7 Final remarks and future work

The main aim of this study was to assess the potential of murine ES cells as tools for probing interactions between pathogens and mammalian cells. ES cells could potentially form the foundation of novel cell-based assays employing either normal or mutant ES cell lines. The study demonstrates for the first time that murine ES cells can be infected independently by the two pathogenic bacteria, *Shigella flexneri* and *Salmonella* Typhimurium. Using Gentamicin assays, similar invasion rates were observed in ES-cell based *in vitro* models and in previously characterized terminally differentiated cell lines for both the bacteria *S. flexneri* and *S.* Typhimurium. This report showed that both pathogens were able to invade ES cells in a manner that was dependent on the expression of bacterial pathogenicity genes that contribute to active cellular invasion. From the observations, the patterns of invasion and intracellular trafficking seemed quite similar in ES cells compared to terminally differentiated cells, although more detailed investigations are required to confirm this observation. Confocal observation revealed that *Shigella* entered murine ES cells via the induction of cytoskeleton rearrangements as previously reported (Adam *et al.*, 1995) and no co-localization was observed with either EEA-1 or LAMP-1 markers suggesting that the bacteria live free inside the cellular cytosol. Observations at 2 hours infection also revealed that *Shigella* produced actin tails once inside ES cells. *Salmonella*-ES cell interaction however was less easy to decipher due to the lack of co-localization with the EEA-1 marker of early endosomes. Nevertheless, it was possible to observe a co-localization of SCV with LAMP-1 and LAMP-2 markers characteristic of lysosomes. A limited number of intracellular markers was used in this study to probe the intracellular location of these pathogens but in the future it would be interesting to investigate the involvement of both the Rho protein family and Rab proteins in the invasion of ES cells as has been suggested for other cell types (Patel & Galan, 2006; Smith *et al.*, 2007). The identification and comparison of the pathogenic mechanisms used during ES cell invasion was useful as so little is known about their interactions and will help in future research to highlight differences in mutated ES cells.

After exploring the basic cellular interactions between murine ES cells and pathogenic bacteria, the transcriptomic profiles of ES cells were determined during *Salmonella*

infection. Perhaps the most striking feature of these profiles was an almost total absence of an immunological response in murine ES cells during bacterial invasion. This is perhaps not surprising since the cell model investigated does not represent an immunological cell line. However, these results may have been a little unexpected since ES cells do produce some cytokines even in an uninfected state (Guo *et al.*, 2006) and a stronger immune reaction after invasion was a possibility. Nevertheless, some interesting genes were revealed by the different type of analyses, especially GeneSpring and the time course analyses highlighted a few interesting genes. For example STAT2 was up-regulated at 4 hours post-infection. STAT2 is involved in the IFN type I and II signal transduction of innate immunity against microorganisms (Decker *et al.*, 2002). Up-regulation of SOCS3 was also detected, this gene is involved in *Salmonella* responses induced by SPiC, a SPI-2 secreted protein. SOCS3 is also involved in the inhibition of cytokine signaling via the JAK/STAT activation pathway (Croker *et al.*, 2008). All of the different analyses identified the up-regulation of cytochrome P450 at 2 hours, followed by a strong down-regulation at 4 hours post-infection, which is intriguing. This could be linked to a stress response in the cells or to a mechanism of auto-defense. The identification of transcription factor XBP1 also reported in other studies is interesting as this factor is known to be involved in the differentiation of myeloid cells (Iwakoshi *et al.*, 2003). This study was restricted to a limited number of post-infection time-points due to the rapid onset of cell detachment. One possible reason for the low immunological signature may be that being undifferentiated, ES cells might not have immunological defense machinery capable of a rapid response which could have an effect on the ‘reaction time’ of the cell. In a previous study conducted on haematopoietic stem cells, it was observed that bacterial infection can induce differentiation and expression of components of immune response (Kolb-Maurer *et al.*, 2004). However, in those experiments there was no observable difference in surface marker expression at 4 hours post-infection. Consequently, the haematopoietic stem cells were incubated with bacteria for longer periods of three and six days in order to detect the expression of immunological genes. In the present study ES cells were treated in a similar manner to epithelial cells and they were incubated with *Salmonella* for shorter times of 2 and 4 hours. However, this approach could be reconsidered and other treatments such as longer incubation times or employing different attenuated bacteria could provide additional insights. Unfortunately several of the mutated bacterial strains

used in this study were restricted in their ability to enter or replicate in the ES cells. This is one of the challenges presented by the use of a new model.

In order to further explore if ES cells can be realistically applied to studies of infectious disease, they were differentiated into dendritic cells, a cell type relevant to the host's fight against infection. The approach also provides a means to possibly generate large quantities of DCs to further advance research on antigenic presentation and immunization. Overall, DCs are perhaps less well studied compared to macrophages since they are less easily produced or differentiated *ex-vivo*, because they can be involuntarily activated during manipulation. The majority of DCs employed in research are obtained from bone marrow, derived from the femurs of mice. These cells have a limited life span and the outcome of experiments can vary depending on the age of the mice, the mouse strain and the differentiation protocol used. The interactions between *Salmonella* and bone marrow derived DCs have been the object of previous studies. However, there is still some controversy on how *Salmonella* and DCs interact due to the contradictory results obtained by different laboratories focusing on the localization of lysosomal markers and the SCV. In this study *Salmonella* intracellular localization within esDCs coincided with both the early endosome marker EEA-1 and the lysosomal markers LAMP-1 and 2. Also, the contribution that DCs bring to the host fight against this intracellular pathogen is not completely defined. It is speculated that DC's cross-epithelial protrusions and by sampling the lumen content might inadvertently help spread the pathogen. The protocol used in this study offers an efficient method to produce DCs *in vitro* thus providing an opportunity to further study DC-*Salmonella* interactions. In addition, the protocol to differentiate ES cells into esDCs has the potential to be further optimized in order to obtain different subpopulations of DCs. This will require further investigation into the dynamics of different DC subpopulation differentiation *in vivo* and *in vitro*.

In this report a transcriptomic analysis of esDCs during infection with *Salmonella* Typhimurium highlighted several distinct pathways that were differentially expressed. Some of these observations confirmed interactions previously described between *Salmonella* and DCs. For example, up-regulation of the IL-12 and IL-4 pathways and the activation of SOCS proteins and Cyclin D1 was reported here and in other similar studies. However, one of the most interesting aspects of this data was the relatively high

number of down-regulated pathways revealed by the analysis. This was a little unexpected since the statistical method used to analyze the data has a very low rate of false discovery for negatively-regulated genes. However, using my data the number of pathways down-regulated was still higher than those up-regulated. Many of these down-regulated pathways are involved in oxidative processes. Perhaps there is more to explore in terms of the pathways down-regulated during infection than in those up-regulated. Obviously, computational analysis plays a pivotal role in functional genomic research. To advance in this field new powerful analytical tools need to take into consideration complex cell signaling-dynamics in time and space allowing for special relocations, organization of different proteins complexes, kinetic data and post-translational modifications (Kholodenko, 2006). In the mean time it is important to integrate gene transcription observations with other techniques such as proteomic analysis.

Taken together the results obtained in this work provide encouragement for the applicability of ES cell models to infectious disease research for several reasons. Firstly, ES cells present a normal karyotype and they can replicate *in vitro* for a potentially infinite number of passages whilst still maintaining 'normal' characteristics. Secondly, ES cells can be genetically manipulated and therefore new genes important in host-pathogen interactions can be highlighted. In addition, it has been proposed that ES cells represent the best type of cell to use for this purpose because, by mutating one cell type, the effect of the same mutation can be investigated in either *in vitro* differentiated cells or *in vivo* produced chimeric mice. Following in this direction, conditional mutations will have an important role to play with the formation of a public mutagenesis library opening the possibility of applying these mutants to any kind of research (Sparwasser & Eberl, 2007). The usefulness of mutant knockout mice in host-pathogen interaction analysis has already been proven by the discovery of the NRAMP gene (Lara-Tejero *et al.*, 2006).

In today's medical and therapeutic scene, DCs are assuming a growing importance as 'targeting' vaccines allowing the medical research community to forge immunotherapic strategies around applications that directly regulate immunity (Plotkin, 2005). For this reason the elucidation of pathways induced by specific molecules that stimulate DC maturation is becoming more important and these advances have the potential for use in

genetically engineered vaccines. In the future, vaccines based on the targeting and manipulation of DCs will find application in infectious diseases as well as in the fight against autoimmune disorders and cancer (Pal *et al.*, 2007). Microarray technology will have an important role to play in this research.

Future directions that this research can take are numerous, including ES cell differentiation, mutation and the development of new cellular techniques. The ability of ES cells to differentiate into any somatic cell lineage opens up many options. For example, the differentiation of gut-like structures from *in vitro* EBs has already been described (Torihashi, 2006). This technique could be applied to study host-pathogen interactions by rebuilding, *in vitro,* the structures found *in vivo*. Also, from a mutational point of view, it will be very interesting to see if the ‘genetics-squared’ (Persson & Vance, 2007) approach that combines host and pathogen genetic manipulation will prove to be a key player in the study of host-pathogen interactions. Finally, one of the next goals in this field is to knockout not just a single gene but a whole pathway to investigate its relationship to pathogenicity. It is possible to imagine the induction of pathways normally down-regulated during infection in order to investigate their contra-effects on the progression of the disease. For this purpose conditional genetic manipulation will be a key factor in understanding the mechanisms that regulate genomic expression.

Lastly, the growing interest in immunomics reflects the urgency of researchers and society to see research move forward in this field (Braga-Neto & Marques, 2006). The application of DNA microarrays, used to identify genomic regulatory networks gives remarkable insight into the host systems. However this represents only one aspect of immunomics which makes use of antibody, peptide, peptide-MHC and cell microarrays. For further advancements in the battle against pathogens a deeper understanding of host-pathogen interactions is needed and future research will benefit from the introduction of new techniques and technologies. In relation to the study reported here it positively paves the way for future applications of ES cells in the field of infectious diseases and host-pathogen interactions.