5. Conclusion
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1. THP-1 cell is one of the best model systems to study all ten TLR molecules as it is expressing these molecules constitutively (Zarember KA and Godowski PJ, 2002). The THP-1 cells maintained in complete RPMI 1640 medium were used for infection assays with *H. pylori* strain P12 and its isogenic mutants such as ∆cagA, ∆vacA, ∆flaA, ∆cgt and ∆cagPAI. The dynamics of mRNA level expression of TLR-1 to TLR-10 were determined by Taqman Quantitative RT-PCR relative to expression of house keeping gene GAPDH. TLR expression pattern at a multiplicity of infection (MOI) 1:50 of *H. pylori* and isogenic mutant strains were analyzed at 6h and 24h after infection. Quantitative RT-PCR data showed that TLR-3, TLR-5, TLR-6, TLR-7, TLR-8 and TLR-10 were highly expressed in THP1 cells after infection with *H. pylori* wild type. TLR-1 and TLR-9 mRNA expressions were induced moderately, whereas TLR-2 and TLR-4 were at very low level after 24h of infection. All the isogenic mutants used in this study were less potent on inducing TLR mRNA expression in comparison with wild type strain. Of which, ∆flaA and ∆vacA mutants were shown to be the least inducer of TLR mRNA expression in THP-1 cells.

2. THP-1 derived DCs represent monotypic DCs and function as antigen presenting cells to activate T cells when matured through stimulation (Berges C et al, 2005). THP1 derived DCs were used to study mRNA expression of TLRs during infection with *H. pylori* and ∆flaA isogenic mutant. TLR-3, TLR-7 and TLR-10 were prominently expressed in THP-1 derived DCs during infection with *H. pylori*. ∆flaA mutant was again found to be the least inducer of TLR mRNA expression. Taken together the data obtained in THP-1 and THP-1 derived DCs showed that there exists differential induction of TLRs in different populations of immune cells during *H. pylori* infection.

3. The protein level expressions of TLR-5 and TLR-10 were studied using whole cell extract immunoprecipitation and Western blot. *H. pylori* wild type and ∆flaA
isogenic mutant, the least inducer of TLR mRNA expression, were used to infect THP-1 cells to study the variation in protein level expression. TLR-5 was found to be moderately increased in expression at protein level as similar to mRNA expression after 6h of infection with *H. pylori* when compared to uninfected control cells. TLR-10 was highly expressed at the protein level in *H. pylori* infected cells which correlated with mRNA expression as shown in Fig.4 of the results. ∆flaA mutant of *H. pylori* was a least inducer of TLR-5 and TLR-10 proteins in THP1 monocytes after 6h of infection when compared to wild type strain. These results clearly ascertain that *H. pylori* virulence and pathogenicity factors have an important role on quantitative and qualitative properties of TLR molecules and that will be the determining factors for successful adaptive immune response.

4. HEK-293 cells stably transfected with TLR-2 (HEK293-TLR-2 cells), TLR-5 (HEK293-TLR-5 cells) and TLR-10 (HEK293-TLR-10 cells) were used for *H. pylori* infection. HEK-293-TLR2 cells infected with *H. pylori* for 6h have also exhibited moderate increase of TLR-2 mRNA expression when compared to uninfected control. ∆flaA and ∆vacA isogenic mutant strains were the least inducers of TLR-2 mRNAs in HEK-293-TLR2 cells. *H. pylori* wild type and ∆cagA isogenic mutant strain have induced 5 and 3 fold increases in TLR-5 mRNA expression, respectively, in HEK-293-TLR-5 cells after 6h of infection. ∆flaA, ∆vacA, ∆cgt and ∆cagPAI isogenic mutants have not shown the induction of TLR-5 mRNA expression in HEK-293-TLR-5 cells after 6h of infection. HEK-293-TLR-10 cells infected with *H. pylori* have also exhibited an increase in the TLR-10 mRNA expression to 4 fold level after 6 h of infection. Infection of ∆cgt isogenic mutant infection resulted an increase of 2.5 fold of TLR-10 mRNA expression in HEK-293-TLR-10 cells. However, ∆flaA, ∆vacA and ∆cagPAI isogenic mutants were not able to increase TLR-10 mRNA expression more than two folds in HEK-293-TLR-10 cells after 6h of infection.
5. The IRAK-1 phosphorylation is a critical step in the signalling of TLR proteins. After analysing mRNA expressions of all ten TLRs, qualitative changes in the TLR signalling during *H. pylori* infection were also studied. It has been revealed that *H. pylori* virulence and pathogenecity factors such as CagA, VacA, FlaA, Cgt and cagPAI status are essential for IRAK-1 massive phosphorylation in THP-1 cells. IRAK-M level, negative regulator of TLR signalling, was also examined. It was found that IRAK-M levels were not changed significantly in THP-1 cells after infection with *H. pylori*. HEK293-TLR2 cells infected with *H. pylori* and mutants have induced a low level IRAK-1 phosphorylation. In HEK293-TLR5 cells infection, Δcgt and ΔcagPAI mutants did not induce IRAK-1 phosphorylation at all, however, wild type, ΔcagA, ΔvacA and even ΔflaA induced low level of IRAK-1 phosphorylation. Interestingly, HEK293-TLR10 cells had induced massive IRAK-1 phosphorylation during infection with *H. pylori*, whereas very low level of phosphorylation was noted with ΔflaA and ΔvacA mutants. HEK293 cells having no or less TLR expression did not activated IRAK-1 phosphorylation during *H. pylori* infection. This implies that *H. pylori* induce IRAK-1 massive phosphorylation through TLR molecules in a host cell type dependent mechanism.

6. Nod-like Receptors are the cytoplasmic pattern recognition receptors for detecting microbial molecular patterns and transducing signals to mount a pro-inflammatory reaction. NLR family of proteins are recognized with a modular organization of a C-terminal leucine rich repeats (LRR), central Nucleotide binding domain (NACHT) and an N-terminal CARD domain, pyrin domain or Bir domain for protein-protein interaction (Tschopp J et al, 2003). These proteins are able to form the high molecular weight structures called ‘inflammasome’ and recruit caspase1 that leads to proteolytic activation of pro IL-1 β and pro IL-18 (Martinon F and Tschopp J, 2004; Martinon F et al, 2002). Apoptosis-associated speck-like protein containing a CARD (ASC) or PYCARD is a protein that is essential for LPS-
induced activation of caspase-1, because ASC knockout mice fail to process procaspase-1 or produce IL-1β and IL-18 following LPS and ATP stimulation, which is consistent with results in Caspase-1 null mice (Mariathasan S et al, 2004; Li P et al, 1995; Kuida K et al, 1995). NLRs such as NOD-1, NOD-2, NALP-1, NALP-2, NALP-3, IPAF and inflammasome adaptor ASC mRNA expression molecules were not up-regulated more than 2 fold in THP-1 cells during H. pylori infection, except IPAF. IPAF was significantly increased by Δcgt, ΔcagA and wild type in the descending order after 6h of infection. However, wild type and isogenic mutants infected cells have significantly increased the IPAF expression after 24h. The ΔvacA mutant was the least inducer of IPAF in H. pylori infected THP-1 cells. This reveals that IPAF might be playing an important role in the intracellular detection of H. pylori or its derived molecules in THP-1 cells.

7. IL-1β and IL-18 are proinflammatory cytokines important in the host defence against infection and in the pathogenesis of various inflammatory disorders. Both IL-1β and IL-18 are synthesized as inactive cytoplasmic precursors that are proteolytically processed to biologically active mature forms in response to various proinflammatory stimuli by caspase-1 (Yu HB and Finlay BB, 2008). Although the intracellular signalling pathways leading to caspase-1 activation remain poorly defined, studies have suggested involvement of members of the NLRs in the regulation of caspase-1 activation. NALP-3 NALP-1 and IPAF can form an endogenous multiprotein complex ‘inflammasome’ containing ASC and caspase-1, that promotes caspase-1 activation and processing of pro-IL-1β. ASC is critical for caspase-1 activation and secretion of mature IL-1β and IL-18 in response to several purified microbial components as well as intracellular bacteria (Martinon F and Tschopp J, 2004; Martinon F et al, 2002). The IL-1β mRNA expression was high during H. pylori and isogenic mutants infection of THP-1 cells and IL-18 mRNA expression was significantly down-regulated. The protein
level expression of mature IL-1β was not changed significantly, after 8h of infection with *H. pylori* and isogenic mutants. Pro-caspase-1 cleavage was inhibited during infection with *H. pylori* and isogenic mutant strains ΔvacA and ΔflaA. On the contrary, Pro-caspase-1 cleavage was not inhibited in the case of ΔcagA, Δcgt and ΔcagPAI infected cells (Fig.15). However, there was no significant change in the process of Pro-IL-1β cleavage to mature IL-1β in THP-1 cells during infection with *H. pylori* and all isogenic mutants tested. The level of IL-1β secretions was very low, although ΔcagA and ΔvacA isogenic mutants infected cells significantly increased the secretion of IL-1β than wild type infected cells. The other isogenic mutants such as ΔflaA, Δcgt, ΔcagPAI infected THP-1 cells secreted less amounts of IL-1β than wild type infected cells and ΔcagPAI exerted the least induction of IL-1β secretion. This implicates that inflammasome mediated Pro IL-1β processing was severely deranged in THP-1 cells during *H. pylori* infection.

8. The influence of known *H. pylori* induced kinase activities in host cells and apoptosis on IL-1β processing and secretion by treating the cells with specific inhibitors, such as c-SRC kinase inhibitor-PP2, EGFR kinase inhibitor-AG1478 and pan-caspase inhibitor-Z-VAD-FMK, thirty minutes prior to infection with *H. pylori* wild type was investigated. The IL-1β secretion from THP1 cells, treated with inhibitors as stated above, after 8h, 24h and 32h of infection with *H. pylori* wild type was measured. c-SRC inhibitor-PP2 treated cells had secreted significantly higher amount of IL-1β after 8h of infection, whereas, levels of IL-1β remain unchanged in cells treated with EGFR kinase inhibitor-AG1478, pan-caspase inhibitor-Z-VAD-FMK and wild type *H. pylori* treated cells. This suggests that c-SRC is involved in the retardation of IL-1β secretion during *H. pylori* infection.
9. Pro-inflammatory cytokines such as IL-6, TNFα and chemokine IL-8 were reported to be increased in the gastric tissues of *H. pylori* infected patients. There are no reports regarding the influence of *H. pylori* virulence and pathogenicity factors on cytokine and chemokine gene expression and secretion during infection. In this study, the IL-6 and TNFα gene expression in THP1 cells during infection with *H. pylori* and isogenic mutants ∆cagA, ∆vacA, ∆flaA, ∆cgt and ∆cagPAI were analyzed. TNF secretion can be induced by conserved structural elements common to microbial pathogens, including cell wall moieties such as PGN, LPS, and CpG DNA motifs on binding with TLRs (Aderem A and Ulevitch RJ, 2000). TLRs transcriptionally induce proinflammatory cytokines, including TNF, through the convergence of NF-kB and NF-AT activating pathways, and enhance translational efficiency by a mechanism targeting consensus 39-untranslated AU-rich elements (ARE) in mRNA (Dumitru CD et al., 2000). THP-1 cells infected with *H. pylori* and isogenic mutants had significantly increased the mRNA expression of TNFα after 6h of infection. However, the level of mRNA expression of TNFα has been significantly decreased in ∆vacA and ∆flaA mutants after 24h of infection when compared to wild type infected cells. TNFα secretion from THP-1 cells after 24h of infection was also analyzed. The data showed that ∆vacA and ∆flaA mutants significantly reduced the secretion of TNFα from THP-1 cells after 24h of infection when compared to wild type infected cells and that correlated with mRNA expressions after 24h. In addition, ∆cagPAI mutant also reduced TNFα secretion from THP-1 cells when compared to wild type infected cells. These data suggests that VacA and FlaA molecules, which have previously been shown to induce significantly less TLR expression and signalling, may be partially responsible for the induction of TNFα secretion from THP-1 cells during infection with *H. pylori*. 
10. IL-6 is traditionally considered an activator of acute phase responses and a lymphocyte stimulatory factor (Kishimoto T et al, 1995). IL-6 belongs to a family of cytokines that promote cellular responses through a receptor complex consisting of at least one subunit of the signal-transducing glycoprotein gp130 (Heinrich PC et al, 2003). Many of the biological activities assigned to IL-6 are mediated through naturally occurring soluble IL-6 receptors (IL-6R). This soluble receptor forms an agonistic complex with IL-6 that binds gp130 to trigger cellular responses. Regulation of this activity is termed “IL-6 trans-signaling” (Jones SA and Rose-John S, 2002; Jones SA et al, 2005). The mRNA expression of IL-6 in THP-1 cells during H. pylori infection was studied and it was found that ΔcagA and ΔcgA infected cells significantly increased the expression after 6 of infection. However, after 24h of infection not only the mutants but also the wild type increased the mRNA expression of IL-6 and the induction by ΔcagA and ΔcgA were more than wild type. ΔvacA, ΔflaA and ΔcagPAI infected cells were the least inducers of IL-6 mRNA expression in THP-1 cells.

11. Interleukin-8 (IL-8) is a potent neutrophil-activating chemokine, central to the immunopathogenesis of H. pylori induced tissue injury (Crabtree J and Lindley I, 1994) and high tissue expression is dependent on the presence of a complete cagPAI (Nilsson et al, 2003). In gastric cell lines, co-culture with H. pylori induces IL-8 via activation of NF-κB a transcriptional regulator (Sharma et al, 1998; Nozawa et al, 2002). H. pylori wild type strain induced very high level of IL-8 secretion from THP-1 monocytes. However, ΔflaA and ΔcagPAI mutants infection with THP-1 cells significantly reduced IL-8 secretion to 74% and 40%, respectively, in comparison with wild type. It can be concluded that reduced TLR expression and signalling by ΔflaA and ΔcagPAI mutants may be one of the reasons for the reduced IL-8 expression and secretion in THP-1 cells.
12. HEK293-TLR2 cells substantially enhanced the mRNA expression and secretion of TNFα in comparison with HEK293 cells when infected with *H. pylori* and isogenic mutants. IL-8 secretion from HEK293-TLR2 cells during *H. pylori* infection was reached more than 20 fold of the secretion by HEK293 cells. TNFα and IL-1β mRNA expressions were significantly induced in HEK293-TLR2 cells when compared to HEK293 cells during infection with *H. pylori* and isogenic mutants. However, IL-6 and IL-18 mRNA expression were increased not more than two folds in both cell lines during *H. pylori* infection.

13. HEK293-TLR5 cells infected with *H. pylori* and isogenic mutants enhanced IL-8 secretion almost seven folds when compared to infected HEK293 cells. Δ*vacA*, Δ*flaA* and Δ*cgt* mutants infected cells have significantly reduced the IL-8 secretion from HEK293-TLR5 cells when compared to the wild type strain. TNFα mRNA expression increased in HEK293-TLR5 cells in comparison with HEK293 cells during infection with *H. pylori* and isogenic mutants, however, this enhancement was not enough to make changes in TNFα secretion when compared to HEK293 cells. IL-1β, IL-6 and IL-18 mRNA expressions were not increased in HEK293-TLR5 and HEK293 cells during infection with *H. pylori* infection. It can be concluded from the above data that TLR-5 signalling is not enough to induce enhanced IL-1β, IL-6 and IL-18 mRNA expressions and TNFα secretion in HEK293 cells.

14. The present study disclosed the fact that TLR-10 is highly expressed during *H. pylori* infection. HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants have significantly increased the IL-8 secretion, which was observed to be more than 5-7 folds in comparison with HEK293 cells. TNFα mRNA expression has significantly increased in HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants. However, TNFα secretion from HEK293-TLR10 in comparison with HEK293 cells was significantly increased in wild type, Δ*cagA* and Δ*cagPAI*
infected cells only. IL-1β, IL-6 and IL-18 mRNA expressions in HEK293-TLR10 cells were increased not more than two fold in HEK293-TLR10 cells in comparison with HEK293 cells during *H. pylori* and isogenic mutants infection. This indicates that TLR-10 is a functional receptor and enhancing the IL-8 and TNFα secretion significantly, but not IL-1β, IL-6 and IL-18 mRNA expression, during infection with *H. pylori*.

15. *In vivo* analysis of high risk host and bacterial genotypes using 42 biopsy samples from patients colonized with *H. pylori* revealed that VacA S1/CagA/IL-1RN*2/*2-allele in 14%, VacA S1/CagA/IL-1RN*1/*1 allele in 7%, and VacA S1/CagA/IL-1RN*1/*2 allele in 16% of patients. IceA1 and IceA2 status were observed in all four cases of gastric ulcer diagnosed endoscopically (3 duodenal ulcer and 1 antral ulcer), however, two DU and one AU are also associated with VacA S1 genotype, while remaining DU is infected with VacA S2 genotype. Of the 42 samples studied, 81% infected with IceA1 genotype, whereas only 19% exhibited IceA2 genotype. In this group of patients 31% (13/42) were infected with both IceA1 and IceA2 genotypes of *H. pylori*. 
