating toxin. In The Comprehensive Sourcebook of Bacterial Protein Toxins, 4th ed. (eds. About, J., Ladant, D. & Popoff, M. R.) 515–557 (Elsevier, 2015).

6. Backert, S. & Tegtmeyer, N. The versatility of the *Helicobacter pylori* vacuolating cytotoxin VacA in signal transduction and molecular crosstalk. *Toxins* 2, 69–92 (2010).

7. Ricci, V. Relationship between VacA toxin and host cell autophagy in *Helicobacter pylori* infection of the human stomach. A few answers, many questions. *Toxins* 8, 203 (2016).

8. Utsch, C. & Haas, R. VacA's induction of VacA-containing vacuoles (VCVs) and their immunomodulatory activities on human T cells. *Toxins* 8, 190 (2016).

9. Gauthier, N. C. et al. *Helicobacter pylori* VacA cytotoxin: a probe for a clathrin-independent and Cdc42-dependent pinocytic pathway routed to late endosomes. *Mol. Biol. Cell* 16, 4852–4866 (2005).

10. Gauthier, N. C. et al. Early endosomes associated with dynamic F-actin structures are required for late trafficking of *H. pylori* VacA toxin. *J. Cell Biol.* 177, 343–354 (2007).

11. Calmiseche, A. et al. The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J.* 23, 6361–6370 (2000).

12. Kern, B. et al. Characterization of *Helicobacter pylori* VacA-containing vacuoles (VCVs), *VacA* intracellular trafficking and interference with calcium signalling in T lymphocytes. *Cell. Microbiol.* 17, 1811–1832 (2015).

13. Djekic, A. & Müller, A. The immunomodulatory VacA promotes immune tolerance and persistent *Helicobacter pylori* infection through its activities on T-cells and antigen-presenting cells. *Toxins* 8, 187 (2016).

14. Molinari, M. et al. Selective inhibition of li-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *J. Exp. Med.* 187, 135–140 (1998).

15. Bennett, K. et al. Antigen processing for presentation by class II major histocompatibility complex requires cleavage by cathepsin E. *Eur. J. Immunol.* 22, 1519–1524 (1992).

16. Finley, E. M. & Kornfeld, S. Subcellular localization and targeting of cathepsin E. *J. Biol. Chem.* 269, 31259–31266 (1994).

17. Chain, B. M. et al. The expression and function of cathepsin E in dendritic cells. *J. Immunol.* 174, 1791–1800 (2005).

18. Samloff, I. M. et al. Slow moving proteasine. *Isolation, characterization, and immunohistochemical localization in gastric mucosa. Gastroenterology* 93, 77–84 (1987).

19. Fiocca, R. et al. Epithelial cytotoxicity, immune responses, and inflammatory components of *Helicobacter pylori* gastritis. *Scand. J. Gastroenterol.* 29(Suppl. 205), 11–21 (1994).

20. Terebiznik, M. R. et al. Effect of *Helicobacter pylori* vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. *Autophagy* 5, 370–379 (2009).

21. Raju, D. et al. Vacuolating cytotoxin and variants in Atg16L1 that disrupt autophagy promote *Helicobacter pylori* infection in humans. *Gastroenterology* 142, 1160–1171 (2012).

22. Fiocca, R. et al. Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *J. Pathol.* 188, 220–226 (1999).

23. Necchi, V. et al. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori* *Helicobacter pylori*. *Gastroenterology* 132, 1009–1023 (2007).

24. Necchi, V., Sommi, P., Ricci, V. & Solcia, E. In vivo accumulation of *Helicobacter pylori* products, NO1, ubiquitinated proteins and proteasome in a novel cytoplasmic structure. *PLoS ONE* 5, e9716 (2010).

25. Necchi, V. et al. Proteasome particle-rich structures are widely present in human epithelial neoplasms: Correlative light, confocal and electron microscopy study. *PLoS ONE* 6, e21317 (2011).

26. Sommi, P. et al. PaCS is a novel cytoplasmic structure containing functional proteasome and inducible by cytokines/trafic factors. *PLoS ONE* 8, e82560 (2013).

27. Newton, K. et al. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell* 134, 668–678 (2008).

28. Ricci, V. et al. Cytotoxicity of *Helicobacter pylori* on human gastric epithelial cells in vitro: role of cytotoxic(s) and ammonia. *Eur. J. Gastroenterol. Hepatol.* 6, 687–694 (1995).

29. Ricci, V. et al. *Helicobacter pylori* vacuolating toxin accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolating activity of ammonia. *J. Pathol.* 183, 453–459 (1997).

30. Papini, E. et al. Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proc. Natl. Acad. Sci. USA* 91, 9720–9724 (1994).

31. Johannes, L., Parton, R. G., Basereau, P. & Mayor, S. Building endocytic pits without clathrin. *Nat. Rev. Mol. Cell Biol.* 16, 311–321 (2015).

32. Ricci, V. et al. High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol. Biol. Cell* 11, 3897–3909 (2000).

33. Kwok, T., Backert, S., Schwarz, H., Berger, J. & Meyer, T. F. Specific entry of *Helicobacter pylori* into cultured gastric epithelial cells via a zipper-like mechanism. * Infect. Immun.* 70, 2108–2120 (2002).

34. Olofsson, A. et al. Biochemical and functional characterization of *Helicobacter pylori* vesicles. *Mol. Microbiol.* 77, 1539–1555 (2010).
35. Calore, F. et al. Endosome–mitochondria juxtaposition during apoptosis induced by *H. pylori* VacA. *Cell Death Differ.* 17, 1707–1716 (2010).
36. Phillips, M. J. & Voeltz, G. K. Structure and function of ER membrane contact sites with other organelles. *Nat. Rev. Mol. Cell Biol.* 17, 69–82 (2016).
37. van der Goot, F. G. & Gruneberg, J. Close encounter of the third kind: The ER meets endosomes at fission sites. *Dev. Cell* 31, 673–674 (2014).
38. Neeftes, J., Jongsm, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11, 823–836 (2011).
39. Chatterjee, B. et al. Internalization and endosomal degradation of receptor-bound antigens regulate the efficiency of cross presentation by human dendritic cells. *Blood* 120, 2011–2020 (2012).
40. Yee, C. S. K. et al. Cathepsin E: A novel target for regulation by class II transactivator. *J. Immunol.* 172, 5528–5534 (2004).
41. Tizone, C., Tsang, J., Prokopowicz, Z., Groevenor, N. & Chain, R. Natural cathepsin E deficiency in the immune system of C57BL/6 mice. *Immunogenetics* 59, 927–935 (2007).
42. Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G. & Morgan, D. R. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J. Med. Microbiol.* 26, 93–98 (1988).
43. Cover, T. L., Dooley, C. F. & Blaser, M. J. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. *Infect. Immun.* 58, 603–610 (1990).
44. Kimura, M. et al. Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb. Pathog.* 26, 45–52 (1999).
45. Willhite, D. C. & Blanke, S. R. *Helicobacter pylori* vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. *Cell. Microbiol.* 6, 143–154 (2004).
46. De Bernard, M. et al. Cell vacuolization induced by *Helicobacter pylori* VacA toxin: cell line sensitivity and quantitative estimation. *Toxicol. Lett.* 99, 109–115 (1998).
47. Maituri, M. C., Zalckvar, E., Kimchi, A. & Kroemer, G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 8, 741–752 (2007).
48. Sommi, P. et al. Persistence of *Helicobacter pylori* VacA toxin and vacuolating potential in cultured gastric epithelial cells. *Am. J. Physiol.* 275, G681–688 (1998).
49. Molinari, M. et al. Vacuoles induced by *Helicobacter pylori* toxin contain both late endosomal and lysosomal markers. *J. Biol. Chem.* 272, 25339–25344 (1997).
50. Catrenich, C. E. & Chestnut, M. H. Character and origin of vacuoles induced in mammalian cells by the cytotoxin of *Helicobacter pylori*. *J. Med. Microbiol.* 37, 389–395 (1992).
51. Saeta, J. et al. ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy. *Autophagy* 2, 189–199 (2006).
52. Kantiuk, N. A. et al. *Citrobacter rodentium* infection induces MyD88-dependent formation of ubiquitinated protein aggregates in the intestinal epithelium. *J. Innate Immun.* 3, 83–98 (2011).
53. Kondylis, V. et al. Endosome-mediated autophagy: an unconventional MHC-driven autophagic pathway operational in dendritic cells. *Autophagy* 9, 861–880 (2013).
54. Montagna, D. et al. Different polyubiquitinated bodies in human dendritic cells: IL-4 causes PaCS during differentiation while LPS or INFα induces DALIS during maturation. *Sci. Rep.* 7, 1844 (2017).
55. Solcia, E. et al. Particle-rich cytoplasmic structure (PaCS): Identification, natural history, role in cell biology and pathology. *Biomolecules* 4, 848–861 (2014).
56. Satin, R. et al. *Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins*. *J. Cell Biol.* 164, 667–675 (2004).
57. Hetzer, S. et al. Dendritic cell aggresome-like induced structure formation and delayed antigen presentation coincide in influenza virus-infected dendritic cells. *J. Immunol.* 175, 891–898 (2005).
58. Bjorkas, G. et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* 171, 603–614 (2005).
59. Pankiv, S. et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* 282, 24131–24145 (2007).
60. Tan, J. M. M. et al. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. *Human Mol. Genet.* 17, 431–439 (2008).
61. Satin, R. et al. Effect of *Helicobacter pylori* vacuolating toxin on maturation and extracellular release of procathespin D and on epidermal growth factor degradation. *J. Biol. Chem.* 272, 25022–25028 (1997).
62. Fujimura, M., Sawada, H. & Yokosawa, H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett.* 349, 173–180 (1994).
63. Monn, N. & Trombetta, E. S. Cross-talk between the endocytic pathway and the endoplasmic reticulum in cross-presentation by MHC class I molecules. *Curr. Opin. Immunol.* 19, 66–72 (2007).
64. Necchi, V., Manca, R., Ricci, V. & Solcia, E. Evidence for transepithelial dendritic cells in human *H. pylori* active gastritis. *Helicobacter* 14, 208–222.
65. Cover, T. L. & Blaser, M. J. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.* 267, 10570–10575 (1992).

**Acknowledgements**

This study was supported by grants from the Italian Ministry of Health to Fondazione IRCCS Policlinico San Matteo and University of Pavia (Grant n. RF-2010-2310098) and from Fondazione Cariplo (Milan, Italy; grants n. 2011-0485 and 2012-0529). We thank Dr. T.L. Cover (Vanderbilt University, Nashville, TN) for kindly providing us with the anti-VacA 123 serum and the VacA-defective *H. pylori* strain 60190:v1.

**Author Contributions**

V.R. and E.S. designed and guided the project. V.N., P.S., A.V., and R.F. performed the experiments. V.R. and E.S. analyzed the data and wrote the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
