2 Materials and methods

2.1 Materials

2.1.1 Bacterial strains

Salmonella enterica serovar Typhimurium SL1344 (naturally streptomycin resistant) was the wild type strain used in this study. Also used was an SL1344 Δ*InvA* derivative lacking the ability to invade epithelial cells (streptomycin and kanamycin resistant) [114].

2.1.2 Oligonucleotides

2.1.2.1 Sequencing of 16S rRNA genes

The following oligonucleotides were used in PCR of bacterial V1-V2 16S rRNA gene regions for microbial community analysis.

27F = AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT CC AGMGTTYGATYMTGGCTCAG

1 = 5' Illumina adapter, 2 = Forward primer pad, 3 = Forward primer linker, 4 = Forward primer.

338R = CAAGCAGAAGACGGCATACGAGAT nnnnnnnnnnnn AGTCAGTCAG AA GCTGCCTCCCGTAGGAGT

1 = Reverse complement of 3′ Illumina adapter, 2 = Golay barcode, 3 = Reverse primer pad, 4 = Reverse primer linker, 5 = Reverse primer.

The Golay barcode is unique for each sample, as described previously [195].

2.1.2.2 Primers used in quantitative polymerase chain reaction (qPCR)

IL10rb spanning exons 1 and 2: Applied biosystems TaqMan® Gene Expression Assay Mm00434153_m1

IL10rb spanning exons 3 and 4: Applied biosystems TaqMan® Gene Expression Assay Mm00434155_m1

IL22ra1 spanning exons 4 and 5: Applied biosystems TaqMan® Gene Expression Assay Mm01192943_m1

2.1.3 Antibodies

Target	Clone	Host	Fluorochrome/ conjugate	Source	Experiment(s) and dilution(s)
C3c	-	Rabbit	FITC	Dako	Western blotting (1 in 2000)
C3d	-	Goat	-	R & D Systems	Western blotting (1 in 5000) Fluorescence microscopy (1 in 10)
C3	-	Rabbit	-	LSBio	Fluorescence microscopy (1 in 10)
CD34	MEC 14.7	Rat	-	Abcam	Fluorescence microscopy (1 in 50)
β-tubulin	-	Rabbit	-	Abcam	Western blotting (1 in 50)
Salmonella common structural antigens (CSA)	-	Goat	FITC	KPL	Fluorescence microscopy (1 in 50)
CD4	RM4-5	Rat	BV510	Biolegend	Flow cytometry (1 in 3000)
CD8a	53-6.7	Rat	APC-H7	BD	Flow cytometry (1 in 200)
CD19	1D3	Rat	PE-CF594	BD	Flow cytometry (1 in 2000)
Ly6G	1A8	Rat	V450	BD	Flow cytometry (1 in 600)
Ly6B	7/4	Rat	FITC	Serotec	Flow cytometry (1 in 1000)
CD115	AFS98	Rat	APC	Biolegend	Flow cytometry (1 in 500)
CD3	145-2C11	Armenian Hamster	PerCP-Cy5.5	Biolegend	Flow cytometry (1 in 400)
CD11b	M1/70	Rat	PE-Cy7	Biolgened	Flow cytometry (1 in 2000)
CD45	30-F11	Rat	Alexa Fluor 700	Biolegend	Flow cytometry (1 in 600)
Goat IgG H & L	-	Donkey	Alexa Fluor 594	Abcam	Fluorescence microscopy (1 in 1000)
Rat IgG H & L	-	Goat	FITC	Abcam	Fluorescence microscopy (1 in 1000)
Rabbit IgG H & L	-	Goat	Cy3	Abcam	Fluorescence microscopy (1 in 1000)
Rabbit immunoglobulins	-	Goat	HRP	Dako	Western blotting (1 in 2000)
Goat IgG	-	Chicken	HRP	R & D systems	Western blotting (1 in 5000)

Table 2.1. Antibodies used in this study

2.1.4 Mice

All experiments were performed using 6 - 10 week old mice maintained in specific pathogen-free conditions, matched by age and sex within experiments. All mice were of C57BL/6 genetic background. Mice were housed in sterilised cages with food and water available *ad libitum.* All mice were killed by cervical dislocation. The care and use of all mice was in accordance with UK Home Office regulations (United Kingdom Animals Scientific

Procedures Act 1986). Mice were monitored throughout experiments for clinical symptoms including piloerection, hunched gait and lethargy, and mice displaying signs of severe disease were killed to prevent further suffering.

2.2 Methods

2.2.1 *S.* Typhimurium culture and preparation of inoculums

Bacteria were picked from Luria-Bertani (LB) agar plates containing appropriate antibiotic (30 µg/ml kanamycin and/or 50 µg/ml streptomycin sulphate) and grown overnight in 20 ml LB broth in a non-baffled flask at 37 °C with constant shaking at 190 rpm with appropriate antibiotics (concentrations as for plates). For infection of mice overnight cultures were serially diluted in sterile phosphate-buffered saline (PBS) to achieve a final dilution of 1 in 100,000. Following infection inoculum was serially diluted in PBS and plated on LB agar to confirm the CFU administered.

2.2.2 *In vivo* experiments and tissue harvesting

2.2.2.1 Streptomycin pre-treatment and infection with *S.* Typhimurium

Mice were treated 24 h prior to infection with 50 mg streptomycin: 200 µl of 250 mg/ml streptomycin sulphate in sterile water delivered via oral gavage. Mice were infected via oral gavage with between 8 x 10^3 and 2 x 10^5 CFU of *S.* Typhimurium in a 200 µl inoculum while naïve controls received 200 µl PBS. Oral gavage was performed with a sterilised blunt-tipped gavage needle under anaesthesia with isoflurane.

2.2.2.2 Enumeration of *S.* Typhimurium counts in tissue

Caecum, colon, small intestine, liver and spleen were removed from mice following confirmation of death for analysis of tissue-associated *S.* Typhimurium CFU. Caecum was cut open longitudinally and content removed by gentle scraping with tweezers. Colon and small intestinal content were removed by gently dragging tweezers along the length of the intact tissue. Tissue was mechanically disrupted (Steward stomacher 80, 2 min at high speed) in 5 ml of sterile water. Serial dilutions were made in PBS and plated on LB agar plates containing streptomycin (50 µg/ml). Colonies were counted following overnight growth at

37 °C. Implements used for the collection of material were cleaned thoroughly with 70% ethanol between samples.

2.2.2.3 Harvesting of tissue for RNAseq

Following confirmation of death caecum was removed from mice and divided into sections for further processing including RNA extraction for RNAseq analysis. 2 mm x 2 mm pieces of caecal tissue were cut reproducibly and placed in microtubes containing 1 ml RNAlater for RNA stabilisation. Tissue pieces were stored at -20 °C prior to RNA extraction.

2.2.2.4 Harvesting of intestinal content and tissue for microbiota analysis

All samples for microbiota analysis were placed in cryotubes and flash-frozen in liquid nitrogen immediately upon collection. For collection of faeces mice were placed in sterilised beakers and 1 - 2 faecal pellets removed and combined for analysis. Mice were killed at day 4 PI and following confirmation of death caecum and colon were removed. Caecum was cut open to allow gentle scraping of content from the surface of the tissue directly into a cryotube. A piece of caecal tissue approximately 1 cm in length from the region adjoining the colon was removed for analysis. For collection of colon content the colon was cut open longitudinally and content material closest to the proximal end removed for analysis. Care was taken to use sterile implements for the collection of samples for microbiota analysis, and implements were cleaned thoroughly with 70% ethanol between samples.

2.2.2.5 Harvesting and culture of peritoneal macrophages

4% w/v thioglycollate (Sigma) solution was prepared using ddH_2O water, autoclaved and stored at 4 °C. 250 µl thioglycollate was delivered to mice by intraperitoneal (IP) injection. At day 4 post-injection mice were killed and the peritoneal cavity washed with 8 - 10 ml of cold PBS. Isolated cells were centrifuged at 220 g for 15 min at room temperature and resuspended in warmed media (RPMI-1640 (Sigma), 10% heat-inactivated foetal calf serum (FCS) (Biosera), 2 mM L-glutamine (Invitrogen), 10,000 U/ml penicillin-streptomycin (Invitrogen)). Peritoneal cells were counted and seeded in 24-well plastic plates with two wells per mouse. Cells were allowed to fix to the culture dish overnight at 37 °C, 5% CO_2. The following day cells were washed with warmed PBS (37 °C) to remove non-adherent cells and peritoneal macrophages were used for RNA or protein extraction. For RNA extraction buffer RLT from an RNeasy Mini kit (Qiagen) was added directly to cells in the 24-well plate. Removal and lysis of cells was performed using a plastic cell scraper followed by transfer of lysate to a 1.5 ml microtube and vortexing. RNA was extracted according to the directions provided in the RNeasy kit accompanying handbook. For protein extraction chilled Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris pH 8, cOmplete protease inhibitor cocktail (Roche), in ddH_2O) was added directly to cells in the 24-well plate at 1 ml/10^7 cells. Adherent cells were removed and lysed using a plastic cell scraper and lysate transferred to a pre-cooled 1.5 ml microtube. Lysate was maintained under constant agitation for 30 min at 4 °C followed by centrifugation at 16,000 g for 20 min. Supernatant was transferred to chilled microtubes for protein concentration determination and further analysis.

2.2.2.6 Harvesting and culture of bone marrow-derived macrophages (BMDM)

Bone marrow cells were obtained by flushing marrow from bones of the hind legs with cold PBS using a 25-gauge needle. Cells were dissociated by gently passing the bone marrow suspension through an 18-gauge needle. Cells were centrifuged at 220 g for 15 min at room temperature and re-suspended in warmed media (RPMI-1640, 10% heat-inactivated FCS, 2 mM L-glutamine, 10,000 U/ml penicillin-streptomycin, 10% filtered supernatant from mouse L929 cell culture as the source of macrophage colony-stimulating factor (M-CSF)). Cells were plated in sterile 10 cm plastic petri dishes, two dishes per mouse, and incubated for 1 week at 37 °C and 5% CO_2. On day 3 post-harvest fresh warmed media was added and on day 5 post-harvest cells were washed with warmed PBS (37 °C) to remove non-adherent cells followed by addition of fresh media. On day 7 post-harvest fully-differentiated BMDM were used for RNA or protein extraction as described for peritoneal macrophages (in each case the solution for cell lysis was added directly to the petri dish).

2.2.3 Peripheral blood leukocyte analysis

Blood was obtained by cardiac puncture performed under anaesthesia with isoflurane and followed immediately by cervical dislocation. Harvested blood was transferred to 100 µl EDTA coated blood tubes and inverted to mix. 25 µl of whole blood was incubated in a 96-well plate for 20 min with a combination of antibodies for cell surface markers (CD4, CD8a, CD19, Ly6G, Ly6B, CD115, CD3, CD11b and CD45) diluted in 25 µl FACS buffer

(5% heat-inactivated FCS, 0.1% sodium azide in PBS) as listed in section 2.1.3. Cells were fixed for 5 min by addition of three volumes of CellFIX solution (BD Biosciences) followed by centrifugation at 400 g for 3 min, and the cell pellet resuspended in Pharm Lyse solution (BD Biosciences) for red blood cell lysis. Subsequently cells were incubated for 5 min, centrifuged again at 400 g for 3 min, and again resuspended in Pharm Lyse solution and incubated for 5 min. Finally cells were washed once in FACS buffer prior to resuspension in FACS buffer for flow cytometry analysis. All incubations were at room temperature. Compensation controls were prepared with UltraComp eBeads (eBioscience). Samples were analysed on a FACS Aria II (BD) and subsequent data analysis performed using the software FlowJo v7.6.5.

2.2.4 Microscopic analysis

2.2.4.1 Histopathological analysis

Tissue Processor and Stainer and Coverslipper machines were operated by Yvette Hooks (WTSI).

Following confirmation of death a 0.5 cm tubular section of caecum, close to the blind end and adjacent to the caecal patch, was excised, caecal content gently removed, and the tissue placed in 4% formaldehyde for 24 h. A 0.5 cm section of proximal colon and pieces of liver and spleen were placed in formaldehyde also. Permanent fixation was performed using a Sakura Vacuum Infiltration Processor 5 which moves samples through formaldehyde, a series of mixtures of ethanol and water for dehydration, and xylene to clear samples before immersion in molten paraffin wax. Samples were embedded in paraffin wax using an embedding station (Leica), and 5 µm sections cut using a RM2125 rotary microtome (Leica) and floated on a 37 °C water bath for 3 - 5 min. Sections were then transferred to Superfrost plus glass slides (VWR International).

Paraffin sections were deparaffinised, rehydrated, stained with Gill’s 2 hematoxylin (Leica) and eosin (Leica), dehydrated and mounted using a Leica Stainer and Coverslipper machine. Briefly, the machine moves slides through a series of solutions; xylene to deparaffinise, a series of mixtures of ethanol and water for rehydration and dehydration, and finally sections were mounted with and Leica mountant. Slides were visualised using a

LSM510 confocal microscope (Carl Zeiss Ltd.). Histopathological analysis was performed by Professor Mark Arends (University of Edinburgh Division of Pathology).

2.2.4.2 Immunofluorescence staining

For immunofluorescence staining caecal tissue was removed as described in section 2.2.4.1 and placed in 4% paraformaldehyde (PFA) for 1 h. Tissue underwent three washes of 10 min in PBS before transfer to 0.075 M glycine in PBS for 30 min for quenching of unreacted aldehyde groups. Fixed caecum pieces were embedded in optimal cutting temperature compound and frozen with cryofreeze aerosol (Agar scientific) before flash-freezing in liquid nitrogen. 5 μm cross sections of OCT-embedded caecum were cut using a cryostat and air-dried on poly-l-lysine-coated glass microscope slides for 2 h. Sections were outlined with a wax pen and incubated with blocking solution (10% FCS in RPMI 1640 culture medium) for 30 min. Blocking solution was replaced with primary antibody diluted in blocking solution as described in section 2.1.3. Unstained and 'secondary antibody-only' controls were also prepared with blocking solution only. Slides were incubated with primary antibody for 1 h and underwent three washes of 5 min in PBS. Secondary antibody diluted in blocking solution as described in section 2.1.3 was added to sections for 1 h after which sections again underwent three washes of 5 min in PBS. For staining of multiple antigens in a single section antibodies were added to consecutively as follows: primary 1 → wash → secondary 1 → wash → primary 2 → wash → secondary 2 → wash. Prolong Gold antifade reagent with DAPI (Life technologies) was applied before mounting and visualisation of sections with a Zeiss LSM510 confocal microscope.

2.2.4.3 Three-dimensional confocal imaging

Following removal caecum was cut open longitudinally and flushed with cold Hank's balanced salt solution containing 0.4 M N-acetyl-L-cysteine. Tissue was placed in 4% PFA in PBS for 2 h at room temperature for fixation and stored in 1% PFA in PBS at 4 °C prior to further processing. Tissue was washed three times in PBS before transfer to blocking and permeabilisation solution for an incubation of 4 h at room temperature (2% Triton X-100, 10% FCS, in PBS). Tissue was again washed three times in PBS before incubation with primary antibodies in 0.2% Triton X-100, 5% FCS, in PBS at 16 °C with gentle agitation overnight. Tissue was again washed three times in PBS before incubation with secondary antibodies in 0.2% Triton X-100, 5% FCS, in PBS at 4 °C with gentle agitation overnight.

Tissue was again washed three times in PBS followed by nuclear counterstaining with 10 nM DAPI dilactate in PBS for 1h at room temperature with gentle rocking. Tissue was washed six times in PBS and immersed in FocusClear (CelExplorer) and covered for 4 hours. Tissue was mounted in Prolong Gold prior to imaging with a Zeiss LSM510 confocal microscope.

2.2.5 RNA methods

2.2.5.1 RNA extraction

RNA was extracted from tissue using an RNeasy Mini kit (Qiagen) according to the directions provided in the accompanying handbook. Tissue disruption and cell lysis were performed in buffer RLT in a GentleMACS Dissociator (Miltenyi Biotec) using the pre-set program for total RNA extraction. The concentration and quality of extracted RNA were assessed using a Bioanalyzer (Agilent). Samples with an RNA Integrity Number RIN < 8 were excluded from further processing and analysis.

2.2.5.2 Reverse transcription and qPCR

Complementary DNA (cDNA) was synthesized from extracted RNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR performed for the validation of RNAseq (Section 3.3.6.1) was carried out using ABsolute Blue QPCR SYBR Green ROX mix (Thermo Scientific), and SYBR green primers. qPCR performed for analysis of gene expression in tm1a mutant mouse lines (Section 6.3.1) was carried out using ABsolute Blue QPCR ROX mix (Thermo Scientific) and TaqMan probe-based gene expression analysis.

2.2.5.3 RNAseq

2.2.5.3.1 Library preparation and sequencing

Multiplexed RNA libraries were prepared using the Truseq RNA sample prep kit (Illumina). Briefly, the kit uses oligodT beads to enrich for mRNA prior to fragmentation using divalent cations under elevated temperature. Random primers were used for reverse transcription of mRNA fragments, producing cDNA to which Illumina adapter sequences with indexing barcodes; allowing multiple samples to be pooled for sequencing; were ligated. Samples were multiplexed such that all samples within an experiment were equally divided between flow cell lanes to avoid lane bias. Libraries were quantified on a Bioanalyzer high sensitivity DNA chip (Agilent) and sequenced on a HiSeq 2000 sequencer (Illumina) to produce 100 bp paired end reads. The target sequencing output was 5 Gb/sample.

2.2.5.3.2 RNAseq data analysis

Sequencing reads were aligned with Tophat [196] version 2.0.8 to the mouse reference genome version MM10/GRCm38. The read counts per gene were generated with featureCounts version 1.4.5-p1; the annotation for which was from ENSEMBL 77. Read counts were used to represent gene expression levels. R version 3.1.1 was used to import count data, and the DESeq2 package used to normalise read counts between samples based on the sample sequencing depth, and detect differentially expressed genes [197]. DESeq2 uses the Benjamini-Hochberg adjustment for multiple testing. Regularised logarithm (rlog)-transformed data was used for construction of principal component analysis (PCA) plots and heatmaps using the R package ggplot2.

2.2.6 16S rRNA gene sequencing for microbiota analysis

2.2.6.1 DNA extraction, library preparation and sequencing

DNA was extracted from faecal pellets, colon content, caecal content and caecal tissue using the FastDNA Spin Kit for Soil and FastPrep Instrument (both MP Biomedicals). 16S rRNA genes were amplified by PCR with Q5 High-Fidelity DNA Polymerase (New England Biolabs) using primers as described in section 2.1.2. Four separate PCR reactions were performed per sample and successful amplification was verified by gel electrophoresis of PCR products. Products of the four PCR reactions were pooled and DNA precipitated by ethanol precipitation as follows. 0.3 volumes (relative to the pooled sample volume) of 1 M sodium chloride were added to the sample. 2 volumes (relative to the combined volume of sample and sodium chloride) of chilled ethanol were added and mixed by inversion, and samples placed at -20 °C overnight. Samples were spun at 16,000 g, 20 min, 4 °C, and the DNA pellet washed with 600 µl of chilled 70% ethanol, then re-spun and dried using an Eppendorf Concentrator before resuspension in water. DNA was quantified by Qubit 2.0 Fluorometer using a dsDNA HS Assay Kit (both Life Technologies) and an equimolar mix of the DNA samples prepared. Equimolar mix was run on a 1% agarose gel and gel purification performed using a Wizard SV Gel and PCR Clean Up Kit (Promega). DNA was sequenced by paired-end sequencing on a MiSeq sequencer (Illumina). The number of clusters sequenced per sample was 48,068 ± 20,647 (mean ± standard deviation).

2.2.6.2 16S rRNA sequencing data analysis

FASTQ files were processed using the MOTHUR software [198]. The software platform R was used to remove low abundance bacterial groups (density < 0.5%) and generate plots. The web-based tool Interactive Tree Of Life (iTOL) was used to display the tree of sample relatedness alongside microbiome composition data [199]. MOTHUR was used to generate the within-sample α-diversity estimates.

2.2.7 Proteins

2.2.7.1 Analysis of tissue extracts by MS

2.2.7.1.1 Extraction of protein from caecal tissue

350 µl of lysis buffer (4% SDS, 150 mM sodium chloride, 50 mM Tris buffer pH 7.6, 2 mM EDTA, 40 mM TCEP, in HPLC-purified H_2O) was chilled on ice in GentleMACS M-tubes (Miltenyi Biotec). 40 - 60 mg pieces of flash-frozen caecal tissue were added to tubes and homogenisation performed in a GentleMACS Dissociator using the pre-set program for protein extraction. Tubes were spun briefly and content transferred to 1.5 ml microtubes. Samples were heated at 95 °C for 10 min before sonication with an ultrasonic probe (Fischer Scientific); 40% energy, 30 cycles (or until cleared) of 1 s pulses at 1 s intervals. Sonicated samples were centrifuged at 16,000 g for 15 min at 18 °C and supernatants transferred to clean microtubes.

2.2.7.1.2 Protein concentration determination

Protein concentration determination was performed by 660 nm protein assay (Pierce) with addition of Ionic Detergent Compatibility Reagent (Pierce).

2.2.7.1.3 Preparation for MS

400 mM iodoacetamide was added to samples at 200 µl/ml protein extract and samples incubated in the dark for 1 h at room temperature. Protein concentrations of IAA-treated extracts were normalised to that of the least concentrated sample by addition of lysis

buffer, then diluted 10-fold in 8 M urea in 0.1M Tris/HCl pH 8.5 for filter-aided sample preparation (FASP) [200]. All FASP centrifugation steps were performed at 20 °C. Urea-diluted extract volumes containing 80 μg total protein were transferred to Amicon UIltra-0.5 Centrifugal Filter Units, nominal molecular weight limit 30 kDa, (Merck Millipore) and centrifuged at 14,000 g until the dead volume was below the lowest marked level (~ 15 min). Flow-through was collected for the analysis of < 30 kDa proteins. Loading and centrifugation were repeated as required for transfer of the entire volumes containing 80 μg of protein. 200 μl of 8 M urea in 0.1 M Tris/HCl, pH 8.5, was pipetted into the filter units followed by centrifugation as before, three times consecutively. As required, waste liquid was pipetted carefully out of the collection tube for disposal. 200 μl of 0.05 M ammonium bicarbonate in HPLC-purified H_2O was pipetted into filter units with centrifugation as before, three times consecutively. Filters were then transferred to fresh collection tubes containing 20 μl of 0.05 M ammonium bicarbonate to aid chamber humidification, and 350 μl of 4.6 ng/μl Trypsin Gold (Promega) in 0.05 M ammonium bicarbonate was pipetted into filter compartment for protein digestion. Filter units were placed in a thermomixer at 600 rpm for 1 min before overnight incubation at 37 °C. Following incubation filter contents were transferred to a clean microtube, acidified to pH 3 - 4 with 25% trifluoroacetic acid, and diluted in HPLC-purified H_2O to a final protein concentration of 0.2 μg/μl. Yeast enolase (Waters) was added at a final concentration of 1 pmol/μl to provide an internal standard.

The retained flow-through fractions from centrifugation of extracts in Amicon UIltra-0.5 Centrifugal Filter Units were combined in naïve and infected sample pools. Pooled samples were applied to Vivsapin centrifugal concentrators, nominal molecular weight limit 5 kDa (Sigma) and washed with 8 M urea in 0.1 M Tris/HCl, pH 8.5 and 0.05 M ammonium bicarbonate as for the > 30 kDa protein fractions. Samples were collected and acidified for MS analysis without prior trypsin digestion.

2.2.7.1.4 MS analysis

MS analysis and database searching was performed by Dr Lu Yu (WTSI).

Quantitative liquid chromatography − tandem mass spectrometry (LC-MS/MS) was analysed on a nano-Acquity UPLC system (Waters Corp) coupled to a Synapt G2-S HDMS mass spectrometer (Waters Corp) with a nanoelectrospray source. Briefly, 300 ng of each sample was loaded and desalted at 5 μl/min for 5 min onto a trap column at 5 μm Symmetry

C18 180 μm × 20 mm column (Waters). Peptides were separated on a nanoACQUITY UPLC HSS T3 column at 75 μm id x 150 mm (Waters) using a linear gradient of 1 to 40% MeCN with 0.1% formic acid over 90 min and clcle time 120 min, at a flow rate of 0.3 μl/min and column temperature of 35 °C. Data collection was performed in ion mobility-assisted data independent acquisition ($HDMS^E$) modes, used a 0.6 s alternating cycle time between low (4 V) and high (15 - 45 V) collision energy (CE). m/z at 785.8426 from [Glu1]-Fibrinopeptide (Sigma) at 100 fm/μl was used as lockmass and acquired at every 60 s. Each sample was analysed in four runs.

2.2.7.1.5 MS data analysis

MS data analysis was performed by Dr James Wright (WTSI).

For the > 30 kDa protein fractions raw spectrum files were processed and identified using Protein Lynx Global Server 3.0 (Waters), against a combined mouse, *Salmonella* and contaminate database (UniProt Proteomes April 2014). The Ion Accounting (IA) results were exported from PLGS and further processed using in house software. Peptides were filtered at a 4% false discovery rate (FDR) before being clustering into a definitive list of proteins using an Occam's razor approach. Protein quantification was performed using a High3-MSMS intensity based approach, where for each protein the summed fragment ion intensities of the most intense three peptides were summed together. Only unique PepFrag1 peptides were allowed in the quantification. These quantification values were normalised using a multistage approach. Firstly, the protein quantifications were normalised by a spiked in protein standard (Yeast Enolase), the %CV was then calculated for each normalised protein across all samples. Using this %CV the 10 least variable proteins across the samples were selected and used in a secondary normalisation. The median normalised quantification values for each sample types were then used to calculate log2 fold changes between the different sample types. Fold changes were assessed for significance using a moderated t-test.

The data from analysis of < 30 kDa protein fractions was processed with MaxQuant. Potential contaminants, protein groups with a Q-value of > 0.015, and protein groups with an MS/MS count of ≤ 1 were excluded. The ratio of protein group intensities in naïve and infected pooled samples were used to reflect the difference in protein abundance between these conditions.

2.2.7.2 Western blotting

2.2.7.2.1 Preparation of intestinal content and faecal samples

Faeces and intestinal content was immediately homogenized upon collection in chilled RIPA buffer using a sterile pipette tip, followed by vortexing for 30 s. Samples were centrifuged at 16,000 g for 20 min at 4 °C and supernatants removed for protein concentration determination.

2.2.7.2.2 Preparation of tissue samples

RIPA buffer was added to flash-frozen tissue at 40 µl/mg and tissue homogenized in a GentleMACS Dissociator (Miltenyi Biotec) using the pre-set program for protein extraction. Homogenised tissue was maintained under constant agitation for 2 h at 4 °C followed by centrifugation at 16,000 g for 20 min. Supernatants were removed for protein concentration determination.

2.2.7.2.3 Preparation of plasma

Whole blood in EDTA-coated collection tubes was centrifuged at 2,000 g for 15 min and supernatant removed for protein concentration determination.

2.2.7.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein concentration of plasma and protein extracts was determined by bicinchoninic acid (BCA) assay (Pierce). Plasma and protein extracts were diluted for blotting in RIPA buffer and prepared for gel electrophoresis by addition of 4X laemmli buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue in 0.125 M Tris HCl pH 6.8). Samples were heated to 95 °C for 5 min and allowed to cool before electrophoresis by SDS-PAGE. Samples were loaded onto 12% Mini-PROTEAN TGX precast gels (Biorad) and run at 175 V for 45 min with Tris/glycine-running buffer.

2.2.7.2.5 Blotting and protein visualisation

Proteins were blotted onto ethanol-activated polyvinylidene difluoride (PDVF) membrane using a semi-dry transfer system (70 mA, 75 min) in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol in ddH_2O). Membranes were blocked by incubation in 5% milk in PBS-T (0.1% Tween-20 in PBS) for 1 h. Primary antibody in 2% milk in PBS-T was added for 1 h followed by three washes of 5 min in PBS-T. Appropriate HRP-conjugated secondary antibody was added for 45 min in 2% milk in PBS-T, followed by three washes of 15 min in PBS-T. Amersham Enhanced Chemiluminescence (ECL) Western Blotting Detection reagent was used for detection of protein bands with Amersham Hyperfilm ECL (both GE Healthcare) as per the manufacturer's instructions. All membrane incubations were carried out at room temperature on a rocking platform. ImageJ software was used for densitometry analysis of protein bands.

2.2.8 Statistical tests

Testing for differences in mouse weight loss was performed using one-way ANOVA. Mann Whitney U-tests were performed for analysis of organ *Salmonella* CFU and blood leukocyte populations. Pathway analysis was performed using the analysis tools available on the InnateDB website (http://www.innatedb.com/index.jsp). The hypergeometric analysis algorithm and Benjamini Hochberg p-value correction methods were used in all pathway analysis. Densitometry analysis of signal intensity in Western blotting was performed with a Student T-test.

Venn diagrams were produced using Venny and BioVenn [201, 202].

In all cases, a p-value of ≤ 0.05 was considered to be significant. Tests were performed using GraphPad Prism 6 graphing and statistical software (GraphPad Software, Inc.).

Star symbols were used to indicate statistical significance as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.