CHAPTER V

Role of shsp18 in the survival dynamics of *M. smegmatis* in THP-1 derived macrophages

INTRODUCTION

Macrophage is a key cell of the mammalian innate immune system that mediates phagocytosis of microbial pathogens and is responsible for the destruction and clearance of both intracellular and extracellular pathogens. Apart from the early destruction of pathogens, macrophages also elaborate innate immune response. In general, macrophage employs a variety of oxygen dependent and independent mechanisms to kill the invading microorganism. Oxygen dependent mechanisms involve generation of superoxide ions and hydrogen peroxide. In addition, the lysosomal enzyme myeloperoxidase produces the potent anti-microbial hypochlorous ions (Babior, 2000). Oxygen independent mechanisms include digestion by lysozymes, proteases or lipases, chelation of iron, exposure to granular cationic proteins or reactive nitrogen intermediates. The phagocytosed pathogen encounters these microbicidal agents following the acidification of phagosome on fusion with lysosomes (Krause, 2000).

Intracellular pathogens have not only evolved strategies to overcome these bactericidal mechanisms but are also capable of multiplying within the phagocytic cells. These include the bacterial pathogens such as *Mycobacteria, Brucella, Legionella, Salmonella* and the protozoans such as *Leishmania, Toxoplasma, Trypanosoma* (Mundayoor and Shinnick, 1994). Strategies generally employed by these pathogens include

1. Destruction of the phagocytic cells
2. Production of enzymes such as superoxide dismutase, catalase and peroxidase that neutralize the effects of free radicals (Edwards *et al.*, 2001; Samuel *et al.*, 2003; Melillo *et al.*, 2009).
3. Inhibition of the fusion of phagosome with lysosome (Buchmeier and Heffron, 1991; Malik et al., 2003; Porte et al., 2003; Shotland et al., 2003; Scherr et al., 2009).

4. Interference with phagosome maturation through the use of specific macromolecules in the cell wall (Frehel and Rastogi, 1987; Fratti et al., 2001; Fratti et al., 2003; Welin et al., 2008)

5. Generation of an electron transparent zone that shields the pathogen from the effects of degradative enzymes and free radicals (Ryter et al., 1984; Frehel et al., 1986)

6. Divergence and impairment of the macrophage functions involved in establishing the cellular immune responses (Esquivel-Solis et al., 2009; Modlin, 2010)

*M. leprae* resides primarily in the mononuclear phagocytes. The means by which *M. leprae* resist the intracellular killing is an area of great interest. A key step in sorting out these survival mechanisms is to identify the gene(s) and gene product(s) of *M. leprae* which are essential for or involved in the resistance to killing by macrophages and also those that are involved in the replication of the pathogen inside the host macrophages. Characterizing the *M. leprae* genes that are specifically expressed during its infection of the macrophages will throw light on the survival tactics of this pathogen. *shsp18* of *M. leprae* is one such gene which has been shown to be specifically activated during intracellular growth and is therefore likely to be involved in the survival of *M. leprae* within macrophages (Dellagostin et al., 1995).

The inherent difficulty faced by researchers working with *M. leprae* is the non-cultivability of this organism in any defined culture media. Consequently, surrogate cloning hosts have played a very important role in advancing our understanding of mycobacterial biology. Initial characterization of *M. leprae* genes was carried out with the aid of surrogate hosts such as *E. coli* and *S. lividans* (Clark-Curtiss et al., 1985; Kieser et al., 1986). Study of the mycobacterial genes in *E. coli* has provided a great deal of information about the selected genes and gene products. However, the general usefulness of this kind of molecular approach is limited because of the genetic and structural differences that exist between enterobacteria and mycobacteria. The truly important biological questions can be answered only by studying the mycobacterial genes in closely related mycobacteria.
With the discovery of the transformation efficient mutant strain of *M. smegmatis*, mc²155 (Snapper *et al.*, 1990), cloning and characterization of genes from pathogenic and non-pathogenic mycobacteria has become a common practice. *M. smegmatis* has been widely used as the cloning host and is well established for the study of a variety of pathogenic mycobacterial genes and promoters (Fiss *et al.*, 1994; Bannantine *et al.*, 1997; Etienne *et al.*, 2005; Santhosh *et al.*, 2005; Schneider *et al.*, 2008; Joon *et al.*, 2010). The advantage in using *M. smegmatis* is that it is a fast growing species with a generation time of approximately 3 hours at 37 °C and can form colonies in 2-3 days (Shinnick *et al.*, 1995). Most importantly, it is non-pathogenic that enables easy handling and manipulation for a variety of studies.

In the present study, *M. smegmatis*, mc²155 was used as the host to understand the role of *shsp18* in the intracellular survival of mycobacteria in macrophages. One of the commonly used human cell line for mycobacterial infection studies is the THP-1 cell line. THP-1 is a human leukemic cell line with distinct monocytic markers (Tsuchiya *et al.*, 1980). They are promonocytic cells committed to the monocytic cell lineage. Treatment with phorbol-12-myristate-13-acetate (PMA) differentiates THP-1 cells into a macrophage-like cells with respect to morphology, loss of proliferation, expression of CD11b, phagocytic activity and an enhanced capability to release mediators such as TNF-α (Schwende *et al.*, 1996).

In this study, *shsp18* gene with its upstream promoter sequence was cloned and introduced as an integrated copy in the genome of *M. smegmatis*. The objective of integrating the *shsp18* gene was to maintain it as a single copy and simulate the environment found in *M. leprae*. Using this recombinant *Mycobacterium*, the ability of *shsp18* to confer survival advantage to *M. smegmatis* during its infection in the macrophages was assessed.

**RESULTS**

Cloning of *shsp18* gene with its upstream promoter region (*shsp18Su*) in the integrative vector, pSET152 was discussed earlier in chapter IV. The recombinant plasmid, pSET-18Su, was used for the studies described in this part of the work.
Integration of *shsp18* in the genome of *M. smegmatis*

A schematic representation on the integration of pSET-18Su in *M. smegmatis* genome is shown in figure 5.1. The φC31 integrase acts on the *attP* site in the vector to bring about a site-specific recombination with the *attB* site in the genome. This results in the integration of the entire vector along with the cloned gene in an orientation as shown in the figure 5.1. The *attB* and *attP* sites after integration form the *attL* and *attR* sites on either side of the integrated vector. *M. smegmatis*, mc²155 which is inherently susceptible to apramycin develops resistance after this integration event and hence, apramycin resistant transformants were selected.

*M. smegmatis*, mc²155 competent cells was transformed with pSET-18Su by electroporation and the transformants, mc²155::pSET-18Su were selected on apramycin plates. Single colonies of the transformants were patched and were passed through a minimum of three generations on the selection plate. After three generations, the transformant was taken for confirming the integration of *shsp18* in the mycobacterial genome. For all the studies described in this part of the work, *M. smegmatis* integrated with pSET152 (mc²155::pSET152) was used as the vector control.

**Confirmation of *shsp18Su* integration**

Integration of *shsp18Su* in *M. smegmatis* genome was confirmed by a plasmid rescue strategy (Paranthaman and Dharmalingam, 2003). This strategy illustrated in figure 5.2A, exploits the fact that after the integration, digestion of *M. smegmatis* genome with an enzyme in the multiple cloning site of the vector would generate a fragment with a segment of the integrated vector and this fragment can be rescued as plasmid in *E. coli*. The enzyme chosen here was *BamHI*, which is present only in the vector and not in the cloned gene, *shsp18Su*. Genomic DNA isolated from *M. smegmatis* transformant was digested completely with *BamHI*. This generates a large number of fragments from all possible *BamHI* sites. If the integration had taken place, amongst these fragments there will be one fragment carrying the *shsp18Su*, apramycin resistance gene, *E. coli* ori and *attR* along with a portion of the mycobacterial genome as shown in figure 5.2B. Since all the fragments had *BamHI* cohesive ends, they were self-ligated and transformed to *E. coli*. Except the fragment shown in figure 5.2B carrying the pUC replicon, none of the other fragments would
Figure 5.1: Schematic representation of the integration of pSET-18Su in the genome of *M. smegmatis*. * - For illustration purpose, only a part of the circular genome is shown. The locus information of *attB* shown in this figure is based on the study by Santhosh et al. (2005). Genes are represented by their locus ID in the figure where MS represents *M. smegmatis*. 
Figure 5.2: Plasmid rescue strategy. A. Schematic representation of the steps involved in this strategy to confirm the integration of \textit{shsp18Su} in \textit{M. smegmatis} genome. B. Details of the rescuable fragment which is shown in figure 5.2A with a dashed rectangle.
would have a combination of *E. coli* ori and apramycin gene and hence would not be propagated in *E. coli* on apramycin selection.

**Isolation of rescued plasmids**

Plasmid DNA was isolated from the apramycin resistant *E. coli* transformants obtained from the plasmid rescue strategy with mc^{2}155::pSET-152Su. Plasmids were screened from 15 transformants and these plasmids could be grouped into two sets based on the difference in their migration pattern in the agarose gel. This difference in the migration indicates a difference in their sizes. A representative plasmid from each group, S1 and S2, is shown in figure 5.3A. Similar size difference was observed with those plasmids isolated from the plasmid rescue transformants of mc^{2}155::pSET152 (figure 5.3B).

**Restriction analysis of rescued plasmids**

To further confirm the integration event and to determine the basis of size difference in the rescued plasmids, a restriction analysis was carried out. The rescued plasmids, S1 and S2, were digested with *BamHI*, *BglII* and *XhoI* and the restriction pattern is shown in figure 5.4. Since the rescued plasmids resulted from self-ligation of fragments with *BamHI* cohesive ends, digestion of these plasmids with *BamHI* should linearize it. In figure 5.4, lanes 3 and 7 are the *BamHI* digest of S1 and S2, respectively which shows the linearized DNA. Generation of a single linear band with *BamHI* confirms that the rescued plasmid was the result of self ligation of a single fragment and not due to concatenation of multiple fragments.

The two plasmids were restricted with *BglII*, a unique site present in *shsp18Su* gene but not in the pSET152 vector. Digestion with *BglII* also linearized these plasmids as seen in lane 4 and 8 of figure 5.4. This indicates that *shsp18* was integrated in *M. smegmatis* genome and is, hence, present in the rescued plasmid. The absence of extra bands on *BglII* digestion of either of the plasmids indicates that there are no *BglII* sites in the *M. smegmatis* genome region of the rescued plasmid.

The difference in the size of S1 and S2 was obvious when these plasmids were linearized with *BamHI* or *BglII*. This size difference can arise only if the mycobacterial genomic region present in these plasmids is different. To confirm this, these plasmids were analyzed with yet another restriction enzyme, *XhoI*, a unique site
Figure 5.3: Rescued plasmids. Plasmids rescued from the genomic DNA of *M. smegmatis* integrated with pSET-18Su (A) or pSET152 (B) were of two different sizes. The representative plasmids varying in sizes are shown.

Figure 5.4: Restriction analysis of the rescued plasmids. Plasmids, S1 and S2, were digested with various restriction enzymes and electrophoresed on a 0.7% agarose gel. Lanes 1, λ *HindIII* marker; 2, S1/uncut; 3, S1/BamHI; 4, S1/BglII; 5, S1/XhoI; 6, S2/uncut; 7, S2/BamHI; 8, S2/BglII; 9, S2/XhoI.
present in vector. Digestion of S1 with XhoI yielded three bands (lane 5 of figure 5.4) while S2 resulted in a single band (lane 9). A linear band of S2 indicates that no extra XhoI site is present in the rescued plasmid except the one present in the vector. On the other hand, three bands resulting from XhoI digestion of S1 indicates the presence of three XhoI sites. Apart from the one in vector, two other sites should be present in the \textit{M. smegmatis} genome region. Hence, the difference in the restriction pattern obtained with XhoI clearly reveals that the two plasmids differ in size because of the difference in the \textit{M. smegmatis} genome region which was rescued. This in turn indicates that pSET-18Su has integrated at different sites in the \textit{M. smegmatis} genome.

From the above restriction analysis, with the aid of DNA marker, the size of S1 and S2 plasmids, were calculated as 7.0 kb and 6.2 kb, respectively. As shown in figure 5.5, only one portion of the integrated pSET-18Su is rescuable while the other portion is not due to the lack of \textit{E. coli} origin of replication. The rescued plasmids carry this rescuable portion along with a portion of \textit{M. smegmatis} genome present beyond the \textit{attR} region. Since the pSET-18Su sequence is known, the size of the rescuable portion (which is common to both S1 and S2) was determined to be 3.94 kb. From the size of the plasmid and that of the rescued region, length of the mycobacterial genome region was calculated as 3.06 kb for S1 and 2.26 kb for S2.

Hence, from the XhoI restriction analysis and subsequent calculations, it was confirmed that the two rescued plasmids differ from each other only in the \textit{M. smegmatis} genome region. The rescue of two different \textit{M. smegmatis} genome region clearly suggests that the integration of pSET-18Su has taken place at two different sites in the genome.

\textbf{Infection assay using THP-1 cell line}

For analyzing the role of \textit{shsp18} in mycobacterial survival in macrophages, human monocytic leukemia cell line, THP-1 was used for the infection studies. THP-1 monocytic cells were differentiated to macrophage-like cells using PMA (Schwende \textit{et al.}, 1996) and were infected with \textit{M. smegmatis} carrying \textit{shsp18Su} (mc\textsuperscript{2}155::pSET-18Su) or pSET152 (mc\textsuperscript{2}155::pSET152) at a MOI of 1:1 as described in materials and methods. In brief, PMA treated THP-1 cells were infected with \textit{M. smegmatis} for 2 hours after which the unphagocytosed bacteria were removed.
Figure 5.5: Schematic representation depicting the difference in the size of the rescued plasmids S1 and S2. Dotted arrow indicates the *M. smegmatis* genome region which is different in S1 and S2.
Infected THP-1 cells were incubated for one hour with fresh RPMI medium containing an antibiotic to kill the bacteria bound to the surface of the macrophages. Subsequently, the cells were cultured for the duration of the assay and at selected time points, macrophages were lysed to determine the number of bacteria surviving inside the macrophages. The survival kinetics was followed by plotting the number of *M. smegmatis* surviving inside the macrophages against time. In addition, percentage of survival was calculated as follows

\[
\% \text{ Survival} = \left( \frac{\text{Number of viable bacteria at } t_x}{\text{Number of viable bacteria at } t_i} \right) \times 100
\]

where \( t_x \) is the time point at which the macrophages were lysed to recover the intracellular bacteria and \( t_i \) is 3 hours after the start of infection, that is, the initial time point when the number of phagocytosed bacteria is determined. The kinetics as well as the percentage of survival was compared between *M. smegmatis* carrying *shsp18Su* and the vector control.

**Survival assay during the early infection period**

Survival of recombinant *M. smegmatis* in the macrophages was assessed during the early hours, *i.e.* at the 3rd, 4th, 5th and 6th hour post infection. The survival kinetics of *M. smegmatis* during this period is shown in figure 5.6A and the percentage survival in figure 5.6B.

From figure 5.6A, it is clear that neither mc2155::pSET-18Su nor mc2155::pSET152 were killed rapidly by the macrophages during the early infection period. By the 6th hour post infection, only less than 20% of the phagocytosed bacteria were killed. Moreover, there was no significant difference in the number of phagocytosed bacteria (viable bacteria after 3 hours from the start of infection) between *M. smegmatis* carrying *shps18su* and the control. This in turn indicates that *shsp18 per se* does not seem to have a role in the uptake of bacteria by the macrophages.

**Survival assay till 36 hours of infection**

*M. leprae* has been shown to inhibit phagosome-lysosome fusion to survive within the macrophages (Frehel and Rastogi, 1987). However, a non-pathogenic strain such
Figure 5.6: Comparison of the survival of *M. smegmatis* in macrophages. Infection of THP-1 derived macrophages with mc²155::pSET-18Su or mc²155::pSET152 was carried out as described in materials and methods. The number of bacteria surviving inside the macrophage was determined at 3, 4, 5 and 6 hours after infection. Comparison of the survival kinetics (A) and the percentage of survival (B) of *M. smegmatis* carrying *shsp18Su* with that of the vector control is shown.
as *M. smegmatis* may not be able to inhibit this fusion and will be exposed to the antibacterial mechanisms of the phagolysosome. To determine the role of *shps18* in preventing the killing by macrophages, survival of recombinant *M. smegmatis* was assessed from the 3rd to the 36th hour after infection. From the survival kinetics shown in figure 5.7A, it is clear that *M. smegmatis* is killed progressively by the macrophages irrespective of the presence or absence of *shsp18Su*. The percentage of viable intracellular bacteria in the macrophage falls to less than 40% by the 36th hour of infection (figure 5.7B). From this, it can be inferred that *shsp18Su* does not help in the survival of *M. smegmatis* in THP-1 cells till 36 hours of infection.

**Survival assay during extended period of infection**

van der Wel and co-workers (2007) have studied *M. leprae* infection in macrophages for an extended time period of up to 7 days and have shown that the pathogen moves into the cytosol by the 4th day after infection. Based on this work, the survival of *M. smegmatis* was determined in the macrophages till 120 hours of infection and the results of this assay are shown in figure 5.8.

From the survival kinetics shown in figure 5.8A, it is clear that the number of surviving *M. smegmatis* decreases with increase in the time of infection till the 48th hour. However, during the next 24 hours, there was a sudden increase in cell number followed by a decrease again by the 96th and 120th hour of infection. This trend in the survival was observed with both mc2155::pSET152 and mc2155::pSET-18Su. Although both displayed an increase in cell number by the 72nd hour, a significant difference was observed in the magnitude of increase. For the control strain, the intracellular bacterial number which was at 11% at the 48th hour increased to only 23% by the 72nd hour of infection. On the other hand during this same time period, *M. smegmatis* carrying *shsp18Su* showed an increase from 14% to 58%. An increase of approximately one log number of cells was seen at the 72nd hour for *M. smegmatis* carrying *shsp18Su*. These results indicate that the presence of *shsp18Su* enables an enhanced multiplication of *M. smegmatis* in the macrophages at a specific phase of infection.
Figure 5.7: Intracellular survival assay during 36 hours of infection. PMA stimulated THP-1 cells were infected with mc²155::pSET-18Su and mc²155::pSET152 and cultured for 36 hours as described in materials and methods. Number of bacteria surviving inside the macrophage was determined at 3, 12, 24 and 36 hours after infection. The survival kinetics (A) and the percentage of survival (B) of M. smegmatis carrying shsp18Su are compared with that of the vector control.
Figure 5.8: Survival of *M. smegmatis* during an extended period of infection in macrophages. THP-1 derived macrophages were infected with mc²155::pSET-18Su and mc³155::pSET152 and cultured for 120 hours as described in materials and methods. The number of bacteria surviving inside the macrophages was determined every 24 hours for 120 hours. Comparison of the survival kinetics (A) and percentage of survival (B) of *M. smegmatis*, carrying shsp18Su with the vector control is shown.
DISCUSSION

An important aspect of the pathogenesis of *M. leprae* is its interaction with the host macrophages. This interaction is critical in determining the establishment or containment and the progression or regression of infection in the human host. This also determines the host immune response to the invading pathogen (Modlin, 2010).

One of the hallmarks of *M. leprae* infection is its ability to survive and replicate in the macrophages. Several mechanisms have been hypothesized in an attempt to explain how these mycobacteria avoid the killing mechanisms in the macrophages. A key step in sorting out these evasion mechanisms will be to identify and characterize the mycobacterial genes and gene products required for intracellular survival and/or replication in the macrophages. The approaches generally adopted (Shinnick *et al.*, 1995) are given below

1. Generation of mutated pathogens defective in intracellular survival.
2. Characterization of the mycobacterial genes that confer the ability to resist the bactericidal activities of macrophages
3. Study of genes that are specifically expressed or induced during infection

Generation and characterization of mutants is not feasible due to the uncultivability of *M. leprae*. Hence, the present study combines the second and third approach to examine the role of *shsp18* in mycobacterial survival in the macrophages using *M. smegmatis* as the surrogate host.

Dellagostin and co-workers (1995) had shown *shsp18* to be specifically upregulated during the intracellular growth in macrophages. These authors used recombinant BCG strains carrying the *shsp18* promoter fragments (136 bp and 256 bp upstream of the first codon) to infect murine macrophages and have observed that the highest relative intracellular activation occurred for *shsp18* gene amongst others analyzed. Hence, in this study, the role of *shsp18* was assessed in the presence of its native promoter (168 bp upstream of the start codon). One of the main objectives of this study was to analyze the role of *shsp18* from the mycobacterial perspective. Since *M. leprae* has only one copy of this gene, analysis of *shsp18* as a single copy in the surrogate host will greatly simulate the real situation in the pathogen. One of the best ways to stably clone a single copy of a gene is to integrate the gene of interest in the
chromosome itself. Hence, the integrative vector, pSET152 was chosen for this study. pSET152 is a broad range mobilizable vector which is non-replicative in *Streptomyces* but could integrate into the chromosomal attachment site of bacteriophage φC31 (Bierman et al., 1992). Although this is a *Streptomyces* vector, studies from our lab have shown that transformation of this plasmid into *M. smegmatis* generates stable transformants carrying the pSET152 vector as an integrated copy (Santhosh et al., 2005). Therefore, *shsp18* along with the upstream 168 bp region was cloned in the pSET152 vector and the recombinant plasmid, pSET-18Su, was transformed into *M. smegmatis*, mc²155.

Electroporation has been described as the most reliable method for introducing foreign DNA into mycobacteria (Shinnick et al., 1995) and hence, pSET-18Su was introduced into *M. smegmatis* by electroporation. Since this vector does not carry any mycobacterial origin of replication, antibiotic resistant transformants could be generated only by integration into the genome by site-specific recombination using the *attP* in the vector. Plasmid rescue strategy was employed to confirm this integration event in the *M. smegmatis* genome. Restriction analysis of the rescued plasmids not only confirmed the integration event but also the presence of *shsp18* in the *M. smegmatis* genome. In addition, this analysis revealed that pSET-18Su has integrated at two different sites in the genome. Earlier work reported from our lab has shown that site specific integration of pSET152 in *M. smegmatis* occurs at the 5´TTG as the cross over site, which is the *attB* site (Santhosh et al., 2005). Therefore, one of the integration observed in this study must have occurred at this *attB* site. Combes et al., (2002) have reported a similar observation on multiple integration events in *Streptomyces*. They have identified three preferred pseudo-*attB* sites for the integration of pSET152 in *S. coelicolor* and *S. lividans*. Based on the data reported by Combes and group, it can be speculated that *M. smegmatis* also has such a pseudo-*attB* site where pSET-18Su could have integrated. Sequence information of the hybrid attachment site present in the rescued plasmids would throw more light on the preferred integration sites for pSET152 in the *M. smegmatis* genome.

Dellagostin et al. (1995) have demonstrated that in *M. smegmatis*, *shsp18* gene utilizes a single transcriptional start point located 65 bp upstream of the start codon. The 168 bp upstream region cloned in this study includes this transcriptional start site and hence the gene expression was expected to occur. The expression of *shsp18*
driven by its native promoter was demonstrated in *E. coli* (in chapter IV). However, this protein could not be detected in the total cell lysate of the recombinant *M. smegmatis* even by western blotting. One probable reason could be that sHsp18 was expressed in too low levels to be detected. In *E. coli*, although *shsp18Su* was present in multiple copies as plasmid, the protein could be detected only by immunoblot analysis. In *M. smegmatis*, with just an integrated copy of *shsp18Su*, the sHsp18 expression was probably below the detection limit. Such low level expression of *shsp18* in *M. smegmatis* has been reported earlier (Dellagostin et al., 1995). Moreover, the expression of α-crystallin like shsp18s in general are negligible under normal growth conditions but when stressed their expression can be increased greatly (Narberhaus, 2002). Therefore, despite our inability to detect the protein, the expression and role of sHsp18 was examined in macrophage studies.

The main objectives of the macrophage infection assay was to determine if *shsp18* has any role in
- uptake of mycobacteria by the macrophages.
- resisting the killing mechanisms by macrophages
- improving the replication efficiency of mycobacteria within the macrophage.

Hence, the survival kinetics of *M. smegmatis* carrying *shsp18Su* in the macrophages was followed for different infection periods. THP-1 cell line was used for the infection studies and the choice of these cells was based on the fact that PMA treatment induces differentiation of THP-1 cells into a macrophage like cell line that displays most of the human monocyte-derived macrophage phenotypes with regard to morphology, expression of membrane receptors, cytokine secretion and induction of several proto-oncogenes (Tsuchiya et al., 1980;1982;Schwende et al., 1996). The PMA stimulated THP-1 cells were infected with *M. smegmatis* integrated with *shsp18Su* and its survival was assessed from the 3rd hour to the 120th hour of infection. Comparison was done with the survival observed for *M. smegmatis* integrated with the vector alone.

The number of *M. smegmatis* recovered at the initial time point (that is, 3 hours after the start of infection) is the total number of bacteria phagocytosed by the macrophages. From the survival assays, it is apparent that the number of *M. smegmatis* phagocytosed does not vary significantly between the test and control
strain. This indicates that shsp18 does not enhance the uptake of *M. smegmatis* by the macrophages under the conditions of this study.

During the early hours and up to 36 hours post infection, the presence of shsp18 does not alter significantly the survival of recombinant *M. smegmatis* when compared to the control strain. As soon as the macrophage phagocytoses the mycobacteria, it employs its antibacterial mechanisms to clear the invading organisms. And, the 36 h infection period analyzed indicate this period of intense macrophage activity and during this phase, the survival of *M. smegmatis* carrying shsp18Su and the vector control was similar. These results imply that the shsp18Su may not have a role in resisting the killing mechanisms of the macrophage.

During the time when this study was carried out, van der Wel and group (2007) had published their studies on the localization of *M. leprae* and *M. tuberculosis* in the macrophages using an extended infection period of up to 7 days. They have shown that *M. leprae* resides in the phagolysosomes during the first 48 hours of infection and subsequently moves into the cytosol by the 4th day after infection, where it multiplies. Based on this work, infection assays in the present study was also carried out for an extended time point of up to 120 h. During this period, a specific pattern in survival was observed for the intracellular *M. smegmatis*. The phagocytosed bacteria were progressively killed till the 48th hour of infection, and then there was a sudden increase in the number till the 72nd hour. This increase was only transient as the bacterial number decreased again by the 96th and 120th hour. This pattern in survival was seen for *M. smegmatis* irrespective of the presence or absence of shsp18Su.

The specific pattern of survival observed in this study for *M. smegmatis* has been reported by other groups as well. Anes et al. (2006) have carried out a systematic analysis on the interaction and fate of *M. smegmatis* in macrophages. They have shown that the survival of *M. smegmatis* in J774 (mouse macrophage cell line) exhibits four phases - killing phase 1, where the active killing of bacteria occurs, intracellular growth phase with a sudden growth of the intracellular *M. smegmatis*, killing phase 2 involving rapid killing of bacteria and a final killing phase 3 which is a slower one. Subsequently Jordao et al. (2008) reported the kinetics of survival of *M. smegmatis* in various macrophages. In J774 and BMM (bone marrow macrophages from mouse), *M. smegmatis* exhibited the four phase survival kinetics.
which were superimposable to the one reported by Anes et al. (2006). However, in RAW cells (mouse leukemic monocyte-macrophage cell line) and HMDM (monocyte derived macrophages from human peripheral blood), *M. smegmatis* was killed in a continuous fashion.

In this study, *M. smegmatis* infecting THP-1 cells also exhibited a four phase survival kinetics similar to that observed for *M. smegmatis* in J774 and BMM (Anes et al., 2006; Jordao et al., 2008). Although the kinetics were superimposable in terms of the phases involved, there was a difference in the time range of infection during which this kinetics was observed for *M. smegmatis*. A comparison of these four phases of *M. smegmatis* survival in the three macrophages is shown in figure 5.9. In the present study, the four phases of intracellular bacterial survival was observed over an infection period which lasts for 120 hours. On the other hand, the kinetics reported for *M. smegmatis* in J774 (Anes et al., 2006) and BMM (Jordao et al., 2008) was only till 24 and 48 hours of infection, respectively. Unlike in other macrophages, *M. smegmatis* was not killed in 24 h by THP-1 cells and these four phases were observed only during an extended infection time. Similar to the THP-1 derived macrophages, adherent cultures of human peripheral blood monocytes/macrophages were shown to harbor *M. smegmatis* during longer infection period (up to 10 days) and intracellular growth was seen only after 1-4 days (Barker et al., 1996). *M. smegmatis* exhibits different kinetics of survival depending on the type of macrophage used (Jordao et al., 2008) and this statement is further substantiated by this study which demonstrates the survival of *M. smegmatis* in yet another type of macrophage, the activated THP-1.

Although, *M. smegmatis* carrying *shsp18Su* and the vector control exhibited superimposable survival kinetics, there was a significant difference between the two in the intracellular growth phase. *M. smegmatis* carrying *shsp18Su* showed a four-fold increase in the number of live bacteria while the control strain showed only a two-fold increase. This difference in the bacterial number can be attributed to the presence of *shsp18Su* and its activation in the macrophage. Thus, when *M. smegmatis* encounters a favorable environment for its replication, the activation and/or expression of *shsp18* facilitated its enhanced replication. Thus, it is evident that *shsp18* does have a role to play in the intracellular replication of mycobacteria.
Figure 5.9: Comparison of the survival kinetics of *M. smegmatis* in different macrophages. The four phases of survival of *M. smegmatis* in PMA stimulated THP-1 observed in this study is compared with that reported in J774* (Anes et al., 2006) and BMM# (Jordao et al., 2008).
Studies of this nature have been carried out earlier to demonstrate the ability of *M. leprae* gene and gene products in the enhanced survival of non-pathogenic mycobacterium in the macrophages. Mundayoor and Shinnick (1994) have shown that at least three different genetic elements might be involved in the intracellular survival of *M. leprae*. Wieles *et al.*, (1997) have reported that Trx system of *M. leprae* can deal with the oxidative stress within the human mononuclear phagocytes and represents one of the survival strategies of the intracellular *M. leprae*. However, detailed studies on the dynamics of survival as shown in this study have not been attempted so far using *M. smegmatis* carrying *M. leprae* genes and studies of this nature will open up new vistas in understanding the survival strategies of pathogenic mycobacteria.

The presence of *shsp18* (with its promoter sequence) thus endows a non-pathogenic mycobacterium with an enhanced ability to replicate inside the macrophages. However, detailed characterization of the fate of the recombinant *M. smegmatis* in macrophages at the light and electron microscopic levels should enable the identification of the possible mechanisms for such increased survival. This in turn would help elucidate the role of *shsp18* in the replication of *M. leprae* in the macrophages.

Although all pathogenic mycobacteria are capable of intracellular growth inside the macrophages, the mechanisms involved in their intracellular survival might differ between the species and the macrophages also. *M. tuberculosis* mainly escapes the host bactericidal mechanisms by avoiding the fusion of lysosome with the phagosome (Armstrong and Hart, 1975). It has also been shown to replicate after escaping into the cytosol, a process dependent on the Esx-1 secretion (van der Wel *et al.*, 2007). *M. avium* survives by both fusion inhibition and electron transparent zone formation around the phagocytosed bacteria (Frehel *et al.*, 1986). *M. leprae* uses inhibition of phagosome-lysosome fusion as one of the strategies to survive in macrophages. This inhibition is an early event and has been demonstrated in bone marrow derived macrophages (Frehel and Rastogi, 1987), infected resident peritoneal macrophages (Sibley *et al.*, 1987) and RAW macrophages (Alves *et al.*, 2004). van der Wel *et al.* (2007) has shown that *M. leprae* moves into the cytosol by the 4th day after infection and could be detected till the 7th day, where it multiplies. Mor (1983) has also demonstrated that the natural site of multiplication of *M. leprae* is the cytoplasmic
matrix of the host cells. Based on the above information combined with the results from the present study, it is tempting to speculate that shsp18 might have a role to play in the replication of M. leprae in the cytosol of the macrophages. However, further studies are required to confirm this speculation.

*M. leprae*, while preserving the genes required for its transmission, establishment and survival in the host, has lost those that can be compensated for by its host-dependent parasitic lifestyle (Vissa and Brennan, 2001). *shsp18* is the only small heat shock protein gene which *M. leprae* has retained during its reductive evolution. Hence, sHsp18 is probably amongst the many important macromolecules in the mycobacterial cell which are vital for the survival of the pathogen in host cells and for progression of the disease. Such macromolecules may either be constitutively expressed or their expression may be upregulated within the host. And, *shsp18* and its regulation possibly fall in the latter category. Genes that are selectively expressed during intracellular growth may be involved in the virulence of *M. leprae*. Alterations in the gene expression take place both in the mycobacteria and the macrophages that in turn yield a favorable environment for the mycobacteria to survive. *shsp18* is one such gene whose expression is triggered intracellularly (Dellagostin *et al.*, 1995) and favors replication of the mycobacteria in the macrophage as shown in this study. Studies with other pathogens have also shown that genes which are upregulated during infection are often important for pathogenicity. Therefore, the identification of mycobacterial genes like *shsp18* that are upregulated in infected macrophages may provide targets for drug design and or vaccine development. Understanding microbial survival strategies and the immune mechanisms that result in killing of intracellular pathogens will deepen our insight into the pathogenesis of infection that could be applied towards the development of effective vaccination and immunotherapy.

**CONCLUSION**

In this part of the study, *shsp18* gene with its native promoter was cloned and integrated into the genome of the surrogate host, *M. smegmatis*. Macrophage killing assays with the recombinant *M. smegmatis* clearly revealed that *shsp18* has a role in the replication of mycobacteria during infection in THP-1 derived macrophages. And this function was evident only during an extended period of infection. Hence, the
results from this part of the study strongly suggest a role for shsp18 in the multiplication of mycobacteria in the macrophages. And, such enhanced replication might be crucial for the intracellular M. leprae to establish an infection in the host.