2 Materials and Methods

2.1 Materials

2.1.1 Bacteria

Strains

Shigella flexneri wild type M90T serotype 5a.

Shigella flexneri M90T mutant Sh42 *dsbA33G* Str^r (Yu *et al.*, 2000) and harboring the plasmid pJKD18 expressing GFP constitutively, produced by Dr. Yu.

Shigella flexneri M90T mutant ΔMxiD ($MxiD^-$) kindly donated by Professor Sansonetti's laboratory.

Salmonella enterica serovar Typhimurium virulent wild type SL1344 and expressing GFP on p1C/1 vector (SL1344/p1C/1) under the control of the promoter *ssaG* (McKelvie *et al.*, 2004).

S. Typhimurium SL1344 Mutant *Sip*B obtained by insertion of a Kanamycin (Kan) resistance cassette, produced by Dr. Yu.

Bacterial Medium

Luria-Bertani (LB) broth: in 1L of water, 10g of Tryptone/Peptone, 5gr Yeast Extract, 10gr Sodium Chloride (NaCl);

LB agar plate, 15gr/L of Agar in LB broth;

Congo red (SIGMA) 1% stock solution;

Ampicillin (Roche) 100μg/ml final concentration;

Kanamycin (Roche) 50μg/ml final concentration.

2.1.2 Cell lines

AB2.2 murine embryonic stem cells, from mouse 129/Sv/EvBRD-Hprtb-m2, a gift from Professor Allan Bradley's Laboratory (WTSI);

J774A.1, mouse hybridoma macrophages-like cells ECACC number 91051511;

Hep2, human hybridoma epithelial cell line ECACC number 86030501;

MF2.2d9 T cell hybridoma recognizing $OVA_{265-280}$ peptide presented on $I\text{-}A^b$, a gift from Dr. Kenneth L. Rock (University of Massachusetts Medical School);
STO's/SNL76/7 murine embryonic feeder cells, a gift from Professor Allan Bradley's Laboratory (WTSI).

2.1.3 Tissue culture

Dimethyl Sulphoxide Biotechnology performance certified (DMSO) (SIGMA);
Dulbecco's Phosphate buffered saline solution (DPBS) $CaCl_2^-$ $MgCl_2^-$ (GIBCO);
Gelatin 2% Solution Type B: from bovine skin (SIGMA);
Cell Dissociation Solution (1x) non-enzymatic (SIGMA);
Trypan blue solution (0.4%) (SIGMA);
25cm^2, 75cm^2 Culture Flasks (Corning);
Cell Culture Dishes, 100mmx20mm (Corning);
EDTA tetrasodium salt (SIGMA);
Trypsin 2.5% solution (Invitrogen);
Chicken serum (Invitrogen);
Glasgow's Minimum Essential Medium (GMEM) (SIGMA);
Knockout Dulbecco's Modified Eagle's Medium (DMEM) Optimized for ES cells 1X (+4.5g/L D-Glucose, +Sodium Pyruvate, GIBCO);
Eagle's Minimum Essential Medium (EMEM) (SIGMA);
Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO);
Foetal Bovine Serum (FBS) (Hyclone);
Foetal Calf Serum (FCS) (SIGMA);
L-Glutamine 200mM (GIBCO);
Sodium Pyruvate 100mM (GIBCO);
MEM-NEAA 100x (GIBCO);
2-Mercaptoethanol (2-ME) 99% (SIGMA);
Penicillin/streptomycin solution 100x (SIGMA);
Leucocyte Inhibitory Factor (LIF) produced in house.

2.1.4 *In vitro* infection

Gentamicin solution 10mg/ml (SIGMA), TRITON-X100 (SIGMA), DPBS $CaCl_2^+$ $MgCl_2^+$ (SIGMA), LB-agar plates.

2.1.5 Flow cytometry

Becton Dickinson FACsAria Cell-Sorting System operated by DIVA software v4.

Buffers were all made in DPBS $CaCl_2^-$ $MgCl_2^-$ (GIBCO).

Calibride beads (BD Biosciences).

- Staining Buffer: 5% Heat Inactivated (HI) (56ºC for 30min) Fetal Calf Serum (SIGMA), 0.1% Sodium Azide (SIGMA), sterile-filtered;
- Sorting Buffer: 5% HI-FCS filtered sterile;
- Fixing Buffer: 1% Para-formaldehyde (SIGMA) sterile-filtered;
- Saponin Buffer: 0.5% Saponin (SIGMA), 1% Bovine Serum Albumin (BSA) (SIGMA), 0.1% Sodium Azide (SIGMA), sterile-filtered.
- RNA*later*® solution (Ambion)
- BD Cytometric Bead Array for cytokine quantification: Mouse/Rat soluble protein master buffer kit (BD Biosciences); BD CBA Mouse Inflammation kit (BD Biosciences); BD CBA Mouse IL-2 Flex Set (BD Biosciences).

For antibodies used see Table 2.1 and Table 2.2 for relative isotype antibodies.

Table 2.1 Antibodies for FACS analysis of murine ES and esDC characterization

Target Antigen	Source	Isotype	Conjugated to	Manufacture
h/b/m Integrin α6 (CD49f)	Rat	IgG2a	allophycocyanin	R&D Systems
hOct 3/4	Rat	IgG2b	phycoerythrin	R&D Systems
CD11b	Rat	IgG2b	allophycocyanin	BD Pharmingen
CD11c	Hamster	IgG1	phycoerythrin	BD Pharmingen
H-2K^b	Mouse	IgG2a	fluorescein isothiocyanate	BD Pharmingen
I-A/I-E	Rat	IgG2a	fluorescein isothiocyanate	BD Pharmingen
CD40	Hamster	IgM	fluorescein isothiocyanate	BD Pharmingen
CD44	Rat	IgG2b	phycoerythrin-Cy5	BD Pharmingen
CD45	Rat	IgG2b	phycoerythrin	BD Pharmingen
CD54	Hamster	IgG1	fluorescein isothiocyanate	BD Pharmingen
CD80	Hamster	IgG2	phycoerythrin	BD Pharmingen
CD86	Rat	IgG2a	fluorescein isothiocyanate	BD Pharmingen
F4/80	Rat	IgG2b	fluorescein isothiocyanate	AbDserotec
DC-SIGN	Rat	IgG2a	phycoerythrin	eBioscience
CD205	Rat	IgG2a	allophycocyanin	MACS
Ly-6C (GR1)	Rat	IgG2b	phycoerythrin	BD Pharmingen
CD4	Rat	IgG2a	allophycocyanin	BD Pharmingen
CD8	Rat	IgG2a	phycoerythrin	BD Pharmingen
TLR2	Rat	IgG2b	fluorescein isothiocyanate	Santa Cruz Biotechnology
TLR4	Rat	IgG2a	phycoerythrin	AbCam
TLR5	Mouse	IgG2a	fluorescein isothiocyanate	AbCam
TLR9	Mouse	IgG2a	fluorescein isothiocyanate	AbCam

Table 2.2 Isotype control antibodies for FACS analysis

These antibodies were tested on the mouse ES cells AB2.2. The isotype controls showed not cross reaction and specificity.

Target Antigen	Source	Isotype	Conjugated to	Manufacture
Isotype control	Hamster	IgG1	phycoerythrin	BD Pharmingen
Isotype control	Hamster	IgG1	fluorescein isothiocyanate	BD Pharmingen
Isotype control	Hamster	IgG2	phycoerythrin	BD Pharmingen
Isotype control	Hamster	IgM	fluorescein isothiocyanate	BD Pharmingen
Isotype control	Mouse	IgG2a	fluorescein isothiocyanate	BD Pharmingen
Isotype control	Rat	IgG2a	allophycocyanin	BD Pharmingen
Isotype control	Rat	IgG2a	fluorescein isothiocyanate	BD Pharmingen
Isotype control	Rat	IgG2a	phycoerythrin	BD Pharmingen
Isotype control	Rat	IgG2b	allophycocyanin	BD Pharmingen
Isotype control	Rat	IgG2b	phycoerythrin-Cy5	BD Pharmingen
Isotype control	Rat	IgG2b	phycoerythrin	BD Pharmingen
Isotype control	Rat	IgG2b	fluorescein isothiocyanate	BD Pharmingen

2.1.6 Bone marrow extraction materials

70% ethanol in water, 2 Petri dishes, 1ml syringes, 25 gauge needles, scissors and forceps, 25cm^2 flasks, RPMI-1640 (SIGMA), 100x penicillin/streptomycin solution

(SIGMA), HI-FCS, Iscove's Modified Dulbecco's Medium (IMDM (SIGMA)), 2-ME 99% (SIGMA), rmGM-CSF (R&D Systems), rmIL-4 (R&D Systems).

2.1.7 Cytokines

Recombinant Mouse Granulocyte-Macrophage Colony Stimulating Factor (rmGM-CSF) (R&D Systems), Recombinant Mouse Interleukin Mouse (rmIL-3) (R&D Systems), Recombinant Mouse Tumor Necrosis Factor alpha (rmTNFα)/TNFSF1A (R&D Systems), Recombinant Mouse Interleukin 4 (rmIL-4) (R&D Systems).

2.1.8 Confocal microscopy

Confocal ZEISS LSM-510;

Phalloidin TexasRedX (Invitrogen);

ProLong Gold antifade reagent with DAPI (Invitrogen);

Poly-L Lysine 0.01% solution (SIGMA);

DQ-OVA (Invitrogen).

For antibodies used see Table 2.3.

Table 2.3 Antibodies used for confocal analysis

Target Antigen	Source	Type	Conjugated to	Manufacture
EEA-1	Rabbit	polyclonal	primary antibody	AbCam
LAMP-1	Rat	monoclonal	primary antibody	AbCam
LAMP-2	Rat	monoclonal	primary antibody	AbCam
Rabbit IgG, F(ab')2	Goat	NA	APC-Cy7	Santa Cruz Biotechnology
Rat IgG, F(ab')2	Goat	NA	APC-Cy7	Santa Cruz Biotechnology

2.1.9 Antigen presentation assay

Concanavalin A from *Canavalia ensiformis* (SIGMA); *Salmonella* LPS (SIGMA); rmTNFα (R&D Systems), whole OVA (SIGMA); DQ-OVA (Invitrogen); Mitomycin C (SIGMA).

Cells employed esDC, BMDC and MF2.2d9.

2.1.10 RNA extraction

RNeasy Midi kit (QIAGEN); RNeasy Mini kit (QIAGEN).

2.1.11 RNA quantification and quality

Agilent RNA 6000 Nano Reagents (Agilent)

Agilent 2100 Bioanalyzer

NanoDrop1000 (Thermo Scientific)

2.1.12 Real Time RT-PCR

Stratagene Mx3000P Real-Time machine and system software version 2;

QuantiTect Reverse Transcription kit (QIAGEN);

SensiMix Plus SYBR® plus Fluorescein (QUANTACE);

For primers used see Tables 2.4 and 2.5.

Table 2.4 RT-PCR Primers to confirm microarray data from murine ES cells uninfected and infected

Gene Symbol	Forward primer	Reverse primer
β-act	CCGTGAAAAGATGACCCAGATC	CACAGCCTGGATGGCTACGT
Lrpap1	CAGGAGTACAATGTGCTGCTAGAC	CAGGAGTACAATGTGCTGCTAGAC
Ccng2	GTGAAAGTGAGGACTCTGGTGAAGA	CAAAGAAGAAGGTGCACTCCTGAT
Cyp1b1	CCTTTCCTTGGCCACTGATC	CTGGAAAACGTCGCCATAGC
Xbp1	AAGAACACGCTTGGGAATGG	CCGGCCACCAGCCTTACT
Herpud1	GGAGTGTGAGTCGCCTCAA	CAACAGCAGCTTCCCAGAAT
Ier3	CGGCGCCAGCTACCA	GATGGCGAACAGGAGAAAGAG
Lyst	CCAGTGCACTCGCCTTCTG	TGCGAGAACCAGCAATGCT
Pou4f2	GGCGATGCGGAGAGCTT	GCACGGGCCAGCAGACT
Socs3	CCACCCTCCAGCATCTTTGT	TCCAGGAACTCCCGAATGG
SpiC	CACGTCAGAGGCAACGCTAA	GGATTGGTGGAAGCCTCCTT
Stat2	GAACCGCTTGGAGAATTGGA	GGCTGTCAAGGTTCTGCAACA
Apaf1	GGCTGCTTTCTTTCGATTATCA	ATGACGAGCAACAGGATGTG
Bfar	TGCAGAGAAAAATGGGAAGG	TGAATGTCTTCAACTCGCATTC
Fst	GGCTGGATGGGAAAACCTAC	TTGGTCTGATCCACCACACA
Lamp2	CCTGACTCCTGTCGTTCAGA	GTAGTCGACGGGGCAGTG
RhoD	ACAACCTGCGGAAGAAAAGA	AGCCGAGCTGAACACTCAAG
Socs3*	CCTCGGGGACCATAGGAG	AACTTGCTGTGGGTGACCAT

* used in ASCA confirmation RT-PCR

Table 2.5 RT-PCR Primers to confirm microarray data from esDC uninfected and infected

Gene Symbol	Forward primer	Reverse primer
S18	GAACACCGAAAAATCGAGGA	CGGTTGAGCTTGGGTTTATC
Tyki	ATCTCGTGGCTTCTGAAATAGC	ACCTCAGTAGCTATGGCGTAGG
Oasl1	AGCGAAACTTCGTGAAGCA	GCTTCCCAGGCATAGACAGT
Cxcr4	ACGGCTGTAGAGCGAGTGTT	AGGGTTCCTTGTTGGAGTCA
H2-DMa	TGACAAAAGCTTCTGCGAGAT	GCTGATGAAACAGACCAACG
Lypla3	TGGCCTCCTGTTACCTCTGT	GTCCGTCTTCTTGGAGCAAA
IL6	GAGCCCACCAAGAACGATAG	GTGGTTGTCACCAGCATCAG
H2-T9	ACAGCTGTCTGAAAGGAATCTG	CTCCACATCGCAGCCTTG

2.1.13 Microarrays

2.1.13.1 Affymetrix array

GeneChip® Expression 3'-Amplification One-Cycle cDNA Syntesis kit (Affymetrix);

GeneChip® Expression 3'-Amplification Reagents for IVT labeling (Affymetrix);

RNeasy Mini Kit (QIAGEN);

Affymetrix GeneChip® Eukariotic Poly-A RNA Control Kit (Ambion for Affymetrix);

Sheared Salmon Sperm DNA (Ambion);

Bovine Serum Albumin (Invitrogen);

GeneChip® Mouse Genome 430 2.0 Array (Affymetrix);

GeneChip® IVT cRNA Clean up kit (QIAGEN for Affymetrix);

Non-stick RNase Free 1.5 and 2 ml Microfuge tubes (Ambion);

Nuclease-free water (Ambion);

DEPC-treated water (Ambion);

5M NaCl (Ambion);

Tween20 (SIGMA);

MES hydrate Sigma Ultra >99.5% titration (SIGMA) ;

20x SSPE Buffer (Ambion);

Affymetrix GeneChip Scanner 3000.

2.1.13.2 Illumina array

Illumina TotalPrep -96 RNA Amplification Kit (Ambion);

Whole-Genome Expression kit containing Mouse WG-6 v1.1 expression BeadChip with 45,000 spot of 50bp mer probes (Illumina);

Illumina Bead Scanner.

2.1.13.3 Microarray analysis tools

Bioconductor [www.bioconductor.org];

GeneSpring;

GEPAS: ASCA [www.gepas.bioinfo.cipf.es].

2.2 Methods

2.2.1 *In vitro* culture of mouse ES cell lines

Murine ES cell line AB2.2 (129/Sv/EvBRD-Hprtb-m2) produced by Professor Allan Bradley from the mouse strain 129/Sv/EvBrd was employed in this study. A description of this murine ES cell line can be found in these references (Adams *et al.*, 2005; Bradley *et al.*, 1984; Ramirez-Solis *et al.*, 1995). The cells were maintained in GMEM media with 10% HI-FBS (Hyclone), 2mM L-Glutamine, 1mM Sodium Pyruvate, 1x MEM-NEAA, 1:1000 of 2-ME stock solution (70µl of 2-ME 99% in 20ml water) and 500-1000U/ml Leukocyte Inhibitory Factor (LIF) (Williams *et al.*, 1988) produced in house, unfiltered. The ES cells were grown on a layer of 0.1% gelatin in DPBS. To split the culture, the cells were first washed with warm DPBS and then trypsinized. The trypsin solution was made with 100mg EDTA tetrasodium salt in 500 ml DPBS containing 10ml 2.5% trypsin solution and 5ml chicken serum and stored at -20°C. The cells were sub-cultured every two days at about 1:5 dilution. To freeze murine ES cells, culture medium was mixed with 10% DMSO and filtered.

2.2.2 *In vitro* culture of Hep2 and J774A.1 and MF2.2d9

The Hep2 cell line is a HeLa derived cell line (human cervix carcinoma). They are considered similar to human epithelial cells. They grow in EMEM media supplemented with 10% HI-FCS (SIGMA), 2mM L-Glutamine and 1x MEM-NEAA, filtered. These cells are adherent forming a monolayer on culture flasks, to passage them they were washed with warm DPBS and trypsinized and sub-cultured 1:3 every two days.

The J774A.1 cell line was cultured in DMEM with 10% HI-FCS, 2mM L-Glutamine, 1mM Sodium Pyruvate, 1x MEM-NEAA, 1:1000 of 2-ME stock solution prepared (70µl of 2-ME 99% in 20ml water), filtered. J774A.1 cells adhere to the culture plastic and they were sub-cultured 1:3 every two days and detached by scraping.

The MF2.2d9 cell line grows in suspension in the same media used for J774A.1. They were sub-cultured 1:5 every 5 days.

2.2.3 Bacterial strains

This study utilised the mutant strain *Shigella flexneri* Sh42 derived from the wild type M90TS, serotype 5a. This strain is attenuated as a result of a substitution at A33G of the active site of the protein DsbA, a periplasmic disulfide bond catalyst (Yu *et al.*, 2000). This strain also harbours the plasmid pJKD18 expressing constitutively the GFP protein and Ampicillin resistance. The plasmid pJKD18 (constructed by Dr. Derek Pickard, Team 15, WTSI) was introduced by electroporation into the mutant strain *Shigella flexneri* Sh42 *dsbA33G*. Also a *Shigella flexneri mxiD*$^{-}$ mutant was employed in this study (a gift from Prof. Sansonetti laboratory) carrying a Kanamycin resistace (Allaoui *et al.*, 1993). These strains were grown on LB-agar plates containing 0.01% Congo Red with or without 100µg/ml Ampicillin or 50µg/ml of Kanamycin, at 37ºC and in liquid culture in LB broth supplemented with or without Ampicillin or Kanamycin at 37ºC shaken at 225 rpm/min.

Salmonella Typhimurium stains employed in this study are all derived from the virulent strain SL1344. The wild type strain SL1344 carrying the plasmid pJKD10 integrated with 166bp of the *ssaG* promoter and cloned into a GFP/LacZ reporter Vector p1C/1 is referred to as SL1344/p1C/1 (McKelvie *et al.*, 2004). *SsaG* is a gene from SPI-2 and therefore is activated once inside the host cell to generate expression of GFP. Also, a SL1344 *SipB* mutant was employed and it was constructed by Dr. Yu (Strathclyde University) by insertion of a Kanamycin resistance cassette disrupting the gene. The *Salmonella* strains were grown from frozen stocks on LB-agar plates and a few single colonies were grown in suspension at 37ºC shaking at 225rpm/min for 4½-5h and then a 1:50 diluted subculture was grown overnight at 37ºC in static conditions.

2.2.3.1 Invasion assay using *Shigella flexneri*

Human Hep2 cells were grown adherent on 24 wells plate whilst murine ES cell line AB2.2 was grown in 6 well plates coated with 0.1% gelatin solution in DPBS, to optimal condition. On the day of infection, the over night bacterial culture was diluted 1:100 in LB broth and grown until log phase (OD_{600}~0.3) at 37ºC with shaking at 225 rpm in an Innova 44 Incubator Shaker (New Brunswick Scientific). The bacteria were then inoculated into the 6 well plate, containing ES cells, at the MOI (Multiplicity of Infection) of 10 or 100 per well. In order to reach MOI 10, 100µl of bacterial culture

was used per well. In order to reach MOI 100, 1ml of bacteria culture was pelleted at maximum speed on a top bench microcentrifuge (Eppendorf), re-suspended in tissue culture medium and inoculated. Half the volumes were used to infect Hep2 cells in 24 well plates. The plate was centrifuged at 2000 rpm (671g) in a Sorvall Legend RT centrifuge rotor 75006445 (Sorvall Heraeus, Thermo Fisher Scientific) for 10 min at room temperature in order to assist/coordinate bacterial contact with the cells. After incubation for 30 min at 37ºC, 5% CO_2 the cells were washed with warm DPBS three times and incubated for 2 or 4 hours in complete media supplemented with 50µg/ml of Gentamicin.

The infected cells were then used either for viable count analysis (colony forming units CFU), immunohistochemistry or flow cytometric analysis. To confirm the MOI the seeded bacteria were serially diluted and each dilution was plated in triplicate on LB-agar plates.

2.2.3.2 Invasion assay with *S.* Typhimurium

Mouse J774A.1 macrophage-like cells and murine AB2.2 ES cells were seeded at $2.5x10^5$ and $2x10^5$ respectively in 6 well plates and grown until 90% confluent and in uncontaminated conditions. *Salmonella* SL1344, SL1344/p1C/1 or mutant *Sip*B, were grown for 4½-5 hours in 5ml LB, with or without Ampicillin selection, at 37ºC in shaking condition (225rpm/min) from a few single colonies. A further 1:50 dilution was cultured over night at 37ºC in static conditions. The overnight culture was diluted to have an OD_{600} of ~ 0.6, a further 1:10 dilution in culture medium was used to seed the murine cells. MOI ~ 10 was reached using 100µl and MOI 100 was achieved by seeding 1ml of the bacterial suspension. The cells were then incubated at 37ºC 5% CO_2 for 30 minutes before being washed with warm DPBS twice and incubated for 2 or 4 hours with complete medium containing 50µg/ml of Gentamicin antibiotic. After incubation the cells were washed with warm DPBS before being used for flow cytometric analysis, immunohistochemistry, colony forming units (CFUs) count or total RNA extraction. In order to confirm the MOI, the seeded bacterial suspension was serially diluted in DPBS and three replicates for each dilution were plated on LB-agar plates.

2.2.3.3 Viable count analysis or Gentamicin invasion assay

Infected human Hep2, mouse J774A.1 and murine AB2.2 ES cells, after incubation with Gentamicin, were washed twice with warm DPBS and lysed with either 500µl per well 0.1% TRITON-X100 solution in water or DPBS with shaking (110mov/min) for 5 min. To count *Shigella* or *Salmonella* CFUs, cell suspension lysates were serially diluted in DPBS and each dilution was plated in triplicate onto LB-agar plated with or without 100µl/ml Ampicillin or Kanamycin and incubated at 37°C over night. Inoculated bacteria were also plated in serial dilution to confirm the MOI applied to the cells.

2.2.4 Flow Cytometric Analyses

2.2.4.1 *In vitro* bacterial infection (Gentamicin invasion assay)

In vitro infection with *Shigella flexneri* M90T mutant Sh42/pJKD18 or *S.* Typhimurium SL1344/p1C/1 was followed by flow cytometric analysis monitoring the production of GFP using the 488nm Argon laser and recording in the FITC channel. Cultured J774A.1, AB2.2 ES cells, and esDCs once infected and incubated under Gentamicin selection for the indicated times, were washed twice with DPBS, fixed with 1% paraformaldehyde in DPBS for 20 min on ice, trypsinized and analyzed on a BD FACSAria (fluorescence-activated cell sorter) machine. Uninfected cells were used as negative control.

2.2.4.2 Murine ES cells characterization

Murine ES cells were washed with warm DPBS and trypsinized for 2min at 37ºC, complete medium was added and the cells were centrifuged at 1200rpm (424g) in a Sorvall Legend RT centrifuge rotor 75006445 (Sorvall Heraeus, Thermo Fisher Scientific) for 7 min, washed with DPBS, pelleted and fixed with Fixing Buffer for 20min on ice. The cells were divided in aliquots and incubated with the chosen antibody at the dilution suggested by the manufacturer, either in FACS Staining Buffer or Saponin Buffer if the marker was exposed on the surface or intracellularly respectively. The cells were washed twice before a final wash with DPBS and read on the FACSAria.

2.2.4.3 EsDC and BMDC surface markers

EsDCs or BMDCs were collected and seeded in 96 well plates at 10^4 cells per well and incubated over night with or without activation supplements, LPS 10μg/ml, OVA 10μ/ml or rmTNFα 5000WHOSU/ml, spun for 5 min at 2000rpm (671g) before aspirating the supernatant, washed with 100μl of DPBS Mg^+Ca^+, treated with cell dissociation buffer for 10min at 37ºC, pelleted and fixed with 1% paraformaldehyde in DPBS for 20 min on ice. The cells were pelleted once more, washed once with DPBS and stained in FACS Staining Buffer using the suggested manufacturers concentrations of antibody. See antibodies table 2.1.

2.2.4.4 Cell Sorting

In order to sort infected cells from uninfected cells, the FACSAria was first sterilized, washed and aligned as per the manual. Sorting was performed in sorting buffer at low pressure 30phi using a 100μm nozzle and the sorting mask 16-16-0. The uninfected and infected cells were sorted into sterile 15ml Falcon tubes containing 2ml of RNA*later*® solution from Ambion. The cells were pelleted at 671g or 2000rpm in a Sorvall Legend RT centrifuge rotor 75006445 (Sorvall Heraeus, Thermo Fisher Scientific) for 10min and total RNA was extracted using a QIAGEN RNeasy Mini kit. Also an uninfected sample of cells was sorted as negative control.

2.2.4.5 Cytometric Bead Array (CBA) Analysis

EsDC or BMDC were seeded in 96 well flat bottom plates at $1x10^5$ with 1:5, 5:1 and 1:1 ratios (each one in triplicate) of MF2.2d9 T cells. Whole OVA protein was added at 10μg/ml and incubated over night; as negative control DCs were seeded either alone or with T cells 1:1 without antigen and with rmTNFα 5000WHOSU/ml.

The concentrations of cytokines produced were measured utilising CBA kits and manufacturer's instructions were followed. Briefly, the standard curve dilutions, prepared freshly for each experiment, and the experimental culture supernatants were incubated with cytokine specific beads. After incubation, the beads were washed and incubated with the detection antibody, washed again and read on the FACSAria. The data obtained was processed with BD FCAP Array software which extrapolates the concentration of the sample in relation to the standard curve for each cytokine.

2.2.5 Immunofluorescence labeling

2.2.5.1 Murine ES cells infected by *Shigella flexneri* or *S.* Typhimurium

To observe *Shigella* Sh42 *dsbA33C* mutant or *S.* Typhimurium infection of murine AB2.2 ES cells, the latter were grown in 24 well plates for two days on sterile glass cover-slips pretreated with acetone and coated with 0.1% gelatin solution. The Gentamicin invasion assay was performed and the infected ES cells washed with warm DPBS and fixed in 1% paraformaldehyde solution (20 min) on ice. After one rinse with DPBS, the ES cells were permeabilised with Saponin Buffer for 2 min at RT and stained with primary antibodies rabbit anti-mouse EAA-1, rat anti-mouse LAMP1 or LAMP2 at the concentration suggested by the manufacturer and incubated for 30-40 min at RT. Following two washes with Saponin Buffer, the cells were further stained with secondary antibody, goat anti-rabbit or goat anti-rat APC-Cy7 conjugate, at the concentration suggested by the manufacturer and the cells were incubated for 30-40 min in the dark. Next, the infected ES cells were washed with Saponin solution twice before one last wash in DPBS and they were mounted on a glass slide with ProLong Gold antifade reagent with DAPI. To stain actin filaments the cells were permeabilised and incubated with TexasRed-X phalloidin in the dark before been washed with Saponin Buffer and mounted on the glass slide.

2.2.5.2 EsDC immunofluorescence labeling

To observe antigen processing, esDCs were grown for two days on glass cover-slips treated with Poly-L-Lysine solution, then treated with DQ-OVA for 2 hours, washed with Hanks' Balanced Salt Solution (HBSS, SIGMA), followed by the addition of mitomycin-C 25μg/ml for 30 min before 4 hours incubation, they were then washed with DPBS, fixed and mounted on glass slides ready for imaging. Alternatively, esDCs were incubated with OVA, LPS or rmTNFα overnight, washed with DPBS, fixed and permeabilised with Saponin Buffer or not as indicated, before been stained with MHC class II antibody FITC conjugated.

To track bacterial cellular location, infected esDCs were treated as ES cells and at the indicated time the cells were washed and fixed with paraformaldehyde buffer. They were then washed, permeabilised with Saponin buffer and stained with primary antibodies against endosomal and lysosomal components EEA-1, LAMP-1 or LAMP-2 respectively, at the manufacturer's recommended dilutions, for 30-40 min, prior to being washed again. Finally they were stained with secondary antibodies goat anti-rabbit or goat anti-rat conjugated to APC-Cy7 at the manufacturer's recommended dilution. After washing they were mounted on glass slides with ProLong Gold containing DAPI.

2.2.6 Total RNA extraction, quantification and Bioanalyzer analysis

The RNA from uninfected and infected ES cells line AB2.2 was extracted following the QIAGEN RNeasy Midi kit instructions.

The RNA from esDCs uninfected and infected with *S.* Typhimurium was extracted with the QIAGEN RNeasy Mini Kit.

The total RNA quantity was measured by Spectrophotometer NanoDrop-1000 v 3.1.0 and the RNA quality was analyzed using an Agilent 2100 Bioanalyzer following manufacturer's instructions.

2.2.7 Affymetrix Microarray

Affymetrix technical manual instructions for GeneChip® expression analysis were followed.

Five μg of total RNA extracted from murine ES cells uninfected and infected were employed for cRNA synthesis. First, double stranded cDNA was synthesized and cleaned using the eukaryotic One-Cycle cDNA Synthesis Affymetrix protocol. This was used as the template to synthesize Biotin-Labeled cRNA using the GeneChip IVT Labeling Kit following the Affymetrix protocol. The cRNA was cleaned, quantified (NanoDrop1000) and analyzed by Bioanalyzer (Agilent) before being fragmented (GeneChip Expression Analysis, Technical Manual Rev. 5, AFFYMETRIX, 2004). Then 15μg of fragmented biotin-labeled cRNA was hybridized onto Affymetrix GeneChip® Mouse Genome 430 2.0 arrays at 45ºC in a rotator mixer at 60rpm, overnight. The chips were washed with Non-Stringent wash buffer and stained with

SAPE Stain Solution and the Antibody solution automatically by the Fluidics Station 450. Also, the fluidics station automatically washed the chips before they were scanned on the GeneChip Scanner 3000, both supported by the software GCOS. Affymetrix Mouse GeneChip® 430 2.0 array comprises 45,000 probe sets representing over 34,000 well-substantiated mouse genes.

2.2.8 Real Time RT-PCR

RT-PCR was performed to confirm the microarray expression data obtained by bioinformatics analysis. cDNA was produced using the same total RNA samples used in the microarray analysis. For cDNA synthesis, QuantiTect Rev. Transcription kit was used and 1μg of total RNA from murine AB2.2 cells uninfected and infected and 30ng of total RNA obtained from esDCs uninfected and infected was transcribed. Manufacturers instructions were used and basically, sample RNA was mixed with water in the presence of RNase and incubated at 42ºC for 2 minutes, this was followed by the addition of polymerase and nucleotide mix plus 10x buffer. The cocktail was incubated for a further 20 min at 42 ºC.

Primers were designed using the primer3 website [http://frodo.wi.mit.edu/primer3] in a way such that they would overlap between two consecutive exons, they would have GC content > 45% and result in a product size between 150 and 200bp. The primers were ordered from SIGMA at 100μM concentration. The RT-PCR reactions were performed using SYBR green dye from Quantace SensiMixPlus SYBR kit. SYBR green binds to double stranded DNA and absorbs light at 488 nm and emits green light at 522nm. A STRATAGENE Mx3000P RT-PCR machine was used. The RT-PCRs were performed following the manufacturers instructions using temperatures and cycle times as indicated i.e. one cycle of 95ºC 10min, followed by 40/45 cycles of 95ºC 15 sec, 58/60ºC 30sec, 72ºC 30sec and then one cycle of each 95ºC 60sec, 55ºC 60sec, 95ºC 60sec.

Each sample was run in triplicate for each biological replicate. To test the primers for specificity and quality, efficiency curves were performed and dissociation products were obtained.

The Ct (theshold cycle) values obtained were analyzed for relative quantification. The method employed uses ΔΔCt value to compare the Ct value of a target gene to the Ct value of the control gene.

2.2.9 Dendritic cell differentiation

2.2.9.1 Differentiation of murine ES cells into dendritic cells

Murine AB2.2 ES cells were induced to differentiate into dendritic cells following the protocol published by Dr. Fairchild (Fairchild *et al.*, 2000; Fairchild *et al.*, 2003; Fairchild *et al.*, 2007). AB2.2 (Bradley *et al.*, 1984) mouse ES cell line at passage 9 were grown with Knockout Dulbecco's Modified Eagle's Medium (DMEM) optimized for ES cells, 15% HI-FBS (Hyclone # CPC0285), 2mM L-Glutamine; 1mM Sodium Pyruvate, 1x MEM-NEAA, 1:1000 of 2-ME stock solution (70µl of 2-ME 99% in 20ml water), unfiltered. After thawing, the cells were grown for 2 passages on irradiated feeder cells STO's/SNL 76/7 and then for two passages in media containing 500-1000U/ml LIF in order to eliminate the feeder cells from the culture. ES cells were then seeded at $4x10^5$ cells in 20ml medium without LIF in Petri dishes and grown for 14 days until Embryoid Bodies (EBs) were formed. Fourteen day old EBs were seeded in 20 ml of medium containing recombinant mouse GM-CSF 25ng/ml and recombinant mouse IL-3 200WHOSU/ml in culture dishes. EBs appeared as cystic or non-cystic floating cluster of cells. After 48 hours on culture dishes, the EBs attached to the culture plastic and some of them started to beat as a sign of 'good health', some did not attach and were discarded during subsequent medium change. In the presence of rmGM-CSF and rmIL3, EBs started to produce, from the edge of the attached site, DC-like cells that looked lightly attached to the plastic named here esDC. EsDCs were harvested by gently rinsing the surface of the EBs with media.

2.2.9.2 Bone marrow extraction and dendritic cells differentiation

Mice 129/Sv were killed at 5-10 week of age and sprayed with 70% ethanol, the back legs were freed by cutting the skin and the femur and the tibia bones were cleaned from the muscle. The bone marrow was extracted from these bones. In a new Petri dish containing 10ml of RPMI-1640 media supplemented with 1x penicillin/ streptomycin

antibiotic and 2% HI-FCS, a 25g needle was inserted inside the bone and the cavity flushed with about one ml of media. The resulting threads of bone marrow were broken by flushing with a 5ml syringe minus a needle. Finally, the 10 ml of media containing isolated cells was transferred into a 15ml tube and allowed to settle for 5min. The supernatant was aspirated without touching the bottom of the tube and transferred into a 50ml falcon tube and the cells were centrifuged at 1200rpm (242g) for 10min. The cells were resuspended in filtered IMDM media containing 10% HI-FCS, 2mM L-Glutamine, 1mM Sodium Pyruvate, 1x MEM-NEAA, 1:1000 of 2-ME stock solution prepared (70µl of 2-ME 99% in 20ml tissue culture water) and 1x of penicillin/streptomycin and counted. The volume was adjusted to give ~$2x10^6$ cells in 10ml of media per $25cm^2$ flask. To the media was added rmGM-CSF 500 WHO Standard Units/ml and rmIL-4 at 150 WHOSU/ml. The cells were incubated for two days at 37ºC prior to changing 75% of the medium. Another incubation period of 4 days at 37ºC without disturbing led to the development of BMDC which were harvested from day 6 to 9 of incubation.

2.2.9.3 Antigen presentation assay

Murine esDCs and BMDCs were seeded at $1x10^5$ cells per well in 96 well plates with flat bottoms with ratios of 1:5, 5:1 or 1:1 (each one in triplicate) of T cell line MF2.2d9 cells in 150µl of medium specific for each DC cell. Whole OVA protein was added at 10µg/ml and incubated over night. As a negative control, either DCs were seeded alone or with T cells 1:1 either without specific antigen or with TNFα 5000 WHOSU/ml.
As positive control $3x10^5$ MF2.2d9 T cells were incubated with 5µg/ml of ConA. All cells were incubated for 24 or 48 hours at 37ºC and the supernatants obtained without disturbing the cells were stored at -20ºC.

2.2.9.4 Cytometric Bead Array

The concentration of cytokines was measured with Cytometric Bead Array (CBA) Mouse inflammation kits which detects the cytokines IL-6, IL-10, IL-12, TNF, MCP-1 and IFNγ. The detection limits are different for each cytokine and they are 5pg/ml, 17.5pg/ml, 10.7pg/ml, 7.3pg/ml, 52.5pg/ml and 2.5pg/ml respectively. Also, Mouse CBA IL-2 Flex Kits were used to measure T cell activation. Manufacturer's instructions were followed. Briefly, standard curves for each cytokine were prepared and then the

control dilutions of experimental supernatants were incubated with cytokine specific beads. After washing and incubation with detection Antibody, the beads were washed once more and read on the FACSAria. The data obtained was processed with FCAP Array software which quantifies the concentration of the sample in relation to the standard curve of each cytokine.

2.2.10 Illumina microarray hybridization

Manual instructions were followed. Briefly, Biotin-labeled cRNA was synthesized from 300ng total RNA with the TotalPrep RNA Amplification kit (Ambion, Foster City, CA, USA). The hybridization mix containing 1500ng of labeled cRNA was prepared according to the Illumina BeadStation500x System Manual (Illumina, San Diego, CA, USA). The hybridization of Illumina MouseWG-6 v1.1 Expression BeadChip array was done incubating the chips at 58ºC over night on the BeadChip Hyb Wheel. The BeadChips were washed with E1BC solution for 15 min, then washed in 100% Ethanol for 10 min and a final wash in E1BC for 2min. The blocking reaction was performed using Block Buffer E1 on a rocker mixer for 10 min. The staining reaction was performed using 2ml of buffer E1 with 1μg/ml of Cy3-streptavidin followed by a 5min wash with buffer E1BC. The BeadChips were then dried by centrifugation and scanned using the Illumina BeadStation 500 platform operated by Illumina BeadArray Reader software.