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Host cell resistance to CagA translocation is as variable as *Helicobacter pylori*

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Abstract
Since its discovery in the human stomach, the bacterium *Helicobacter pylori* has been branded as the cause of gastric diseases. This association is linked to the oncogenic toxin CagA produced by certain *H. pylori* strains, which causes severe damages but needs to be injected into the host cells to exert its toxic effect. Injection is achieved by a special bacterial transport mechanism, the Cag Type IV secretion system (Cag-T4SS). However, in nature, not all *H. pylori* strains infecting a patient contain the CagA toxin and the Cag-T4SS. In accordance with this, we have performed pre- and co-infection experiments of human cells *in vitro* with several strains of *H. pylori*. These experiments revealed that host cells can build up a resistance to the injection of CagA and to cellular damages associated with it. In order to further understand the mechanisms involved in this behavior, we analyzed the scope of it by looking at single aspects of infection. These included an evaluation of time of pre-infection and its effect on CagA translocation and cytokine response of the host cells to the Cag T4SS. Additionally, because of the high genetic variability of *H. pylori*, it was necessary to study the outreach of this phenomenon during the combination of different wild-type strains. It is remarkable that this phenomenon was not only observed in the epithelial host cell model but as well in primary cells from a hematopoietic origin, suggesting a relevance of the resistance mechanism to the CagA toxin and the Cag T4SS in the way the immune response is triggered during infection.

Introduction
Self-infection experiments with *Helicobacter pylori* performed by Barry Marshall labelled *H. pylori* as the pathogen responsible for gastric pathologies [1] [2] with the *cagA* gene as one of the first genes correlated with severe gastric pathologies [3] [4]. Its product, the CagA toxin, is injected into human cells by a Type IV secretion apparatus, encoded by *H. pylori* in the cag pathogenicity island (Cag-PAI). Inside the host cell CagA is phosphorylated by host cell kinases [5] [6]. A functional Cag T4SS is not only able to inject CagA into host cells but it also induces a strong pro-inflammatory chemokine response that includes IL-8 and IL-1ß [7]. Since CagA found inside the host cell, either phosphorylated or not, is able to disturb several signaling pathways [8] [9], it is necessary to understand under which conditions the bacterium injects the toxin into the host cells. In nature, a human stomach can contain multiple *H. pylori* strains at the same time. Some strains may contain a functional CagA T4SS, denominated as Type I strains, while others are free of it, and are classified as Type II strains [10] [11]. During competition experiments between different *H. pylori* strains, the amount of CagA injected into the host cells is strongly reduced. This effect is independent of the strain's binding capacity but highly specific for a given *H. pylori* strain.

Objective
Two *Helicobacter pylori* strains infecting a host cell reduce the amount of CagA toxin injected by the type I strains. The objective of the study is to discern some aspects of the resistance response of host cells to CagA translocation induced by *H. pylori* strains. For this, several experiments were performed addressing the following questions: i) How fast can the resistance be formed? How does it behave over time? ii) Is it a specific response of epithelial cells or do immune cells resist as well CagA intoxication? and iii) Can all -Type I strains be blocked by all *H. pylori* strains in the same level? Since *H. pylori* CagA translocation is closely related to the production of pro-inflammatory cytokines, all analyses were complemented with measurements of the cytokine IL-8 during the different assays.

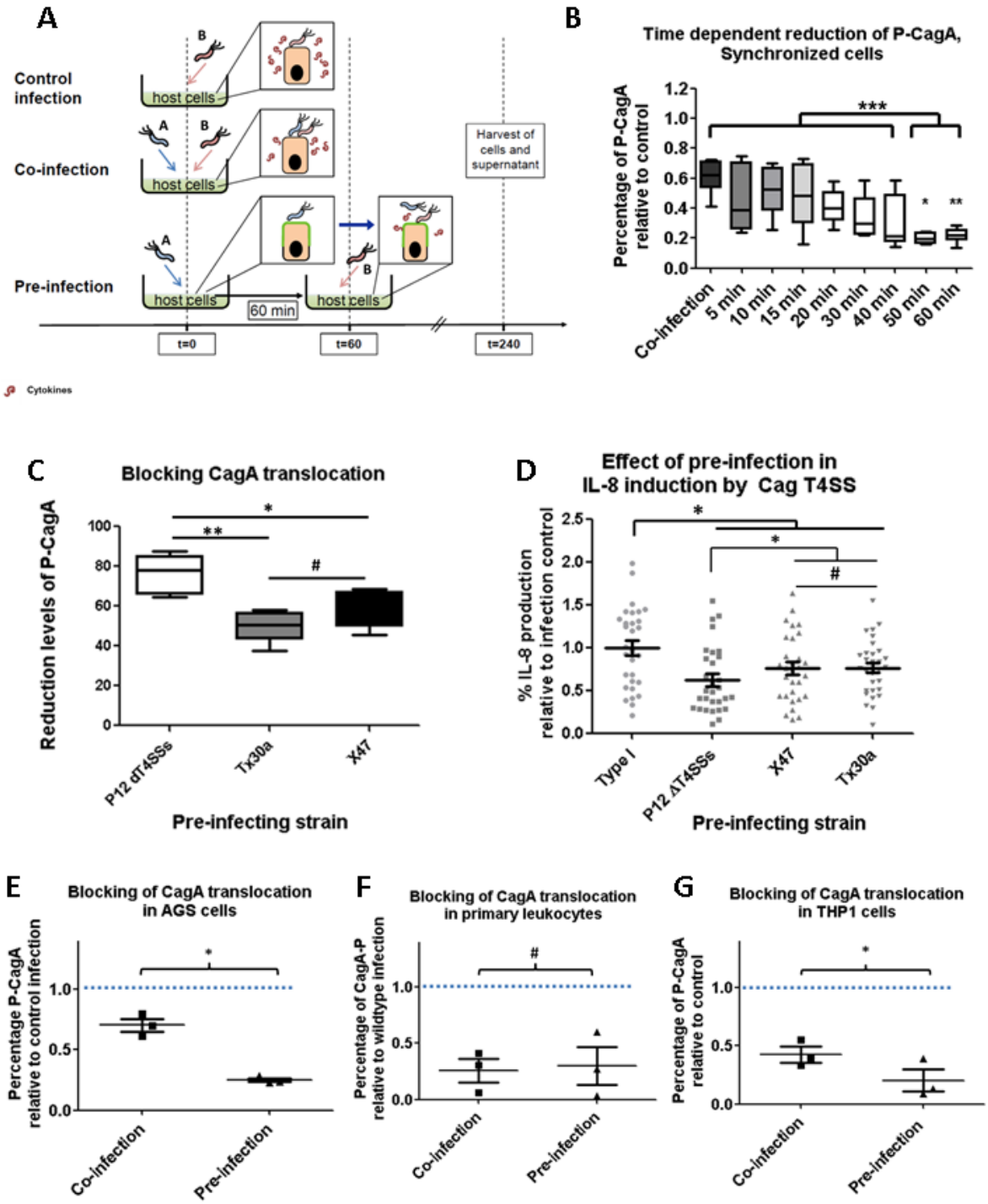

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Figure Legend

Figure 1. *Helicobacter pylori* in multiple infections.

A. Experimental setup.

Three different infections are being evaluated for CagA translocation: Control infec-

tion, Co-infection and Pre-infection. In co- and pre-infection two different strains are involved: pre-infecting or co-infecting strain (Strain A) and CagA translocating strain (Strain B). The control infection is performed with only the Strain B (CagA translocating). This infection establishes the fitness of the bacteria for CagA translocation at the time of infection and defines the reference value for semi-quantitative analysis of the CagA phosphorylation signal.

B. Time lapse response of cells to co- and pre-infection to CagA translocation.
CagA phosphorylation levels were compared to the time point control infections (t=0 (co-infection), t = 5, 10, 15, 20, 30, 40, 50 and 60 minutes (pre-infections), as explained in Figure 1A, using P12ΔT4SSs (Strain A) and P12 wild type (Strain B). The data summary is shown as Boxes (Median and quartiles) with whiskers (Min-Max values). All statistical analysis was performed as described in Methods. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

C. Effect of different pre-infecting strains (Strain A) on wild type I strains (Strain B) and its capacity to translocate CagA.
Cells were pre-infected for 60 min with strain A (P12ΔT4SSs, X47 or Tx30a), then infected with one of the wild-type Type I strains (P12, P145, G27, P217 and 26695) as Strain B (n=20). Each Strain B time point control was used as normalization control (100% CagA translocation). Reduction levels of CagA translocation achieved by pre-infections with the three different strains of all Type I strains are shown here. The data summary is shown as Boxes (Median and quartiles) with whiskers (Min-Max values). Statistical analysis was performed as described in Methods. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

D. IL-8 production by AGS cells after pre-infection experiments using different combinations of Strains A and Strain B.
General effect on IL-8 caused by different pre-infecting strains (Strain A: P12ΔT4SSs, X47 or Tx30a) in infections with Type I wild-type strains (Strain B: P12, P145, G27, P217 or 26695) after 60 min pre-infection (n=20). (Scatter dot blots (mean and SEM) graphs). Statistical analysis was performed as described in Methods. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

E-F. Cellular resistance CagA translocation by the Cag T4SS in co- and pre-infections.
AGS, human blood leucocytes and THP-1 and human blood leucocytes where co- and pre-infected with P12ΔT4SSs (Strain A) and P12 wild-type (Strain B). Levels of CagA translocation normalized to the control infection (dotted line represents normalized value). Scatter dot plot with mean and SEM. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

Results & Discussion

The simultaneous infection of epithelial cells *in vitro* using two bacterial strains with and without a Cag T4SS resulted in a fast reduction of CagA translocation into the host cells. (see Experimental setup, Figure 1A). This reduction in CagA translocation of the second strain (type I) was even stronger with a 60 min pre-infection of the first (type II) strain. Two possible scenarios for the differences in resistance are i) the co-infection effect is the first effect visible and should increase linearly with longer pre-infection times, or ii) both cellular reactions to pre- and co-infections are independent events. Our experiments evaluated the first 15 min of pre-infection (Lapses of 5 min), up to 60 min response, with lapses of 10 min (Figure 1B). The effect observed at simultaneous infection was maintained up to 40 min; and even though the variability of the results diminished with time, the differences (mean value/SEM) to the previous time points were not statistically significant. However, the CagA translocation is drastically reduced at the time points 50 min and 60 min, raising the possibility that at 50 min a new mechanism is activated resulting in a stronger blocking of CagA translocation.

For CagA translocation to occur, the Cag T4SS has to be functional, which implies that it also will induce the secretion of IL-8 by epithelial cells [12]. Therefore the question arises whether or not a pre- or a co-infection situation has an effect on the IL-8 production induced by a functional Cag T4SS. These experiments show that both pre- or co-infection situations caused a reduction of IL-8 secretion as a response to the function

of the Cag T4SS. The differences in IL-8 production starts to be visible at pre-infections of 15 min and maintained at 60 min pre-infection (Supplement Figure 2A). These results are similar to the ones observed with CagA translocation (Figure 1B). The previous experiments were performed with strains having an identical genetic background using as pre-infecting strain *H. pylori* P12 lacking all T4SS (P12ΔT4SSs). This kind of experiments may represent the behavior of an *H. pylori* strain living in the host that turns off their Cag T4SS by genetic switch [13] [14]. However, in human infections, genetically different strains would be expected. For this reason, we compared different wild-type trains, using as a control the P12ΔT4SSs (originally a type I strain) and two type II strains (CagA (-); Tx30a and X47) as pre-infecting strains. Although all pre-infecting strains (P12ΔT4SSs, Tx30a and X47) caused up to 80% reduction in CagA translocation by the secondary strain (P12, P145, G27, P217 and 26695), there was a significant difference between the effect caused by the P12ΔT4SS strain and the natural type II strains (Figure 1C).

However, when looking at each CagA-translocating strain, it is visible a different response to the pre-infecting strain. While P12, P145 and 26695 strains (Supplement Figure A-E) show no significant differences between the amount of translocated CagA blocked by the different pre-infecting strains, the strains G27 and P217 respond differently. G27 can translocate less CagA if the P12ΔT4SSs is the pre-infecting strain (Supplement Figure 1C), and P217 capability to translocate CagA is much less affected by pre-infections with the Tx30a strain (Supplement Figure 1D).

The effect of a co- or pre-infection on IL-8 secretion was similar to the one observed with CagA considering all different strains tested (Figure 1D), with a significant difference observed between the control infection and the pre-infections, dependent on the type of pre-infecting strain. However, the host cell responded differently to the pre-infection with different strains combinations. In this case, the different levels of IL-8 secreted are stronger with strains P145 and G27 (Supplement Figure 2B and 2D), while P12 was affected only by the P12ΔT4SSs (Supplement Figure 2B), and strains P217 and 26695 could induced similar levels of IL-8 induction in presence of the pre-infecting strains as they do alone (Supplement Figure 2E and 2F).

Comparing these data, it is visible that there is no specific correlation between lower CagA translocation and lower IL-8 induction in pre-infecting assays for each strain. Variations might be explained by Cag T4SS independent factors on IL-8 production, such as OipA [15] or others, which have not been analyzed.

The blocking assays performed so far used epithelial-like cells; however, the Cag T4SS affects as well immune cells [16]. And although the specific effect of CagA in these cells is not know, in the host, there is a high probability that *H. pylori* will have a direct contact with leucocytes in damaged gastric tissue. We, therefore, tested the effects of co- and pre-infection in the standard cell line THP-1, being our model for immune cell and CagA translocation, and verified the observation in primary leucocytes. Co- and pre-infection experiments of primary leucocytes and THP-1 cells (monocytic leukemia cell line) using AGS as control showed that both cell cancer lines (AGS and THP-1) behave similar, while isolated primary leucocytes respond very strong to both treatments without differences between co- and pre-infection conditions (Figure 1E to 1F). Although cancer epithelial cells are excellent models, the analysis of immune cells response to this phenomena shows us that i) it is not only an *in vitro*- cancer-cell type effect and; in this specific case, ii) that immune cells will respond strongly to multiple infections.

To our surprise, a pretreatment effect on IL-8 could not be evaluated for THP-1 cells and primary leukocytes, since they showed no difference on IL-8 induction between the T4SSs mutant and the wild-type (Supplement Figure 3B and 3C), suggesting that IL-8 induction in these cells is independent of the Cag T4SS. This is in contrast to the response from AGS cells (Supplement Figure 3A) and previously published association of IL-8 induction and T4SS [12].

Conclusions

CagA and its associated Cag T4SS are responsible for the injection and phosphorylation of CagA in the host cell, together with the induction of inflammatory chemokines, like IL-8 [17]. By measuring both CagA translocation and IL-8 induction as a parameter to

evaluate the effectiveness of Cag secretion system in co- and pre-infection assay, our results show that i) different levels of resistance to CagA translocation and IL-8 induction involve fast processes in the host cell, which highlights the importance of host-bacteria interaction in the dynamics of pathology. ii) Although the resistance is a prevalent response to the contact of *H. pylori* to epithelial or immune cells, each combination of strains has a different effect on CagA translocation resistance and IL-8 induction, making the already complicated life of *H. pylori* in the stomach fascinating and the outcome unpredictable in cases of multiple infections, and iii) although immune cells respond differently to the multiple infection setup in levels of IL-8 secretion, the effect on CagA translocation is conserved and even stronger than in the epithelia cell line model.

Limitations

The knowledge we have about the injection of CagA toxin is limited to the use of cell culture. Although mice and gerbils are used as animal models, *Helicobacter pylori* has adapted to humans. In humans, most of the data about colonization did not contribute to a real picture about the levels of multiple *H. pylori* strains in one host. Our studies in cells are just an approximation of what could happen in the human mucosa upon having two different strains, but better *in vitro* models, such as primary polarized gastric cell culture models or human gastric organoids [18] could bring us closer to a better understanding of the real interaction taking place in the host tissue.

The search for the molecular trigger on the bacterial-host interaction is complicated because of the remarkable variability of *H. pylori*, not only in their genetic level but as well their capacity of changing their proteins through variations, which complicates the work with different strains.

Since the resistance to CagA translocation seems to be caused by two different mechanisms, one early-onset and another delayed, we will evaluate the involvement of different cellular processes separately. As fast responses (shown by co-infection) the best candidates will include membrane processes and membrane lipid composition. For the 60 min response, we will concentrate on "slow" processes, such as de novo protein synthesis, protein recycling processes and protein modification systems. Because of the strong variations between different wild-type strain combinations, it will be necessary to evaluate the cellular processes relevant for each type of CagA translocation event.

With respect to bacteria, we need to verify the validity of the mutations of outer membrane proteins that we have found play a role in the resistance of the host cell. We are, as well, verifying the relevance of the *in vitro* data to the development of gastric diseases in humans. If confirmed, we would have found a new factor in the development of gastric pathologies in the presence of *H. pylori*. This will open the possibility that disease only is present when a single Type I *H. pylori* colonizes its host, and medical treatment can be changed from the actual eradication treatment to a supplement with a type II strain fitted for the strain found in the patient's stomach.

Additional Information

Methods and Supplementary Material

Please see https://sciencematters.io/articles/201706000006.

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Ethics Statement

The primary leukocytes were isolated from human blood (Permission 114-16, Ethic commission approval to Dr. Wolfgang Fischer) from human volunteers.

Citations

[1] Marshall et al. "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration". In: *The Lancet* 1.8390 (1984), pp. 1311–1315. URL: PM:6145023.

[2] Marshall et al. "Attempt to fulfil Koch's postulates for pyloric Campylobacter". In: *The Medical journal of Australia* 142.8 (1985), pp. 436–439. URL: PM:3982345.

[3] Tummuru et al. "Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production". In: *Infection and Immunity* 61.5 (1993), pp. 1799–809.

[4] Blaser and M. J. "Helicobacter pylori Phenotypes Associated with Peptic Ulceration". In: *Scandinavian Journal of Gastroenterology* 205 (1994), pp. 1–5.

[5] Mueller et al. "c-Src and c-Abl kinases control hierarchic phosphorylation and function of the CagA effector protein in Western and East Asian Helicobacter pylori strains". In: *The Journal of Clinical Investigation* 122.4 (2012), pp. 1553–66. URL: http://www.ncbi.nlm.nih.gov/pubmed/22378042.

[6] Odenbreit et al. "Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion". In: *Science* 287.5457 (2000), pp. 1497–1500. URL: PM:10688800.

[7] Lindholm et al. "Local Cytokine Response in Helicobacter pylori-Infected Subjects". In: *Infection and Immunity* 66.12 (1998), pp. 5964–71. URL: http://www.ncbi.nlm.nih.gov/pubmed/9826379.

[8] Hatakeyama and M. "The Role of Helicobacter pylori CagA in Gastric Carcinogenesis". In: *International Journal of Hematology* 84.4 (2006), pp. 301–308.

[9] Wroblewski et al. "Helicobacter pylori targets cancer-associated apical-junctional constituents in gastroids and gastric epithelial cells". In: *Gut* 64 (2015), pp. 720–30. URL: http://gut.bmj.com/content/early/2014/08/14/gutjnl-2014-307650.abstract.

[10] Xiang et al. "Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin." In: *Infection and Immunity* 63.1 (1995), pp. 94–8. URL: http://www.ncbi.nlm.nih.gov/pubmed/7806390.

[11] Censini et al. "cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors". In: *Proceedings of the National Academy of Sciences* 93.25 (1996), pp. 14648–53. URL: http://www.ncbi.nlm.nih.gov/pubmed/8962108.

[12] Fischer et al. "Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8". In: *Molecular Microbiology* 42.5 (2001), pp. 1337–1348. URL: PM:11886563.

[13] Linz et al. "A mutation burst during the acute phase of Helicobacter pylori infection in humans and rhesus macaques". In: *Nature Communications* 5 (2014), p. 4165. URL: http://www.ncbi.nlm.nih.gov/pubmed/24924186.

[14] Barrozo et al. "Functional plasticity in the type IV secretion system of Helicobacter pylori". In: *PLOS Pathogens* 9.2 (2013), pp. 1–16. URL: http://www.ncbi.nlm.nih.gov/pubmed/23468628.

[15] Yamaoka et al. "Role of interferon-stimulated responsive element-like element in interleukin-8 promoter in Helicobacter pylori infection". In: *Gastroenterology* 126.4 (2004), pp. 1030–43. URL: http://www.ncbi.nlm.nih.gov/pubmed/15057743.

[16] Odenbreit et al. "Interaction of Helicobacter pylori with professional phagocytes: role of the cag pathogenicity island and translocation, phosphorylation and processing of CagA". In: *Cellular Microbiology* 3.1 (2001), pp. 21–31. URL: PM:11207617.

[17] Backert Steffen and Naumann Michael. "What a disorder: proinflammatory signaling pathways induced by Helicobacter pylori". In: *Trends in Microbiology* 18.11 (Nov. 2010), pp. 479–486. DOI: 10.1016/j.tim.2010.08.003. URL: https://doi.org/10.1016/j.tim.2010.08.003.

[18] Fatehullah et al. "Organoids as an in vitro model of human development and disease". In: *Nature Cell Biology* 18.3 (2016), pp. 246–54. URL: http://www.ncbi.nlm.nih.gov/pubmed/26911908.

[19] Schindele Franziska et al. "Quantitative analysis of CagA type IV secretion byHelicobacterpylorireveals substrate recognition and translocation requirements". In: *Molecular Microbiology* 100.1 (2016), pp. 188–203. DOI: 10.1111/mmi.13309. URL: https://doi.org/10.1111/mmi.13309.