CHAPTER 4: TIME-LAPSE VIDEO MICROSCOPY COMPARING SALMONELLA-INDUCED, CASPASE-1 MEDIATED MACROPHAGE DEATH WITH YERSINIA-INDUCED, CASPASE-1-INDEPENDENT APOPTOSIS.

Chapter 4 Summary

Salmonella and *Yersinia* both induce macrophage death. We show here by live time-lapse video microscopy that there are distinct differences between the mechanisms of *Yersinia*- and *Salmonella*-induced macrophage death. *Salmonella* induces a very rapid caspase-1-dependent death and is associated with small membrane 'blisters', whereas *Yersinia*-induced death has slower kinetics and is associated with dramatic membrane 'blebs'. We monitored the phosphatidyl asymmetry with annexin V conjugated to a fluorescent dye and entry of the DNA-binding dye SYTOXblue to measure the loss of membrane integrity. The amount of time between annexin V binding and the DNA dye entering was significantly greater for *Yersinia*-induced death, 60 minutes, compared to *Salmonella*-induced death, 2 minutes. Thus, *Salmonella* induces a macrophage death that is unique and dependent on the host molecule caspase-1, whereas *Yersinia* induces a slower death that we describe as portraying a more 'classical' apoptosis.

Introduction

Recently, studies have shown that programmed cell death, apoptosis, is triggered in macrophages in response to *Yersinia* infection {Monack, 1997 #1;Mills, 1997 #8}. The genus *Yersinia* includes three species which are pathogenic for humans and rodents, and carry the virulence plasmid, pYV (Carter, 1975; Cornelis *et al.*, 1987). *Y. pestis* is the causative agent of plague. The enteropathogenic *Yersinia* species, *Y. enterocolitica* and *Y. pseudotuberculosis*, cause gastrointestinal syndromes, lymphadenitis, and septicemia (Cornelis *et al.*, 1987; Bottone, 1977). Furthermore, systemic infections can occur with formation of abscesses in the liver and spleen as well as immunopathologic sequelae such as reactive arthritis (Ahvonen *et al.*, 1969; Bouza *et al.*, 1980).

Similar to *Salmonella*, *Yersinia* binds to and invades the M-cells within the follicle-associated epithelium (FAE) overlying the lymphoid follicles of the Peyer's patches (PP), which are components of the gut-associated lymphoid tissue (GALT)(Grützkau *et al.*, 1990). Several virulence factors have been implicated in the ability of *Yersinia* to multiply in the PP and then spread to deeper tissues. Many of the genes encoding virulence are contained on the pYV plasmid. The plasmid-encoded proteins comprise a host cell contact-dependent or type III secretory pathway that shares a number of homologous sequences with the type III secretory components seen in *Salmonella* pathogenicity islands SPI1 and SPI2, as well as in the *Shigella* virulence plasmid. The fundamental infection strategies of *Yersinia* initially appear to be markedly different than that of *Salmonella* or *Shigella*. Virulent *Yersinia* do not actively enter host cells, including normally phagocytic macrophages. Rather, virulent *Yersinia* rapidly inhibits the phagocytic machinery (Rosqvist *et al.*, 1990; Fallman *et al.*, 1995). Thus,

Yersinia, unlike *Salmonella* and *Shigella*, does not induce host cell ruffling and does not invade host cells; *Yersinia* is not a facultative intracellular parasite. Yet, like *Salmonella* and *Shigella*, the coordinate activity of the secretion machinery and the adherence factors allows the bacteria to translocate pYV-encoded proteins called Yops (Cornelis and Wolf-Watz, 1997). Several Yops are translocated into host cells where they interfere with normal cellular processes and set the stage for the bacteria to extend their range beyond the PP (Hanski *et al.*, 1991; Brubaker, 1991).

The exact mechanism by which *Yersinia* induces apoptosis is not known, but recent studies have shown that YopJ (YopP in *Y. enterocolitica*), one of the *Yersinia*-secreted proteins, is necessary for inducing apoptosis of macrophages *in vitro* (Monack *et al.*, 1997; Mills *et al.*, 1997). YopJ causes a variety of additional effects, such as suppression of tumor necrosis factor α (TNF-α) and interleukin-8 production, as the result of blockade of the inactivation of mitogen-activated protein kinase (MAPK) kinases (MKKs), MAPK, and nuclear factor κB (NF-κB) (Boland and Cornelis, 1998; Palmer *et al.*, 1998; Schesser *et al.*, 1998). We have recently shown that *Y. pseudotuberculosis*-induced death does not require caspase-1, as does *Salmonella* and *Shigella* (Monack and Falkow, 2000).

Although the exact host cell molecular mechanisms involved in *Salmonella*- and *Yersinia*-induced macrophage death are not completely known, they do appear to involve different host cell molecules and/or pathways. We decided to compare by live time-lapse video microscopy these two apparently different mechanisms of pathogen-induced macrophage death. We followed several parameters in order to characterize the host cell death. We looked at the morphological changes in the macrophage membrane by DIC

and epifluorescent microscopy. Early during the process of apoptosis in some cell types, cells lose their phospholipid membrane asymmetry and expose phosphatidylserine (PS) at the cell surface while maintaining their plasma membrane integrity (van Engeland *et al.*, 1998). Annexin V has been shown to specifically bind PS exposed on the surface of the plasma membrane (van Engeland *et al.*, 1998). We measured the time after infection of annexin V binding to the plasma membrane. We also measured the delay in time between annexin V binding and the ultimate loss of membrane integrity.

Materials and Methods

Live time-lapse video microscopy of infected macrophages

Either RAW264.7 macrophage cell line or bone marrow-derived macrophages were cultured overnight on round glass coverslips. *S. typhimurium* containing the green fluorescent protein, SL1344 pgfp, or *Y. pseudotuberculosis*, YPIIIpYV pgfp, were grown as indicated previously (chapter 1.4.1 and {Monack, 1997 #1}) and added to the macrophages at a final MOI of 10 bacteria per macrophage. Infected cells grown on glass coverslips were observed 0.5-6 h after infection after mounting on a stage whose temperature was maintained at 37° C by a circulating water bath. Cells were overlaid with phenol red-free DMEM with 10% FBS and buffered with 20mM Hepes (pH7.3), and containing 2.5 mM $CaCl_2$, 30 μM annexin V-alexa568 (Molecular Probes), 0.1 μM SYTOXblue, and covered with a thin layer of silicone DC-200 fluid (Serva) to prevent evaporation. Observations were performed on a Nikon Diaphot-300 inverted microscope equipped with either phase contrast or DIC and epifluorescence optics. Time-lapse video microscopy was acquired with a cooled CCD camera (NDE/CCD; Princeton Instruments) and Metamorph (Universal Imaging) software. DIC/fluorescence image pairs were recorded every 2 m for *Salmonella* infections and every 10 m for *Yersinia* infections, with 30 ms bright field, 200 ms at 480nm, 200 ms at 578 nm, and 300 ms at 436 nm exposures.

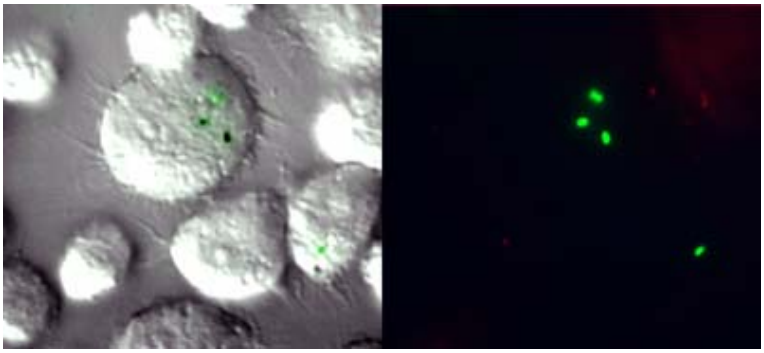
Results

Salmonella-induced death is rapid compared to Yersinia-induced death

We conducted all of our live imaging of infected macrophages, which were grown on coverslips, in the presence of annexin V conjugated to the fluorescent dye alexa568 to detect PS exposure to the outer leaflet of the plasma membrane and the DNA-binding dye SYTOXblue to detect loss of plasma membrane integrity. One of the first observations we made was the time after bacterial infection that the macrophages were dieing. Consistent with previously published results, the macrophages infected with *S. typhimurium* that were grown such that they are highly invasive (see Experimental Procedures) began to die approximately 2 hours post-infection (Monack *et al.*, 1996; Chen *et al.*, 1996a). In contrast, the macrophages infected with *Y. pseudotuberculosis* began to die 5 hours post infection ($p=0.01$ comparing the two strains). Thus, there is a significant difference between *Salmonella* and *Yersinia* in the time after infection that death occurs, with *Yersinia*-induced death occurring greater than four hours after *Salmonella*-induced death.

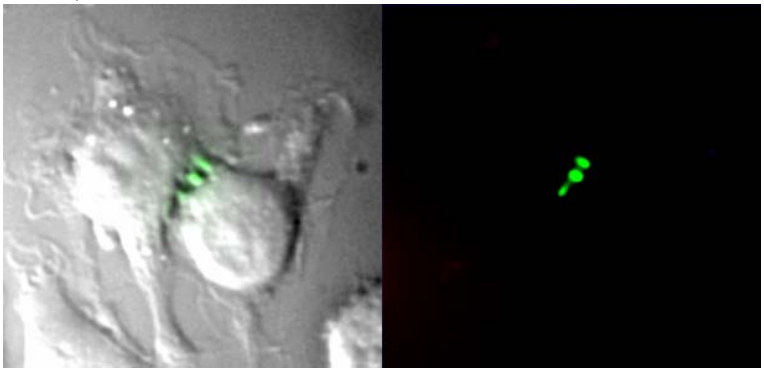
In addition to the dramatic difference in the onset of host cell death, we observed that *Yersinia* induced very dramatic membrane blebs (Fig. 4-2 and Fig. 4-3B) approximately 60 minutes prior to macrophage death whereas *Salmonella* induced small blebs that looked similar to blisters (Fig. 4-1 and Fig. 4-3A).

Fig.4-1 Movie of **Salmonella typhimurium** *infecting RAW264.7 macrophages (double click to open with Quicktime)*



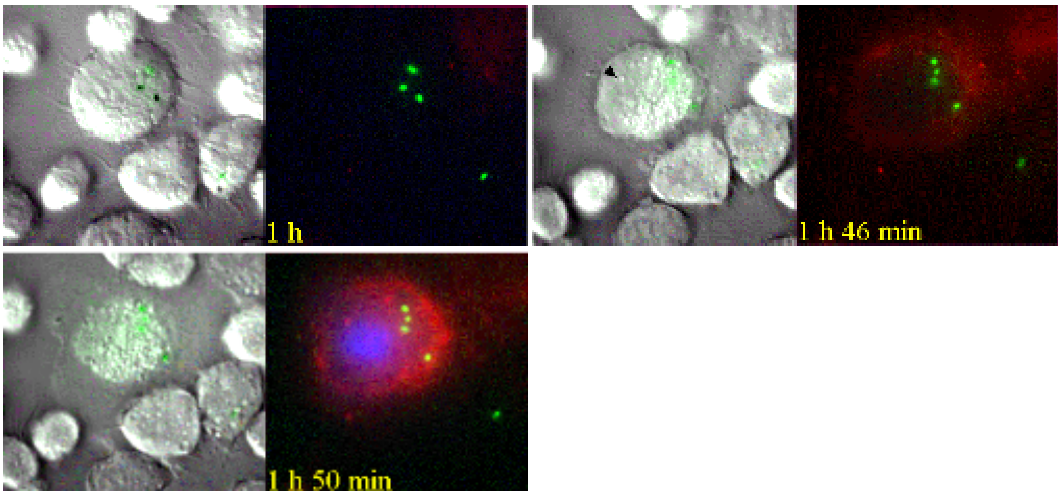
Salm2.mov

Fig. 4-2 Movie of **Yersinia pseudotuberculosis** *infecting RAW264.7 macrophages (double click to open with QuickTime)*



Yers2.mov

A.



B.

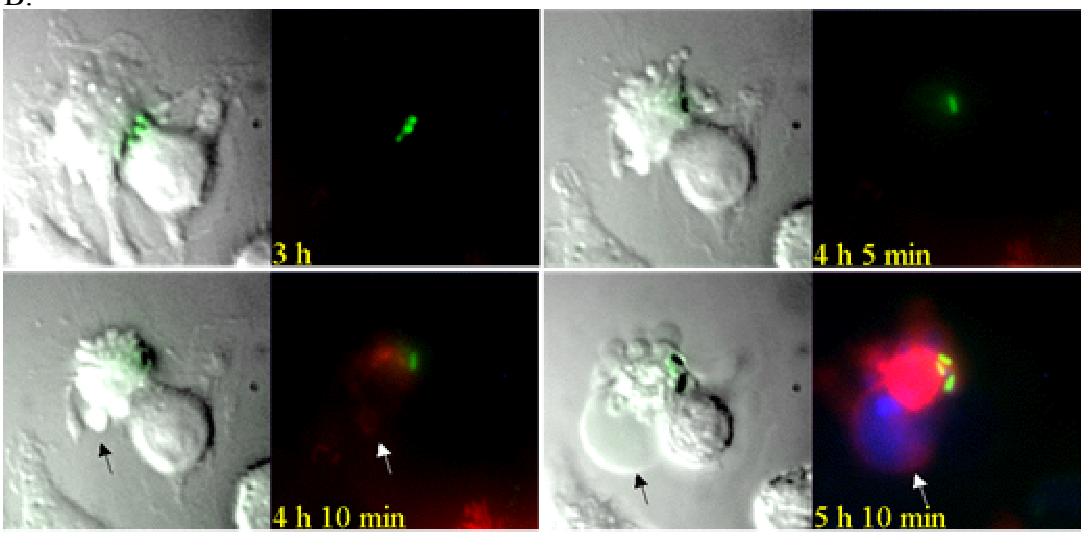


Fig. 4-3* S. typhimurium *caspase-1-dependent death occurs faster than* Y. pseudotuberculosis *caspase-1 independent macrophage death.

Individual frames (times indicated in yellow) from the movies in Figures 4-1 and 4-2. Wild-type macrophages were infected with either (A) *S. typhimurium* SL1344 or (B) *Y. pseudotuberculosis* YPIIIpYV at a MOI of 10:1 and imaged by live time-lapse video microscopy in the presence of annexin V-alexa568 (red) and the DNA dye SYTOXblue (blue). The DIC image is shown to the left of the corresponding fluorescence image. Arrowhead points to small blister on the surface of *S. typhimurium*-infected macrophage. Arrows point to a large membrane bleb on *Y. pseudotuberculosis*-infected macrophage.

The delay between annexin V binding and the loss of macrophage membrane integrity is greater for Yersinia-induced apoptosis than Salmonella-induced death.

One parameter that has been used to characterize host cell death is the delay between the loss of PS asymmetry in the plasma membrane and the subsequent destruction of the membrane integrity (van Engeland *et al.*, 1996). Early apoptotic cells display PS in their outer leaflet while maintaining membrane integrity. We detected the loss of PS asymmetry as the time in which the flourescently labeled phospholipid-binding protein, annexin V-alexa568, bound the surface of the infected macrophage. The loss of membrane integrity was detected by measuring the influx of the membrane-impermeant DNA-binding dye, SYTOXblue, into the cytosol and nucleus of infected macrophages. We measured the amount of time between annexin V binding to PS and SYTOXblue entering the host cell and subsequently binding the DNA for both *S. typhimurium* and *Y. pseudotuberculosis* infected macrophages. The amount of time between annexin V binding to *S. typhimurium*-infected macrophages and SYTOX entering the host cell was 1.73 ± 2.83 minutes (Fig. 4-4). This was in stark contrast to *Y. pseudotuberculosis*-infected macrophages, where the time between annexin V binding and SYTOXblue entering cells was 61.33 ± 8.76 minutes. Thus, the difference between *Salmonella*- and *Yersinia*-induced delays in phosphatidyl serine exposure on the surface of macrophages and the ultimate loss of plasma membrane integrity highlights the fact that these pathogens are triggering different host cell pathways that lead to death.

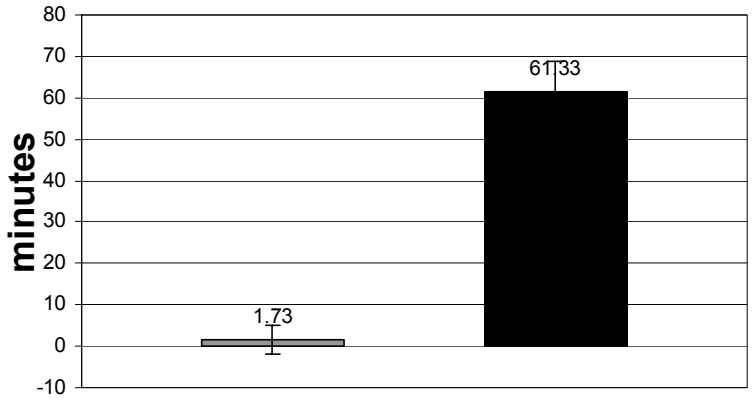


Fig. 4-4 The d*elay between annexin V binding and SYTOXdye entering is much greater for* **Yersinia** ***compared to*** **Salmonella.**

The lack of phosphatidyl asymmetry in the macrophage plasma membrane was detected by annexin V-alexa568 binding and the loss of membrane integrity was detected with entry of SYTOXblue and binding to host cell DNA. The delay in the binding of these two fluorescent dyes was calculated for *S. typhimurium*-infected (gray) and *Y. pseudotuberculosis*-infected (black) RAW264.7 macrophages. N= 18 for