Summary and Conclusion.
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Toll-like receptors (TLRs) recognize conserved pathogen associated molecular patterns (PAMPs) and participate in the induction of inflammatory and innate immune responses during microbial infections. These responses constitute a critical component of immunity against pathogenic microorganisms. TLR-5 recognizes flagellin, the primary protein component of bacterial flagella. Flagellin is the major proinflammatory determinant of pathogenic *Salmonella*. The inflammatory responses produced upon infection of human intestinal epithelial cells (IECs) with this pathogen *in vitro* can be almost totally reproduced by stimulating these cells with flagellin. A unique feature of the flagellin-TLR-5 interaction is that signaling through TLR-5 can be initiated only with monomeric flagellin because the receptor-binding site is not accessible on flagella present on the surface of bacteria. In this study, the mechanism by which proinflammatory flagellin monomers are made available to the innate immune system by pathogenic *Salmonella* has been investigated. The results showed that:

- *Salmonella* releases proinflammatory monomeric flagellin upon interaction with human or mouse IECs. This secretion was also seen when bacteria were incubated with culture supernatant derived from a model human intestinal epithelial cell line, Caco-2. The host molecule(s) in Caco-2 culture supernatant capable of triggering release of flagellin from *Salmonella* were resistant to treatment with proteinase K suggesting that the host stimulus was non-proteinaceous in nature.

- The induction of flagellin secretion was also observed following incubation of *Salmonella* with fetal calf serum or bovine serum albumin but not with another serum protein, transferrin.

- Treatment with the protein synthesis inhibitor, gentamycin, abrogated the ability of bacteria to secrete flagellin in response to activation with the host stimulus.
suggesting that this process required metabolically active bacteria. This data also revealed that flagellin released by Salmonella upon interaction with the host stimulus was not derived from flagella which were expressed in abundance on the surface of gentamycin-treated Salmonella, but was secreted afresh by bacteria.

- The ability to activate flagellin release from Salmonella could be efficiently reproduced with lysophospholipids such as lysophosphatidic acid (LPA) or lysophosphatidylcholine (LPC) but not with phospholipids. Consistent with these results, inhibition of biogenesis of these lipids in IECs by treating cells with inhibitors of calcium-independent phospholipase A\(_2\) (iPLA\(_2\)) or calcium-dependent secretory phospholipase A\(_2\) (sPLA\(_2\)) abrogated the ability of cells to trigger release of flagellin from Salmonella, confirming the identity of the host stimulus capable of inducing flagellin secretion as lysophospholipids. LPC was identified as the major lysophospholipid released by Caco-2 cells.

- Lysophospholipid-mediated activation of flagellin was not restricted to IECs in vitro but was also seen during interaction of Salmonella with host cells in vivo. Inhibition of iPLA\(_2\) by feeding mice with the iPLA\(_2\) enzyme inhibitor, bromoenol lactone (BEL), abrogated release of flagellin from bacteria without affecting release of LPS.

- Investigation into the mechanism involved in host-stimulus mediated release of flagellin revealed that lysophospholipid sensing by Salmonella activated transcription from the flagellin promoter. Furthermore, flagellin secretion from Salmonella in response to lysophospholipids involved cAMP-dependent intracellular signaling in the pathogen.
• The secretion of flagellin from pathogenic *Salmonella* was also observed during infection of macrophages with these bacteria. This secretion was seen even with bacteria which expressed flagellin but did not assemble flagella on the surface, demonstrating that, as seen with IECs, flagellin secreted upon interaction with the host stimulus was not derived from surface flagella.

• Flagellin release during infection of macrophages with *Salmonella* was dependent on pathogen-induced cell death. Infection of macrophages in the presence of the pan-caspase inhibitor, zVADfmk, abrogated their ability to activate release of flagellin from the pathogen. Moreover, infection with SipB-deficient *S.typhimurium* which does not induce cell death in macrophages, was not associated with secretion of flagellin from the pathogen.

• Induction of cell death following infection with *Salmonella* activated secretion of a stimulus from macrophages that triggered release of flagellin from the pathogen. The secretion of this host stimulus was dependent upon cellular iPLA₂, demonstrating that as seen with IECs, the host stimulus that activated release of flagellin from *Salmonella* was lysophospholipid in nature. Inhibition of cellular iPLA₂ with BEL abrogated generation of the flagellin-inducing host stimulus from macrophages. Interestingly, this stimulus was also released by cells in response to UV exposure, a trigger that has been shown to induce secretion of LPC from cells in caspase-3-dependent fashion.

• Remarkably, the induction of apoptosis in macrophages and the release of lysophospholipids following infection with *Salmonella* was dependent on the expression of flagellin in the bacterium. This suggests that intracellular sensing of flagellin by macrophages results in cell death-dependent release of
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lysophospholipids that can modulate TLR-5-mediated responses by activating expression of its ligand (i.e. flagellin) from the pathogen.

These results demonstrate that induction of cellular responses through TLR-5 during infection of cells with pathogenic Salmonella is a regulated process that involves two way sensing. Salmonella sense host lysophospholipids and release flagellin monomers not via depolymerisation of surface associated flagella but through induction of fresh flagellin expression that is coupled to its secretion. This flagellin is then recognized by TLR-5 expressed on host cells and inflammatory/ innate immune responses are initiated. These findings not only provide a mechanism by which pathogenic Salmonella makes a key TLR ligand available to the innate immune recognition system but also identify lysophospholipids as novel regulators of innate immunity. These results also provide a link between pathogen-induced cell death and innate immunity.

The mode of induction of inflammatory responses as well as the mechanism of invasion of IECs are more or less conserved amongst various Salmonella species. In spite of this conservation, many Salmonella species exhibit a high degree of host specificity and produce different clinical outcomes even when they infect the same host; the reasons for these differences are not clear. S.typhi causes typhoid in humans while S.typhimurium produces only self-limiting gastroenteritis. To understand possible differences in the interaction of these two Salmonella species with host cells, experiments were carried out to analyze if infection of human IECs with these two closely related pathogens triggers secretion of different mediators from these cells which might have different modulatory effects on cellular responses from dendritic cells (DCs) infected with Salmonella. The results demonstrated that:
Infection of human DCs with *S. typhi* or *S. typhimurium* brings about secretion of a number of cytokines from these cells as well as induction of co-stimulatory molecules. There were no significant differences in the degree of these responses induced by the two *Salmonella* species.

Conditioning of these DCs with soluble mediators released by IECs, prior to infection with *Salmonella*, reduced secretion of IL-12 from these cells without affecting TNF-α. This modulation by IECs did not change if preconditioning of DCs was done with soluble mediators derived from *S. typhimurium*-infected IECs. On the other hand, preconditioning with mediators derived from *S. typhi*-infected IECs abrogated the ability of IECs to downregulate IL-12 secretion from *Salmonella*-infected DCs. These results suggest an important role for IEC-DC crosstalk in modulating the quality of immune response during infection with different *Salmonella* serovars.

The *S. typhi*-specific modulation reported here might have important implications for *S. typhi* pathogenesis or immunity against this pathogen, considering that IL-12 is a crucial inflammatory cytokine that is secreted by DCs upon infection with pathogens or stimulation with potent TLR agonists. This cytokine is vital for the differentiation of T cells to Th1 type of cells that are characterized by secretion of IFN-γ that plays a critical role in immunity against *Salmonella*. Future studies should investigate this host-pathogen interaction in detail.