Uptake of Shigella-containing pseudopodia by neighboring epithelial cells at tricellular junctions via non-canonical clathrin-dependent trafficking pathway

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Many invasive intracellular bacterial pathogens such as Shigella and Listeria monocytogenes can spread from cell to cell during infection, and this spreading is an important pathogenic mechanism to expand their replicative niches. However, it is still unclear how motile bacteria are able to cross epithelial cell-cell junctions. We present evidence that Shigella-containing pseudopodia target tricellular junctions, and that tricellulin is functionally important to promote bacterial cell-cell spreading. We also showed that a Shigella cell-cell spreading dependent on phosphoinositide 3-kinase, clathrin, Epsin-1 and Dynamin-2, which localized beneath the plasma membrane of the engulfing cells. Since knocking down tricellulin, Epsin-1, clathrin or Dynamin-2 expression reduced Shigella cell-cell spreading, whereas AP-2, Dab2 and Eps15 were not required for this process, it is likely that Shigella-containing pseudopodium that is fully engulfed by a neighboring epithelial cell undergoes non-canonical clathrin-dependent endocytosis.

In eukaryotic cells, the internalization of extracellular cargo via the endocytic machinery is an essential process for many cellular functions, including nutrient uptake, membrane trafficking, recycling extracellular receptors, cell migration, cell-cell communication and microbial infection. Intercellular bacterial movement, such as that mediated by Shigella, is a sequential process that partially resembles the intercellular trafficking of double-membrane vesicles such as during connexin and claudin trafficking. This process has been shown to require many bacterial and host factors, but the molecular basis remains still partly speculative.

The tight cell-cell junctions (TJs) in the intestinal epithelium are essential for maintaining epithelial integrity, which also act as an intrinsic barrier against microbial invasion as well as bacterial cell-cell spreading. Nevertheless some cytosolic invading bacterial pathogens such as Shigella and L. monocytogenes can move from one epithelial cell to another. This process consists of at least three distinctive stages. First, the motile bacterium attaches to the plasma membrane and impinges upon the membrane so that it protrudes as a pseudopodium. Second, the protruding pseudopodium is engulfed by a neighboring cell. Finally, the double plasma membranes are lysed, allowing the bacterium to disseminate into the cytoplasm of the neighboring cell. TJs are a network of transmembrane and peripheral proteins that form a semipermeable barrier to paracellular flux, and thus function as the main determinants of the epithelial and endothelial barriers. The distinct composition of different transmembrane proteins includes occludin and claudin family members. In addition, tricellulin, which is a basic element of tricellular tight junctions (tTJs) and discovered as the first tight junction protein and characterized by Schoichiro Tsukita’s group as distinct tetraspan transmembrane protein, mainly localizes to tricellular cell contacts and is present to a lesser extent in bicellular tight junctions (bTJs) (Ikenouchi et al. J Cell Biol 2005).

We thus undertook investigation of the mechanism that allows Shigella to move from one epithelial cell to neighboring epithelial cells. We used time-lapse imaging to monitor the fate of motile bacteria from the onset of bacteria-induced membrane protrusion until the bacteria disseminated into an adjacent cell using MK2 cells, which was formally termed as LLC-MK2 cells (rhesus monkey kidney epithelial cells). When MK2 cell monolayers were infected with Shigella, 47% of the bacteria moved into adjacent cells via tTJs, while 72% of bacteria spread via bTJs. The same tendency was observed with other epithelial cell lines, such as Caco2 (human colon carcinoma) and MDCK (Madin-Darby canine kidney) cells. Electron microscopic analysis confirmed that the Shigella-containing pseudopodium extended around the tTJ, which was then engulfed by a neighboring epithelial cell.

Of note this highly selective bacterial cell-cell movement at tTJs was not predominant when MK2 cell monolayers were infected with L. monocytogenes. Only 51% and 49% of L. monocytogenes cell-cell spreading occurred via bTJs and tTJs, respectively, suggesting that tTJs are not a preferential site of L. monocytogenes cell-cell spreading. Interestingly, Rajabian et al. (Nat Cell Biol 2009) reported that the L. monocytogenes virulence protein internalin C (InIC) plays an important role in cell-cell spreading. InIC inhibits Tuba, which perturbs the tension between the apical junctions and facilitates protrusion of Listeria-containing pseudopodia from bTJs. Indeed, the authors showed that ectopic expression of InIC in epithelial monolayers perturbed apical junctions via interactions with Tuba, which interfered with N-WASP binding and reduced tension at bTJs (Rajabian et al., Nat Cell Biol 2009). A previous study showed that Tuba is concentrated at bTJs via interactions with ZO-1, and that knocking down Tuba expression caused membrane curving and slack between cell-cell junctions (Otani et al., J Cell Biol 2006). We, therefore, speculate that InIC-mediated perturbation of bTJs allows L. monocytogenes to protrude from pseudopodia at both bTJs and tTJs.

Because tricellulin is highly expressed at tTJs and is an essential component of tTJs, we used polarized MDCK monolayers due to the feasibility of a plaque formation assay to examine Shigella cell-cell movement, and investigated whether tricellulin is functionary involved in Shigella cell-cell spreading. shRNA-mediated knockdown of tricellulin expression...
reduced the diameter of plaques in the cell monolayer that were due to bacterial cell-cell spreading compared with that of the control epithelial cells, and the fraction of bacteria-containing pseudopodia that protruded from tTJs was also reduced compared with the mock control. Since the number of bacteria-containing pseudopodia per epithelial cell was similar in the tricellulin knocked down and control cells, we concluded that Shigella dissemination into neighboring epithelial cells depends on the integrity of tTJs.

It has been shown that apical junctional complexes are markedly plastic under physiologic and pathophysiologic conditions. Tight junctions are frequently remodeled under physiologic and pathophysiologic conditions. Tight junctions are frequently remodeled under physiologic and pathophysiologic conditions. These processes are characterized by the exchange of apical junctional complex proteins from junctional and cytoplasmic pools (Edelblum and Turner, Curr Opin Pharmacol 2009; McMahon and Boucrot, Nat Rev Mol Cell Biol 2011; Shen et al., J Cell Biol 2008; Shen et al., Neurochem Res 2009; Shen et al., Annu Rev Physiol 2011). In the intestinal epithelium, the plasticity of tight junctions is critically important for epithelial integrity, the intestinal barrier, and homeostasis, because bTJs and tTJs are constantly needed as dying cells are shed and the epithelium is rapidly sealed. Remodeling adhesive cell-cell contacts, including adjusting the junctional length and correctly localizing new epithelial cells, requires endocytosis and recycling of adhesion molecules (Madara, J Membr Biol 1990; Troyanovsky et al., Mol Biol Cell 2006). At present, the molecular mechanisms behind tTJs formation remains poorly understood, and how tricellulin per se contributes to the bacterial spreading also remains unclear. Since some studies indicated that tTJs are exploited as “window” for protrusions from epithelial cells to elongate into the lumen to sense the outer environment (Kubo et al., J Exp Med 2009; Shum et al., Cell 2008), it is likely that the plasma membrane around tTJs could be a frequent source and destination of endocytotic vesicles, which may facilitate protrusion and engulfment of pseudopodia during Shigella cell-cell movement.

Remarkably, it has been shown that a bacterium that is enclosed within a pseudopodium will not be released into the free space surrounding the host cell in the absence of neighboring cells, implying that a bacteria-containing pseudopodium must directly contact the neighboring cell to trigger pseudopodium engulfment by the neighboring cell membrane. Phosphoinositide (PI) 3-kinase activity is required to remodel the membrane surface architecture and regulate membrane trafficking, cytoskeletal dynamics, and signal transduction (Lindmo, J Cell Sci 2006). Hence, we first investigated the potential role of PI 3-kinase in the formation of Shigella-containing pseudopodia. To this end, we fused GFP to the pleckstrin homology (PH) domain of Akt (GFP-Akt-PH), which binds to PtdIns(3,4,5)P3 and PtdIns(3,4)P2, generated by PI 3-kinase activity. When a MK2 cell transiently expressing GFP-Akt-PH engulfed a Shigella-containing pseudopodium that was protruding from a neighboring MK2 cell that lacked GFP-Akt-PH expression, the GFP-Akt-PH signal was detected as early as 2 min after pseudopodium engulfment using time-lapse imaging. Indeed, when the PI 3-kinase activity in epithelial cells was blocked with LY294002 (PI 3-kinase inhibitor), the degree of bacterial intercellular spreading was markedly diminished, although the protrusion of Shigella-containing pseudopodium was not affected. Giemsa staining of a Shigella-infected Caco-2 cell monolayer confirmed the LY294002 treatment results and showed that Caco-2 cells were filled with bacteria that had replicated but failed to spread into adjacent cells. These data suggested that the PI 3-kinase activity of epithelial cells is essential to trigger the engulfment of Shigella-containing pseudopodia by adjacent epithelial cells but not for pseudopodium formation per se. Therefore, it appears that the mechanisms underlying the internalization of bacteria-containing pseudopodia and clathrin-dependent recycling of transferrin, epidermal growth factor (EGF) and low-density lipoprotein (LDL) appear to differ. Additionally, studies are needed to characterize this noncanonical endocytosis pathway during Shigella cell-cell spreading.

There are three major endocytic membrane trafficking pathways in mammalian cells, including clathrin-dependent endocytosis, caveolin-dependent endocytosis and macropinocytosis (McMahon and Boucrot, Nat Rev Mol Cell Biol 2011). Therefore, we wished to determine which of these trafficking pathways is involved in Shigella cell-cell spreading. To this end, we treated Caco2 monolayers with phenylarsine oxide (PAO), methyl-β-cyclodextrin (MβCD) and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), which respectively inhibit clathrin, caveolin and macropinocytosis. Caco2 cells that were treated with PAO, MβCD or EIPA and infected with Shigella were examined for plaque formation as the consequence of Shigella cell-cell spreading. The plaque assay showed that Caco2 cells treated with PAO (and partially MβCD), but not EIPA, diminished Shigella cell-cell spreading. Indeed, the fraction of Shigella-positive plaques in the monolayer decreased to 21% of the untreated control, strongly suggesting that Shigella-containing pseudopodia are taken up by neighboring cells through a clathrin-mediated trafficking pathway. This was ensured by using shRNA to knock down clathrin expression in epithelial cells infected with Shigella in a plaque formation assay. Clathrin knockdown decreased the diameter of plaques to less than one-third of the mock control. The same was also true for dynamin-2 knockdown. The number of Shigella-containing pseudopodia in clathrin knocked down cells and dynamin-2 knocked down cells was 4.4 ± 0.2 and 4.2 ± 0.2, respectively, and pseudopodium formation was similar to that of mock control cells (4.4 ± 0.3), suggesting that the presence or absence of clathrin and dynamin-2 did not substantially affect pseudopodium formation by motile Shigella. Furthermore, when Caco-2 cell monolayers were infected with Shigella and stained with anti-human clathrin and anti-human dynamin-2 antibodies, both clathrin and dynamin-2 were detected around bacteria-containing pseudopodia. To further confirm that clathrin and dynamin-2 accumulate beneath the cell membrane of neighboring cells that engulfed bacteria-containing pseudopodia, MK2 cells transiently expressing clathrin-GFP (or dynamin-2-GFP) that had taken up Shigella-containing pseudopodia, were examined by immunofluorescence microscopy. When we analyzed a bacteria-containing pseudopodium that protruded from a clathrin-GFP-negative MK2 cell (or dynamin-2-GFP-negative) into a neighboring cell expressing clathrin-GFP (or dynamin-2-GFP), we detected GFP signals around the bacteria-containing pseudopodium. Importantly, time-lapse movies further showed that clathrin accumulated around a long bacteria-containing pseudopodium that was engulfed by a clathrin-GFP-expressing epithelial cell. These GFP signals were detected after the 30 min time point, when clathrin-GFP was abundant at the tip of the elongated...
pseudopodium. In contrast, clathrin-GFP only minimally accumulated around the bacteria-containing pseudopodium at time points earlier than 30 min, which clearly indicated that clathrin-dependent engulfment of a Shigella-containing pseudopodium is the latest event during the engulfment by a neighboring cell. It was previously shown that the clathrin coat assembles within a few minutes during canonical clathrin-mediated endocytosis. Therefore, we examined the localization of early endosome markers on Shigella-containing pseudopodia since clathrin-coated pits ultimately pinch off from the endocytic membrane and translocate to early endosomes. We found that EEA1 (early endosome antigen 1) and the FYVE domain of EEA1, which binds to PtdIns(3)P, accumulated around the Shigella-containing pseudopodium. We also found that Rab5 accumulated as early as 2 min after the bacteria-containing pseudopodium entered a neighboring cell. However, shRNA-mediated knockdown of Rab5 did not impair Shigella cell-cell spreading. Based on this series of experiments, we speculated that neighboring cells use non-canonical clathrin-dependent endocytosis during the late stage of engulfment, followed by scission of the bacteria-containing pseudopodium.

We further characterized clathrin-dependent engulfment of Shigella-containing pseudopodia by neighboring cells in relation to the functional involvement of clathrin coat assembly. MDCK cells were treated with shRNA to knockdown AP-2, Eps15, Epsin-1 and Dab2, which are initiation adaptors of clathrin-coated pits. After knocking down these components, MDCK monolayers were infected with Shigella and then examined for Shigella plaque formation. Although Epsin-1 knockdown had no effect on the subcellular localization of tricellulin, occludin and E-cadherin, the knockdown of Epsin-1, but not AP-2, Eps15 and Dab2, resulted in decreased plaque size. The number of bacteria-containing pseudopodia that protruded from Shigella-infected MDCK cells with or without Epsin-1 knockdown was approximately 4 pseudopodia per cell for 3 h post-infection, indicating that knocking down each of these adaptors had no effect on pseudopodium formation during Shigella cell-cell spreading. These results suggest that the mechanisms underlying internalization of bacteria-containing pseudopodia and clathrin-dependent recycling of transferrin, EGF and LDL, in which AP-2, Eps15 and Epsin-1 rapidly accumulate beneath the endocytosed plasma membrane, appear to somewhat differ. When the localization of GFP-Epsin-1 in Shigella-infected MK2 cells transiently expressing GFP-Epsin-1 was examined by time-lapse imaging, the GFP-Epsin-1 signal around the pseudopodium was detected after 25 min during the protrusion of a Shigella-containing pseudopodium. shRNA-mediated knockdown of Epsin-1 in MDCK cells resulted in less clathrin accumulation around the Shigella-containing pseudopodium compared with the mock control. However, AP-2 knockdown in epithelial cells did not alter the accumulation of clathrin around the pseudopodium. Together, these results indicate that Epsin-1 plays a functional role in recruiting clathrin to Shigella-containing pseudopodia.

To confirm that the Epsin-1-clathrin-dependent endocytic pathway is involved in the late stages of the engulfment of Shigella-containing pseudopodia, we identified the domain in Epsin-1 that is required for Shigella cell–cell spreading. Epsin-1 consists of the ENTH region [required to bind to PtdIns(4,5)P2 and PtdIns(3,4,5)P3 and induce membrane curvature], UIMs region (interacts with polyubiquitins and ubiquitinated cargo receptors for internalization), and COOH region (required to interact with clathrin, Eps15 and AP-2). We created in-frame deletions of Epsin-1 that lack the ENTH (ΔENTH), UIMs (ΔUIMs) or COOH (ΔCOOH) domains. We then infected MK2 cells expressing each of the Epsin-1 deletions with Shigella and examined the accumulation of Epsin-1 along bacteria-containing pseudopodia that were engulfed by neighboring cells. The results showed that MK2 cells expressing ΔENTH and ΔUIMs, but not ΔCOOH, failed to recruit Epsin-1 to the endocytosed pseudopodia. To ensure this, each of the Epsin-1 deletion derivatives were ectopically expressed in MDCK monolayer cells, and each of the MDCK cells infected with Shigella were investigated for the effect on the formation of plaques. The results showed that either of the deletions containing the ENTH, UIMs or COOH domains of Epsin-1 reduced the size of plaques formed by Shigella cell–cell spreading to less than half of the control level. Furthermore, we showed that all of the Epsin-1 deletion mutants prevented clathrin from accumulating around Shigella-containing pseudopodia. Together, these results suggest that Epsin-1 is functionally important in mediating the accumulation of clathrin around Shigella-containing pseudopodia and Shigella cell-cell spreading. In addition, when MK2 cells transiently expressing clathrin-GFP (or GFP-Epsin-1) were infected with Shigella and then treated with LY294002, the accumulation of clathrin or Epsin-1 around Shigella-containing pseudopodia was barely detected, assuring that PI 3-kinase activity is essential to recruit Epsin-1 and clathrin to the plasma membrane where the bacteria-containing pseudopodium was engulfed.

Aside from the well-documented role in vesicle endocytosis, clathrin has also been implicated in the internalization of large particles, such as bacteria, viruses and even double-membrane intercellular vesicles (Piehl et al., Mol Biol Cell 2007; Matsuda et al., J Cell Sci 2004). We discovered that the initial protrusion and subsequent penetration of Shigella-containing pseudopodia occur through a clathrin-independent pathway via tTJs, which may be directed by the bacteria-containing pseudopodium that protrudes due to the force of the motile bacterium (Cossart and Sansonetti, Science 2004; Ashida et al., Curr Top Microbiol Immunol 2009). Indeed, Shigella that lack the virG (icsA) gene, which is essential to mediate actin polymerization at one pole of the bacterium, are unable to induce pseudopodium protrusion. Thus, our results highlighted in the study that when an elongating pseudopodium is fully engulfed by a neighboring epithelial cell, this neighboring cell undergoes noncanonical clathrin-dependent endocytosis (Fig. 1).

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A bacterial effector targets the TRAF6-NFκB pathway to modulate the acute inflammatory response to bacterial invasion of epithelial cells

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Upon infection of many bacterial pathogens, bacterial invasion is quickly sensed by the innate immune system, resulting in acute inflammatory responses. However, it is still unclear how pathogens modulate host inflammatory responses to bacterial internalization into epithelial cells. Here we provide evidence that the Shigella flexneri effector Ospi delivered via the type III secretion system dampens acute inflammatory responses during bacterial invasion of epithelial cells by targeting TNF receptor-associated factor 6 (TRAF6). We found that Ospi can bind to UBC13, an E2 ubiquitin-conjugating enzyme, and act as a unique glutamine deamidase by selectively deamidating Gln100 to Glu100 in UBC13. Consequently, the E2 ubiquitin-conjugating activity that is required for TRAF6 activation was inhibited, allowing S. flexneri Ospi to modulate the diacylglycerol-CBM complex-TRAF6-NFκB signaling pathway. We determined the 2.0 Å crystal structure of Ospi, which contains a putative Cys-His-Asp catalytic triad. A mutational analysis showed that this catalytic triad was essential for the deamidation activity. Our results highlight a unique bacterial tactic that modulates acute inflammatory responses to bacterial invasion of epithelial cells by targeting the UBC13-TRAF6 complex.

Many bacterial pathogens can enter various host cells where they promote their intracellular survival, transiently evade humoral immunity and further disseminate into other cells and tissues. When bacteria enter host cells and replicate intracellularly, the cells use various pattern recognition receptors (PRRs) to sense the invading bacteria either through damage-associated molecular patterns (DAMPs) or various pathogen-associated molecular patterns (PAMPs). As a result, the host cells induce alarm signals that activate the innate immune system to target and eliminate these pathogens. For example, upon invasion of the intestinal epithelium, some bacterial pathogens, such as S. flexneri and Listeria monocytogenes, induce the disruption of vacuolar membranes to facilitate their entry into the host cytoplasm. This membrane rupture is quickly sensed as DAMPs, which subsequently trigger acute inflammatory responses and occasionally autophagy by activating various cell signaling pathways. In addition, after S. flexneri enters host cells, the bacteria multiply therein and then spread to neighboring cells, during which the bacteria release peptidoglycan, lipopolysaccharide and flagellin. These bacterial components are recognized as PAMPs by cytoplasmic PRRs, such as Nod-like receptors (NLRs), that induce NFκB-mediated and MAPK-mediated inflammatory responses. Therefore, bacterial pathogens deploy various mechanisms to modulate the host immune responses. Indeed, S. flexneri delivers several effectors that modulate inflammatory signaling, including IpAH9.8, OspF, OspG and OspZ. However, it is still unclear what mechanisms bacteria use to modulate host responses, especially early innate immune responses to bacterial invasion of epithelial cells in which vacuolar membrane rupture is detected as DAMPs. In order to gain further insight into these mechanisms, we searched for S. flexneri effectors that are delivered via the type III secretion system (T3SS) and modulate acute inflammatory responses to bacterial invasion.
of epithelial cells. In our current study, we identified Ospl, which is an uncharacterized T3SS-secreted effector encoded by ORF1669 on the large (230 kb) virulence plasmid of *S. flexneri*. We determined that Ospl is a key player in dampening acute inflammatory responses to bacterial invasion of epithelial cells.

We initially performed a comprehensive microarray analysis of HeLa cells infected with YSH6000 (wild-type *S. flexneri*), ΔospI (ospI-deficient mutant) or S325 (T3SS-deficient mutant) in order to assess the role of Ospl in infection. We found that the levels of various chemokines (e.g., IL-8, CCL20, CXCL2 and CCL2) and cytokines (TNF and IL-6) in ΔospI-infected cells were markedly increased at 60 min after infection. This elevated chemokine and cytokine production was detected as early as 30 min post-infection and persisted until 60 min and 120 min post-infection. These changes in chemokine and cytokine levels during ΔospI infection were also observed in Caco-2 cells, a human colonic carcinoma cell line. Since these chemokines and cytokines are induced via activation of the NFκB and MAPK pathways, we subsequently examined immune signaling in ΔospI-infected HeLa cells. As expected, increased phosphorylation of IkBα and JNK1/2 was detected in HeLa cells that were infected with ΔospI but not YSH6000. Importantly, increased IkBα phosphorylation was detected as early as 10 min after infection during the early stage of *S. flexneri* invasion of epithelial cells. At 20 min post-infection, NFκB (p65) nuclear translocation was 4-fold higher in cells infected with ΔospI than YSH6000. Based on these results, we reasoned that Ospl acts as a novel effector that dampens acute inflammatory responses to *S. flexneri* infection.

To establish the in vivo role of Ospl, we exploited a guinea pig rectum infection model, which is the most reliable model to evaluate *S. flexneri* pathogenesis. Guinea pigs were intrarectally inoculated with 1 × 10^7 cfu of YSH6000 or ΔospI and then the intestines were examined at 24 h after intrarectal challenge. ΔospI-infected rectums had increased IL-8 mRNA levels compared with YSH6000-infected rectums. Importantly, there were fewer ΔospI bacteria than YSH6000 bacteria in the rectal tissue at 24 h after intrarectal challenge. ΔospI infection also resulted in severe inflammation in the rectal epithelium layer with monocytes infiltrating into the lamina propria and this phenotype was much more profound compared with YSH6000 infection, suggesting that Ospl contributed to *S. flexneri* pathogenesis.

We subsequently determined whether Ospl inhibits NFκB activation upon *S. flexneri* infection of HeLa cells and found that ectopic Ospl expression further inhibited NFκB activation. Previous studies showed that *S. flexneri* infection of epithelial cells resulted in activation of the Nucleotide-binding oligomerization domain 1 (NOD1)-RIP2-dependent and NOD1-RIP2-independent NFκB signaling pathways. The NOD1-RIP2-dependent pathway can be stimulated via PAMPs, while the NOD1-RIP2-independent pathway is stimulated via DAMPs. Therefore, we investigated how Ospl suppressed NFκB activation using HeLa cells transiently expressing Ospl (HeLa/Ospl) or a mock control. When HeLa/Ospl cells were stimulated with TNFα, NOD1 or phorbol 12-myristate 13-acetate (PMA) and then examined for NFκB activation, it was determined that Ospl suppressed PMA-mediated, but not TNFα- or Nod1-mediated, NFκB activation. Since PMA is a substitute for diacylglycerol (DAG) in the activation of the protein kinase C (PKCs)-NFκB pathway and DAG in the host membrane acts as an important cue to trigger antibacterial autophagy against *Salmonella enterica serovar Typhimurium*, we hypothesized that Ospl may selectively target a DAG-dependent NFκB signaling pathway during *S. flexneri* invasion of epithelial cells. Therefore, we used confocal immunofluorescence microscopy to examine membrane ruffles protruding around *S. flexneri* entry sites in HeLa cells expressing PKC-C1-GFP (PKC-C1 region fused to GFP as a DAG sensor) and found that DAG accumulated around the bacterial entry site. Indeed, increased IL-8 production in ΔospI-infected cells was suppressed by treating with Propranolol, a DAG inhibitor. The DAG-NFκB pathway is mediated through the CARD (CARD9, 10, 11 and 14)-BCL10-MALT1 (CBM) complex in lymphoid, myeloid and non-myeloid cells. The CBM complex is a major regulator of NFκB signaling in both innate and adaptive immunity. Thus, we examined if Ospl modulates CBM complex-mediated NFκB signaling by knocking down BCL10 levels with siRNA and then found that IL-8 levels were greatly decreased compared with the control siRNA. Furthermore, we found that GFP-MALT1 was recruited to the *S. flexneri* entry point in HeLa/GFP-MALT1 cells, since MALT1 functionally interacts with BCL10. These results suggested that DAG that colocalized with *S. flexneri*-induced membranes stimulates the DAG-CBM complex-NFκB pathway and that Ospl specifically dampens this pathway.

To gain further structural and functional insight, we determined the crystal structure of recombinant *S. flexneri* Ospl at 2.0 Å resolution (PDB ID code 3BZ2). Ospl had an α/β fold with four β-strands (β1–β4), seven α-helices (α1–α7) and one 310 helix. The structure was organized around a central anti-parallel β-sheet, with α-helices packed on both sides of the sheet (Fig. 1A). A search of known structures in the Protein Data Bank revealed that Ospl shared structural homology with a cysteine protease family and was most closely related to AvrPphB with a root mean square deviation (r.m.s.d.) value of 3.3 Å. AvrPphB is a *Pseudomonas syringae* T3SS effector and a member of a superfamily of related enzymes containing papain-like cysteine proteases, acetyl transferases, deamidases and transglutaminases. While there is considerable divergence in the overall fold across this superfamily, a core anti-parallel β-sheet and an α-helix containing the active site cysteine, which packs against the β-strands, are conserved across this family. A potential catalytic triad (cysteine (C, Cys) 62, histidine (H, His) 145 and asparagine (D, Asp) 160) in Ospl was identified based on a comparison with the active site of AvrPphB. Superimposing His145 and Asp160 of Ospl onto His212 and Asp227 of AvrPphB or other members of this superfamily revealed remarkable similarity (Fig. 1B). However, Cys62 of Ospl existed in three discrete conformations in the crystal structure, and the Sγ position was located on the opposite side of the active site in AvrPphB. The fractional occupancy of each conformer was estimated to be 0.55 (conformation A), 0.35 (conformation B) and 0.1 (conformation C). The highest occupancy site of Cys62 appeared to form a disulfide bond with Cys65 at 2.05 Å (Fig. 1B).

To confirm that the C-H-D triad is the catalytic center within Ospl, we substituted C62, H145 and D160 with serine (S) (for 62) or alanine (A) (for C62, H145 and D160), and these Ospl mutants were examined for their ability to suppress NFκB activation. Complementing the ΔospI mutant with plasmids encoding the ospI (C62S), ospI (H145A) or ospI (D160A) genes did not reduce the increased IkBα phosphorylation and IL-8 induction that were observed in ΔospI-infected HeLa cells. Consistent with this result, an NFκB reporter assay showed that...
Ospl (C62S), Ospl (H145A) and Ospl (D160A) lost the ability to suppress NFkB activity upon S. flexneri infection or PMA stimulation. To investigate the possible involvement of Cys65 in Ospl activity, we substituted Cys62 with serine (S) and then examined the effects of Ospl C65S on IL-8 expression during S. flexneri infection. Ospl Cys65 had no effect on Ospl activity. Together these results indicated that C62, H145 and D160 in Ospl are the catalytic triad that suppresses NFkB signaling.

We subsequently determined which steps in the DAG-CBM complex-NFkB pathway are targeted by Ospl by examining NFkB activity in HeLa/Ospl or HeLa/Ospl (C62S) cells that were induced with BCL10, TRAF6, TAK1/TAB1, IKKβ or NFkB (p65). Ospl but not Ospl (C62S) suppressed NFkB activity when HeLa cells were induced with BCL10 and TRAF6 but not with TAK1/TAB1, IKKβ or p65, suggesting that Ospl targets TRAF6 or an upstream step. Thus, we examined the possibility that Ospl modulates TRAF6 activation during S. flexneri infection. We used rescued Tra6-deficient mouse embryo fibroblasts (MEFs) [Tra6−/− WT-TRAF6, Tra6−/−/TRA6 (C70A; E3 ligase-deficient mutant) and Tra6−/−/mock] and examined the effects of YSH6000 (wild-type S. flexneri) or ΔospI infection on Cxcl2 mRNA production. When Tra6-deficient MEFs were rescued with TRAF6 (C70A) or the mock control, the Cxcl2 levels induced upon ΔospI infection were greatly decreased compared with that rescued by wild-type TRAF6, suggesting that the ΔospI phenotype depends on TRAF6 E3 ligase activity. Based on these results, we concluded that Ospl interferes with TRAF6 activation during S. flexneri infection.

TRAF6 is an E3 ubiquitin ligase that cooperates with ubiquitin (Ub)-activating E1 and Ub-conjugating E2 enzymes (UBC13 and UEV1A), which are required for TRAF6 self-ubiquitination and TRAF6-induced NFkB activation. Therefore, we investigated the effects of Ospl and Ospl (C62A) on TRAF6 using an in vitro self-ubiquitination assay and found that Ospl, but not Ospl (C62A), dampened TRAF6 polyubiquitination. However, Ospl did not affect the formation of E2–Ub thioester intermediates, suggesting that Ospl modifies TRAF6, UBC13, UEV1A or ubiquitin. We incubated Ospl with each of these putative targets and examined their electrophoretic mobility using native PAGE and found that the mobility of UBC13 but not the other proteins shifted in an Ospl dose-dependent manner. Furthermore, Ospl could interact with His-UBC13, suggesting that Ospl targeted UBC13 to alter its negative surface charge. Hence, we used LC-MS/MS to examine how Ospl post-transcriptionally modifies UBC13. We determined that two overlapping tryptic-digested peptides (WSPALQiR and DKWSPALQiR) of UBC13 were deamidated at glutamine (Q, Gln) 100 to glutamic acid (E, Glu) 100 by Ospl. To confirm that Ospl deamidates UBC13, we created UBC13 (Q100E), which underwent the same mobility shift as UBC13 that was modified at Gln 100 by Ospl, but not Ospl C62A, H145A or D160A. Consistently, endogenous UBC13 was deamidated in HeLa cells at 10 min after infection with YSH6000 and ΔospI/ospI but not ΔospI or ΔospI/ospI (C62S). Of note the co-crystal structures of UBC13 and the Zn-finger of TRAF6 indicated that Gln100 of UBC13 was proximal (13 amino acids behind) to the catalytic pocket, but also located near the interface between UBC13 and the TRAF6 Zn-finger. Thus, we further characterized the Ub-conjugating E2 activity of UBC13 (Q100E) using an in vitro ubiquitination assay. The efficiency of ubiquitin chain formation catalyzed by UBC13 (Q100E) with TRAF6 was greatly decreased compared with wild-type UBC13. In an NFkB reporter assay, UBC13 (Q100E) acted as a dominant-negative, in which UBC13 (Q100E) suppressed the NFkB pathway.

In summary, we identified Ospl as a new T3SS effector that specifically targets TRAF6-dependent acute inflammatory signaling during S. flexneri invasion of epithelial cells. Ospl selectively deamidates UBC13 to inactivate the E2 ubiquitin ligase activity that is required for TRAF6 polyubiquitination, allowing S. flexneri to block acute NFkB-mediated inflammatory responses at the early stage of epithelial invasion (Fig. 2). Recent studies reported that the cycle inhibiting factor (Cif) from enteropathogenic E. coli and the Cif homolog from Burkholderia pseudomallei (CHBP) selectively deamidate ubiquitin-like protein NEDD8 and ubiquitin, which abolishes Cullin-RING ubiquitin ligase activity. The selective Cif-mediated deamidation of NEDD8 is linked to the ability of some enteropathogenic E. coli strains to induce cell cycle arrest and actin stress fiber formation. In our study, we performed LC-MS/MS analysis and showed that Ospl and Cif do not have deamidase activity against ubiquitin and UBC13, respectively. We also performed a native PAGE analysis to further confirm the
substrate specificity and dose-dependency of the deamidation activity of Ospi and Cif. We found that Cif deamidates UBC13 and ubiquitin, but only at high concentrations. Although Cif preferentially deamidates NEDD8, Cif also deamidates ubiquitin, but only at high concentrations. Therefore, together these studies suggest that Ospi and Cif have distinct substrate specificities. Our structural and functional analyses of Ospi strongly indicate that Ospi is a unique T3SS effector that dampens TRAF6-dependent inflammatory signaling in response to bacterial internalization in epithelial cells.

Molecular insight into how MRSA is becoming increasingly dangerous
Comment on: Li M, et al. Nat Med 2012; 18:816-9; PMID:22522561; http://dx.doi.org/10.1038/nm.2692
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Why does methicillin-resistant Staphylococcus aureus (MRSA) remain a dangerous pathogen, particularly in hospital-associated infections? In part, this is due to the fact that MRSA infectious outbreaks occur in “epidemic waves,” meaning that this pathogen constantly produces new clones that are better adapted than previous ones to infect people and/or persist in the hospital environment. However, we only have a very limited understanding of the molecular processes and determinants underlying the rise of new, better-adapted MRSA clones. In a recent study, Li et al. describe a novel surface protein that promotes MRSA colonization and virulence and is transferred via a mobile genetic element. Notably, they provide evidence for a significant spread of that protein among infectious MRSA isolates, analyzing a large series of MRSA hospital infections in China. These findings shed light on the molecular rearrangements occurring in MRSA that lead to the constant creation of more successful and dangerous MRSA clones.

Staphylococcus aureus is a major human pathogen that causes frequent and often severe infections throughout the world. What makes S. aureus infections difficult to treat is the fact that many S. aureus strains are resistant to antibiotics. Most importantly, S. aureus has become increasingly resistant to methicillin, an antibiotic of first choice against S. aureus infections. Methicillin is a penicillinase-resistant penicillin derivative that was developed as a response to the global spread of the penicillinase gene among S. aureus strains, which started in the 1950s. However, methicillin-resistant S. aureus was detected only about one year after the introduction of methicillin into clinical use; and nowadays many countries report methicillin resistance rates among invasive hospital isolates of S. aureus that reach and exceed 50%. More recently, MRSA has also appeared in the community (community-associated MRSA, CA-MRSA), posing an additional threat to public health systems. Nevertheless, hospital-associated cases still represent the by far greater source of morbidity and death among MRSA infections.

Over time, we have seen the disappearance of the original 1960s and the surge of novel MRSA clones, which was accompanied...
by the appearance of new types of SCCmec, the mobile genetic element that harbors the methicillin resistance gene, mecA. The "archaic" 1960s clone was first found in the United Kingdom and had SCCmec type I. Infections with that clone were limited mostly to Europe. The hospital-associated (HA-) MRSA clones that caused the worldwide MRSA pandemic that started in the 1980s have SCCmec types II and III. These clones still are among the most frequently isolated HA-MRSA clones today and belong to only about five clonal groups. CA-MRSA isolates typically have SCCmec types IV or V. These types are smaller compared with other types of SCCmec, cause less of a fitness cost, and thus likely contribute to the capacity of CA-MRSA to spread and infect healthy people. The molecular basis of virulence and spread of MRSA has recently received much attention. However, it has remained largely unknown why MRSA infections occur in "epidemic waves," or more specifically, which molecular factors contribute to the rise and epidemiological success of novel HA-MRSA clones.

In contrast to CA-MRSA, extraordinary virulence is not necessarily considered the major driving force behind the success of HA-MRSA. Indeed, despite being less aggressive and showing lower virulence characteristics in animal infections models than CA-MRSA isolates, HA-MRSA clones such as USA100 continue to be a major cause of hospital-associated infections. This suggests that molecular factors other than only such contributing to aggressive virulence are behind the epidemiological success of HA-MRSA.

What makes HA-MRSA clones so well adapted to the hospital environment and hospital-associated infections? It has frequently been proposed that HA-MRSA clones thrive in the hospital environment due to an exceptional capacity to colonize patients and hospital personnel. *S. aureus* predominantly colonizes the nose and many molecular factors involved in nasal colonization have been identified. However, researchers have not yet been able to link a colonization factor to the spread and success of an MRSA clone.

The importance of a recent study by Li et al., who have identified a novel *S. aureus* surface protein, SasX, that promotes nasal colonization, lies therefore not only in the molecular and functional analysis of that new protein, but mostly in the evidence they provide for the spread of the corresponding gene among HA-MRSA infective isolates. Evaluating data from three large teaching hospitals in eastern China, they showed that the frequency of *sasX*-positive HA-MRSA infections increased significantly over the last decade. The *sasX* gene was linked mostly to HA-MRSA strains belonging to sequence type (ST) 239, the most frequent ST in most parts of Asia. Thus, SasX may contribute to the high prevalence of ST239 among hospital MRSA isolates in this geographical area. Interestingly, no ST239 clones were found among community isolates from healthy individuals in the same region, underscoring that this ST is specifically adapted to the hospital environment. These findings shed light on the epidemiology of MRSA in Asia, which is still not as well understood as that in the US or Western Europe. More importantly, however, they exemplify how a colonization factor may contribute to the epidemiological success of a newly arising MRSA clone.

The *sasX* gene is part of a large mobile genetic element (MGE), namely a $\Phi$5Pb-like prophage of 127 kb. In accordance with the capacity of prophages to be mobilized and transferred to other strains, Li et al. observed a more recent spread of *sasX* to STs other than ST239. The transfer of a surface protein gene promoting colonization represents a quite unique MGE-associated feature, as compared with the more common transfer of toxin or antibiotic resistance genes via such elements. Notably, it may significantly enhance the recipient strains’ capacity to colonize patients and spread in the hospital environment. In fact, animal colonization studies performed with the frequent *sasX*-recipient ST5 suggest this is the case. Naturally, the large prophage contains many genes that are transferred together with *sasX*. However, the lack of other obvious pathogenesis-associated genes in the prophage indicates that the spread of the phage is due to a major extent to *sasX* and its properties. Furthermore, it is interesting that the *sasX* gene is at the very end of the prophage, suggesting that it may have been included in the prophage by incorrect phage excision during evolution. It needs to be added that the prophage contains some antibiotic resistance genes potentially conferring resistance to aminoglycoside antibiotics. These may theoretically also play a role in the spread of the prophage. However, supporting the importance of *sasX* for the spread of ST239 and the distribution of the prophage among MRSA clones, Li et al. found that some strains had prophage deletions in the region containing antibiotic resistance genes, while the *sasX* region was maintained.

As for the mechanisms underlying the phenotypes associated with *sasX*, the authors showed that nasal colonization is due to an enhanced capacity of *sasX*-positive clones to adhere to human nasal epithelial cells. Adhesion could be blocked with purified SasX protein in a competitive fashion, indicating a specific interaction. Interestingly, *sasX*-positive clones also revealed enhanced virulence in animal lung and skin infection models. This is surprising, given that virulence-promoting properties are usually associated with toxins rather than colonization factors. As a plausible explanation for the observed enhanced virulence, the authors found increased capacity of *sasX*-positive clones to evade neutrophil phagocytosis and survive in human serum.

Many bacteria evade phagocytosis by forming aggregates. When they form on surfaces, these bacterial aggregates are called biofilms. SasX promotes aggregation and biofilm formation, phenotypes that therefore presumably form the mechanistic basis of the immune evasion and virulence-enhancing properties associated with *sasX*. Furthermore, biofilm formation may contribute to prolonged survival on abiotic surfaces, which possibly also contributes to the spread of MRSA in the hospital environment.

Many important questions regarding *sasX* remain. First, where did it come from? The finding that there is a very similar prophage in an *S. epidermidis* strain, also harboring *sasX*, indicates that it was acquired from coagulase-negative staphylococci by horizontal gene transfer, as has been proposed for a series of virulence and resistance determinants. Second, what are the mechanisms underlying adhesion to nasal epithelial cells and biofilm formation? Usually, adhesion to eukaryotic cells that is facilitated by a bacterial surface protein is dependent on a specific receptor on the eukaryotic cell. Furthermore, many MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which connect bacteria to eukaryotic matrix proteins, contain large parts whose function it is to extend the protein through the cell wall, in order to make a terminal part accessible for receptor interaction. In that regard, it is interesting that the SasX protein is rather small in its secreted, processed form, only ~15 kDa, which is likely too small to protrude...
through the cell wall and expose a domain for interaction. It appears as if SasX uses another mechanism to achieve interaction with the eukaryotic cell. Thus, the eukaryotic SasX receptor and the mechanism by which SasX connects to it remain to be identified. Third, how does SasX accomplish bacterial aggregation and biofilm formation? Several surface proteins were recently described to promote biofilm formation, in addition to their primary function, which often is to facilitate adherence to tissue. Thus, a double function in promoting adherence to eukaryotic cells and bacterial aggregation, as found for SasX, is not uncommon. However, it is not clear whether bacterial aggregation in those cases is also due to a specific, receptor-type interaction, or dependent on a non-specific aggregation phenomenon, possibly based on the physico-chemical properties of exposed protein domains.

In conclusion, the identification of sasX as a driving force of an MRSA epidemic represents an important example helping us to understand how novel MRSA clones arise. By acquiring novel colonization factors they may replace other MRSA clones that are less optimally adjusted to persist in the hospital environment. While research on S. aureus toxins and their potential use as targets for anti-staphylococcal drug therapy has recently been very intense, especially regarding CA-MRSA, this finding suggests that there should also be a strong focus on the molecular factors promoting colonization and the spread of MRSA in hospitals. Given that most MRSA infections originate from colonizing strains, MRSA decolonization has often been proposed as a promising approach to reduce MRSA infection rates. Whether SasX may be used as a target for efforts aimed to reduce colonization or virulence remains to be shown. As a first step in that direction, the use of anti-SasX antibodies in animal models should be tested. Clearly, the use of any drug targeting SasX will be limited to sasX-positive clones. However, it is not uncommon to explore targets that are only present in very successful MRSA clones, such as in the often-discussed case of MRSA harboring Panton-Valentine-leukocidin genes. Probably, all such virulence or colonization-targeted approaches to find anti-MRSA therapeutics will require a mixture of drugs or antibodies targeting different bacterial virulence or colonization determinants.

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Can surveillance of the influenza virus PB1-F2 gene be used to predict the severity of secondary bacterial infections?

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The influenza virus genes that will dictate the severity of the next influenza virus pandemic currently exist in nature. However, most deaths associated with a future pandemic may likely be due to secondary infections caused by Gram-positive respiratory pathogens. Previous reports have demonstrated that certain versions of the influenza virus protein PB1-F2 play a role in the development of secondary bacterial pneumonia associated with Streptococcus pneumoniae, but our current surveillance efforts do not typically include efforts to sequence this viral gene. Surveillance strategies must be expanded to include genes associated with virulence in laboratory models of primary influenza virus infection and influenza virus:bacteria super-infection. Identification of potentially deadly reassign-ant virus strains before these circulate within humans may allow adequate time to implement plans for treatment and/or prevention.

The World Health Organization estimates that influenza virus epidemics are associated with 250–500,000 deaths and 3–5 million illnesses worldwide on an annual basis. It is well-accepted that influenza virus infection predisposes hosts to secondary bacterial pneumonia, and the Gram-positive pathogens Streptococcus pneumoniae, Staphylococcus aureus and Streptococcus pyogenes have all been associated with excess mortality during influenza epidemics and pandemics. The virulence associated with a circulating influenza virus appears to be a multi-genic trait, with specific reassortment events yielding viruses with the potential to cause devastating pandemics, like the one that occurred in 1918. Currently, the highly pathogenic avian influenza A viruses of the H5N1 subtype represent a significant pandemic threat that could be realized if these viruses acquire the ability to transmit from human-to-human. In addition to identifying the genetic changes associated with adequate transmissibility of these viruses, we must also understand the viral genes that modulate virulence in the context of both primary influenza virus infection and influenza virus:bacteria super-infection. One way to accomplish this goal is to identify viral genetic signatures associated with virulence that occur in the natural setting, ideally in the dominant intermediate mammalian host, the swine.

Influenza virus virulence factors have been largely defined using models of primary influenza virus infection, with relatively few studies evaluating the contribution of these factors toward disease within models of influenza virus:bacteria super-infection. The influenza virus protein PB1-F2, discovered in 2001 (Chen et al., Nat Med 2001), has been studied as a viral virulence factor that contributes to morbidity and mortality within models of influenza virus:S. pneumoniae super-infection. Viruses that circulated during the 1918, 1957 and 1968 influenza pandemics expressed full-length, virulent variants of this 87–90 amino acid viral protein, while future seasonal virus isolates expressed variants of this protein that were less virulent due to the loss of specific amino acids through either mutation or truncation (McAuley et al., PLoS Pathog 2010). Using experiments designed to focus solely on the PB1-F2 protein, prior studies have identified the key amino acids expressed by this protein that predispose the host to lethal super-infections. While studies designed to focus on individual viral virulence factors are critical for evaluating the mechanisms through www.landesbioscience.com Virulence 9
which a given virulence factor affects overall morbidity and mortality, one must also consider whether variant forms of these genes contribute to virulence when expressed by naturally-occurring virus isolates.

In a recent manuscript, our group tested the hypothesis that the laboratory-defined virulence associated with the PB1-F2 gene could be used to predict the outcome of secondary bacterial infections initiated with naturally-occurring swine influenza virus isolates. To test this hypothesis, natural swine influenza virus variants were incorporated into murine models of influenza virus:bacteria super-infection, with either S. pneumoniae, S. aureus, or S. pyogenes acting as secondary bacterial invaders. One unique aspect of this study is its evaluation of the virulence associated with naturally-occurring influenza virus variants, using the PB1-F2 genotype as the sole criterion for virus selection. In addition, this is also the first study where three distinct Gram-positive respiratory pathogens were directly compared in a model of influenza virus:bacteria super-infection. The major findings from this study are summarized here, with emphasis on the association between laboratory-defined virulence and naturally-occurring virus reassortants that circulate within pigs.

To date, the virulence associated with PB1-F2, defined in mice using both models of primary influenza virus infection and models of influenza virus:bacteria super-infection, has been mapped to five specific amino acid residues (62L, 66S, 75R, 79R and 82L). Naturally occurring swine influenza viruses that were selected for inclusion in our studies were grouped based on the number of virulence-associated amino acids expressed. In addition, one group of viruses expressed truncated forms of PB1-F2 that were either 11 amino acids or 57 amino acids in length. Based on the length of these truncated proteins, none of the virulence-associated amino acids indicated above were present in the PB1-F2 proteins expressed by these viruses.

In general, our findings demonstrate that outcomes after super-infection in mice are directly associated with an increasing number of virulent PB1-F2 amino acids expressed, regardless of the bacterial species introduced as the secondary invader. Of note, the virus that demonstrated the most lethal phenotype within our model was the only virus that expressed an asparagine at position 66 instead of a serine. This N66S mutation has demonstrated increased virulence in models of primary influenza virus infection with the 1918 H1N1 virus, and may have contributed to the high mortality observed during this pandemic (Conenello et al., J Virol 2011). Furthermore, one surprising observation was that viruses expressing truncated PB1-F2 proteins, with predicted avirulent phenotypes, demonstrated a particular preference for S. pyogenes as a secondary invader, compared with S. pneumoniae and S. aureus. This demonstrates that variation in the bacterial species can also contribute to the severity of the super-infection, providing evidence that all Gram-positive respiratory pathogens cannot be considered equivalent in their ability to induce lethal outcomes in murine super-infection models. Thus, survival after super-infection can differ greatly based on host, viral, and bacterial contributions to these polymicrobial infections.

The obvious caveat to our findings is that additional virulence factors expressed by these naturally-occurring swine influenza virus variants have the potential to contribute to the phenotype observed. However, our ability to use a laboratory-characterized virulence factor to predict the outcomes of a super-infection supports current efforts to expand the surveillance of naturally-occurring influenza virus variants to include the sequencing of virulence-associated genes, like PB1-F2. Because whole-genome sequencing of viruses can be done rapidly and inexpensively now, I propose that this replace current strain typing methods. Alternatively, either microarray or PCR-based methods designed to detect specific amino acid residues that have been identified as molecular signatures of virulence could also be employed.

Since the genes expressed by prior pandemic influenza viruses are known to have evolved within intermediate mammalian species during the pre-pandemic phase (Vijaykrishna et al., Science 2010), pigs represent an excellent target for such surveillance efforts. In fact, our model specifically incorporated primary swine influenza virus isolates that already express virulent PB1-F2 variants, which demonstrates that reassortment events could occur at any time, with potentially deadly outcomes predicted. Experiments designed to evaluate the contribution of additional viral genes toward the virulence observed with these specific viruses have been initiated, with full consideration for contributions of more than one gene toward severe super-infections. At the present time, interspecies transmission of H3N2 variant (H3N2v) influenza viruses from swine to humans has increased in the United States (CDC, MMWR 2012). An assessment of the PB1-F2 sequences present in five of these recent H3N2v human isolates indicates that the PB1-F2 gene expressed is full-length (90 amino acids), it contains just two amino acids associated with virulence (62L and 82L), and there is an N present at position 66. Thus, focusing solely on the PB1-F2 genotype, I would predict that these H3N2v viruses would be less likely to predispose toward severe secondary bacterial infections with Gram-positive respiratory pathogens, at least in a murine model.

In summary, our group recently evaluated naturally-occurring swine influenza viruses within murine models of influenza virus:bacteria super-infection. The swine influenza viruses utilized were selected based on the predicted virulence associated with the PB1-F2 gene expressed. This study was not designed to identify the specific genotypes associated with virulence, rather our intent was to determine whether increased surveillance efforts could provide insight into outcomes from influenza virus:bacteria super-infection. Although some exceptions to the general rule were identified, we were able to broadly predict severity of secondary bacterial infection based solely on the PB1-F2 expressed by these influenza viruses. Since secondary bacterial infections contribute significantly to the morbidity and mortality observed after primary influenza virus illness, our ability to use surveillance to predict the incidence of these complicated infections will allow us to improve future approaches toward treatment and prevention.
Sensing the enemy: New role for a bacterial secretion system in activation of an innate immunity-associated microRNA

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Both pathogenic and non-pathogenic microbes express common microbe-associated molecular patterns (MAMPs) that can activate innate pro-inflammatory responses. There is evidence to suggest that host immune systems are adapted to minimize unwarranted, potentially damaging pro-inflammatory responses, e.g., to commensals at mucosal surfaces where very high microbe loads are found, while maintaining the ability to mount aggressive responses to invading pathogens. The recognition of bacterial secretion systems by host innate immune pathways may represent one mechanism used by host immune cells to discriminate between pathogen and non-pathogen, or strains with reduced virulence potential. Most recently, it has been shown that the type IV secretion system (T4SS) encoded by virulent cagPAI-positive strains of the pro-carcinogenic pathogen *Helicobacter pylori* induces the expression of microRNA-155, a known mediator of innate immunity that is also implicated in oncogenesis. The T4SS-specific activation of miR-155 occurred independently of TLR and NOD1/2 pattern-recognition receptor (PRR) signaling. We discuss the potential role of the T4SS-dependent activation of miR-155 as a signaling pathway (Rad et al., Gastroenterol 2009; B) pro-inflammatory responses (Viala et al., Nat Pathol 1995; Parsonnet et al., New Engl J Med 1991). Infection is also associated with B cell mucosa associated lymphoid tissue (MALT) lymphoma (0.2% of infected individuals) (Capelle, Eur J Can 2008). Thus far, there is no vaccine that can induce protective immunity and although drug regimes that include antibiotics can clear infection and peptic ulcer disease and regress MALT lymphoma, individuals that had evidence of histological progression to intestinal metaplasia prior to infection clearance probably remain at increased risk of gastric cancer (Zullo et al., W J Gastroenterol Oncol 2012). An immune response is mounted by the host to infection, but this is almost always inadequate in terms of infection clearance and, the chronic inflammatory response that can result is widely accepted to induce immunopathology and underlie oncogenesis. The pathological outcome of *H. pylori* infection is likely dependent on a number of factors, host and environmental determinants, and the complex interplay between these factors (Pritchard and Crabtree, Curr Opin Gastroenterol 2006). There is much still to be clarified as our current understanding of *H. pylori* virulence and the role of deregulated inflammatory responses during pathogenesis is incomplete.

*H. pylori* virulence is associated with an ~40-kb horizontally acquired genomic pathogenicity island of unknown origin, the cagPAI, which encodes a type four secretion system (T4SS) and the prototypical *H. pylori* secreted virulence protein, CagA, among others (Amieva et al., Science 2003). In epithelial cell lines, experimental animal infection models and clinically, infection with cagPAI-positive strains is associated with increased inflammatory responses and risk of gastric cancer (Crabtree et al., J Clin Pathol 1995; Parsonnet et al., Gut 1997; Rieder et al., Gastroenterol 2005).

The host immune response to *H. pylori* infection is also strongly implicated in pathogenesis of infection (Roth et al., J Immunol 1999). An unresolved inflammatory response most likely serves to increase infiltration of monocytes and neutrophils from the circulation, creating an environment that primes T cells for a Th1/Th17 dominant T cell adaptive immune response that appears to be responsible for attempts to control and clear infection, and at the same time, for immunopathology and associated disease (Sayi et al., J Immunol 2009). Prominent early innate defense mechanisms are directed against *H. pylori* by gastric epithelial cells and tissue resident innate immune cells such as macrophages and dendritic cells (DCs). Detection of microbe associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD) receptors and the retinoic acid inducible gene 1 receptor (RIG-I) is an important component of the host innate immune response to *H. pylori* in the gastric mucosa and results in the induction of pro-inflammatory cytokines, particularly in macrophages and DCs. Isolated DCs have been shown to upregulate inflammatory cytokines such as IL-6 in response to *H. pylori* infection in a MyD88-dependent manner. *H. pylori* MAMPs activate extracellular TLR-2 and -4 (LPS), and endosomal TLR-9 (bacterial DNA) to induce the nuclear factor kappa B (NFκB) pro-inflammatory pathway (Rad et al., Gastroenterol 2009; Obonyo et al., Infect Immun 2007) (Fig. 1). *H. pylori* RNA can also activate the soluble cytosolic RIG-I receptor in DCs to induce IFN-γ responsive genes (Rad et al., Gastroenterol 2009) and bacterial peptidoglycan activates the cytosolic NOD1 receptor to induce IL-8 production in gastric epithelial cells in a cagPAI-dependent manner, which contributes to inflammatory responses (Viala et al., Nat Immunol 2004). Also implicated in the innate immune response to *H. pylori*, is infection-induced cellular microRNA (miRNA) expression (Xiao et al., J Infect Dis 2009). MicroRNAs are short non-coding RNAs that are post-transcriptional regulators of gene expression. Typically,
they bind to the 3′-UTRs of their mRNA targets to inhibit gene expression. The modulation of cellular miRNA expression in response to bacterial infection is, potentially, a common theme in host-pathogen systems that has broad implications for immunity and disease. Through their regulation of key inflammatory signaling pathways—in particular NFκB—and cytokine gene expression, miRNAs are increasingly being implicated as important mediators of host immune responses to bacterial and viral pathogens (Gantier, J Interferon Cytokine Res 2010; Eulalio et al., RNA Biol 2012), and as potential disease loci in humans. MicroRNA expression can be activated by PRRs; for example, LPS-mediated stimulation of TLR4 has been shown to induce the expression of miR-132, miR-146a/b and miR-155. The NFκB pathway is also implicated in the expression of miRNAs, including miR-155 (Eulalio et al., RNA Biology 2012), miR-155 may be of particular relevance to infection and immunity as it is induced in macrophages in response to TLR ligands and Gram-negative and Gram-positive pathogens and is widely implicated in inflammatory responses and innate immunity; for example, it was found to be induced in a TLR-dependent manner during infection of macrophages by the Gram-negative intestinal pathogen Salmonella enterica and was crucial for normal immune function during experimental Salmonella infection of mice, and for the induction of protective immunity (Rodriguez et al., Science 2007). miR-155 was also among the most significantly upregulated miRNAs during infection with the Gram-positive pathogen Listeria monocytogenes in macrophages (Schnitger, PLoS One 2011). The induction of miRNAs, including miR-155, has also been reported during L. monocytogenes infection of epithelial cells (Izar et al., Int J Mol Sci 2012). Interestingly, the regulation of miRNA expression in that study was dependent on L. monocytogenes virulence factors and the sub-cellular localization of bacteria, which suggests that, at least for intracellular pathogens, this may provide an additional level of host-pathogen cross talk. miR-155 was also among the most significantly upregulated miRNAs during infection with the Gram-positive pathogen Listeria monocytogenes in macrophages (Schnitger, PLoS One 2011). Interestingly, miR-155 is associated with both pro-inflammatory responses and oncogenesis, warranting further investigation of the role of this miRNA in H. pylori infection-induced pathology.

Recently, we observed that the H. pylori T4SS pathogenicity determinant upregulated miR-155 expression during experimental infection of primary macrophages deficient in TLR and NOD1/2 signaling (Koch et al., Proc Natl Acad Sci U S A 2012). This latest finding that H. pylori modulates the expression of miR-155 by a mechanism partly dependent on the T4SS, and independent of TLR/NOD1/2 receptor recognition is intriguing. Since only the more virulent cagPAI positive H. pylori strains contain the T4SS, this could suggest that the T4SS acts as a MAMP that provides a critical function for the host innate immune system: the ability to

Figure 1. The proposed T4SS-dependent activation of miR-155 expression in macrophages during H. pylori infection. The T4SS, specific to virulent H. pylori strains, induced NFκB dependent miR-155 expression, in addition to the known activation by H. pylori MAMPs via TLR and NOD1/2 pattern recognition receptor signaling. T4SS-dependent miR-155 expression, represented by (?), may be directly dependent on an unidentified cellular receptor, or on an indirect activation pathway. miR-155 was found to target a number of pro-apoptotic genes in macrophages, and conferred an anti-apoptotic effect upon infection in the presence of a DNA damage inducing reagent. It is speculated that miR-155 expression could prolong macrophage survival in the inflammatory microenvironment, which could contribute ultimately to the deregulation of pro-inflammatory responses, increased expression of pro-inflammatory mediators e.g., IL-8 and IL-6 and pathology progression observed during H. pylori infection. MAMP, microbe-associated molecular pattern; NOD, nucleotide oligomerization domain; ROS, reactive oxygen species; RNS, reactive nitrogen species; T4SS, type IV secretion system; TLR, Toll-like receptor.
distinguish between pathogenic strains, and less pathogenic strains and commensals. Both pathogenic and non-pathogenic bacterial populations possess common PRR-activating MAMPs such as lipid A, and so a pivotal question in biology is whether immune systems can discriminate between pathogens and commensals, and if they do, then how? There is mounting evidence to suggest that host cells are able to distinguish between pathogenic and less pathogenic strains and commensals. One possible mechanism may involve exploiting the PRR-independent detection of pathogen-specific features, including secretion systems, to ensure that inflammatory responses are commensurate with threat level, since in addition to common MAMPs, pathogenic species typically also express virulence factors and machinery, specific combinations of which may be unique to pathogenic strains (Blander and Sander, Nat Immunol 2012). It has been postulated that pathogens display particular signatures, or “patterns of pathogenesis,” that can act as PRR-independent activators of immune responses, which may then synergize with PRR-mediated signaling to optimize and target the response to pathogen infection (Fontana et al., PLoS Path 2011; Vance et al., Cell Host Mic 2009). Examples of pathogen-specific patterns of pathogenesis typically involve initiation of general perturbations to cellular function/breaching of cell barriers, and may include deregulation of the actin cytoskeleton, induction of cell death (Vance et al., Cell Host Mic 2009) and inhibition of host translation (Fontana et al., PLoS Path 2011). It has been noted that patterns of pathogenesis may distinguish viable from non-viable bacteria, and the term vita-PAMP was coined to describe bacterial MAMPs that stimulate a host immune response only when pathogens are viable (Sander et al., Nature 2011; Vance et al., Cell Host Mic 2009). The ability to distinguish dead from viable bacteria could be important for scaling of immune responses in accordance with perceived threat level. Bacterial virulence factors such as secretion systems, and secreted effector proteins, have themselves been experimentally implicated as having differing functional roles in the PRR-independent activation of host immune responses, and may act as vita-PAMPs in this capacity. Evidence suggests that secretion systems can directly activate PRR-independent immune responses, i.e., via an interaction between the secretion system and a cellular factor/receptor, or indirectly, which may not involve a cellular receptor. Secretion system, specifically type III secretion system (T3SS) and T4SS, dependent activation of the NLRC4 inflammasome may be important for distinguishing pathogen from non-pathogen, for example; this has been reported to occur indirectly, e.g., via the secretion-system mediated delivery of flagellin into host cells, and directly, e.g., via the interaction of the relatively highly conserved rod region of the T3SS from a number of pathogenic species including Shigella flexneri, S. Typhimurium and Burkholderia pseudomallei (Miao et al., Proc Natl Acad Sci U S A 2010). Recently, it was demonstrated that in murine intestinal phagocyte populations, pathogenic Salmonella and Pseudomonas bacteria induced the expression of the pro-inflammatory cytokine IL-1β through activation of the NLRC4 inflammasome, whereas commensal bacteria did not. The production of IL-1β was dependent on a functional T4SS or T3SS and was required for infection clearance (Franchi et al., Nat Immunol 2012); the authors did not describe whether the secretion systems played a direct or indirect role in inflammasome activation. The molecular mechanism by which the T4SS activated NFκB-dependent miR-155 expression in a TLR- and NOD1/2-independent manner in macrophages during H. pylori infection remains to be resolved, although the secreted H. pylori virulence factor CagA was excluded as having a role—of course this does not rule out the possibility that other secreted factors are involved. Whether the T4SS mediated induction of miR-155 observed in the latest study requires the direct interaction of the T4SS with an as yet unidentified host cell receptor, or whether there is an indirect mechanism responsible for NFκB activation and miR-155 induction, remains to be determined. It is conceivable that there is a direct interaction between the T4SS and a host cell receptor in macrophages. Certainly, the integrin α5β1 has been suggested elsewhere to be a host cell receptor for the H. pylori T4SS (Kwok et al., Nature 2007) and virulence factors from other pathogens are also known to exploit integrin α5β1 as a receptor; for example, the surface protein Td92 of the periodontopathogen Treponema denticola binds to α5β1 and activates the NLRP3 inflammasome and pro-IL-1β transcription via an NFκB dependent pathway (Jun et al., Immunity 2012).

The contribution of the T4SS to miR-155 expression was significant but modest in macrophages in vitro. We speculate, however, that this effect may take on new relevance in tissue resident macrophage populations in the gastric mucosa, which are phenotypically distinct from blood monocytes. Gastrointestinal tissue-resident macrophages—the largest population of macrophages in the body—reportedly exhibit inflammatory “anergy” under conditions of tissue homeostasis. Anergic macrophages are typically down-regulated for PRR signaling, including TLR, but retain phagocytic properties (Smythies et al., J Clin Inv 2005). Inflammatory anergy is assumed to be an immunotolerance mechanism that regulates immune responses at mucosal surfaces, which present particular challenges as they are bombarded with foreign antigens from various sources. We suggest that the ability of macrophages to detect the H. pylori T4SS pathogenicity determinant, independent of TLR signaling, may contribute to the pro-inflammatory response to H. pylori in the context of gastric mucosal macrophage inflammatory anergy. Interestingly, Franchi et al. observed the bacterial secretion-system dependent induction of the inflammasome in anergic murine intestinal phagocyte populations (Franchi et al., Nat Immunol 2012). However, whether the H. pylori T4SS specific induction of miR-155 occurs in anergic macrophages or indeed, whether it contributes to inflammatory responses during H. pylori infection requires further investigation. A number of cellular microRNAs are upregulated, and downregulated, in the gastric mucosa in response to H. pylori infection (Matsushima et al., Int J Cancer 2011; Fassi Fehri et al., PLoS One 2010). Concerning H. pylori-induced pathology, miR-155 is of particular interest as it is widely implicated in inflammatory responses and cancer and, in particular, is observed to be overexpressed in gastric cancer and B cell lymphomas. Moreover, during in vitro and in vivo investigations, miR-155 was consistently upregulated in response to H. pylori infection in a number of non-hematopoietic and hematopoietic cells. In gastric epithelial cell lines and gastric mucosal tissues, miR-155 was upregulated by the NFκB and AP-1 pathways in response to infection (Xiao et al., J Infect Dis 2009); miR-155 overexpression in this system reportedly inhibited the expression of IL-8. We have shown that miR-155 is upregulated in response to H. pylori infection of a human T-cell line and murine macrophages, and in gastric mucosal...
biopsies from human volunteers (Fassi Fehri et al., PLoS One 2010). The role of miR-155 may best be described as “immunomodulatory” during infection since NFκB pathway genes are also miR-155 targets; thus, the regulation of this miR-155 is under negative feedback control, which may ultimately serve to “fine-tune” inflammatory responses. One can only speculate as to the role of miR-155 in macrophages in vivo during infection, particularly as the role of macrophages in the host response to *H. pylori* infection and infection-induced pathology is in general unclear. In the latest study, comparative microarray analysis of miR-155−/− and wild type primary murine bone marrow derived macrophages showed that miR-155 regulated the expression of a large subset of genes that are involved in cell-death. Indeed, when macrophages were stressed with the DNA-damaging reagent cisplatin during *H. pylori* infection, miR-155 protected the cells against apoptosis, and the overall effect of miR-155 in this system was anti-apoptotic. We suggested that this could be potentially biologically significant in vivo for prolonging macrophage survival and, thus, proinflammatory potential in the inflammatory microenvironment. This is anecdotally supported by the observation that mice chemically depleted of macrophages exhibit reduced pathology during *H. pylori* infection, despite having comparable bacterial loads to wild-type mice (Kaparakis et al., Infect Immun 2008). The role of miR-155 expression in other cell types during *H. pylori* infection in vivo is also ambiguous; to date, results in animal models have shown that miR-155 expression in T cells controls bacterial load. Interestingly, in this model miR-155−/− mice were significantly protected against *H. pylori* induced pathology and showed fewer precancerous lesions despite high bacterial loads compared with wildtype mice. Reduced ability to control infection and the decreased pathology in miR-155−/− mice were both largely attributed to defective production of IL-17 and IFN-γ. Together, these data suggest that miR-155 induced during *H. pylori* infection has a key role in both infection control and pathogenesis (Oertli et al., J Immunol 2011); however, the current experimental evidence highlights the need for in vivo clarification of the functional consequences of infection-induced changes in miR-155 expression profiles in the different cell types present in the gastric mucosa.

It is clear that microbes can manipulate cellular miRNA profiles to profoundly alter host biology; moreover, altered or deregulated miRNA profiles in host cells during infection may prove to be a common pathogenesis mechanism, with implications for clinical management of infection-induced pathologies. The latest finding that the T4SS specifically upregulates miR-155 in macrophages during infection with *H. pylori* suggests that, similar to other innate immune responses that are pathogen specific, the T4SS provides macrophages with an additional level of discrimination for mounting a response to *cagPAI*-positive *H. pylori* strains. Mechanistically, how the T4SS activates the cellular miR-155 response, and whether this response in macrophages contributes to deregulated inflammatory responses during infection and disease phenotypes, will be interesting topics for future research into the virulence potential of *H. pylori*.

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