3 Characterization of the interaction of *S. flexneri* and *S.* Typhimurium with undifferentiated murine embryonic stem cells

3.1 Introduction

3.1.1 *Salmonella* interaction with *in vitro* models

In vitro models are important tools for studying biology at the cellular level. Cell-based systems have been applied successfully in many areas of basic and applied research such as the evaluation of novel drugs, for example in target identification. They are also used routinely in the assessment of the safety of new chemical compounds with therapeutic potential. "Compounds can be flagged or classified earlier in drug development and consequently this can save time, money and animals in pre-clinical toxicity studies," writes Silvio Albertini from La Roche (Albertini *et al.*, 2006).

Infectious disease studies utilising *in vitro* cellular models have been largely exploited to investigate the intimate interactions between pathogen and host cells, and such approaches have contributed to our understanding of how pathogens are able to interact with a single cell or groups of related cells. Although host tissues are inevitably much more complex than the *in vitro* systems, the latter give us the opportunity to closely observe host-pathogen interactions in a controlled environment. A further important consideration is the easy availability of *in vitro* systems and their economic accessibility compared to the costs of setting up and maintaining animal facilities, not to mention the ethical issues that *in vivo* animal experimentation pose. Both primary and transformed cells can be examined during *in vitro* studies. However, over the years, certain transformed cell types have emerged as favoured options for cellular studies involving pathogens. This is, in part, because the ease of handling of such cells and the favourable exploitation of a particular cell type stimulates further studies as researchers become familiar with these cell lines. Examples of transformed cell types frequently used to

study host cell-pathogen interactions include HeLa, CaCo, Henle, MHO, and Hep2 cell lines.

Murine embryonic stem (ES) cells have been the subject of intense investigation, as they are readily culturable *in vitro* but retain pluripotency and can be driven down different differentiation pathways using simple stimuli. Indeed, stem cells are potentially exploitable in many areas of biology both in murine and human systems. Mouse ES cells are a 'young' model of investigation and relatively little is understood about them but the expectations in medical applications are quite high. In fact it seems from recent research that ES cells are promising to contribute to curing diseases as far ranging as cancer (Lisowski & Sadelain, 2008), Alzheimer's (Korecka *et al.*, 2007), diabetes (Santana *et al.*, 2006) and Parkinson's (Lindvall, 2003). Stem cells have the potential to play an important role in infectious disease research but their role in basic research and studies on pathogen-host interactions still needs to be explored.

ES cells can be driven to differentiate into cell types which are either challenging to isolate from the body, difficult to maintain *in vitro* or the limiting factor in their application. In fact, primary cells are difficult to produce in sufficient quantity and can be prone to batch-to-batch variability in quality, depending on the source (Thomson, 2007). ES cells are attractive because they can be subjected to gene targeting or random mutagenesis to investigate the impact of a particular genetic alteration or mutation (Brault *et al.*, 2006). Moreover, ES cells are the only truly immortal stem cells and most importantly usually maintain a normal diploid karyotype, which renders them a suitable model for developmental studies. They can also be implanted into female mice and used to generate transgenic or knock out mouse lines (Gorba & Allsopp, 2003).

This chapter reports on investigations into the ability of murine ES cells to be infected by Enterobacteria such as *S. flexneri* and *S.* Typhimurium, and compares these interactions to those observed with 'traditionally' exploited cell lines such as human epithelial cell derived Hep2 cells or J774A.1, a mouse macrophage-like hybridoma cell line. The murine ES cells predominantly used in this study are AB2.2, male (XY) cells derived from the inbred mouse line 129Sv/Ev (Adams *et al.*, 2005; Ramirez-Solis *et al.*, 1995). The cells were originally derived from *hprt*-deficient cells using cre recombination across *loxP* sites embedded in two complementary but non-functional

fragments of the hypoxanthine phosphoribosyl transferase (*hprt*) gene (Ramirez-Solis *et al.*, 1995). For experimental purposes, AB2.2 ES cells were maintained in feeder free culture in medium containing leukaemia inhibitory factor (LIF), a member of the IL-6 family, which maintains the cells in a self-renewing state through interactions with the gp130 receptor and via JAK/STAT pathway activation.

Other cell lines utilised here were Hep2 and J774A.1, which are laboratory adapted and are widely used in the study of infectious disease (Finlay & Falkow, 1988). However, their real karyotype is somewhat dubious and controversial. The Hep2 cell line, for example, was first derived from a laryngeal carcinoma but it seems that it later became 'contaminated' with HeLa cells, giving them an unknown profile that cannot safely be related to any other cells in the body (Nelson-Rees *et al.*, 1981). Nevertheless *in vitro* studies exploiting these cells have proved to be very important in revealing aspects of the complex intracellular survival mechanisms of both *Shigella* and *Salmonella* bacteria (Finlay & Falkow, 1988). It is experimentally well established that *Shigella* can induce an active form of self-phagocytosis through the actions of a (plasmid encoded) Type III Secretion System (TIIISS) (Ohya *et al.*, 2005). Once inside the cell, *Shigella* frequently escape from the endosomal vacuole in Hep2 and other cells to live freely inside the cell cytoplasm (Sansonetti *et al.*, 1986). Here the bacteria can propel themselves through the cell via the synthesis and exploitation of a 'comet' like actin tail (Suzuki *et al.*, 1996). *Salmonella* uses a very similar technique of cell invasion whereby a TIIISS present on Salmonella pathogenicity island-1 (SPI-1) induces active ruffling of the cell membrane and phagocytosis. However, in contrast to *Shigella*, *Salmonella* normally reside inside the cells in a vacuole-limited area called the Salmonella Containing Vacuole (SCV) and replicate through the actions of proteins carried on a second TIIISS present on SPI-2 (Kuhle *et al.*, 2006). *Salmonella* and *Shigella* are able to resist the killing activity of host cells using a number of additional systems (Ohl & Miller, 2001). *Shigella* can induce apoptosis of eukaryotic cells and can mediate cell to cell spread (Hilbi *et al.*, 1998). However, it is still not clear how *Salmonella* escape the eukaryotic intracellular environment and spread inside the host body, although macrophage trafficking does play a key role (Sukhan, 2000). An important reason for studying these pathogens is found in their broad distribution and high disease burden within developing countries and elsewhere. *Shigella* is now more common in developing countries where it is responsible for the death of young children (Sansonetti, 2006). On the other hand,

Salmonella has remained a significant problem in the USA, Japan, and Europe due to transmission in the food chain. In developing countries the incidence has increased thanks to the occurrence of other infectious diseases like HIV and malaria (WHO, 2002).

The attractiveness of any new *in vitro* model will be dependent on the experimental techniques, tools, and reagents available to the research community. Here, a new *in vitro* model based on murine ES cells was investigated for its potential in studies on host-pathogen interactions. Initially, ES cells were examined for their susceptibility to bacterial infections to discover if they could sustain the intracellular survival of invasive bacteria.

3.2 Results

3.2.1 The interaction of Shigella flexneri with murine ES cells

3.2.1.1 The ability of *S. flexneri* Sh42 to invade Hep2 cells

In order to optimize cell based invasion assays, Hep2 cells were exploited as a control cell line with known susceptibility to *S. flexneri* infection. Hep2 cells are a human epithelium cell line originally derived from a laryngeal carcinoma. Initially Hep2 cells were infected independently at a multiplicity of infection (MOI) of ~ 100 with *S. flexneri* Sh42 with or without the plasmid pJKD18 which directs the expression of the green fluorescent protein (GFP) constitutively. *S. flexneri* Sh42 is derived from the virulent wild type strain M90TS serotype 5 and harbours a point mutation of A to G in the gene encoding the protein disulfide bond catalyst DsbA at position 33 in the open reading frame (Yu *et al.*, 2000). The attenuated *S. flexneri* Sh42 was used in all preliminary experiments for safety reasons because wild type *S. flexneri* M90TS is highly infectious. The colony forming units (CFUs) shown in Figures 3.1 and 3.2 are the means of triplicate counts from each of three replicate wells. *S. flexneri* Sh42 was readily able to invade Hep2 cells as deduced by the classical gentamicin susceptibility assay, although the levels of invasion were likely to be reduced compared to wild type *Shigella* due to the impact of the *dsbA* mutation (Figure 3.1). This is most probably due to the defective ability of this mutant to spread from cell to cell as described by Yu *et al* (Yu *et al.*, 2000). Also, the observed reduction in bacterial cell count after 2 hours compared to 4 hours could be due to the heavily infected cells dying by some mechanism releasing bacteria into the media containing 50μg/ml of gentamicin antibiotic .

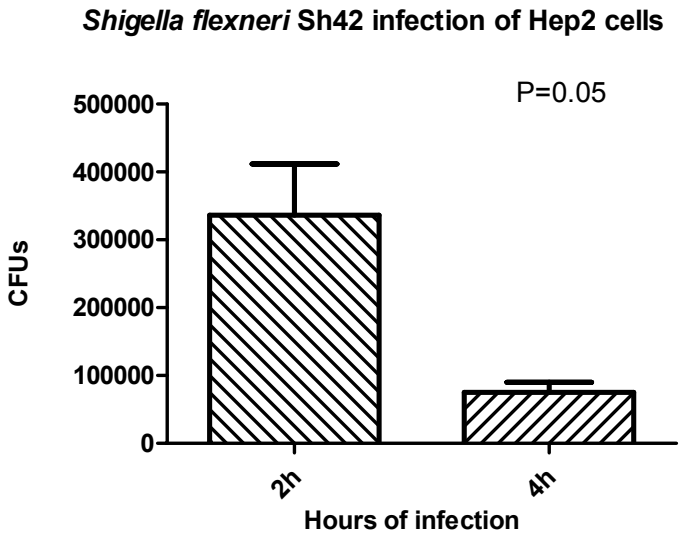

Figure 3.1 Bacterial CFUs from gentamicin assay on Hep2 cells with *S. flexneri* Sh42 at MOI 100

The CFUs are reported as the arithmetic mean of triplicate counts per three wells of a 24 well plate at each time point. The bacteria counts are significantly reduced at 4h infection. This mutant does not exhibit the infection trend expected with the wild type M90TS. The error bars represent 1 standard deviation (SD) and the P value is obtained from a non-parametric Mann-Whitney test.

Figure 3.2 reports the CFU counts resulting from the gentamicin assay performed using *S. flexneri* Sh42 harbouring the plasmid pJKD18 (Sh42/18). These experiments were performed to investigate if the presence of the plasmid significantly affects the behaviour of the *Shigella* bacteria during *in vitro* infection and invasion process. The data indicates that the plasmid does not have a dramatic impact on bacterial behaviour during Hep2 infection.

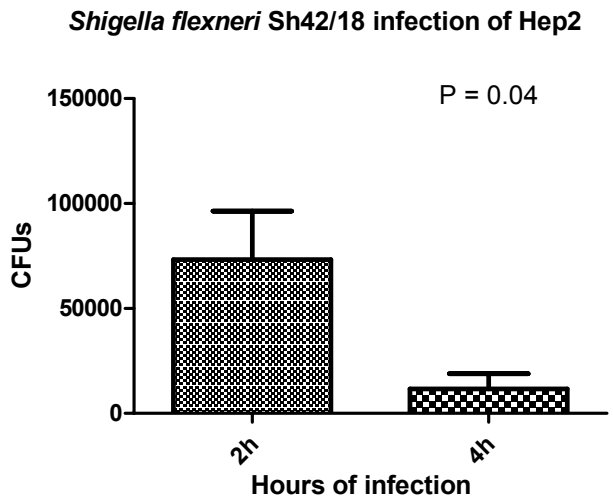

Figure 3.2 Bacterial CFU counts at 2 and 4h infection of Hep2 with *S. flexneri* Sh42 harbouring the plasmid pJKD18 (Sh42/18)

The CFUs are reported as the arithmetic mean of three triplicate counts per three wells of a 24 well plate at each time point. This experiment confirms that the expression of the plasmid pJKD18 does not further attenuate the strain significantly and that the plasmid driving the expression of GFP can be used in flow cytometry and confocal analysis. The error bars represent 1 SD and the P value is obtained from a non-parametric Mann-Whitney test.

These preliminary experiments demonstrate that, under the experimental conditions employed, the behavior of S. flexneri Sh42 during *in-vitro* invasion was equivalent to that reported by Yu 2000, who found that this strain is unable to spread from cell to cell and is attenuated (Yu *et al.*, 2000). *S. flexneri* Sh42 harbours a mutation in DsbA a protein involved in disulphide bond formation (Yu, 1998). Although this strain behave differently from the wildtype strains, it was chosen for safety reasons and because it was previously described. Indeed, it was of particular importance to establish how the techniques and strains used in this study worked in well characterised *in vitro* model cells. Such comparative studies would enable the identification of any differences observed during the infection of a new *in vitro* cellular model such as ES cells. Consequently it should be more feasible and reliable to assign any observed phenotypic differences to the nature of the cell line instead of the experimental design.

3.2.1.2 Flow Cytometric characterization of AB2.2 murine ES cells

Due to an unfamiliarity of working with ES cells and before starting bacterial invasion experiments, AB2.2 ES cells were characterised by a number of methods including flow cytometry and microscopy. AB2.2 ES cells originate from 129Sv/Ev mice as previously described (Bradley *et al.*, 1984). The cells were routinely cultured in GMEM complete medium containing 1000 U/ml of LIF on gelatin coated culture flasks. The cells were passaged less than 35 times by routine enzymatic treatment every two days. After ES cells had been successfully propagated and shown to be intact and uncontaminated they were analysed using FACS analysis, monitoring the expression of well documented typical ES cell markers (Figure 3.3). The ES cells were stained with labelled antibody directed against surface markers such as Integrin α6 (Cooper *et al.*, 1991) conjugated with the fluorochrome APC, Stage Specific Embryonic Antigen-1 (SSEA-1) (Solter & Knowles, 1978) conjugated to PE fluorochrome and with the internal marker Oct3/4 also conjugated to PE. For the latter intracellular marker, ES cells were reversibly permeabilised with saponin buffer before incubation with the antibody and washed twice with the same buffer after staining. ES cells were also analysed for the expression of CD44, a protein that has been reported to interact with pathogens during eukaryotic cell invasion (Garcia-del Portillo *et al.*, 1994; Skoudy *et al.*, 2000). The cells were analyzed on a Becton Dickinson FACSAria Sorter using DIVA software for the data analysis.

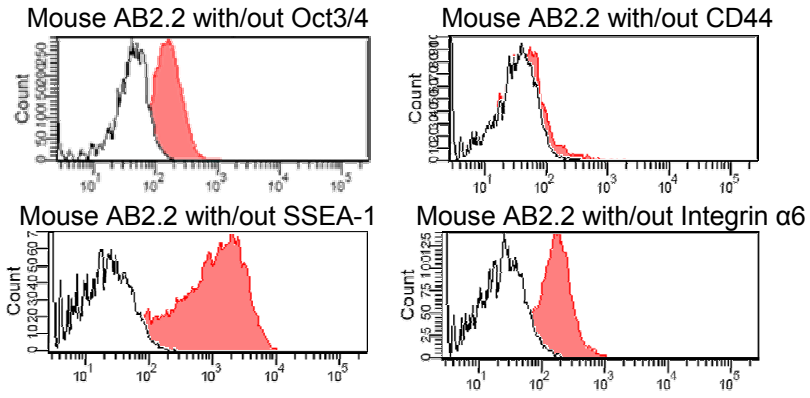

Figure 3.3 Flow cytometric characterization of AB2.2 murine ES cells

AB2.2 murine ES cells, passage 12 (p12) were characterized by flow cytometry using antibodies to the surface markers Integrin α6, SSEA-1, CD44 as well as the intracellular marker, Oct3/4 (red peaks) compared to their relative isotype controls (white peaks). The data clearly indicate that under the growth conditions used the mouse cell line AB2.2 expressed markers characteristic of pluripotent and self-renewing ES cells whilst being negative for CD44.

3.2.1.3 Gentamicin assay on AB2.2 mouse ES cells exposed to *S. flexneri*

AB2.2 murine ES cells were exposed to *S. flexneri* Sh42 with or without pJKD18 at an MOI of ~ 10 or 100 and subjected to a standard gentamicin assay. In Figure 3.4 it can be observed that the CFU counts are lower after 4h infection and are significantly lower if the infection was performed at MOI 100. This is probably due to the fact that at MOI 10 lower numbers of bacteria enter each single cell.

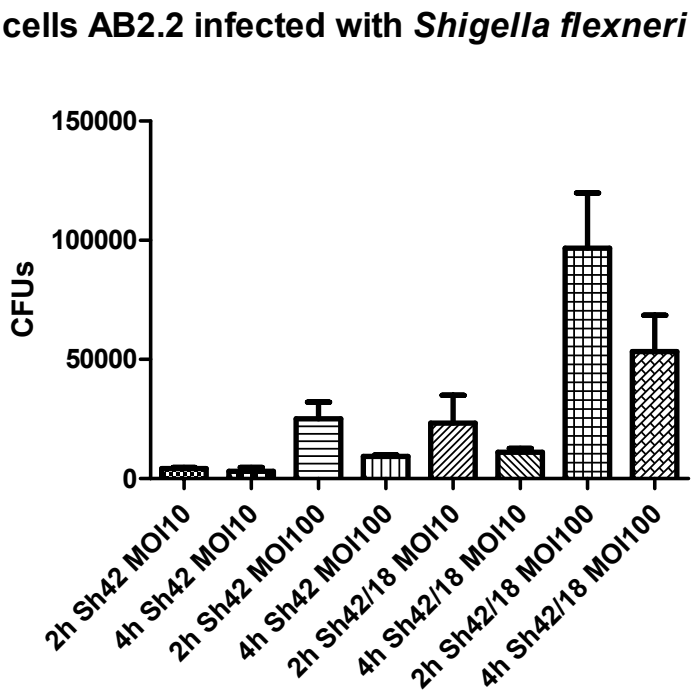

Figure 3.4 CFU counts from gentamicin assays performed on AB2.2 with *Shigella flexneri* Sh42 with and without plasmid pJKD18 at MOI 10 and 100

The histograms show the levels of *S. flexneri* Sh42 and Sh42(pJKD18) CFUs detected after an invasion assay performed on AB2.2 murine ES cells at MOI 10 and 100 after 2 and 4 h incubation time. The data in this histogram demonstrates a difference in the CFUs recovered from Sh42 and Sh42/18 infected cells. This data suggest that the Sh42/18 mutant is more invasive or it is more viable after incubation with ES cells than Sh42. The resons for these differences were not investigated further.The error bars represent 1 SD and the P value is obtained from a non-parametric Mann-Whitney U-test performed on triplicate wells.

3.2.1.4 Gentamicin assay using wild type *S. flexneri* M90TS and AB2.2 mouse ES cells

Wild type *S. flexneri* serotype 5a strain M90TS was used for these experiments, which were performed in a CL3 containment laboratory under the supervision of Dr. Jun Yu. Although *S. flexneri* is a CL2 pathogen in the UK it was deemed prudent to keep these bacteria in a higher containment environment to avoid potentially exposing other workers to this highly infectious pathogen. Mouse ES cells were grown and then moved inside the CL3 facility for the assay. *S. flexneri* M90TS was grown as previously described but, due to the absence of a spectrophotometer, the bacterial culture OD_{600} was not checked before the assay. During this experiment ES cells were maintained in medium without foetal calf serum (FCS) and no antibiotic selection was used other than the gentamicin selection during the last 2 or 4 hours of incubation. The arithmetic mean of CFU counts resulting from three independent experiments is represented in Figure 3.5.

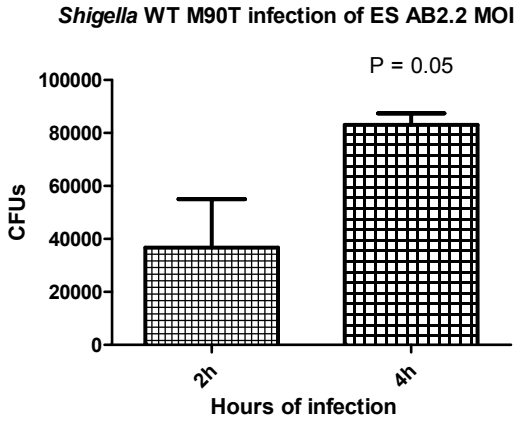

Figure 3.5 CFU counts obtained during a gentamicin assay performed on mouse ES cells AB2.2 using wild type *S. flexneri* M90TS at MOI 100

From this histogram it is clear that, in contrast to the *S. flexneri* Sh42 mutant, the wildtype bacteria are able to enter, replicate, and accumulate inside mouse ES cells as reported before in other cell lines (Yu *et al.*, 2000). The CFUs reported in this histogram are the means of three independent experiments, the error bars represent one SD and the P value is calculated with non-parametric Mann-Whitney test using data from three independent experiments.

3.2.1.5 Flow cytometric analysis of AB2.2 infected by *S. flexneri*

Gentamicin assays are very practical and have been used for many years as a method to quantify and detect bacterial invasion into cells during *in vitro* cellular infections. However gentamicin assays do not provide information on how many eukaryotic cells are infected and how many bacteria are in each cell. In order to have an idea of the dynamics of the *in vitro* infection, parallel flow cytometric analysis of infected cells was performed. For this purpose, *S. flexneri* Sh42/18(pJKD18), expressing GFP, was utilised as this approach permits tracking the intracellular or cell-associated bacteria, giving an estimation of the percentage of infected ES cells.

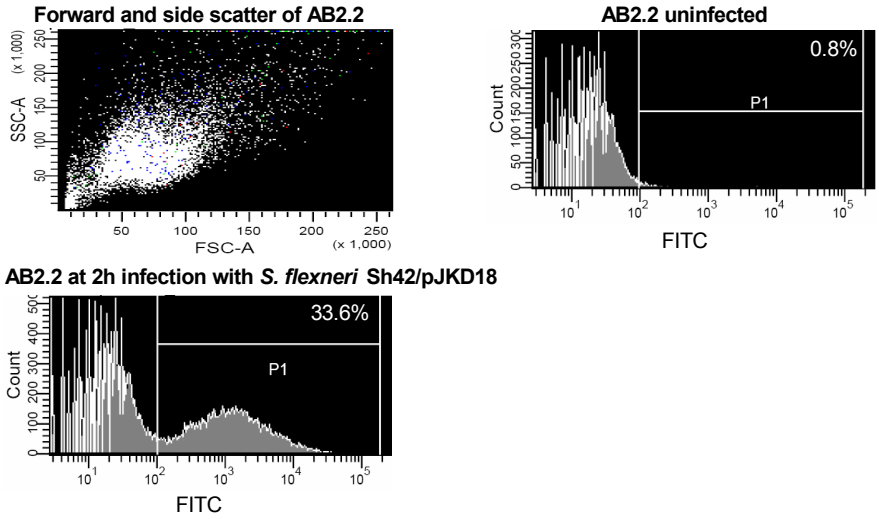

Figure 3.6 Flow cytometric analysis of murine ES AB2.2 cells during infection with *S. flexneri* Sh42(pJKD18)

Mouse ES cells were infected with *S. flexneri* Sh42(pJKD18) constitutively expressing GFP protein as described above. After 2h incubation with medium containing 50µg/ml gentamicin the cells were washed with warm PBS, trypsinized, and fixed with 1% paraformaldehyde. The top right dot-plot histogram represents the side and forward scatter of uninfected murine ES cells. The top left histogram represents the uninfected ES cell profile in the FITC channel. The bottom histogram represents the murine ES cells infected with Sh42(pJKD18) at 2h post-infection, the P1 selection on the right represents the emission of the GFP protein expressed by the internalised bacteria. No light emission was recorded in the FITC channel by cells infected with bacteria no-expressing GFP protein (data no shown).

3.2.1.6 Confocal imaging of AB2.2 murine ES cells infected by *S. flexneri*

Confocal microscopy was used to further observe the behaviour of *S. flexneri* bacteria inside ES cells. *S. flexneri* Sh42(pJKD18) expressing GFP facilitated viewing the bacteria under the microscope. Murine ES cells were grown on glass cover slips pre-treated with acetone and coated with 0.1% gelatin solution for two days to assure cell adhesion. AB2.2 cells adhere very gently to glass cover slips forming clusters of cells. Several well known internal eukaryotic cell vesicle markers were analysed using labelled probes. Actin filaments were stained with Texas red phalloidin and the early endosome marker EEA-1 was also visualized, together with late lysosome markers LAMP-1 and LAMP-2. After staining, the cover slips were mounted in Prolong gold containing DAPI and observed with a ZEISS laser scanning microscope (LSM-510) with 63x optical magnification.

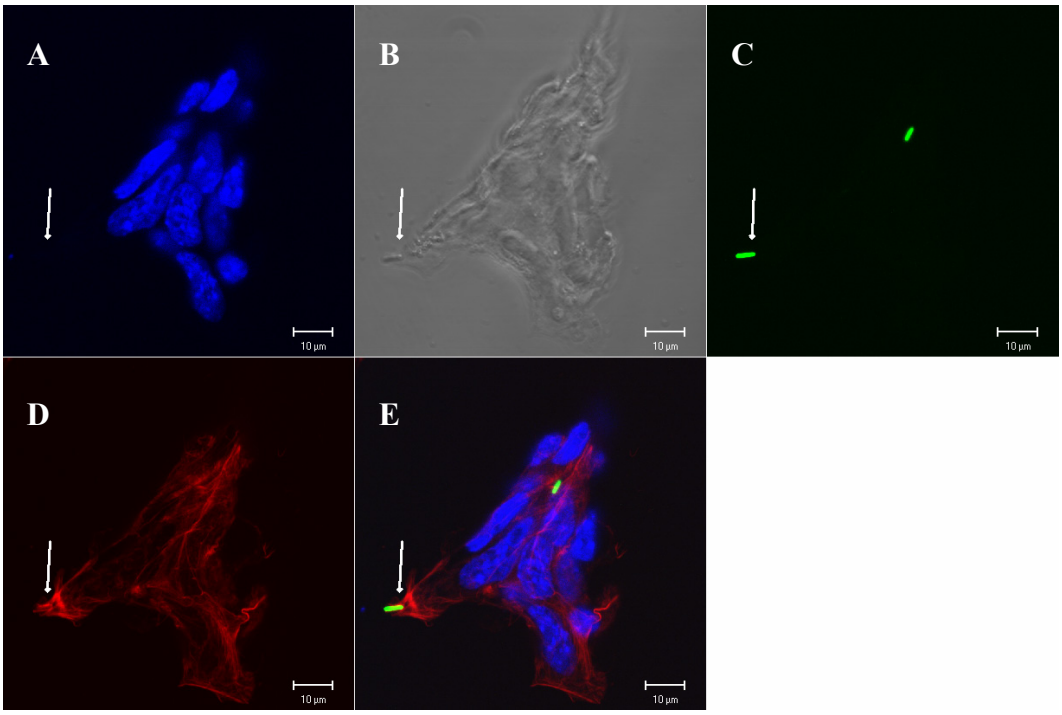

Figure 3.7 Confocal image of AB2.2 murine ES cells infected with *S.* flexneri Sh42 (pJKD18) at time point 0

From confocal observation at time 0 it can be seen that the bacteria preferentially enter cells located at the periphery of the clusters and that the initial interaction between bacteria and ES cells induces actin rearrangements around the entrance point. In this image (63x magnification) can be observed the nuclei stained with DAPI (blue) panel A, the cell cluster shape can be visualized in the phase contrast channel,

panel B; the bacteria *Shigella flexneri* expressing GFP (green) is reported in panel C; the actin filaments were stained with phalloidin TexasRedX (red) panel D; the combination of all the channel can be observed in panel E.

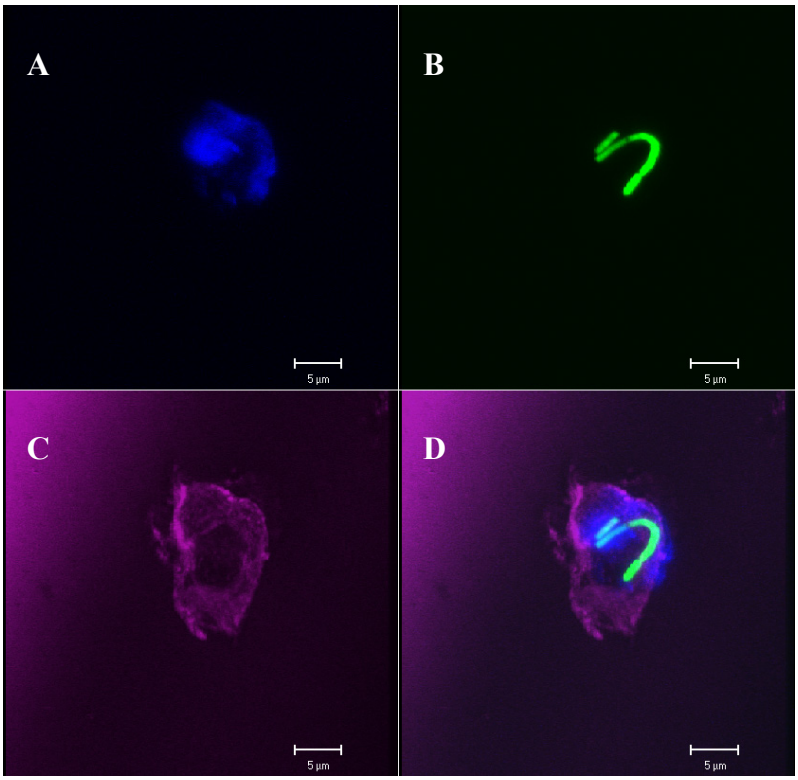

Figure 3.8 Confocal image of AB2.2 murine ES cells after 30 minutes infection with *S. flexneri* Sh42(pJKD18)

AB2.2 murine ES cells were infected with *S. flexneri* Sh42(pJKD18). This image (63x magnification) was obtained after 30 min incubation in media containing 50µg/ml of gentamicin from a Z-stack projection. It can be observed that after 30 min the bacteria have already escaped the vacuole and are multiplying inside the cell cytoplasm. The nuclei were stained with DAPI (blue) panel A, the bacterial express constitutively GFP (green) panel B, the early endosome marker EEA-1 with APC-Cy7 conjugated secondary antibody (purple) panel C; the combination of all the channels can be observed in panel D.

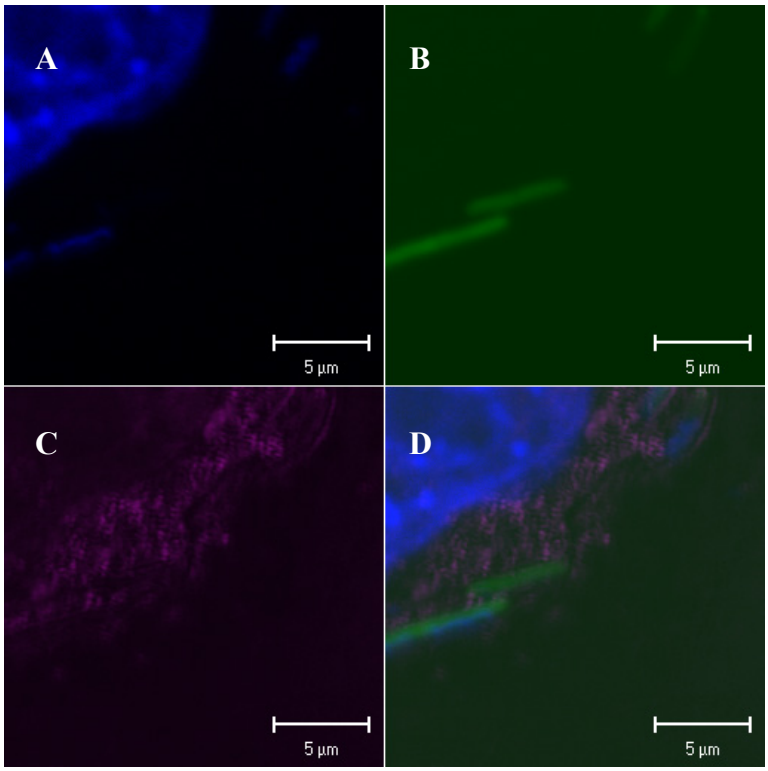

Figure 3.9 Confocal image of AB2.2 murine ES cells at 2h infection with *S. flexneri* Sh42(pJKD18)

After 2h infection of mouse ES cells with *S. flexneri* Sh42(pJKD18), the ES cells were stained with the lysosome marker LAMP-1 then with APC-Cy7 secondary antibody. This experiment investigated whether *Shigella* has the same behaviour inside mouse ES cells as inside other differentiated cells. From the confocal investigation (63x magnification), It is apparent that there is no obvious overlap of the LAMP-1 marker with the green bacteria (GFP). The nuclei are stained blue with DAPI. Panel A, nuclei blue, panel B green bacteria, panel C LAMP-1, panel D overlapping of all the channels.

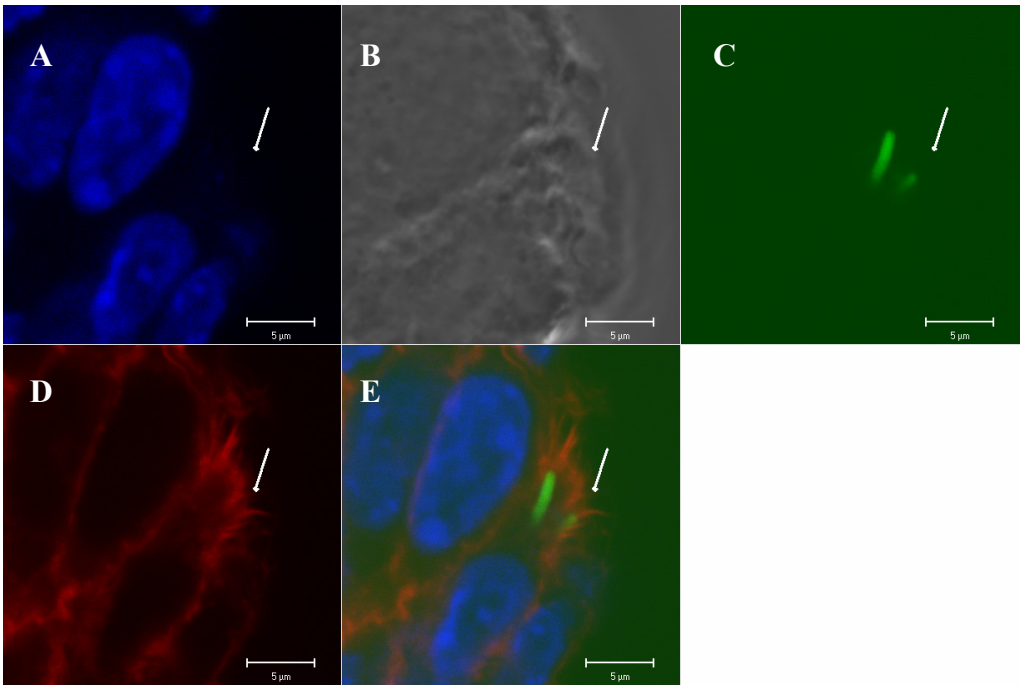

Figure 3.10 Confocal image of AB2.2 murine ES cells at 2h infection with *S. flexneri* Sh42(pJKD18)

ES cells were infected as described with *S. flexneri* Sh42(pJKD18) expressing GFP protein and incubated for 2h with medium containing 50µg/ml of gentamicin. From this image (63x magnification) it can be observed how *Shigella* is able to organize an actin tail even in the limited cytoplasm of mouse ES cells. The nuclei are stained with DAPI (blue) panel A, phase-contrast view in panel B, GFP expressing bacteria (panel C), phalloidin Texas Red stains the actin in red panel D and overlap of the channels is in panel E.

3.2.2 The interaction of *S.* Typhimurium with murine ES cells

3.2.2.1 Gentamicin assay of *S.* Typhimurium interaction with control J774A.1 cells

In order to validate the *in vitro* cellular infection conditions, the mouse J774A.1 hybridoma cell line was initially used as a control. J774A.1 is derived from J774.1 and is a mouse BALB/c monocyte macrophage-like cell line that grows in a semi-adherent manner to the culture flask plastic, predominantly as a monolayer. The cells were grown in 24 well plates until 90% confluent and infected with either wild type *S.* Typhimurium SL1344 or SL1344 harbouring the plasmid vector p1C/1. The vector p1C/1 is described in the reference (McKelvie *et al.*, 2004) and carries the gene for GFP protein expressed under the control of 166bp of the *ssaG*'s promoter. SsaG is normally encoded on SPI-2, a TIIISS involved in pathogen intra-vacuole survival. The gentamicin assay was performed as described in M&M using an MOI of ~ 100. Here are reported the CFU counts as the arithmetic mean of three counts per dilution; the CFU of a representative assay are shown in Figure 3.11.

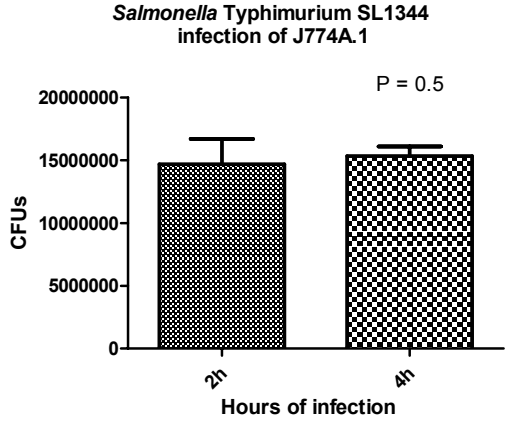

Figure 3.11 Gentamicin assay on mouse J774A.1 cells infected with *S.* Typhimurium SL1344
This histogram reports the mean CFU calculated from three wells during infection with *S.* Typhimurium SL1344 on the mouse J774A.1 macrophage cell line at an MOI of ~100. The CFUs were counted at 2h and 4h post-infection. The histogram shows that the bacteria enter the cells in great numbers and the bacterial counts are similar at 2h and 4h post-infection. J774A.1 cells are able to phagocytose both particles and external pathogens, thus facilitating bacterial entrance. The error bars represent 1 SD of triplicate experiments and the P value was calculated with non-parametric Mann-Whitney U-test.

3.2.2.2 Flow cytometric analysis of *S.* Typhimurium infecting mouse J774A.1 cell line

In order to perform flow cytometric analysis, J774A.1 cells were infected with *S.* Typhimurium expressing GFP under control of the *ssaG* promoter. The protein ssaG is part of the TIIISS present on SPI-2 which is necessary for bacterial survival inside the vacuole and is predominantly expressed by *S.* Typhimurium only after the bacteria have entered host cells. After infection and subsequent incubation with gentamicin, cells were analysed on a Becton Dickinson FACS Aria Sorter utilising DIVA software. The GFP expressing bacteria were detected in the FITC channel excited by the Argon laser.

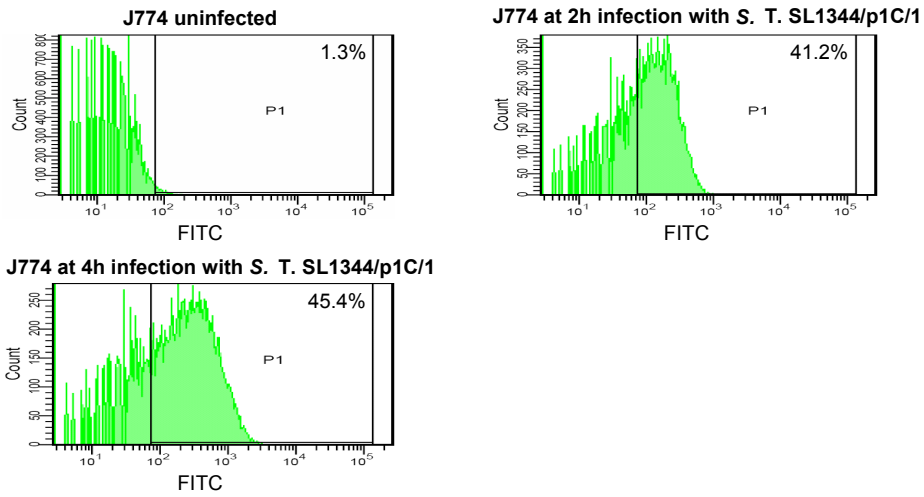

Figure 3.12 Flow cytometric analysis of murine J774A.1 cells infected with *S.* Typhimurium SL1344(p1C/1)

Infection of the mouse J774A.1 macrophage cell line with *S.* Typhimurium SL1344(p1C/1) expressing GFP protein under the control of *ssaG* promoter. The green bacteria infecting the ES cells can be followed by flow cytometry. The histograms show an increase in the percentage of infected cells between 2h and 4h post-infection.. However, this apparent discrepancy with the CFU counts reported in Figure 3.11 could be due to the fact that the CFU counts give the total number of bacteria without taking in account how many cells are actually infected and the number of bacteria per cell. Also, GFP can be very stable protein once expressed and its presence does not necessarily reflect bacterial viability. Also cells infected with bacteria no-expressing GFP protein were investigated for light emissionin the FITC channel.

3.2.2.3 Gentamicin assay monitoring the interaction of *S.* Typhimurium SL1344(p1C/1) with AB2.2 murine ES cells

AB2.2 ES cells were grown on 6 well plates coated with 0.1% gelatin solution and infected with either *S.* Typhimurium SL1344 or SL1344(p1C/1) at an MOI of ~ 100 in standard gentamicin assays. CFUs were enumerated and a representative histogram of these experiments is reported in Figure 3.13. In these experiments the bacterial numbers increase significantly during infection.

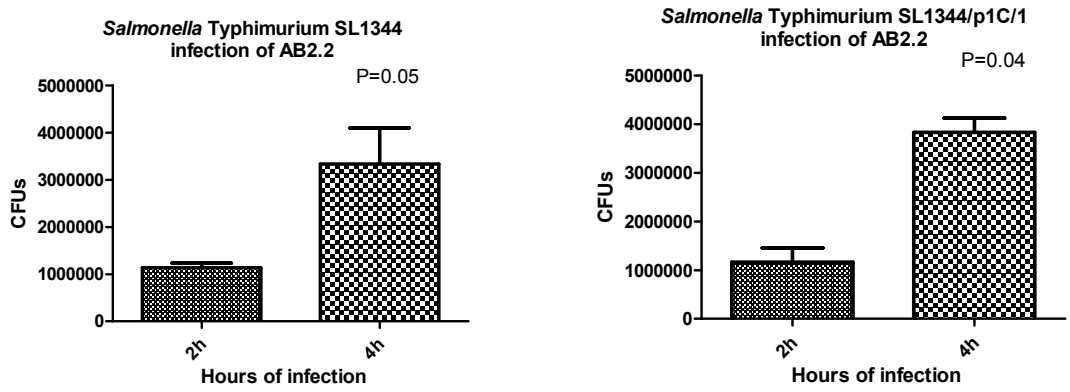

Figure 3.13 Gentamicin assay of mouse ES cells infected by *S.* Typhimurium SL1344 with or without the reporter plasmid p1C/1

The CFU counts reported in these histograms are the arithmetic mean of bacterial numbers counted in three wells detected during the infection of mouse ES cells with *S.* Typhimurium SL1344 or SL1344(p1C/1). The histograms show that *Salmonella* is able to enter and replicate inside mouse ES cells. The presence of the plasmid p1C/1 does not appear to influence the bacterial capacity to infect the cultured cells. The error bars represent 1 SD and the P value is calculated using a non parametric, one tail distribution and the Mann-Whitney test.

3.2.2.4 Flow cytometric analysis of mouse ES cells infected with *S.* Typhimurium using a time course of infection

To investigate the time progression of *S.* Typhimurium infection of AB2.2 murine ES cells, gentamicin assays were performed using the bacteria carrying the plasmid p1C/1 expressing GFP. At each hour up to 5 hours, the cells were analyzed in parallel by FACS (Figure 3.14) and by taking CFU counts (Figure 3.15). The invasion assay was carried out as described in M&M. The aim of this experiment was to investigate the rate of the bacterial entrance, replication and spread between cells. This experiment was repeated 3 times and it was always observed a steady increase of infected cells and CFU counts. The results show how *Salmonella* enter the cells and establish a significant intracellular infection within 2 hours. These results clearly demostrate that these two techniques are complementary and together they give a more complete understanding of the *in vitro* infection profile.

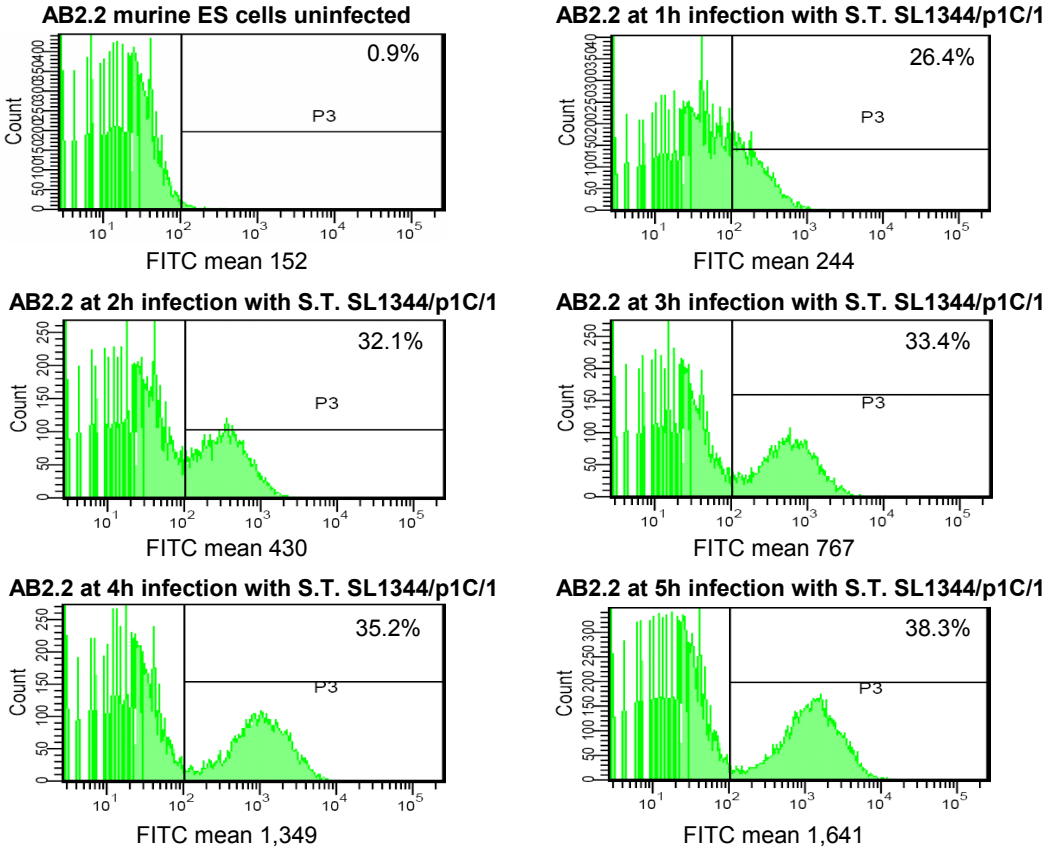

Figure 3.14 Flow cytometric analysis of time course infection of mouse ES cells infected with *S.* Typhimurium expressing GFP protein

Mouse ES cells AB2.2 were infected with *S.* Typhimurium SL1344(p1C/1) expressing the GFP protein under the control of the *ssaG* promoter. The protein ssaG is normally encoded in the SPI-2 locus and is expressed optimally once the bacteria are inside the host cell. The infected cells can thus be tracked by flow cytometric analysis using the 488nm Argon laser which excites GFP. The histograms presented here show the increasing percentage of infected AB2.2 cells during a 5 h post-infection assay. The mean intensity of the FITC channel emission increased over time reflecting the increasing number of bacteria inside the cells.

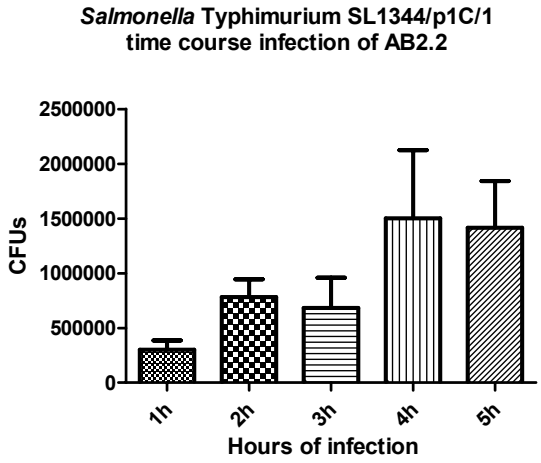

Figure 3.15 CFU counts from time course gentamicin assay of *S.* Typhimurium SL1344 infection of mouse ES cells

The histogram shows the CFUs detected during a 5h infection of ES cells with SL1344(p1C/1). The CFU counts reflect quite closely the FACS data showing a clear increase in numbers of bacteria between 2h and 4h of infection. The CFUs shown are the result of triplicate counts for three wells per time point. The error bars are 1 SD.

3.2.2.5 Confocal imaging of *S.* Typhimurium infecting AB2.2 murine ES cells

To track bacterial intra-cellular localisation, mouse ES cells were grown for two days on glass cover-slips pre-treated with acetone and coated with 0.1% gelatin solution and then infected with *S.* Typhimurium SL1344(p1C/1). After fixation at 30minutes and 1hour after infection, antibodies including rabbit anti-EEA-1, rat anti-LAMP-1 and rat anti-LAMP-2 were utilised as primary reagents on saponin permeabilised cells. Species specific secondary antibodies used were anti-rat APC-Cy7 and anti-rabbit APC-Cy7. The cover-slips were mounted in Prolong Gold containing DAPI and images were captured with the ZEISS LSM510 at 63x magnification.

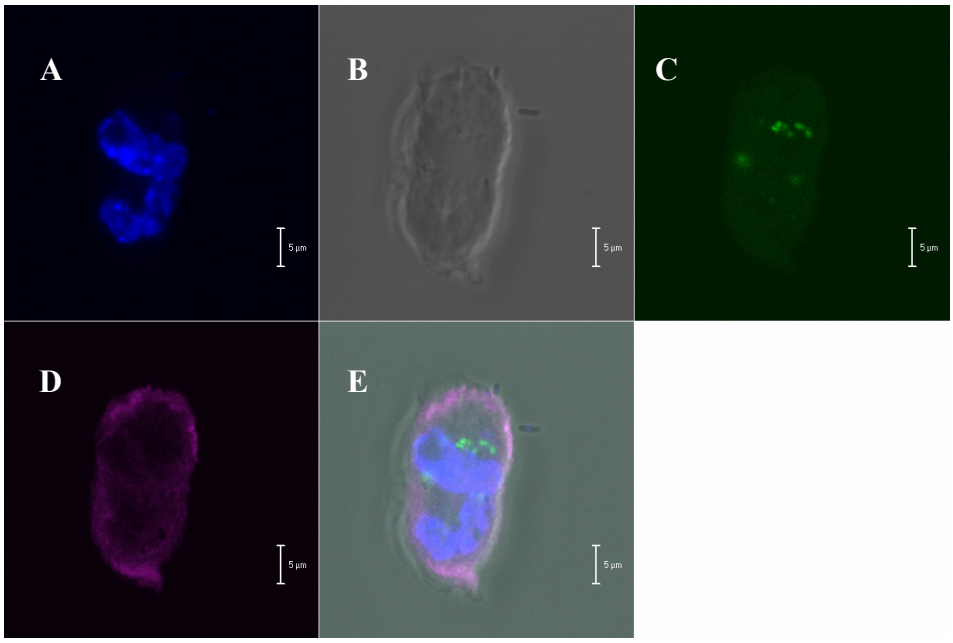

Figure 3.16 Confocal image of AB2.2 cells infected with *S.* Typhimurium at 30 minutes

Murine ES cells were infected with *S.* Typhimurium(p1C/1) detectably expressing GFP protein only when bacteria are inside the host cell. From the picture at 30 min infection, EEA1 has a sub-membrane distribution and an apparent lack of association with bacteria. Note the presence of as few bacteria outside the ES cells which are not expressing GFP. We can observe the nuclei stained with DAPI panel A, a phase contrast panel B, *S.* Typhimurium(p1C/1) in green (GFP), panel C, early endosome marker EEA1 in purple (APC-Cy7) panel D and all the channels overlapped in panel E.

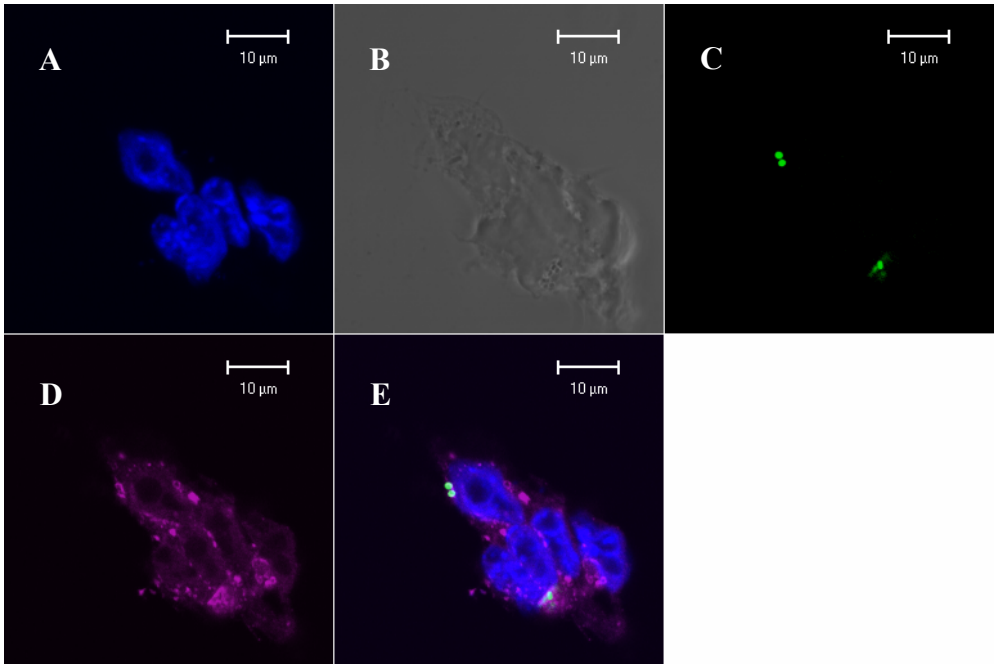

Figure 3.17 Confocal image of AB2.2 mouse ES cells infected with *S.* Typhimurium(p1C/1) at 1 h

After 1 h incubation with ES cells, the bacteria can be clearly seen co-localizing with the LAMP-1 antibody. Here the nuclei are stained in blue panel A, the phase contrast highlights the shape of the cluster panel B, the green *S.* Typhimurium(p1C/1) in panel C, the late lysosomal marker LAMP-1 in purple panel D, and all the channels combined in panel E.

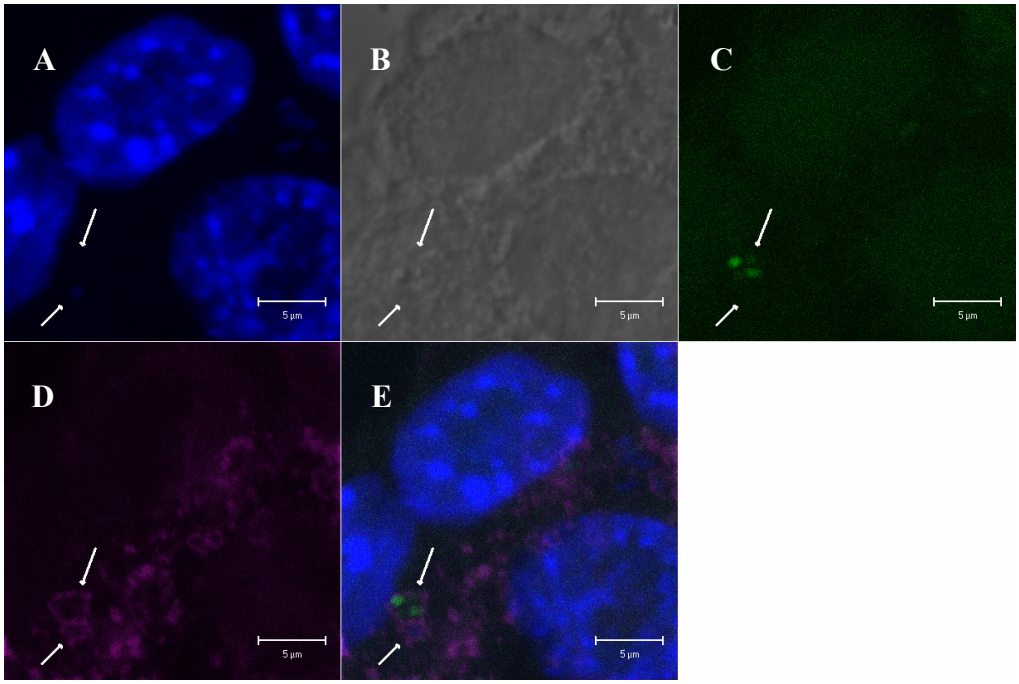

Figure 3.18 Confocal image of AB2.2 mouse ES cells infected with *S.* Typhimurium(p1C/1) at 1 h

After 1 h incubation the cells were washed, fixed and after saponin permeabilisation, stained with rat anti-LAMP-2 and anti-rat APC-Cy7 secondary antibody. LAMP-2 was visible but at an apparently lower level than LAMP-1 staining. In panel A can be observed the nuclei stained with DAPI, panel B report the phase contrast channel, panel C show the green *S.* Typhimurium(p1C/1) bacteria, panel D report late the lysosome marker-2 LAMP-2 (purple), panel E report all the channel combined. Note next to the green bacteria, a blue bacteria also inside a vacuole (white arrows).

3.2.3 Role of Type III secretion systems during infection of mouse ES cells

3.2.3.1 Gentamicin assay using the *S. flexneri mxiD* mutant interacting with AB2.2 mouse ES cells

Shigella invasion of eukaryotic cells requires the expression of a functional type III secretion system, commonly referred to as Mxi-Spa, and a cognate set of secreted Ipa invasins (Schuch *et al.*, 1999). MxiD is an outer membrane protein required for the secretion of the Ipa invasins (Allaoui *et al.*, 1993). Here, a *mxiD* mutant was used to investigate if the *S. flexneri* invasion of mouse ES cells observed in the above studies was dependent on a TIIISS.

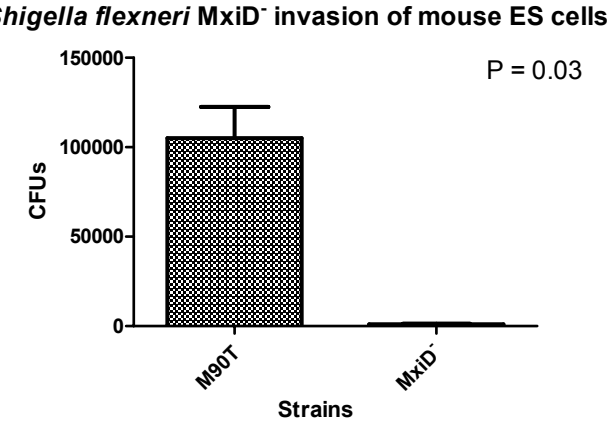

Figure 3.19 Gentamicin assay performed on mouse ES cells using S. flexneri *mxiD*$^-$

CFU detected during exposure of mouse ES cells to potential invasion by the wild type *Shigella flexneri* M90TS on the left and the derived mutant *mxiD* on the right. This result highlights that *S. flexneri* utilizes a TIIISS during the invasion of mouse ES cells (as *Shigella* does during the invasion of epithelial cells). The bars represent the mean of the CFUs from three replicate and the error bars represent 1 SD. This experiment was conducted with Dr. Jun Yu.

3.2.3.2 Gentamicin assay monitoring the levels of invasion of AB2.2 mouse ES cells by a *S.* Typhimurium *sipB* mutant

The *Salmonella* pathogenicity island 1 (SPI-1) is a complex chromosomal locus required for the productive invasion of eukaryotic cells by *S. enterica*. SPI-1 encodes a group of invasion effectors including the Sips (*Salmonella* invasion proteins A-D) that upon contact with the target cell undergo type III export from the bacterial cytoplasm and translocation into the eukaryotic cell membrane or cytosol (Hayward *et al.*, 2000a). Here, the contribution of SPI-1 to *S.* Typhimurium invasion of mouse ES cells was investigated using a TIIISS associated *sipB* mutant. Gentamicin assays was performed and the CFU counts are reported in Figure 3.20.

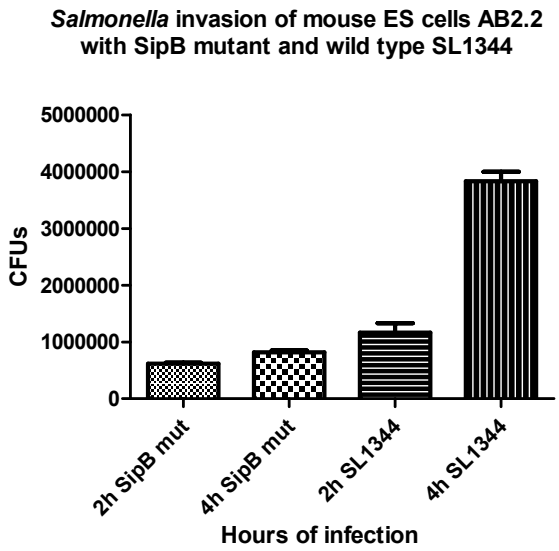

Figure 3.20 Levels of *S.* Typhimurium SL1344 sipB mutant invasion of mouse ES cells AB2.2
CFU counts of *S.* Typhimurium SL1344 *sipB* mutant compared to the levels detected with wild type SL1344 at 2h and 4h after infection of AB2.2 mouse ES cells. The histogram indicates that the mutant *Salmonella* enters the cells in lower numbers even though the difference with the wild type at 2h is not statistically significant using one way ANOVA (non-parametric) and Tukey's multiple comparison test. The CFU counts are reported as the arithmetic mean of triplicate count per time point and the error bars are 1 SD.

3.3 Discussion

This chapter reports data that demonstrate how ES cells can be infected by isolates of the enteropathogenic bacteria *S. flexneri* and *S.* Typhimurium using classical gentamicin assays, flow cytometric analyses, and confocal microscopic observations. Infections with *Shigella* and *Salmonella* derivatives harbouring mutations in specific TIIISS were also analysed to confirm their involvement in the pathogens ability to enter during ES cells. These data confirmed aspects of the theory that mouse ES cells have the potential to be used as an alternative *in vitro* cellular model to study host-pathogen interactions.

Mouse ES cells are immortal cell lines that grow out from the inner cell mass of cultured pre-implantation mouse embryos. They can maintain the potential to support normal development of embryonic and extra-embryonic structures when reinjected into blastocysts and implanted into pseudo-pregnant female mice (Cooper *et al.*, 1991). Currently, we know that these cells have specific surface and internal markers that define their nature. At the beginning of the study a few markers were selected to characterize the murine ES cell line used. The ES cells were characterized by flow cytometric analysis using conjugated antibodies specific for pluripotent cells. Two external markers were used: Integrin α6/CD49f and stage-specific mouse embryonic antigen (SSEA-1), and one internal marker: Oct3/4. Integrin α6 is one of the first extracellular matrix proteins expressed in the developing mammalian embryo and is present throughout the embryonic tissues thereafter (Cooper *et al.*, 1991). SSEA-1 is important in the regulation of cell interactions, cell sorting development and differentiation (Solter & Knowles, 1978). Oct3/4 is a POU transcription factor involved in the regulation of pluripotent and germline cells. Oct3/4 is essential for the development of the mammalian embryo (Loh *et al.*, 2006). As shown in Figure 3.3, 90% of the AB2.2 cells cultured in LIF were positive for the integrin α6/CD49f and for Oct3/4 markers, and 70% of the cells were positive for SSEA-1 markers. In contrast, ES cells were found to be negative for a differentiation marker, CD44, that can play a role in bacterial binding and internalization (Garcia-del Portillo *et al.*, 1994; Skoudy *et al.*, 2000). One of the first theories to be investigated as part of this project, was that mouse ES cells lose their pluripotent characteristics on exposure to bacteria and for this purpose Oct3/4 expression was investigated during infection (data not shown). The

experiment showed that loss of Oct3/4 does not detectably occur during infection, possibly as a consequence of the short time window of infection over which the investigations are taking place, i.e. up to several hours after infection.

The first part of this chapter reported how *S. flexneri* is able to invade murine ES cells in a manner comparable to previously described cellular models. *S. flexneri* Sh42 was described previously by Yu *et al*. 2000. The mutant Sh42 harbours a point mutation in the gene encoding DsbA protein which is required for the oxidative folding of Spa32. Spa32 is an outer membrane protein and is part of the TIIISS involved in eukaryotic cell invasion. However, DsbA also has an intracellular role in Ipa secretion and in the formation of the Mxi-Spa secretion system required for intracellular spread (Allaoui *et al.*, 1993). All togheter it was possible to observe facets of how Sh42 interacts with the control cell line Hep2 (Figures 3.1 and 3.2) and the target cell line AB2.2 (Figure 3.4). In both cases after 4 hours incubation the CFU counted were significantly lower than the 2 hours time point. This implies that the bacteria die between 2 and 4 hours post infection. This behaviour was previously observed and reported by Dr. Yu in his paper in 1998 describing a polar *dsbA* mutant (Yu, 1998) in which he documented that this mutant was barely able to grow inside HeLa cells. As Sh42 lacks the ability to spread from cell to cell, it can be postulated that the bacteria, after intracellular proliferation, are released into the extracellular milieu as a consequence of disruption of bacterial or host cell function and are then killed by the gentamicin. Alternatively, the bacteria may be killed by other undefined mechanisms. From these experiments it can be inferred that the bacteria behave in a similar manner in Hep2 and AB2.2 ES cells in the decreased number of recovered bacteria at 4h versus 2h infection, although SH42/pJKD18 gave generally higher CFUs counts during mouse ES cells infection. Moreover, when wild type *S. flexneri* M90TS was used, an increase in CFU counts between 2 and 4 hours incubation during gentamicin selection (Figure 3.5) was observed. This observation reflects the higher infectivity of the wild-type bacteria.

Usually a plaque assay is performed to measure the entrance, growth, and spread of *Shigella* in cultured cells. Unfortunately, due to the physiology of ES cells, this was not possible since they grow in clusters and they don't form regular monolayers. Instead, in order to monitor entry, invasion and the spread of *Shigella* within ES cells, flow cytometric analysis was used to detect GFP expressing bacteria. *S. flexneri* Sh42

harbouring the plasmid pJKD18 which constitutively directs the expression of GFP protein was used in these studies. Initially, the impact of the plasmid on the behaviour of *S. flexneri* in gentamicin invasion assays was assessed. The CFU counts indicated that the bacteria carrying the plasmid pJKD18 behaved in a similar way to the original mutant strain. The results from these experiments are shown in Figures 3.2 and 3.4 panels 3 and 4. It has been reported that *Shigella* is able to infect Henle cells up to levels as high as 97% (Purdy *et al.*, 2002), such levels of invasion were not observed in our laboratory. However, it was noted that mouse ES cells became very delicate during infection and they detached quite easily from the slides or wells and even the washes with DPBS could affect the outcome of the assay. Flow cytometric analyses indicated that up to 33% and sometimes up to 42% of ES cells were infected by the mutant *S. flexneri* Sh42(pJKD18), see figure 3.6.

Confocal microscope observations were made of *S. flexneri* Sh42(pJKD18) infecting murine ES cell line AB2.2. Generally, it was observed that *Shigella* invades cells that are in a peripheral position on the cluster of cells (Figure 3.7). *Shigella* is able to routinely escape the endocytic vacuole and survive freely inside the cell cytoplasm of epithelial cells. Consequently, a serial staining experiment using antibody against the early endosome marker EEA-1 and the late lysosome markers LAMP-1 was performed. Sh42(pJKD18) did not obviously colocalize with the early endosome marker EEA-1 30 minutes post-infection (Figure 3.8). Also, Sh42(pJKD18) infected mouse ES cells staining with LAMP-1 after 1 hour (not shown) and 2 hours, revealed no obvious overlap of the cellular marker with the green bacteria (Figure 3.9). Nevertheless, mouse ES cells stained for actin with phalloidin Texas Red revealed a clear actin rearrangement during bacterial infection (Figure 3.7). Also, by 2 hours after infection it was noticed that the *Shigella* bacteria were often associated with an actin 'comet' tail of polymerised protein (Figure 3.10). It is also obvious from these observations that *S. flexneri* Sh42 bacteria have some kind of deficit in their ability to divide, as they were frequently seen as long strings of bacteria.

S. Typhimurium was subsequently used to further evaluate bacterial interactions with murine ES cells. *S.* Typhimurium is a promiscuous pathogen that can cause gastroenteritis in cattle and establishes a systemic disease in mice that mimics some aspects of typhoid fever. For this reason wild type SL1344 was used in the *in vitro*

cellular experiments. A control mouse macrophage-like cell line J774A.1 was first used to optimize the *in vitro* infection conditions. From the results of gentamicin assays it was concluded that *S.* Typhimurium SL1344 was able to invade macrophage cell line J774A.1 (Figure 3.11) and that the CFU counts at 2 hours and 4 hours infection were very similar. These data were supported by flow cytometry observations wherein SL1344 harbouring the plasmid p1C/1 expressing GFP under the control of a 166bp DNA fragment encoding the *ssaG* promoter region (McKelvie *et al.*, 2004) was employed. In *Salmonella* bacteria harbouring this plasmid, GFP is expressed optimally only once the bacteria are inside the SCV. Consequently, ES cells containing *S.* Typhimurium bacteria were detected by FACS with a 488nm Argon laser in the FITC channel. Figure 3.12 shows that at 2 hours 41% and at 4 hours 45% of the J774A.1 cells were infected, confirming that the *Salmonella* bacteria is most likely able to replicate inside the macrophage cells.

S. Typhimurium SL1344 *in vitro* infection of mouse ES cells displayed some distinct characteristics. The CFU counts from gentamicin assays doubled between 2 and 4 hours infection ($P = 0.05$). Moreover, there seems to be no difference in the invasive ability between wild type SL1344 and the bacteria carrying the plasmid p1C/1, whose CFUs also doubled between 2 and 4 hours (P value = 0.04) (Figure 3.13). Some of the difference in CFU counts observed in the *Salmonella* gentamicin assay between J774A.1 and AB2.2 cell lines could be due to the phagocytotic nature of J774A.1. This possibility was not investigated further.

In order to determine if *Salmonella* was actually invading and replicating inside mouse ES cells, a time course infection was performed and the data were analysed in parallel by both flow cytometry and CFU counts. The data illustrated in Figures 3.14 and 3.15 revealed that *Salmonella* start to enter the cells within 1 hour, during which time relatively low levels of GFP are produced. The GFP intensity increased at 2 hours post infection and the percent of infected cells increased in parallel with the CFU counts. At 3 hours after infection there was an apparent static phase, reflected also by the CFU counts. Here the bacteria might be spreading from cell to cell prior to re-growth in the new cell environment, as indicated by the increase of CFU at 4 hours. The attempt to extend the observation time of these experiments encountered technical difficulties because after a few hours of infection mouse ES cells began to detach from the culture

plate and it became difficult to obtain a reliable estimate of the percentage of infected cells because they were readily washed away.

To observe the complicated intracellular lifestyle of *S.* Typhimurium during infection of mouse ES cells, confocal observations were performed using antibodies to the early endosome antigen EEA-1, and the late lysosome markers LAMP-1 and LAMP-2. Infected AB2.2 ES cells were stained for EEA-1 at 30 minutes and 1 hour post-infection. No obvious co-localization was observed between the internalized GFP expressing bacteria and the early endosome marker, which was clearly localized under the cellular membrane (Figure 3.16). In order to investigate any potential bacterial fusion with lysosomes, lysosome markers such as LAMP-1 and LAMP-2 were used to stain AB2.2 ES cells infected with *S.* Typhimurium SL1344(p1C/1) at 1 hour. As Figures 3.17 and 3.18 show, some co-localization of these two markers with the bacteria was detected. These observations confirm that during infection of murine ES cells, *Salmonella* follow a pathway with similarities to the previously reported route through internal vacuole membranes (Ramsden *et al.*, 2007).

An important aspect of bacteria-host interaction studies is the assessment of how host cells became infected. Host cell infection (or invasion) can involve two distinct but interacting processes. The host cell can actively phagocyte the pathogen or alternatively the pathogen can actively infect the host cell. Indeed, the two processes can occur at the same time. Both *Shigella* and *Salmonella* are able to invade non-phagocytic cells, such as epithelial cells, using a protein structures present on the pathogen surface called TIIISSs (Hermant *et al.*, 1995). In order to determine if murine ES cells invasion was due to an active process involving the SPI1 TIIISS, mutant bacteria were employed in this study. In particular the CFUs from a gentamicin assay performed using a *S. flexneri mxiD* mutant (Figure 3.14) and a *S.* Typhimurium *sipB* mutant (Figure 3.15). These experiments revealed that both *S. flexneri* and S. Typhimurium actively infected/invaded murine ES cells using TIIISS. The invavion of *S. flexneri mxiD* was significantly reduced by the lack of the MxiD protein, which is involved in the formation of the TIIISS basal body. In contrast, the *Salmonella* SipB mutant exhibited a relatively small drop in relative CFUs at 2 hours post-infection, possibly due to compensation for the lack of SipB protein function by other Sip proteins. Interestingly, murine ES cells are unable to phagocyte 1μm latex beads (our data, not shown) further

supporting the concept that bacterial invasion into ES cells is an active process induced by the pathogen.

In conclusion, this chapter demonstrates how mouse ES cells interact with two representative Gram negative pathogenic bacteria; *S. flexneri* and *S.* Typhimurium. The data confirm that the bacteria invade ES cells at a similar level to terminally differentiate epithelial and macrophage cell lines. This observation partially confirms *in vivo* observation of how these two pathogens are able to interact with different cell types resident on the intestine epithelium and highlight some of the advantages and disadvantages of using *in vitro* cell models. Moreover, the data here generated give hope that in the future ES cells will have a role to play in infectious disease research.