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Role of different receptors and actin filaments on *Salmonella* Typhimurium invasion in chicken macrophages

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ABSTRACT

Bacterial attachment to host cell is the first event for pathogen entry. The attachment is mediated through membrane expressed adhesins present on the organism and receptors on the cell surface of host. The objective of this study was to investigate the significance of Fc receptors (FcRs), actin filament polymerization, mannose receptors (MRs), carbohydrate moieties like N-linked glycans and sialic acid on chicken macrophages for invasion of *S*. Typhimurium. Opsonisation of *S*. Typhimurium resulted in three folds more invasion in chicken monocyte derived macrophages. Cytochalasin D, an inhibitor of actin filament polymerization prevented uptake of *S*. Typhimurium. Pre-incubation of macrophages with cytochalasin D, showed severe decrease (28 folds) in *S*. Typhimurium invasion. Next we attempted to analyse the role of carbohydrate receptors of macrophages in *S*. Typhimurium invasion. Treatment of macrophages with methyl α-D-mannopyranoside, PNGase F and neuraminidase, showed 2.5, 5 and 2.5 folds decrease in invasion respectively. Our data suggest that deglycosylation of N-linked glycans including sialic acid by PNGase F is more effective in inhibition of *S*. Typhimurium invasion than neuraminidase which removes only sialic acid. These findings suggested FcRs, actin filament polymerization, MRs, N-linked glycans and sialic acid may act as gateway for entry of *S*. Typhimurium.

1. Introduction

"In the midst of chaos there is also oppurtunity."

Sun Tzu, The art of war

The enteric pathogen *Salmonella enterica* serovar Typhimurium faces daunting odds during its voyages in the natural environment as well as during infection process. In order to detour host immune response *S*. Typhimurium must employ survival tactics for its growth. It does this by entering into the host cell and establishes its niche within the intracellular environment. The interaction of *S*. Typhimurium with its host cell is a complex process and involves various virulence factors for its invasion. Bacterial binding to the host cell surface provides a selective advantage for its colonization as well as to prevent pathogen elimination from the host. Accordingly, *S*. Typhimurium have developed a battery of molecular manoeuvre to adhere to host cell surface.

Microbial binding to host cell is a key event in the pathogenesis of infectious disease. Surface of the microbes are equipped with adhesins which allows interaction between the bacterial cells and eukaryotic cell membrane. *S*. Typhimurium is a facultative intracellular pathogen of gastrointestinal tract. Salmonellae are usually acquired by oral ingestion of contaminated food and water. Within the intestine, *S*. Typhimurium invades the non-phagocytic cells such as enterocytes. However, this bacterium also initiates uptake by M-cells and dendritic cells as an alternative route of breaching intestinal barrier (Haraga et al., 2008). *S*. Typhimurium infection triggers inflammatory response that affects the competing intestinal flora and generates novel nutritional supply for its growth (Stecher and Hardt, 2011)

Macrophages serve as first line of defense against intracellular bacteria like *Salmonella*. Pathogen associated molecular patterns or the structural determinants on the surface of the bacteria are recognized by the phagocytic receptors (Gordon, 2002). These include non-opsonic and opsonic receptors, where former recognizes the pathogen directly and latter recognizes the antibody or complement opsonised bacteria (Wright et al., 1989). Non-opsonic receptors include Toll like receptors (TLRs) and mannose receptors (MRs) whereas complement receptors (CRs) and antibody receptors (FcRs) are opsonic receptors (Janeway, 1989; Janeway and Medzhitov, 2002; Stuart and Ezekowitz, 2005). MRs recognise mannose and fucose residues of glycoprotein and mediate bacterial uptake by phagocytic cells (Stahl and Ezekowitz, 1998). Like *Escherichia coli*, Type 1 fimbriae are expressed by *S*. Typhimurium

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and bind specifically to the MRs of the host cells for its invasion (Klemm and Krogfelt, 1994; Clegg and Swenson, 1994). Opsonins like antibodies coat bacteria and make them congenial for phagocytes to enhance bacterial uptake through FcR (Ezekowitz et al., 1984). Microbial binding to the host cell is followed by the induction of actin filament rearrangement for subsequent invasion of the organism (Flannagan et al., 2011). Type III secretion system (T3SS) is a specialized organelle in *S*. Typhimurium that transfer effector proteins to the host cell (Galan, 2001). These effector proteins interact with host cell cytoskeleton and triggers actin filament polymerization for *S*. Typhimurium invasion (Hayward and Koronakis, 2002; Lilic et al., 2003).

Host cell surface glycoproteins are targeted by many pathogens (Daya et al., 2015) Carbohydrate moieties present on the mucosal surface of the gastrointestinal tract, serve as route and site of infection. Mucin covering the epithelial cells of gastrointestinal tract is rich in fucose, galactose, sialic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose (Rakoff-Nahoum et al., 2014). Pathogenic bacteria in the gut can exploit these sugars for attachment (Ng et al., 2013). The frequent ligands for binding of microbial adhesins are sialic acid and *N*-acetylglucosamine (Pieters, 2011). Thus, our research aimed to analyse the importance of receptors (FcRs, MRs), actin filament polymerization and also the significant role of N-linked glycans and sialic acid on chicken macrophages in *S*. Typhimurium invasion. We observed enhanced uptake of *S*. Typhimurium upon its opsonisation in chicken macrophages. Invasion was severely hampered when macrophages were treated with cytochalasin D. Further, invasion was significantly reduced when chicken macrophages were pre-incubated with methyl α-D-mannopyranoside, PNGase F and neuraminidase. These findings emphasize the importance of actin filament polymerization, MRs, N-linked glycans and sialic acid respectively in *S*. Typhimurium entry.

2. Materials and methods

2.1. Bacterial strain

S. Typhimurium strain 5591 was procured from the National *Salmonella* Centre (Veterinary type) repository, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar.

2.2. Laboratory animals

All animal experiments were conducted under the guidelines of Institutional Animal Ethical Committee (IAEC), IVRI, Izatnagar, India. One day old White Leghorn layer birds were procured from the ICAR-Central Avian Research Institute (CARI), Hatchery, Izatnagar, India and screened for the presence of *Salmonella* spp. by collecting the cloacal swabs followed by PCR and serotyping (Pesingi et al., 2017).

2.3. Isolation of poultry macrophages

Blood was collected in heparinised tubes from jugular vein. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation method as described previously (Lan, 1999). In brief, Histopaque-1077 (Sigma) was taken in a 15 ml conical centrifuge tube and equal volume of blood was carefully layered over the histopaque along the walls of the tube. The tube was centrifuged at 1300 rpm for 40 min at room temperature. A distinct band of mononuclear cells between the plasma and histopaque was transferred in a 15 ml conical centrifuge tube and centrifuged at 1300 rpm for 10 min at room temperature to remove the residual plasma and histopaque. The cells were washed and re-suspended in RPMI-1640 (HiMedia) containing penicillin/streptomycin, 2% chicken serum and 8% fetal bovine serum (FBS). Total viable cell count was done using trypan blue dye exclusion method.

2.4. Bacterial culture

Single bacterial colony from Hektoen Enteric (HE) agar (HiMedia) plate was picked and inoculated in 10 ml Luria Bertani (LB) broth (HiMedia) and incubated overnight at 37 °C/180 rpm in an orbital shaker. Next day, it was sub cultured in 1:100 ratio and the bacteria were grown up to the mid log phase (OD_{600} = 0.6). From mid log phase culture, 10 ml was centrifuged at 7500 rpm for 5 min at 4 °C. Supernatant was discarded and pellet was washed in PBS at 7500 rpm for 5 min and finally suspended in 10 ml sterile PBS.

2.5. Precipitation of anti-S. Typhimurium antibodies (IgY) by sodium sulfate

Anti-*S*. Typhimurium antibodies were raised in birds. The birds were orally inoculated with 3.7×10^{10} *S*. Typhimurium. Serum was collected on 28th day post inoculation. One ml of serum was diluted with equal volume of distilled water. Anhydrous sodium sulfate (0.18 g/ml) was added gradually to the diluted serum with continuous stirring at room temperature for 30 min. Then the mixture was kept at 4 °C for overnight. The precipitated proteins were pelleted and dissolved in distilled water, adjusted to the original volume. The proteins were re-precipitated with gradual addition of 0.14 g/ml of anhydrous sodium sulfate with continuous stirring for 30 min. The precipitated proteins were pelleted and dissolved in distilled water. The dissolved proteins were dialysed against four changes with normal saline and the dialyzed material was centrifuged at 13,000 rpm for 15 min. The supernatant was collected and protein concentration was estimated by bicinchoninic acid (BCA) assay.

2.6. Bacterial invasion assays with pre-treated anti-S. Typhimurium antibodies

The PBMCs were adjusted to a desired concentration of 2×10^6 cells/ml. Then, 24-well flat bottom cell culture plates were seeded with 1×10^6 cells/well and incubated for 3 h. Cells were stimulated with chicken GM-CSF (abcam, USA) at 50 ng/ml and incubated at 37 °C with 5% CO_2 up to 48 h till monocytes attain macrophage morphology.

S. Typhimurium cultures were pre-incubated with 10 µg/ml anti-*S*. Typhimurium antibodies or control chicken serum for 30 min at 37 °C before being added to the cell culture. Chicken macrophages were infected with the above treated bacteria at MOI 50:1 and incubated for 2 h. Cells were washed and incubated with 50 µg/ml gentamicin for 90 min to remove extracellular bacteria. Then the cells were lysed with 0.1% triton X-100 and different dilutions of the cell lysates were plated on HE agar plates. The plates were incubated overnight at 37 °C and colony forming units (cfu)/ml were recorded.

2.7. Bacterial invasion assays in presence of cytochalasin D

One hour prior to infection, chicken macrophages were incubated with cytochalasin D at a concentration of 1 µg/ml (Elhadad et al., 2016). Pre-incubated cells were infected at MOI 50:1 and cytochalasin D was present throughout the bacterial internalization process. After 2 h of incubation, 50 µg/ml of gentamicin was added and incubated for 90 min. Then the cells were lysed and the lysate plated on HE agar plates.

2.8. S. Typhimurium invasion in presence of methyl α-D-mannopyranoside

Chicken macrophages were incubated with 50 mM methyl α-D-mannopyranoside for one hour (Kisiela et al., 2012). Cells were washed to remove excess mannopyranoside and infected with bacteria for 2 h at MOI 50:1. Subsequently 50 µg/ml of gentamicin was added and incubated for 90 min. Cells were lysed and plated on HE agar plates.