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Altering the balance between pathogen containing vacuoles and lysosomes

A lesson from salmonella

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Intracellular pathogens like Salmonella, Mycobacteria and Listeria has several survival mechanisms to combat the host assault. One of the very interesting strategies observed in case of these intracellular bacteria is their ability to survive and replicate in specialized vacuole inside the infected cells. Salmonella, in its turn, resides in a low pH and nutritionally depleted compartment termed as Salmonella containing vacuole (SCV) which never fuses with the lysosomes. Using macrophage cells we have recently demonstrated a unique characteristic of the SCV. Our data indicates that during Salmonella cell division, the SCV also divides and always a single SCV contains only one bacterium. This actually increases the total SCV number in the Salmonella infected cells. Further, Salmonella infection reduces the lysosome numbers and gives the pathogen an upper hand in the infected cells. Here we will summarize and expand upon our previous findings.

Key words: salmonella, salmonella containing vacuole, lysosome, phagosome

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Introduction

The successful evasion strategy by a pathogen is a key to establishment of infectious diseases. One of the strategies which are most commonly used is the inhibition of targeting a pathogen to lysosomes. However, is inhibition of fusion with lysosome the only mechanism? Lysosomes, organelles which are loaded with degradative enzymes constitute a major innate defence arm of the host. Largely, the bacteria get cleared in the host by the lysosomal degradative pathway. Many successful vacuolar pathogens like Mycobacteria, Brucella and Salmonella are known to avoid the phago-lysosomal fusion. Salmonella is known for its smart strategies to evade the host defence system. Hence, Salmonella evolved as a very successful pathogen with increasing morbidity and mortality. Therefore, the question asked was: Is it just a mere inhibition of fusion between Salmonella phagosome and lysosomes? The answer is No! In depth experiments in our laboratory led to some very important findings. Salmonella is strictly an intracellular pathogen enclosed in a vesicle. Hence, when Salmonella divides, the vesicle divides along.

So the situation now is, growing number of Salmonella all packaged separately. With this growing number of Salmonella, what happens to the acidic lysosomes? Do these organelles also increase in number to combat the pathogen? Our experiments showed that with the growing number of Salmonella vesicles, the number of acidic lysosomes decreased. Hence, perhaps Salmonella actively stops lysosome biogenesis. The decrease in the acidic lysosomal numbers was found to be an active process as the dead Salmonella did not lead to the observed reduction. Furthermore, the "in vivo" *mice* experiments suggested the similar findings where the Salmonella filled splenic cells actually had less acidic lysosomal numbers.[1] Here we will expand our views about the future directions and new ideas.

SCV

Immediately following invasion, individual Salmonella are found within discrete vacuoles characterized by the transient presence of early endocytic markers on the membrane. By 5–10 min post invasion, these proteins are replaced by lysosome-associated membrane proteins (LAMPs), which accumulate further over many hours. Early endosome proteins such as EEA1, Rab5 and transferring receptor with the SCV is short lived and lysosomal membrane proteins, particularly LAMP1 and LAMP2, rapidly replace them. Rab family GTPases regulate membrane-trafficking events and are therefore key targets for hijacking by serovar Typhimurium. Transport between late endosomes and lysosomes is regulated by the GTPase Rab7, which is present on both organelles.[2] Rab7 associates transiently with SCVs after the removal of Rab5 at 30–60 min. Rab7 is important for regulating late endosome to lysosomal transport in cells. It appears to recruit LAMP1 to the SCV and has been demonstrated to link the SCV to dynein/dynactin, promoting the early juxtanuclear trafficking of the SCV, via the adaptor protein RILP (Rab7-interacting lysosomal protein). Acquisition of rab7 by the SCV precedes that of lamp1, suggesting that rab7 may play a role in the biogenesis of the SCV. In the same study, it was observed that expression of the dominant-negative form of rab7 delays acquisition of lamp1 in the SCV.[3] Rab9 is present on Salmonella-induced filaments and is required for their formation.[4] Rab9 antagonizes SifA-induced LAMP1 recruitment and SCV position in cells and thereby plays a very important role in SCV stability.[5] Further, Salmonella secretory protein SopB has been shown to activate host kinases protein AKT1 on the cell membrane which in turn phosphorylate cytosolic AS160. AS160 is a potential rab14-GTPase activator protein which in the phosphorylated form fails to bind to phagosomal membranes and rab14 remains activated in the SCV. Active rab14 then possibly recruits effectors that inhibit SCV fusion with lysosomes.[6]

Several hours after invasion, *S. typhimurium* expresses the SPI-2 T3SS to translocate effectors across the SCV. SPI-2 effectors direct positioning of the SCV in the perinuclear region of host cells in close proximity to the Golgi apparatus. Bacterial replication begins after an initial lag period and is accompanied by the formation of extensive membrane tubules (Salmonella-induced filaments, Sifs), which project from the SCV and extend throughout the cell. From within the SCV, the bacteria alter the host cytoskeleton, regulate actin and microtubule motors to control SCV positioning and modulate the endocytic pathway.[7] SCVs do not acquire the cation-independent mannose-6-phosphate receptor (MPR) or lysosomal hydrolytic enzymes like cathepsins L, which are normally delivered from the trans-Golgi network (TGN) to endocytic compartments via the MPR.[8] The specific constituents of the SCV are not exactly known. Microarray analysis performed with serovar Typhimurium in murine macrophages indicated that magnesium, manganese and iron could be limited in the SCV compared to a nutrient-rich medium like LB.[9]

Other Pathogen Containing Vacuole

Successful pathogens have evolved their own strategy to get enclosed in a specific membrane bound compartment. These compartments are termed as "vacuole". Other intracellular pathogens like Mycobacteria, Legionella, Chlamydia, Brucella and Coxiella are enclosed by special vacuoles which are pathogen specific.[10] Vast numbers of literature are reported which discuss about the biogenesis of vacuoles of different pathogen. The Mycobacteria vacuole contains markers like EEA1 and Rab5 which indicate that it is arrested in the early endosome stage.[11] Brucella customized the vacuole in a very elegant way. Brucella containing vacuole (BCV) displays markers of early endosome like EEA-1, transferring receptor, but it sheds all the markers and displays features of autophagosome and endoplasmic reticulum.[10] The Legionella-containing vacuole (LCV) interacts with ER derived vesicles to mature into a vacuole primarily consisting of rough ER-derived membranes.[12] On the other hand, *Coxiella burnetti*, the causative agent of Q fever thrives in the harsh condition of lysosome.[13] Hence, it is evident that the success behind the pathogen's life is its "Customized Vacuole". According to the evasion strategy, each pathogen modifies its vacuole for successful niche.

Mechanism Exhibited by Various Pathogens to Avoid Fusion with Terminal Lysosome

Different intracellular pathogens have evolved a variety of mechanisms to avoid lysosomal degradation.[14] Mycobacterium stalls the maturation of its vacuole at an early endosomal level.[15] *Escherichia coli* on the other hand modulate the trafficking of its vacuole to avoid fusion with lysosomes[16] and Shigella and Listeria escape from phagosomes and enter the cytoplasm. These mechanisms are summarized in **Figure 1**. BCV rapidly acquires several late endocytic markers, including the guanosine triphosphatase Rab7 and its RILP. BCVs acquire various late endocytic markers, as well as fluid-phase markers preloaded into lysosomes, demonstrating that BCVs requires RILP-Rab7 interaction and fusion with lysosome for successful growth.[17] *Neisseria gonorrhoeae*, a gonococci, which gets cleared acquire Rab7-RILP to their phagosome. LAMP-1 and LAMP-2 double deficient cells lines were unable to clear Neisseria.[18] This indicates that pathogen like Neisseria did not possess evasion strategy like Mycobacteria or Salmonella to prevent killing by targeting to lysosome.

However, the mechanism by which Salmonella evades lysosomal degradation is controversial. Studies have demonstrated that Salmonella blocks the fusion of the SCV with terminal acidic lysosomes.[19,20] However, there are some reports that show that the SCV fuses/interacts actively with lysosomes.[21,22] The uncertainty in this matter led us to study SCV biogenesis from a different angle.

More Number of Pathogen Containing Vacuoles and Decreasing Number of Toxic Lysosomes

In this study, we addressed this problem from a different perspective by

investigating if there is sufficient number of lysosomes inside infected host cell. Our results demonstrate that Salmonella tackles the lysosomal degradation problem in an elegant manner by causing an imbalance in the ratio of SCVs to acidic lysosomes.[1]

Using both confocal laser scanning microscopy and transmission electron microscopy, we observed that each Salmonella was enclosed in a separate vacuole inside Salmonella-infected RAW264.7 cells, resulting in multiple SCVs per cell. In an infected cell harboring many bacteria, the scenario of a single bacterium per SCV arises when the SCV divides. The conclusions were drawn after studying hundreds of infected cells and that was done more than 3 times. The method of classifying SCVs was as follows: SCVs were classified according to the number of bacteria per vacuole (single bacterium per vacuole or multiple bacteria per vacuole) based on LAMP1 staining of SCVs inside RAW 264.7/Intestine 407 cells. Only those cells where SCVs are clearly defined were included in this analysis. At least 50 infected cells were counted in each case.

Further, we stained infected cells with lysotracker (LT), a fluorophore that accumulates in the acidic compartments of the cell. SCVs are not as acidic as lysosomes, and therefore, they do not accumulate LT. Interestingly, we observed an MOI-dependent decrease in the LT fluorescence of infected RAW 264.7 cells. Heat-killed bacteria and a SPI-2 mutant strain were unable to cause any significant change in the LT fluorescence in RAW 264.7 cells, indicating that proliferation of bacteria is essential to reduce the LT fluorescence in infected RAW 264.7 cells. We used acridine orange to confirm the results obtained using LT fluorescence. Flow cytometry demonstrated a significant decrease in the acridine orange fluorescence of RAW 264.7 cells upon Salmonella infection.[1] This might be partially the result of SCV division, which increases the number of SCVs, causing redistribution of molecules, like vacuolar-type ATPase, LAMP1, LAMP2, cathepsin D and acid phosphatase, that are required for lysosomal biogenesis.[1]

Exposure of Salmonella to microbicidal agents like lysosomes and the availability of nutrients to Salmonella are different

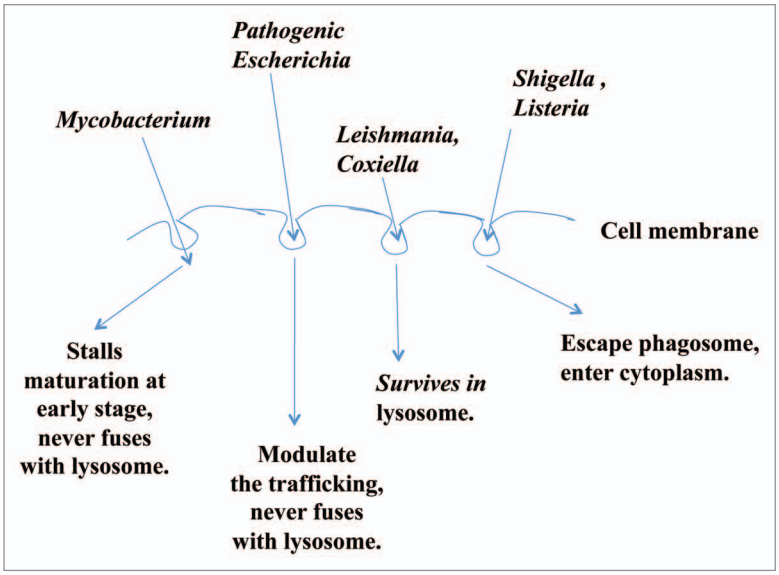

Figure 1. Fate of pathogen containing vacuole with respect to lysosome.

in these two contrasting situations, i.e., multiple bacteria per SCV and a single bacterium per SCV. It is relatively difficult for the host cell to defend itself when there are many SCVs inside it; a host cell has to target each SCV separately with lysosomes, reactive oxygen and nitrogen intermediates, antimicrobial peptides and other microbicidal agents. On the other hand, a host cell has to target only a single or a few SCVs if many bacteria are clustered inside one or a few SCVs, which is advantageous for the host cell. In addition, in the case of a single bacterium per SCV, there is no competition for nutrients and each bacterium. Thus this single bacterium per SCV is a bacterial strategy to counteract host defense mechanisms and the same theory holds good for the decrease in the lysosome number.

Future Direction

There are several unanswered questions. What are the signaling pathways that lead to accumulation of such enormous quantities of membranes towards SCV? Is there specific role of any effector proteins of Salmonella in this regard? The main question which remains to be answered is whether the lysosome biogenesis is inhibited by Salmonella? If so, how the growing number of SCV can inhibit the biogenesis of acidic lysosomes. LAMP-1 and LAMP-2 are the intergral membrane proteins of the lysosomes and are very important for lysosome biogenesis. LAMP 1 and 2 in the trans golgi network (TGN) are packed into vesicles and are then targeted from TGN to lysosomes via the intracellular route.[23] In an interesting finding by David Holden and his group, Salmonella which remained closely associated with the Golgi network were able to multiply. One of the effector proteins, SseG co-localized extensively with markers of the TGN.[24] Hence it is possible that the LAMP1 and 2 vesicles which are in the TGN fuse with the SCV which are also in the TGN. Hence TGN may form an important hub for the SCV to acquire the vacuole markers which are very important to give stability to SCV. Due to the growing numbers of the SCV there may be continuous fusion with the LAMP-1 and LAMP-2 vesicles in the TGN thereby hampering the lysosome biogenesis. Similarly, Rab7 which is a key to lysosomal biogenesis[25] is recruited by growing numbers of SCV. Salmonella smartly utilizes the same components for its SCV which is pivotal for lysosome biogenesis. Hence it can be mere shortage of the integral membrane components which makes successful lysosomes. These possibilities and scope for future work is illustrated in **Figure 2**. Various live imaging microscopy using fluorescent tagged proteins for lysosomal biogenesis may answer this puzzle. One technical difficulty is the unavailability of a marker which is unique to lysosome. All the markers like LAMP1, 2

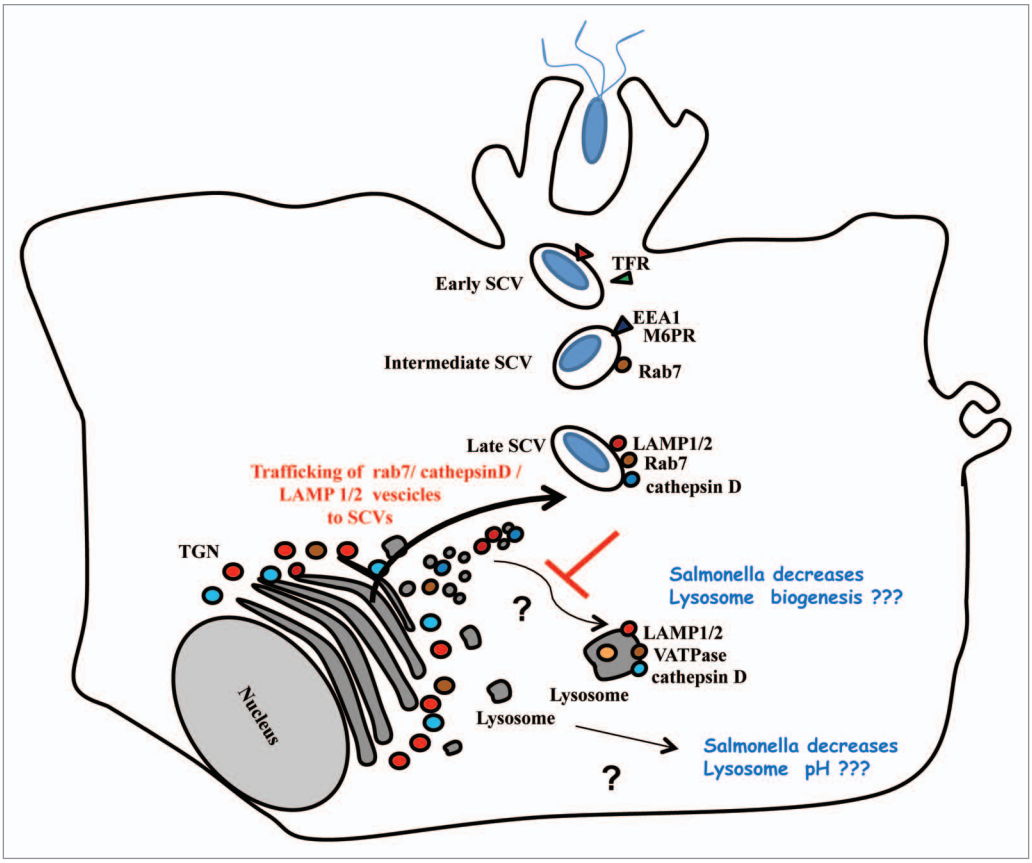

Figure 2. Differential trafficking of Integral lysosomal membrane proteins to SCV: a probable hypothesis.

and others also localize with the late endosome. Fluid tracers which ultimately get localized to the terminal lysosomes along with a pH sensor can be used to demonstrate where the lysosome biogenesis is hampered.

Second important question which remains to be answered is whether it is the total number of the acidic lysosomes which are decreasing or just the pH of the lysosomes are altered and hence the pH tracer used in our study can no longer detect the lysosomes. This can be answered by tagging the lysosomes with LAMP-1 antibody and measuring the pH in those lysosomes using flow cytometry under infected and non-infected conditions which we could not perform in our work.

Summary

Successful pathogens can avoid the fusion with lysosomes, modulate their vacuole to stay in the lysosomes or can decrease the number of lysosomes. Lysosome biogenesis is a very dynamic process and it is very important to study the mechanism by how this can be halted by a pathogen. There is a pressing need to design new drug to combat multidrug resistant Salmonella infection. For this purpose, novel bacterial virulence factors and survival mechanisms need to be identified. Hence, future studies should focus on looking further into the process of lysosome biogenesis under infection conditions with various other pathogens also. Identifying all of the virulence factors and signaling pathways involved in regulating SCV biogenesis might lead to newer therapeutic targets for novel drug design.

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