2 Materials and methods

2.1 Materials

2.1.1 Reagents

All chemicals were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated. Reagents were prepared and stored according to the manufacturer's guidelines.

2.1.2 Bacterial strains

Bacterial strain	Characteristics	Reference/source
S. Typhimurium SL3261	*aroA his* deletion harbours pnir15TetC plasmid that drives TetC expression	Supplied by Derek Pickard[32]
S. Typhimurium SL1344	Wild-type of SL3261	Supplied by Derek Pickard[190]
S. Typhimurium C5	Fully virulent strain	Supplied by Simon Clare[180]
C. rodentium DBS100	Wild type naladixic acid resistant	Supplied by Derek Pickard[191]

Table 1. Bacterial strains used during study

2.1.3 Immunofluorescence antibodies

Target Molecule	Host	Isotype	Conjugate	Source
CD45R/B220 clone RA3-6B2	Rat	$IgG_{2a,\kappa}$	FITC	Pharmingen
PNA	*Arachis hypogaea*	N/A	Rhodamine	Pharmingen

Table 2. Antibodies for immunofluorescence staining

2.1.4 ELISA antibodies

Target Molecule	Host	Isotype	Conjugate	Source
Ig	Goat	Ig	Horse Radish Peroxidase (HRP)	BD Pharmingen
IgG	Goat (Rat absorbed)	IgG	HRP	AbD Serotec
IgG_1	Rat	IgG_1,κ	HRP	BD Pharmingen
IgG_{2a}	Rat	IgG_1,κ	HRP	BD Pharmingen
IgA	Goat	IgG	HRP	AbD Serotec
IgM	Goat	IgG	HRP	AbD Serotec

Table 3. Antibodies Used for ELISAs

2.1.5 Mice

Female and male 5-7 week old C57BL/6 and miR155-deficient mice obtained from the Sanger Institute were used in all animal experiments. miR-155-deficient mice (*Bic*^{m2} allele) were previously described[31]. All animals were given food and water *ad libitum*. Mice were sacrificed by cervical dislocation. Animal husbandry and experimental procedures were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986.

2.1.5.1 Chimeric mice

Wild type- and miR-155-deficient, μMT chimeric mice were produced by Dr Elena Vigorito (Babraham Institute, Cambridge) as previously described. Briefly, μMT (B cell-deficient) mice were irradiated (5.0 Gy) and reconstituted with 3 X 10^6 bone-marrow cells. For the generation of mixed chimeras, irradiated μMT mice received a mixture of 80% bone-marrow cells of μMT origin and 20% wild-type or miR-155-deficient cells.

2.2 Methods

2.2.1 Bacterial growth conditions

All strains were routinely grown at 37°C in Luria Bertani (LB) broth, in air.

Citrobacter Rodentium strain DBS100 was grown at 37°C overnight with shaking (220 rpm) and aeration in LB broth.

S. Typhimurium strains SL1344, SL3261and C5 were grown at 37°C for 16 h as a standing culture without aeration in LB broth.

2.2.2 Animal methods

2.2.2.1 Preparation of inoculum

2.2.2.1.1 Oral *C. rodentium*

C. rodentium cultures were grown overnight as described above. Cultures were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and resuspended in 1/10 of the original volume in Dulbecco's Phosphate Buffered Saline (D-PBS). Mice were orally inoculated, with anaesthetic, using a gavage needle with 200µl (approximately 1 X 10^9 CFU of *C. rodentium* organisms) of the bacterial suspension. The viable count of the inocula was determined by plating on LB agar supplemented with nalidixic acid (100µg/mL).

2.2.2.1.2 Intravenous *S.* Typhimurium SL3261

S. Typhimurium SL3261 cultures were grown overnight as described above. Cultures were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and resuspended in D-PBS. Mice were injected with 200µl (approximately 1 x 10^5 CFU of SL3261 organisms) of solution into their tail vein. Actual numbers of CFU administered were determined by serial dilutions of the inoculum followed by plating on LB plates prior to animal infection.

2.2.2.1.3 Intravenous *S*. Typhimurium SL1344

S. Typhimurium SL1344 cultures were grown overnight as described above. Cultures were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and resuspended in D-PBS. Mice were injected with 200µl (approximately1 x 10^2 CFU) of solution into the lateral tail vein. Inocula were cultured on LB agar to determine the administered dose.

2.2.2.1.4 Oral *S*. Typhimurium C5

S. Typhimurium C5 cultures were grown overnight as described above. Cultures were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and resuspended in D-PBS. Mice were orally inoculated, with anaesthetic, using a blunt-tipped gavage needle with 200µl (approximately 1 x 10^{10} CFU S. Typhimurium C5 organisms). Actual numbers of CFU administered were determined by serial dilutions of the inoculum followed by plating on LB plates prior to animal infection.

2.2.2.2 Determination of number of viable *C. rodentium* in faecal samples

At regular time-points post-infection, mice were placed individually in sterile beakers and faecal samples from individual mice were collected in separate sterile eppendorfs. Faecal samples were weighed and for every 0.01g of faeces 100µL of sterile PBS was added (example – 0.02g faeces add 200µL PBS). Faecal samples were homogenised on a vortex and serially diluted. The number of viable bacteria was determined by viable count on LB agar supplemented with nalidixic acid (100µg/mL). The assay was repeated at least 3 times to ensure reproducibility.

2.2.2.3 Determination of pathogen burden in tissues

At selected time-points post-infection, mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. Colons, caecums, caecal patches, livers, spleens and mesenteric lymph nodes (MLNs) were removed aseptically. The terminal 6cm of colon was removed, and the colon weighed after removal of faecal pellets. Caecums were flushed with Phosphate Buffered Saline (PBS) before being processed further, the flow through was collected in a 15mL falcon tube to determine the number of bacteria not intimately attached to the epithelium. Colons, caecums, caecal patches, livers, spleens and mesenteric lymph nodes (MLNs) were placed into individual sterile Seward Stomacher® 400 classic bags and homogenized in 5mL of sterile double distilled water using a Seward Stomacher 80 (Seward, London UK) for 2 minutes at high speed. The number of viable bacteria in tissue homogenates were enumerated by serial dilution and plating in triplicate on LB agar [for C. rodentium LB agar was supplemented with nalidixic acid (100µg/mL)]. Colonies were counted after overnight incubation at 37°C to determine the Log_{10} CFU per gram of tissue. Lower detection limits were approximately 83 CFU/gram of tissue. The assay was repeated twice for each mouse strain to ensure reproducibility.

2.2.3 Molecular methods

2.2.3.1 Total RNA and miRNA extraction

At selected time-points post-infection, mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. Caecal patches and colons were removed aseptically and a 2.5mm^2 piece of each placed immediately into 5mL of RNA*later*® solution (Ambion). Samples were incubated at 4°C overnight before either being immediately processed or placed at -70°C for long term storage. Surfaces and equipment were thoroughly cleaned with 70% ethanol followed by RNase*Zap*® and DNase*Zap*® (Ambion) before use. Pieces of tissue were then individually removed from RNA*later*® solution

and placed into a 50mL falcon tube containing double the volume of Qiazol lysis reagent recommended in the Qiagen miRNeasy mini kit (Qiagen) handbook. Caecal patch and colon tissues were homogenized using a tissue homogenizer. Total Caecal patch and colon RNAs and miRNAs were then isolated using the Qiagen miRNeasy mini kit (Qiagen) as per the manufacturer's instructions. Eluted RNAs and miRNAs were stored at -70°C until needed.

2.2.3.2 RNA/miRNA quantification and quality control

All RNA/miRNA samples were quantified using a Thermo Scientific NanoDrop™ 1000 spectrophotometer (Thermo Scientific) as per the manufacturer's instructions. The qualities of the samples were checked using an Agilent 2100 bioanalyser (Agilent Technologies) as per the manufacturer's instructions.

2.2.3.3 Microarray

Gene-expression profiling was performed by the Wellcome Trust Sanger Institute Microarray Facility. Total RNA (500 ng) was amplified using the illumina Total Prep RNA Amplification Kit (Ambion) according to the manufacturer's instructions. Briefly the mRNA is reverse-transcribed with oligo (dT) primers. The oligo (dT) primer incorporates a T7 RNA polymerase (pol) binding site at the 5'-end. After this reaction (2 hours (h) at 42°C) the RNA is digested with RNaseH and the cDNA is converted to double strand cDNA with DNA polymerase (single reaction, 2 h at 16°C). The purified cDNA is then incubated overnight at 37°C with T7 RNA pol and rNTPs (including biotin-tagged rUTP) to produce biotinylated single-stranded anti-sense RNA (often called aRNA or cRNA). Once purified, the cRNA is quantified using a Thermo Scientific NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The biotinylated cRNA (1500ng per sample/array) is then mixed with hybridisation buffer and applied to illumina MouseWG-6 v2 Expression BeadChips and incubated in a humidified atmosphere at 58°C for

16-20 h. Chips were washed according to the standard illumina protocol: 10 min in high temp wash buffer at 55°C (this is the high stringency wash), 5 min in E1BC, 10 min in 100% ethanol (this is to remove any unwanted remnants of the hybridization gasket and to 'clean' the array), 2 min in E1BC (another rinse), 10 min in blocking agent (this is a casein block in PBS to prevent non-specific binding in the next step), 10 min in blocking agent containing 1 ug/ml streptavidin-cy3 (the strp-cy3 will bind to the biotinylated cRNA that has hybridized to the probes on the array) and finally a 5 min rinse in E1BC and a spin-dry (4 min at 275 g). All washes were performed at room temperature except the first high temperature wash. The slides are scanned using a BeadArray reader according to the manufacturer's instructions and the scanner output imported into BeadStudio software (Illumina).

2.2.3.4 Analysis and annotation of microarray data

Analysis and annotation of microarray data was performed by Cei Abreu-Goodger, European Bioinformatics Institute (EBI), Wellcome Trust Genome Campus. Briefly the raw expression values for all samples were exported from Illumina BeadStudio version 3.1.8 as a Sample Gene Profile file. All further data processing was performed using open software packages available through R and Bioconductor[192, 193]. The *lumi* package was used to import the raw expression values, perform a Variance-stabilizing Transformation (VST) and quantile normalisation[194, 195]. In order to discover differentially expressed genes, linear models were fit to each probe and an empirical Bayes approach was used to shrink the estimated variance according to the *limma* package[196]. The resulting P-values for differential expression were adjusted for multiple testing by the Benjamini and Hochberg procedure[197].

Probe annotation was derived from a variety of sources. The Bioconductor packages *lumiMouseAll.db*, *GO.db* and *KEGG.db* were used to retrieve information about Entrez and RefSeq identifiers, gene descriptions, gene Symbols, assignment to GO Biological Process terms and KEGG pathways[198-200]. Information about Ensembl gene and transcript annotation and the 3'UTR

sequences was obtained directly from Ensembl[201]. The 3'UTR sequences were queried for seed matching sites of miR-155 using custom Perl scripts. Predicted targets of miR-155 were also retrieved from TargetScan v4 and PITA[202, 203].

Sylamer was used to test for significant enrichment or depletion of 7-nucleotide words complementary to the seed region of all known mouse microRNAs[204]. Two independent gene lists were derived, one for colon tissue and one for ceacal patch, both containing all genes on the microarrays sorted from the most up- to the most down-regulated in the *bic* mice. The expected result when comparing the expression profile of a microRNA knockout against wild type is a clear enrichment of seed-matching words towards the up-regulated portion of the gene list. The Sylamer plots did not reveal a significant enrichment of miR-155 seed words beyond the expected background distribution. A likely explanation is that miR-155 is expressed exclusively in certain cell types, and that the whole tissue samples being analysed on the microarrays contained a large amount of expression changes coming from other cell types. These changes would not be direct targets of miR-155, and thus the expected enrichment signal might be diluted beyond detection.

2.2.3.5 Quantitative, Real-Time PCR (RT-PCR) for RNA

2.2.3.5.1 Reverse-Transcription of total RNA

Total RNA was reverse transcribed using the Qiagen Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Briefly the purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA (according to table 4).

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 µl	1x
Template RNA	Variable (up to 1 µg*)	
RNase-free water	Variable	
Total volume	**14 µl**	–

* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA analyzed.

Table 4. Genomic DNA (gDNA) elimination reaction components

After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT primer mix according to Table 5.

Component	Volume/reaction	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase*	1 µl	
Quantiscript RT Buffer, 5x†‡	4 µl	1x
RT Primer Mix‡	1 µl	
Template RNA		
Entire genomic DNA elimination reaction (step 3)	14 µl (add at step 5)	
Total volume	**20 µl**	–

* Also contains RNase inhibitor.

† Includes Mg^{2+} and dNTPs.

‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5 µl of the premix per 20 µl reaction.

Table 5. Reverse-transcription reaction components

The entire reaction takes place at 42°C for 15 minutes and is then inactivated at 95°C for 3 minutes.

2.2.3.5.2 Real-Time PCR step

Two-Step RT-PCR (standard protocol) was performed with QuantiTect Primer Assays and QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions; using glyceraldehydes 3-phosphate dehydrogenase (GAPDH) for normalisation across samples. Briefly, 2x QuantiTect SYBR Green PCR Master Mix, 10x QuantiTect Primer Assay, template cDNA (50ng/reaction), and RNase-free water were thawed and a reaction mix prepared according to Table 6.

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix*	25 µl	1x
10x QuantiTect Primer Assay	5 µl	1x
Template cDNA (added at step 4)	Variable	≤100 ng/reaction
Optional: Uracil-N-glycosylase†	0.5 µl	0.5 units/reaction
RNase-free water	Variable	–
Total volume	**50 µl‡**	**–**

* Provides a final concentration of 2.5 mM $MgCl_2$.

† Supplied with the QuantiTect SYBR Green PCR +UNG Kit.

‡ If using a total volume other than 50 µl, adjust the amounts of the master mix and the primer assay so that their final concentrations remain 1x, but continue to use 0.5 units of UNG and ≤100 ng of template cDNA.

Table 6. Reverse-transcription reaction components

The reaction mix was mixed thoroughly, and 25 µL dispensed into a MicroAmp™ Fast Optical 96-well reaction plate (Applied Biosystems). Template cDNA (50ng/reaction) was added to the individual wells containing the reaction mix. Data acquisition was performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) using the cycling conditions recommended in the QuantiTect Primer Assay handbook (see Table 7).

Step	Time	Temperature	Additional comments
UNG (optional) Carryover prevention	2 min	50°C	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR			
Initial activation step	**15 min**	**95°C**	This step activates HotStarTaq DNA Polymerase
3-step cycling:			
Denaturation*	15 s	94°C	
Annealing	30 s	55°C	
Extension†	30 s	72°C	Perform fluorescence data collection
Number of cycles	35–40 cycles		The number of cycles depends on the amount of template cDNA and abundance of the target

Table 7. Cycling conditions for two-step RT-PCR

The expression of each target gene was determined by calculating the average of 4 technical replicates for each of 5 biological replicates. The expression of each target gene was determined by the Comparative Ct method ($2^{-[\Delta][\Delta]Ct}$ method), in which Ct is the threshold cycle, ΔCt = [Ct target gene - Ct reference gene (GAPDH)] and the ΔΔCt = [ΔCt experiment – ΔCt control].

2.2.3.5.2.1 QuantiTect Primers

QuantiTect Primer Assays were shipped lyophilized. To reconstitute a tube of 10x QuantiTect Primer Assay, tubes were briefly centrifuged and 1.1 mL TE, pH 7.0 (Ambion®) was added before mixing the tube by vortexing 4-6 times. Aliquots of the reconstituted primers were stored at -20°C until required.

Gene Name Species mouse (mus musculus)	Entrez Gene ID	Detected transcript	Ensembl Transcript ID	QuantiTect Primer Assay
Matrix metallopeptidase 3 (Mmp3)	17392	NM_010809	ENSMUST00000034497	Mm_Mmp3_1_SG QuantiTect Primer Assay (QT00107751)
B-cell leukaemia/lymphoma 6 (Bcl6)	12053	NM_009744	ENSMUST00000023151	Mm_Bcl6_1_SG QuantiTect Primer Assay (QT01057196)
CD86 antigen (CD86)	12524	NM_019388	ENSMUST00000023618	Mm_Cd86_1_SG QuantiTect Primer Assay (QT01055250)
matrix metallopeptidase 10 (Mmp10)	17384	NM_019471	ENSMUST00000034488	Mm_Mmp10_1_SG QuantiTect Primer Assay (QT00115521)
CD19 antigen (CD19)	12478	NM_009844	ENSMUST00000032968	Mm_Cd19_1_SG QuantiTect Primer Assay (QT00108801)
chemokine (C-X-C motif) receptor 3 (Cxcr3)	12766	NM_009910	Not Applicable (N/A)	Mm_Cxcr3_1_SG QuantiTect Primer Assay (QT00249438)
glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	14433	NM_008084	ENSMUST00000073605	Mm_Gapdh_3_SG QuantiTect Primer Assay (QT01658692)

Table 8. Quantitect Primers

2.2.3.6 Quantitative, RT- PCR for miRNA

miR-155 expression was determined using the miScript System (Qiagen), a three-component system which comprises the miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, and miScript Primer Assay.

2.2.3.6.1 Reverse-Transcription Step

The miScript Reverse Transcription Kit (Qiagen) includes miScript Reverse Transcriptase mix and miScript RT Buffer. miScript Reverse Transcriptase Mix is an optimized blend of enzymes comprising a poly(A) polymerase and a reverse transcriptase. miScript RT Buffer has been developed specifically for use with the miScript Reverse Transcriptase Mix. This buffer system enables maximum activity of both enzymes as well as containing Mg^{2+}, dNTPs, oligo-dT primers, and random primers. Unlike mRNA, miRNAs are not polyadenylated in nature. During the reverse transcription step, miRNAs are polyadenylated by poly(A) polymerase. Reverse transcriptase converts RNA (including miRNA, other small noncoding RNA, and mRNA) to cDNA using oligo-dT and random primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a universal tag sequence on the 5’ end. This universal tag allows amplification in the real-time PCR step.

For reverse transcription, template RNA/miRNA was thawed on ice and 5x miScript RT buffer thawed at room temperature. Each solution was mixed by gently flicking the tubes and then centrifuged briefly. The reverse-transcription master mix was prepared on ice according to Table 9.

Component	Volume/reaction	Final concentration
miScript RT Buffer, 5x*	4 µl	1x
miScript Reverse Transcriptase Mix	1 µl	
RNase-free water	Variable	
Template RNA	Variable (up to 1 µg)	
Total volume	**20 µl**	–

* Includes Mg^{2+}, dNTPs, and primers

Table 9. Reverse-transcription reaction components for miRNA or Non-coding RNA

Template RNA (1 µg) was added to each tube containing reverse-transcription master mix and incubated for 60 min at 37°C. tubes were then incubated for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and subsequently stored at -20°C prior to real-time PCR.

2.2.3.6.2 Real-Time PCR for Detection of miRNA or Noncoding RNA

2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, template cDNA, and RNase-free water were thawed and mixed according to Table 10. The miScript Universal Primer allows detection of mature miRNAs in combination with a miRNA-specific primer (miScript Primer assay), an miRNA-specific forward primer which is used for mature miRNA detection. Template cDNA was then dispensed into individual wells of a 96-well plate along with appropriate volumes of the reaction mix, and briefly centrifuged.

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Final conc.
2x QuantiTect SYBR Green PCR Master Mix*	10 µl	25 µl	1x
10x miScript Universal Primer	2 µl	5 µl	1x
10x miScript Primer Assay	2 µl	5 µl	1x
RNase-free water	Variable	Variable	–
Template cDNA (added at step 4)†	≤2 µl	≤5 µl	≤100 ng/ reaction
Total volume	**20 µl**	**50 µl**	–

* Provides a final concentration of 2.5 mM $MgCl_2$.

†The volume of cDNA should not exceed 10% of the final reaction volume.

Table 10. Reaction setup for RT-PCR detection of miRNA and noncoding RNA

Data acquisition was performed on a Stratagene Mx3000P QPCR System (Agilent Technologies, Inc) according to the cycling conditions provided in the miScript System Handbook (see Table 11).

Step	Time	Temperature	Additional comments
PCR Initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension†	30 s	70°C	Perform fluorescence data collection.
Cycle number	35–40 cycles		Cycle number depends on the amount of template cDNA and abundance of the target.

Table 11. Cycling conditions for real-time PCR using block cyclers.

The expression of miR-155 at each time point was determined by calculating the average of 4 technical replicates for each of 2 biological replicates. The

expression of miR-155 was determined by the Comparative Ct method ($2^{-[\Delta][\Delta]Ct}$ method), in which Ct is the threshold cycle, ΔCt = [Ct miR-155 - Ct GAPDH] and the ΔΔCt = [ΔCt experiment (after infection) – ΔCt control (naive)].

2.2.3.6.2.1 miScript Primer Assay

miScript Primer Assays were shipped lyophilized. To reconstitute a tube of 10x miScript Primer Assay, vials were briefly centrifuged and 550 µL TE, pH 7.0 (Ambion®) was added before mixing the tube by vortexing 4-6 times. Aliquots of the reconstituted primers were stored at -20°C until required.

Gene Name	Entrez Gene ID	Transcripts for this gene	miScript Primer Assay
microRNA-155 (Mir155)	387173	NR_029565	Mm_miR-155_1 miScript Primer Assay (MS00001701)

Table 12. miScript primer Assay

2.2.4 ELISA methods

2.2.4.1 Serum extraction

Mice were placed in a heated box at 37°C for 15 minutes prior to tail bleed. 200µL of whole blood was collected in a sterile eppendorfs and centrifuged at full speed for 15 mins, before serum was collected and stored at 4°C until required.

2.2.4.2 ELISA for total Ig, IgG, IgG1, IgG2a, IgA and IgM in mouse sera (General ELISA protocol)

Flat bottomed Nunc Maxisorp plates were coated overnight at 4°C with 50µL of a solution of relevant antigen (2.5µg/mL ESPA or 5µg/mL TetC) in carbonate buffer (pH 9.6, H_2O containing 0.00303% Na_2CO_3 and 0.006%

$NaHCO_3$). After incubation, antigen/carbonate buffer solution was removed from the plate by inversion and the plate washed once with PBS containing 0.01% Tween-20 (wash buffer). Plates were blocked with 100µL of 3% bovine serum albumin (BSA) in PBS (blocking solution) at room temperature for 1 hour. After blocking solution was removed, plates were washed once with wash buffer, and sera from experimental animal groups was added as follows: 3µL of sera was added to 27µL of PBS + 1% BSA (antibody buffer), 12.5µL of this was added to 112.5µL of antibody buffer placed in the top well of the ELISA plate, which is a 1:100 dilution of serum and then diluted 5 fold down the plate. Each plate contained control wells with pre-immune (naive) serum and PBS alone. Then plates were then left to incubate for 1 hour at 37°C. After 3 washes with wash buffer, antibodies conjugated to horse radish peroxidise (HRP) diluted 1:1000 in antibody buffer were added at 100µL per well. Conjugate antibodies were either anti-mouse total Ig, anti-mouse IgG, anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgA or anti-mouse IgM. Plates were incubated at 37°C for 1 hour, washed 5 times in wash buffer. To develop the plates, 50µL of Sigma fast OPD tablet set dissolved in double distilled water was added to each well and allowed to incubate for 15 minutes at room temperature. The reaction was stopped by adding 25µL 3M sulphuric acid to each of the wells. Absorbances were read at OD490nm and titres were determined arbitrarily as the reciprocal of the serum dilution giving an absorbance of 0.3, using a computer based program called Endpoint. Where possible, a minimum of 5 mice of each mouse strain from at least 2 independent experiments were analyzed.

2.2.4.3 ELISA for IgA in faecal samples

Flat bottomed Nunc maxisorp plates were coated overnight at 4°C with 50µL faecal homogenate. After incubation faecal homogenate solution was removed by inverting the plate and washing 3 times in PBS containing 0.01% Tween-20 (wash buffer). Plates were blocked with 100µL of 3% bovine serum albumin (BSA) in PBS (blocking solution) at 25°C for 1 hour. Blocking solution was removed, plates were washed once with wash buffer, and sera

from experimental animal groups was added as follows: 3µL of sera was added to 27µL of PBS + 1% BSA (antibody buffer), 12.5µL of this was added to 112.5µL of antibody buffer placed in the first well of the ELISA plate, which is a 1:100 dilution of serum and then diluted 5 fold down the plate. Each plate contained control wells with naive serum and PBS alone. The plates were then left to incubate for 1 hour at 37°C. After 3 washes with wash buffer, antibodies conjugated to horse radish peroxidise (HRP) diluted 1:1000 in antibody buffer were added at 100µL per well. Conjugate antibodies were anti-mouse IgA. Plates were incubated at 37°C for 1 hour, washed 5 times in wash buffer. To develop the plates, 50µL of Sigma fast OPD tablet set dissolved in double distilled water were added to each well and allowed to incubate for 15 minutes at 25°C. The reaction was stopped by adding 25µL 3M sulphuric acid to wells. Absorbances were read at 490nm and titres calculated based on the reciprocal dilution giving an absorbance of 0.3, using a computer based program called Endpoint. Where possible, a minimum of 5 mice of each mouse strain from at least 2 independent experiments were analyzed.

2.2.5 Tissue staining methods

2.2.5.1 Paraffin embedding tissue and sectioning

Mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. Spleens, livers, mesenteric lymph nodes (MLNs), colons and caecal patches were removed and immediately placed in 4% formaldehyde and incubated overnight at room temperature. Tissues were processed in a Shandon Excelsior Tissue Processor (Thermo Fisher Scientific) and then embedded in paraffin wax. 5µm sections were cut using a Leica RM2125 microtome (Leica) and transferred to superfrost plus slides (VWR international).

2.2.5.1.1 Histology

Paraffin sections were deparaffinised and rehydrated by passing slides through Histoclear for 2 minutes, 100% ethanol for 2 minutes, fresh 100% ethanol for

a further 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes and finally washed with water for 5 minutes. Sections were then placed in Mayer's haematoxylin for 2 minutes and washed with running water for 5 minutes. After washing, sections were placed in 1% ethanol for a few seconds to remove excess haematoxylin dye. Sections were washed with water, incubated in eosin for 5 minutes and washed again with water. Sections were dehydrated; 70% ethanol for 2 minutes, 90% ethanol for 2 minutes, 100% ethanol for 2 minutes, fresh 100% ethanol for 2 minutes and finally 2 minutes in Histoclear. Slides were subsequently mounted with DPX and allowed to air-dry overnight before visualization using a Zeiss LSM510 Meta Confocal Microscope and/or Zeiss Axiovision Wide Field Microscope (Zeiss). Crypt depths in colon were determined microscopically, from at least 5 mice per group. Multiple sites were measured throughout each organ, and used to calculate the average crypt depth for each sample.

2.2.5.2 General Chemical Fixation and fine preservation for Transmission Electron Microscopy (TEM)

Mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. Colon, caecum and caecal patch tissue were removed and 1mm x 1mm pieces were placed immediately in primary fixative (2% paraformaldehyde (PFA) with 2.5% glutaraldehyde (GA) in 0.1M sodium cacodylate buffer at pH7.42 with added 0.1% and 0.05% magnesium and calcium chloride, respectively) and subsequently incubated at 20°C for 15 minutes, then on ice for remainder of 1 hour. Specimens were rinsed three times for 10 minutes each, in sodium cacodylate buffer with added chlorides, as above. Specimens were then placed into 1% osmium tetroxide in sodium cacodylate buffer only, at room temperature for 1 hour. All the following steps were performed at room temperature. Specimens were rinsed 3 times in cacodylate buffer over 30 minutes and mordanted with 1% tannic acid for 30 minutes to 1 hr and rinsed with 1% sodium sulfate for 10 minutes. Samples were dehydrated by passage through an ethanol series: 20%, 30%, 50%, 70%, 90% and 95% ethanol for 20 minutes each, then 100% ethanol for 3 x 20

minutes. A rotator was used throughout the following steps to aid infiltration. Specimens were incubated twice for 15 minutes in propylene oxide (PO) and then for at least 1 hour in 1:1 PO to Epoxy embedding resin kit (TAAB Epon:812 (48g), DDSA (19g), MNA (33g) and DMP30 (2g) weighed out and mixed in a 50ml falcon) mixture. Specimens were then placed over night in neat Epon (with a few drops of PO). Specimens were embedded in fresh Epon, in a flat moulded tray and cured in an oven at 65°C. Subsequently, 500nm semithin sections were cut on a UCT ultramicrotome and stained with toluidine blue on a microscope slide. Images were recorded on a Zeiss Axiovert CCD camera and areas for ultrathin 50nm sectioning were selected. Thin 50nm sections were collected onto copper grids and contrast stained with uranyl acetate and lead citrate before viewing on an FEI 120kV Spirit Biotwin TEM and recording CCD images on an F4.15 Tietz camera. Images are representative of 3 mice per group.

2.2.5.3 Scanning Electron Microscopy

Colon and caecum samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.01M PBS at 4°C for 1 hour, rinsed thoroughly in 0.1M sodium cacodylate buffer 3 times and fixed again in 1% buffered osmium tetroxide for 3 hours at room temperature. For better conductivity the samples were then further impregnated with 1% aqueous thiocarbohydrazide and osmium tetroxide layers separated by sodium cacodylate washes following the protocol for OTOTO[205]. The samples were dehydrated in an ethanol series 30%, 50%, 70%, 90% and then 3 x 100% for 20 minutes each before critical point drying in a Bal-Tec CPD030 and mounted onto aluminium stubs with silver dag. Finally each sample was sputter coated on the luminal surface with a 2nm gold layer in a Bal-Tec SCD050 and examined on a Hitachi S-4800 SEM. Images are representative of 3 mice per group.

2.2.5.4 Frozen sectioning

Mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. Spleens, livers, mesenteric lymph nodes (MLNs), and colons were

removed into separate Corning® External Thread Cryogenic vials tubes (Corning) and immediately snap-frozen in liquid nitrogen. 5µm sections were cut using a Shanndon cryostat. Sections were transferred to HistoBond® slides, allowed to air-dry for 1 hour at room temperature and subsequently fixed in 100% acetone for 10 minutes before use.

2.2.5.4.1. Immunofluorescent staining of frozen tissue sections

Fixed frozen tissue sections were washed 3 times, 5 minute per wash in PBS, sections were subsequently blocked with PBS supplemented with 10% normal goat serum, 5% fish gelatin, 0.01% sodium azide, 0.1% BSA and 0.01% Tween-20 (IHC blocking solution) for 45 minutes at room temperature. Blocking buffer was tapped off, and relevant antibodies added (see Table for antibodies used). For directly conjugated fluorochrome primaries, all antibodies were diluted 1:100 with IHC blocking solution. Control sections for each group did not have any antibody added; only PBS was added to these sections. Slides were incubated for 1 hour at 37°C in darkness. After incubation sections were washed in PBS at room temperature for 15 minutes, in total darkness. Finally slides were mounted with ProLong Gold (Invitrogen) and coverslips, before being left to air-dry in the dark overnight before visualization using a Zeiss LSM510 Meta Confocal Microscope and/or Zeiss Axiovision Wide Field Microscope (Zeiss). The numbers and size of germinal centres within mesenteric lymph nodes were determined microscopically, from at least 4 mice per group. Multiple sites were measured throughout each organ, and used to calculate the average germinal centre size and number.

2.3 Statistical analysis

Statistical analysis was performed with two-tailed Student’s unpaired t-test unless otherwise stated with the graphing and statistical software, GraphPad Prism 5 (GraphPad Software, Inc, USA).