1 Introduction

1.1 MicroRNAs

1.1.1 Discovery

Viktor Ambros and colleagues discovered miRNAs whilst conducting a study of genes involved in the development of the nematode worm, *Caenorhabditis elegans* (*C. elegans*)[1-3]. They identified that the gene *lin-4* was essential for post-embryonic developmental stages in *C. elegans* and functioned by negatively regulating the level of LIN-14 protein[4]. Surprisingly, when the genomic sequence of the *lin-4* gene was analysed for potential open reading frames it was shown not to encode any functional proteins[5]. Further analysis of the transcripts of *lin-4* led to the discovery that they contained complementary sequences to a repeated sequence in the 3' untranslated region (UTR) of the lin-14 mRNA and thus they speculated that lin-4 may function via an antisense RNA-RNA mechanism[5]. Initially, miRNAs were believed to be unique to *C. elegans*, however, it is now known that they are not just an oddity of this worm species. Indeed, miRNAs have now been documented in an incredibly diverse range of organisms from plants and animals to bacteria and viruses.

1.1.2 MicroRNAs post-transcriptionally regulate gene expression

During the preliminary studies looking at how *lin-4* was able to exert an inhibitory effect on the translation of the LIN-14 protein, it was shown that transcripts of the lin-4 gene were able to bind directly to the lin-14 mRNA. Thus, it was hypothesised that it functioned through an antisense RNA-RNA mechanism[5]. The exact action of miRNAs is now commonly known to involve bases within the 5' end of the miRNA, which mediate complementary base pairing to the 3' un-translated region (UTR) of target mRNAs[6-8]. The degree of complementarity between the sequences is thought to determine

whether the mRNA is degraded directly by RNA- induced silencing complex (RISC) or not[9-11]. If the sequences are a perfect or very close to perfect match, the mRNA is cleaved directly by Argonaute2 (AGO2) protein within the RISC (Figure 1)[9-11]. However, perfect complementarity is rare in animals; it is thought to occur mainly in plants. Incomplete or imperfect base pairing leads to accumulation of target mRNAs within discrete cytoplasmic foci called GW bodies (or Processing (P)-bodies) where they are inaccessible to the translational machinery (Figure 1)[12, 13]. The translationally repressed mRNA is then either destroyed by RNA-degrading enzymes or stored[8, 12-14] .

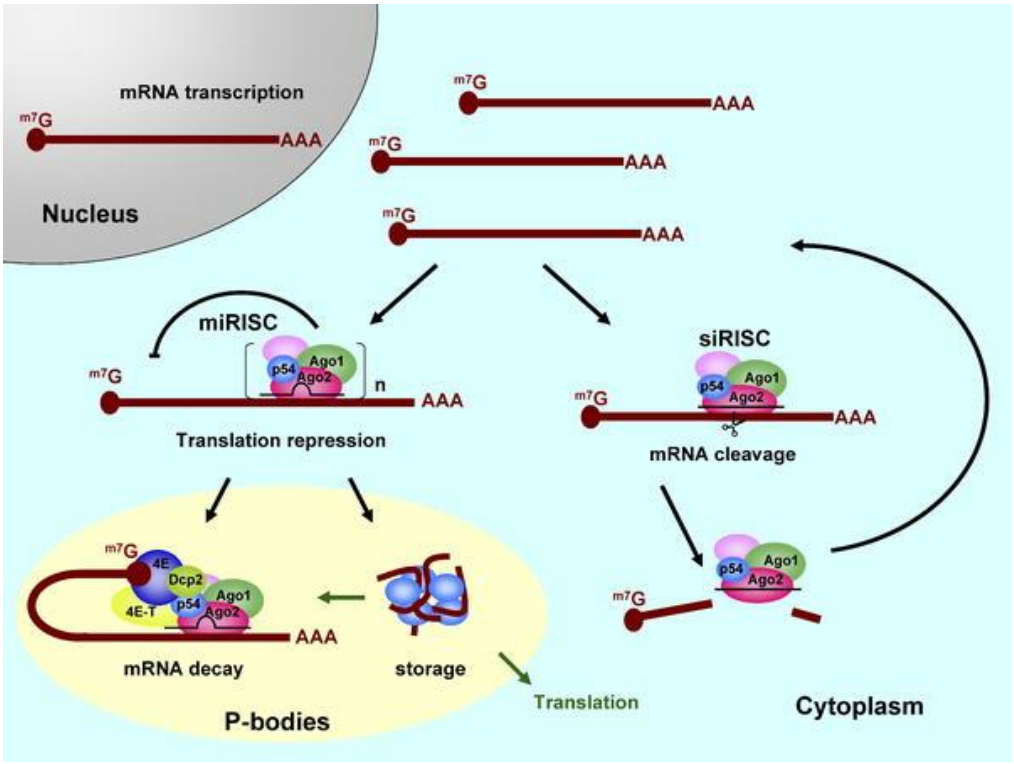

Figure 1. A Model for Human RISC Function Involving miRNA and siRNA

RISC contains Ago2 (red), Ago1 (green), RCK/p54 (blue, labelled p54), and other known (e.g., Dicer and TRBP) and unidentified proteins (pink) and is distributed throughout the cytoplasm. RISC binds to its target mRNA by perfectly matching base pairs, cleaves the target mRNA for degradation, recycles the complex, and does not require P-body structures for its function. Multiple numbers *(n)* of miRISC bind to target mRNA by forming a bulge sequence in the middle that is not suitable for RNA cleavage, accumulate in P-bodies, and repress translation by exploiting global translational suppressors such as RCK/p54. The

translationally repressed mRNA is either stored in P-bodies or enters the mRNA decay pathway for destruction. Depending upon cellular conditions and stimuli, stored mRNA can either re-enter the translation or mRNA decay pathways.

1.2 **miR-155/ B cell integration cluster (BIC)**

One particular miRNA, miR-155, has received a great deal of interest over recent years because it has been implicated as an important player in the development and/or function of a number of different immune cells and has also been shown to be involved in the generation and control of cancer.

1.2.1 Identification of miR-155

The loci encoding miR-155 was indirectly identified by Clurman and Hayward, during a study of the common oncogenes activated during avian leukosis virus (ALV)-induced B-cell lymphoma development[15]. They showed that in the majority of ALV induced lymphomas, dysregulation of *c-myc* expression occurred as a result of proviral integration within or near the *c-myc* gene. However, although disruption of *c-myc* expression occurred early in lymphomagenesis they determined that additional proto-oncogene activations were required for the induction of later stages of the disease. Using an experimental strategy to identify proto-oncogenes they found that ALV commonly integrated into three loci within the chicken genome; *c-myb*, *c-myc* and the newly identified locus which they called *c-bic* (B-cell integration cluster). Comparative sequence analysis of the *c-bic* locus with sequences held within the then GenBank data base revealed that *c-bic* was an unidentified locus. Furthermore, analysis of the genomic sequence for potential reading frames revealed that the locus did not encode any obvious proteins.

Amid a myriad of papers describing how mRNA-like non-coding RNAs can act as 'riboregulators' Tam *et al* pioneered the suggestion that *BIC* may function as a non-coding RNA[16-18]. Unfortunately, none who tried were able to provide a definitive mechanism by which *BIC* worked until in 2002, Lagos-

Quintana *et al* conclusively showed that the *c-bic* locus encoded a microRNA, which later became known as miR-155[19].

1.2.2 miR-155 as an oncogenic microRNA

Several different studies have described the accumulation of miR-155 transcripts in human lymphomas[20-26]. Van den Berg *et al* were first to demonstrate the high expression of miR-155 in the Hodgkin lymphoma (HL)-derived cell line, DEV and other HL derived cell lines[23]. They noted that miR-155 was not detectably expressed in non-Hodgkin lymphoma (NHL)-derived cell lines or normal tissue controls. However, analysis of normal human lymph node and tonsil tissues revealed the presence of miR-155 transcripts in a small number of germinal centre $CD20^+$ B cells. Using the Burkitt lymphoma (BL)-derived cell line Ramos they later demonstrated that the expression of miR-155 could be up-regulated by B-cell receptor (BCR) triggering and consequently hypothesised that miR-155 may play a role in B cell selection and/or function. The BCR-induced expression of miR-155 in Ramos was found to involve protein kinase C (PKC) and the downstream transcription factor nuclear factor-κB (NF-κB)[24]. Metzler *et al* supported Van den Berg hypothesis and further showed that miR-155 was highly expressed in tissues from children with BL[22]. Costinean *et al* generated the first transgenic mice carrying a miR-155 transgene, the expression of which was targeted and overexpressed in B cells (Eμ-mmu-miR155)[27]. The transgenic mice in the study developed preleukemic pre-B cell proliferation in the spleen and bone marrow resulting in B cell malignancy[27].

1.2.3 miR-155 is important for normal immune function

1.2.3.1 The role of miR-155 in innate immunity

Two recent studies looking at miRNA expression in stimulated macrophages have shown that miR-155 may play a significant role in the innate immune response[28, 29]. In the first study, microarray technology was used to probe which miRNAs were induced in primary murine macrophages stimulated with

the virally relevant stimuli polyriboinosinic:polyribocytidylic acid [poly(I:C)] or interferon beta (IFN-β). miR-155 was identified as the only assayed microRNA that was substantially induced upon stimulation. Additionally, when macrophages were stimulated with bacterially relevant Toll-like receptor (TLR) ligands; such as lipopolysaccharide (LPS), hypomethylated DNA (CpG) or a synthetic lipoprotein, Pam3CSK4, miR-155 expression was once again found to be upregulated in response to all TLR ligands. Using myeloid differentiation factor 88 (MyD88)- or TRIF-deficient macrophages lacking the TLR signalling adaptor proteins it was demonstrated that signalling through either of these pathways is sufficient to induce miR-155 expression[29]. During a second study, mir-155 transcript levels were measured in Raw 264.7 macrophages stimulated with LPS. Analogous with the previous analysis, miR-155 was shown to be up-regulated in cells after six hours of stimulation. Further, when splenocytes were extracted from wild-type C57BL/6 mice inoculated intraperitoneally with *S. enteritidis*-derived LPS the levels of miR-155 were not only found to be increased but this up-regulation was shown to enhance the production of tumour necrosis factor-alpha (TNF-α), an important cytokine produced by macrophages in response to LPS. In unstimulated Raw 264.7 macrophages, TNF-α transcripts are known to be expressed but not translated. Translational repression is due to a self-inhibitory element in the 3' UTR of *TNF-α* mRNAs, miR-155 is thought to eliminate this inhibition but as yet the mechanism has not been elucidated.

1.2.3.2 miR-155 function in T cells

Haasch *et al* discovered that miR-155 is rapidly expressed in normal human T cells after activation[30]. Using expressed sequence tag (EST) technology they identified a number of ESTs that were more abundantly expressed in normal $CD4^+$ T lymphocytes activated with anti-CD3 and -CD28 antibodies. One EST was of particular interest because it was copiously expressed in other EST libraries produced from arthritic joint synovial fluids, colon samples from patients with ulcerative colitis as well as lymphoma and thymus samples. The EST identified contained no open reading frames (ORFs) and

was thus deemed to be non-protein coding. Sequence comparison analysis revealed that the EST was miR-155. Subsequently, they were able to show that the expression of miR-155 in activated T cells could be repressed by the administration of immunosuppressive drugs that affect either calcineurin-dependent NFAT activation or AP-1 and NF-κB activation[30]. Thus, it was concluded that miR-155 might play an important role in T cell function.

1.2.3.3 The miR-155-deficient mouse

To further elucidate the role of miR-155 *in vivo*, Antony Rodriguez and colleagues used a gene targeting strategy to create a miR-155-deficient mouse (Figure 2)[31].

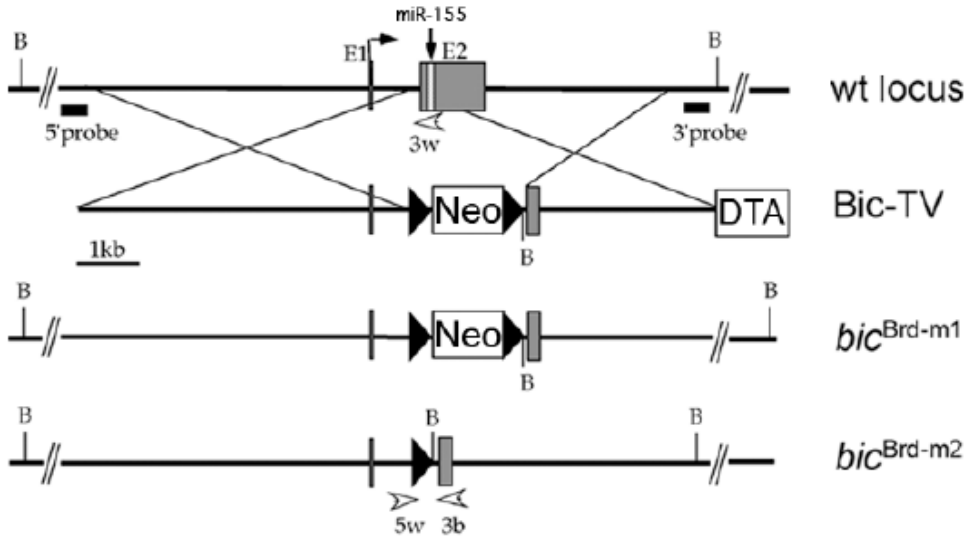

Figure 2. Gene targeting strategy to generate bic/miR-155-deficient mice

Schematic illustration of bic/miR-155 genomic locus and the targeting vector used to generate bic/miR-155 null alleles ($bic^{brd\text{-}m1}$ & $bic^{brd\text{-}m2}$). The two alleles are null for miR-155 stemming from its physical deletion by the PGK-*Neo* (NEO) cassette flanked by *lox*P sites (black triangles).

Whilst the miR-155-deficient mice generated were viable and fertile, at age 320-350 days old approximately half the mice exhibited enhanced remodelling of the lung airway, characterised by a significant increase in bronchiolar subepithelial collagen deposition and in the numbers of

leukocytes in bronchoalveolar lavage fluids. In addition, miR-155-deficient mice were prone to enteric inflammation (symptoms typically seen when the immune response is skewed toward T_h2 differentiation) reminiscent of inflammatory bowel disease (IBD) thus, suggesting that miR-155 may be involved in regulating the homeostasis of the immune system. When miR-155-deficint mice were intravenously (i.v.) immunized with live attenuated *Salmonella enterica* subspecies *enterica* serovar Typhimurium[32] (*S.* Typhimurium) and later challenged orally with virulent *S.* Typhimurium to assess their degree of immunity, miR-155-deficient mice were less readily protected after vaccination compared with wild-type C57BL/6 mice, with approximately 80% of mice succumbing to infection. Furthermore, following immunisation with tetanus toxin fragment C protein (TetC), a T cell-dependent antigen, miR-155-deficient mice produced significantly less antigen-specific antibodies and splenocytes retrieved from immunised mice and restimulated *in vitro* with TetC produced significantly less interleukin (IL)-2 and interferon (IFN)-γ. Similarly, uncommitted naive miR-155-deficient $CD4^+$ T cells cultured with anti-CD3 and-CD28 antibodies were also less able to produce IFN-γ and miR-155-deficient B cells stimulated *in vitro* with LPS or IL-4 proliferated normally but were unable to yield significant levels of IgG1. Dendritic cells (DCs) from miR-155-deficient mice were also tested for their ability to activate T cells in culture and despite their normal expression of major histocompatability complex (MHC)-II and co-stimulatory molecule CD86, miR-155-deficient DCs failed to activate cognate T cells. Thus, it appears that miR-155 is required for the function of a number of different cells of the immune system, including T cells, B cells and DCs.

1.2.3.4 miR-155 is required for normal B cell function and germinal centre formation

T cell-dependent antibody affinity maturation and memory B cell generation occurs within germinal centres (GCs), this is discussed in more detail in chapter 1.3.2.2. miR-155-deficient mice immunized with alum-precipitated 3-hydroxy-4-nitro-phenylacetyl (NP) coupled to chicken gamma globulin

(CGG) produced significantly less and smaller splenic GCs and NP-CGG-specific antibodies than wild-type control mice (Figure 3)[33]. However, sequencing of the antibody V_H186.2 gene segments showed that the absence of miR-155 had no effect on somatic hypermutation rate. Considered together these results suggest that miR-155 may be involved in GC formation. Thai *et al* showed using reverse transcription-polymerase chain reaction (RT-PCR) that miR-155 may partially control the GC response by regulating cytokine production. B cells stimulated in vitro with a BCR cross-linking agent produced significantly less TNF-α and lymphotoxin (LT)-α transcripts.

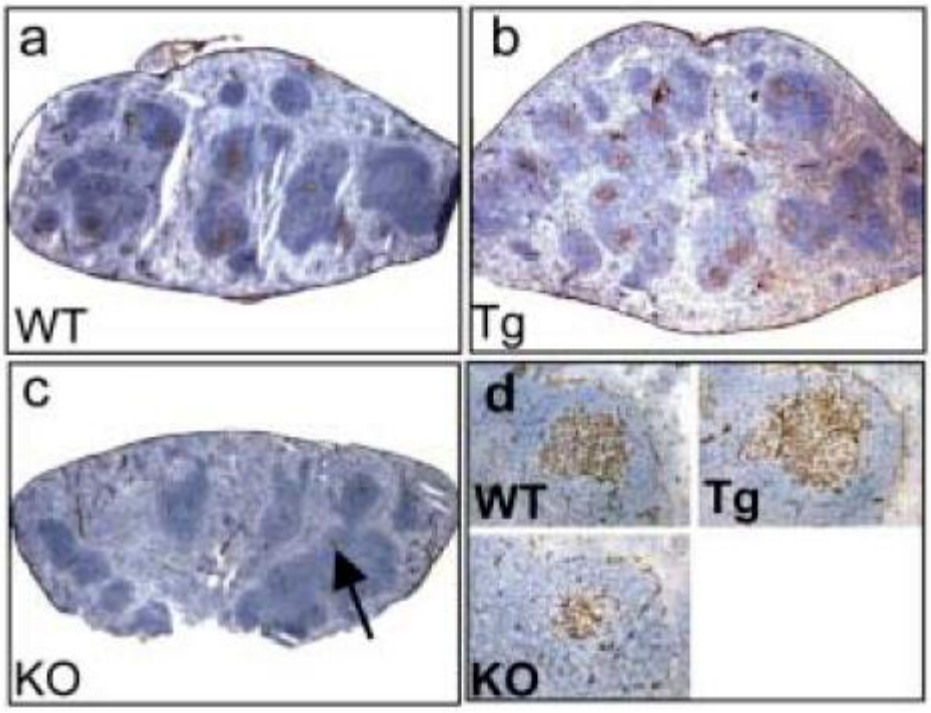

Figure 3. miR-155-/- mice show impaired T cell-dependent antibody responses

Immunohistochemistry performed on day 14 NP-immunized spleen sections from wild-type (a), B cell$^{miR-155}$ (b), and knockout mice (c) to detect GCs (brown, PNA$^+$; blue, haematoxylin). High-magnification image is shown in (d). Images are representative of three mice per group[33].

Vigorito *et al* have further shown that B cells lacking miR-155 generated reduced extrafollicular and germinal centre responses when mice were immunised with a T cell-independent antigen, dinitrophenylated

lipopolysaccharide (DNP-LPS) and a T cell-dependent antigen, 4-hydroxy-3-nitrophenylacetyl conjugated to keyhole limpet hemocyanin (NP-KLH). Immunised miR-155 deficient B cells produced significantly less antigen specific antibodies and exhibited defective antibody class switching[34]. They too showed that miR-155 was not required for somatic hypermutation and that it was dispensable for class switch recombination (CSR)[33, 34]. Furthermore, expression of the enzyme activation-induced cytidine deaminase (*Aicda*) which controls CSR was found to be normal in activated miR-155-deficient B cells. Most recently however, miR-155 has been identified as a direct negative regulator of Aicda[35-37]. The 3' UTR of Aicda mRNA possesses a miR-155 target site, disruption of which results in deregulation of Aicda expression, increased CSR and impaired affinity maturation[35, 37].

What is apparent is that miR-155 affects the function and development of a number of different cells of the immune system.

1.3 **The immune system and infection control**

Humans and vertebrates constantly come into contact with a variety of microbial organisms, many of which have the potential to cause disease, thus they have evolved an array of protective mechanisms to help combat these challenges. Firstly, they possess a number of anatomical barriers such as the outer layer of skin which is rich in keratin, a very tough and insoluble protein which physically prevents infection. Glands within the skin secrete lysozyme and oleic acid, antimicrobial compounds which kill bacteria. Gastrointestinal, genitourinary and respiratory tracts however prove problematic because they provide a means of access into the body which must also be protected. Accordingly these surfaces are covered in a layer of mucus which traps microorganisms, largely preventing them from adhering to the epithelium. Mucus also contains a number of important antimicrobial peptides such as defensins which play a major role in resistance against pathogens. In addition, microbiota in the gastrointestinal and genitourinary tracts limit the outgrowth of pathogenic bacteria by competing with them for vital nutrients and space[38].

Occasionally microorganisms are able to penetrate these anatomical barriers and for this reason many organisms have evolved an immune system. The immune system of mammals can be further divided into two components; the innate and the adaptive immune system.

1.3.1 The innate immune system

The innate immune system provides an immediate and largely non-specific response against any microorganism that is able to breach the physical, chemical and biological barriers[39, 40]. Cells and molecules of the innate immune system recognise small molecular motifs that are conserved between groups of organisms called pathogen associated molecular patterns (PAMPs). PAMPs include microbial DNA, lipids, polysaccharides and flagella proteins. Pattern recognition receptors (PRRs) allow the recognition of PAMPs and once activated the innate immune system responds in two main ways; inflammation and phagocytosis[41-58]. Cells that become infected or damaged release inflammatory mediators such as cytokines and eicosanoids, which

promote increased blood flow, and the chemotaxis of immune cells, leukocytes (white blood cells), into infected tissues. However, after an infection has resolved the innate immune system provides no long-term memory against a specific pathogen. Upon subsequent challenges with the same pathogen the innate immune system responds in the same generic way as it did previously. It is however worth noting that although the innate immune system is non-specific and possesses no immunological memory it is critically important for activating and directing the adaptive immune response.

1.3.2 The adaptive immune system

Vertebrates have evolved an adaptive in addition to an innate immune system which, enables the generation of specific and long-lasting protection against a particular pathogen, so that a stronger and faster attack can be mounted each time the pathogen is encountered. The adaptive immune system is centred upon highly specialised cells called lymphocytes. The two main types of lymphocytes are T cells which are involved in cell-mediated and B cells which are involved in antibody-mediated responses.

1.3.2.1 T lymphocytes

T cells can be distinguished from other lymphocytes because they express T cell antigen receptors (TCR) on their cell surface. TCRs are critical for recognition of foreign antigen associated with major histocompatability complexes (MHC) on the surface of antigen presenting cells. Additionally, T cells express one of two key surface co-receptors, CD8 or CD4 glycoproteins.

$CD8^+$ T cells are able to recognise antigen when it is complexed with MHC class I molecules. They are often referred to as Cytotoxic T lymphocytes (CTLs) because they are capable of destroying cells infected with protozoa, viruses and intracellular bacteria as well as cancer cells via the release of cytotoxins, perforin, granulysin and granzymes. Furthermore, upon activation, CTLs up-regulate expression of Fas Ligand (FasL), a surface transmembrane protein which aids in the recognition of Fas molecules expressed on target

cells. Binding of FasL to Fas initiates a caspase signalling cascade which ultimately results in apoptosis of target cells.

The CD4 surface glycoprotein is expressed on the surface of all T helper cells (T_h cells), so called because they provide 'help' to effector cells such as B cells, macrophages and CTLs. Thus despite having no cytotoxic or phagocytic capabilities T_h cells play an essential role in activating, regulating and directing other immune cells. Unlike $CD8^+$ T cells, $CD4^+$ T cells are capable of recognising antigen complexed with MHC class II molecules expressed on the surface of specialised innate immune cells called professional antigen presenting cells (APCs). Macrophages, B cells and dendritic cells are all examples of professional APCs, although dendritic cells are considered to be the most efficient at presenting antigen, particularly to naïve cells. During an infection, activated APCs positioned within infected tissues phagocytose foreign antigen and up-regulate co-stimulatory molecules including CD40, CD80 (B7.1) and CD86 (B7.2) and chemokine receptors such as CCR7 which, allow them to traffic into the lymph nodes. Once within the lymph nodes, APCs process the phagocytosed material and present derivative peptides complexed with MHC class II to naive $CD4^+$ T cells.

In order to become fully activated $CD4^+$ T cells require two independent signals; the first signal comes as a result of TCR recognition and binding of cognate peptide-MHC II expressed on the APC surface while the second signal is provided by the interaction of CD28 on the $CD4^+$ T cell surface with the APC co-stimulatory molecules, CD80 or CDCD86 (Figure 4). Once both signals have been received, the naive $CD4^+$ T cell is licensed to begin proliferating.

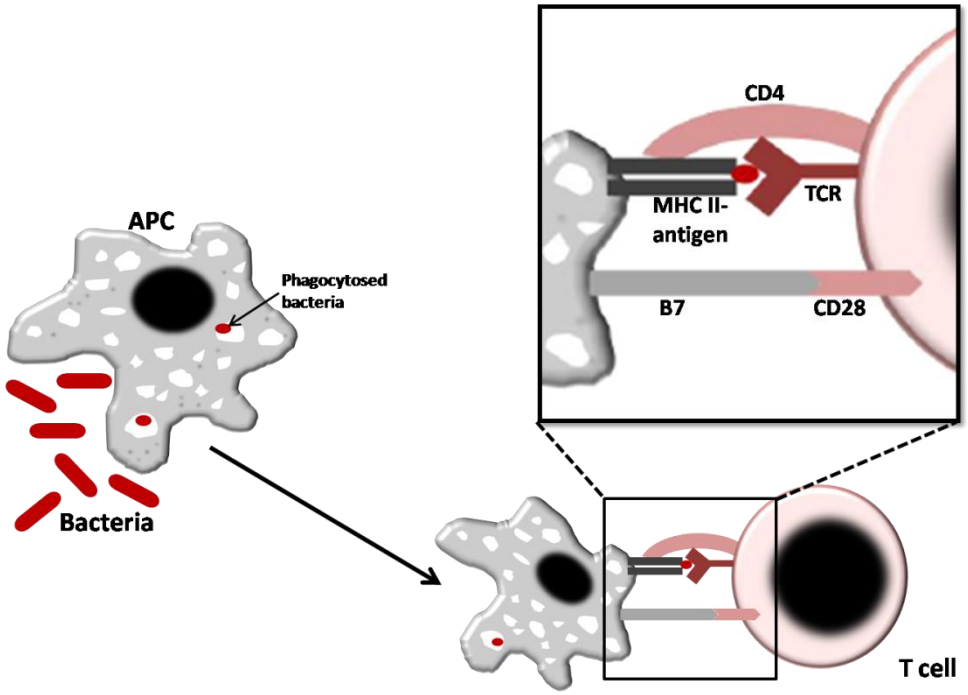

Figure 4. Antigen presentation between a professional APC and naive $CD4^+$ T cell

Professional APCs within infected tissues phagocytose and process foreign material e.g. bacteria and then traffic to the lymph nodes. Within the lymph nodes APCs present pathogenic peptides complexed with MHC class II to naive CD4 T cells. Naive CD4 T cells can only become activated after recognition and binding of cognate TCR to peptide/MHC complex as well as CD28-B7 co-receptor signalling.

After activation, $CD4^+$ helper T cells initially differentiate into T_h0 cells capable of secreting IL-2, IL-4 and interferon-γ (IFN-γ). The decision to further develop along the T_h1- or T_h2- differentiation pathways into either T helper 1 (T_h1) or T helper 2 (T_h2) cells respectively then depends on the milieu of cytokines elicited from pathogen-activated innate immune cells (Figure 5). Both T helper cell subtypes possess specific effector functions appropriate for dealing with different classes of pathogens. For example, T_h1 cells are characterised by their production of IFN-γ, lymphotoxin α (LTα) and IL-2, while T_h2 cells by their production of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), inteleukin-10 (IL-10) and interleukin-13 (IL-13).

T_h1 cells have specifically evolved to aid the clearance of viral and intracellular pathogens, their differentiation is dependent upon the co-operative actions of IFN-γ and IL-12 (Figure 5)[59, 60]. Upon infection, IFN-γ is rapidly produced by natural killer (NK) cells and cytotoxic $CD8^+$ T cells while activated dendritic cells (DCs) and macrophages are the major producers of IL-12. All naive T cell precursors express functional receptors for type I and type II interferons, IFN-γ and IFN-α, respectively. Signalling through these cytokine receptors activates signalling transducer and activator of transcription 1 (STAT1) which, in turn up-regulates T-bet, a T_h1-specific transcription factor[61]. Expression of T-bet simultaneously inhibits T_h2 differentiation whilst inducing T_h1 differentiation. T-bet functions by increasing both the production of IFN-γ and the expression of the IL-12 receptor (IL-12R) signalling subunit, IL-12Rβ2. Consequently, IL-12 signalling through IL-12Rβ2 activates STAT4 and induces IL-18Rα expression. Mature T_h1 cells that express both IL-12Rβ2 and IL-18Rα are able to produce IFN-γ through TCR-dependent and independent pathways, in response to IL-12 plus IL-18[60]. T_h1 cells have been shown to play a vitally important role in macrophage-mediated inflammatory responses[62-64]. The IFN-γ produced by T_h1 cells has two key functions; firstly it enhances the microbial actions of macrophages and is therefore critical for enhancing macrophage killing efficacy. Secondly, it stimulates B cells to produce IgG antibodies which, bind and opsonise microbes thus promoting phagocytosis. IgG2a and IgG3 are the main IFN-γ-dependent antibody isotypes produced in mice[64, 65]. In addition, IFN-γ together with the T_h1 cytokine, IL-2 promote the differentiation of $CD8^+$ T cells into active CTLs[64].

Conversely, T_h2 cells have developed specifically to enhance the clearance of parasites. It is known that signalling through the Interleukin-4 receptor (IL-4R) and subsequent activation of Stat-6 in conjunction with TCR signalling is sufficient to promote T_h2 cell differentiation (Figure 5)[66, 67]. Simultaneous Stat-6 activation and TCR signalling results in the up-regulation of GATA-3, a transcription factor responsible for the transcription of all T_h2 cytokine genes[67]. T_h2 cells are involved in stimulating B cells to proliferate and for

inducing antibody class-switching (Figure 5). For instance, IL-4 a key T_h2 cytokine induces B cells to switch to the production of IgE which, in turn activates mast cells and basophils. T_h2 cells also stimulate B cells to produce high levels of IgM and non-complement-fixing IgG isotypes, such as IgG1 in mice[65].

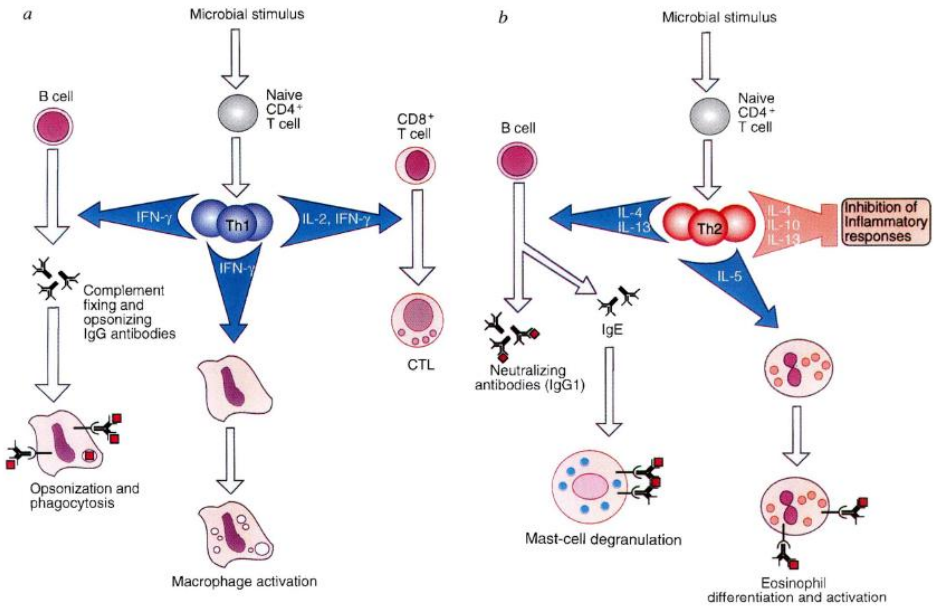

Figure 5. Effector functions of Th1 and Th2 subsets of CD4 helper T lymphocytes.

Th1 cells (a) induce phagocyte and T-cell mediated defence reactions against microbes; Th2 cells (b) induce IgE-dependent mast-cell degranulation and eosinophil activation, two components of the immediate hypersensitivity response[64].

1.3.2.2 B lymphocytes

B cells are the component of the adaptive immune system responsible for the production of antibodies (or immunoglobulins) during humoral immune responses. Antibodies are heavy (~150 KDa) globular plasma proteins consisting of four polypeptide chains; two identical heavy chains and two identical light chains linked by disulphide bonds (Figure 6). Antibodies can be either membrane-bound, as part of the B cell receptor (BCR) or secreted by B cells in a soluble form.

There are five different types of heavy chain; Alpha (α), Delta (δ), Epsilon (ε), Gamma (γ) and Mu (μ) which, determine the antibody isotype IgA, IgD,

IgE, IgG and IgM respectively. Different antibody isotypes occupy distinct functional locations within the body and possess unique effector properties. Each heavy chain has two regions; a constant region which is identical between antibodies of the same isotype and a variable region which is unique to each B cell. In mammals, there are two different immunoglobulin light chains, Kappa (κ) and Lambda (λ) and each antibody will possess two identical light chains of either type. Analogous to the heavy chain, each light chain has both a constant and variable region.

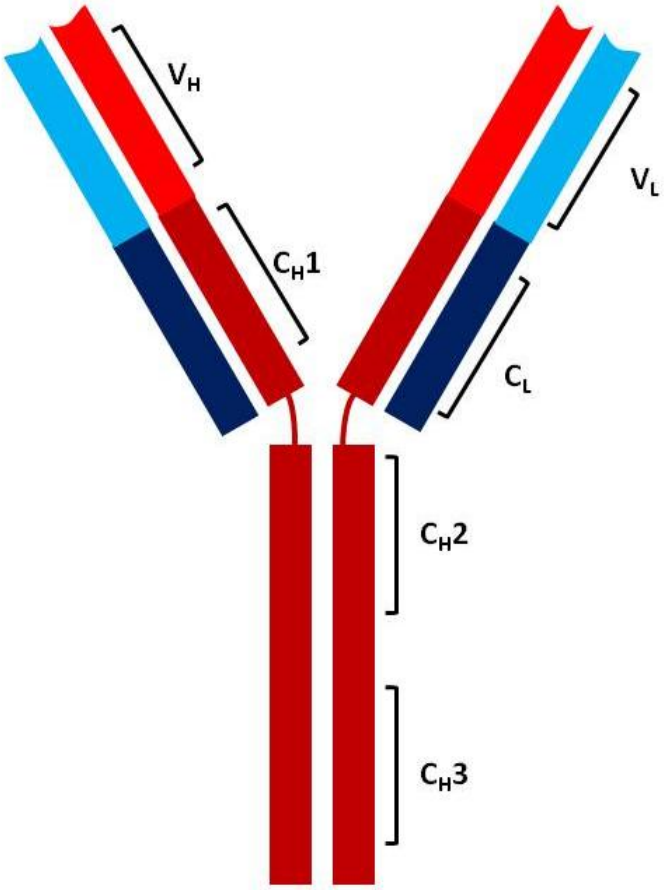

Figure 6. General structure of an antibody molecule

Antibodies consist of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulphide bonds. Each heavy chain has a constant (C_H1, C_H2 and C_H3) and a variable (V_H) region. Each Light chain has a constant domain (C_L) and a variable domain (V_L)

The variable regions of the heavy and light immunoglobulin chains are encoded by multiple genes organised into distinct segments (Figure 7). The genes encoding the heavy chain variable region are segregated into variable (V) genes, Diversity (D) genes and Joining (J) genes whilst the genes encoding the light chain variable region are organised into V and J genes (Figure 7). The process by which recombination of V, D and J gene segments occurs during lymphocyte development is called somatic recombination and it enables the production of a considerable lymphocyte repertoire from a limited number of genes[68]. For example, there is estimated to be approximately 10^{12} unique B cells in the human body, each capable of binding a distinct epitope of an antigen[69].

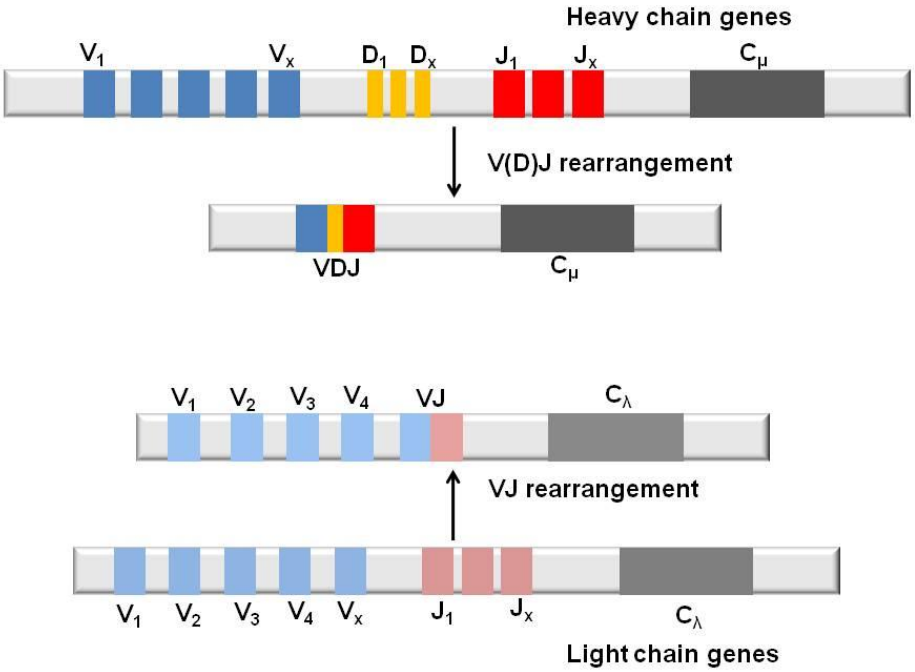

Figure 7. Immunoglobulin Somatic Recombination

The variable regions of immunoglobulin heavy and light chains are encoded by multiple genes arranged into segments. The heavy chain genes are organised into Variable (V), Diversity (D) and Joining (J) genes whilst the light chain genes are arranged into V and J genes.

B cell development is a highly regulated process that occurs in the liver during mid-to-late foetal development and within the bone marrow after birth. B cells are generated from a common lymphoid progenitor that also give rise to T cells, NK cells and lymphoid dendritic cells[70]. Lymphoid progenitor cells can only begin development upon receiving specific developmental signals from stromal cells within the bone marrow. Interactions between the stromal cell transmembrane protein, stem cell factor (SCF) and CD117 (c-Kit receptor) expressed on lymphoid progenitor cells are known to be important signals for initiating the development of both B and T cells, as is the binding of cytokine IL-7 (secreted from stromal cells) to the IL-7 receptor (IL-7R) on developing lymphoid progenitor cells. Co-ordinated signalling through these receptors induces the expression of recombination-activating genes (RAG-1 and RAG-2) and terminal deoxynucleotidyl transferase (TdT) in lymphoid progenitor cells. RAG-1 and RAG-2 are crucial for somatic recombination as mice with disruptions of either gene are completely defective in V(D)J recombination[68]. During recombination, RAG protein complexes bind to recombination signal sequences (RSSs), consisting of a conserved nonamer and heptamer element, separated by a spacer of 12 or 23 nucleotides, proceeding two immunoglobulin gene segments to be joined together[68]. As a consequence of binding between the RAG protein complexes, the two RSSs are brought into close proximity. RAGs then cleave the DNA at the heptamer/coding borders creating hairpin structures at the ends of the segments to be joined. The hairpin structures are subsequently opened, processed and ligated to form an imprecise coding joint. The processing and ligation stages are mediated by terminal deoxynucleotidyl transferases (TdT) and non-homologous end-joining factors which are responsible for the addition and subtraction of nucleotides from the segment ends, thus leading to greater antibody diversity.

The first stages in B cell development involve the joining of the D and J gene segments on the Heavy (H) chain chromosome to form early pro-B cells (Figure 8). At this stage, the cells also begin expressing CD45 (B220) and MHC class II. In the late pro-B cell, the V segment is joined to the already

combined D-J_H genes and if V(D)J rearrangement is successful, cells begin expressing membrane μ chains together with surrogate light chains (which are the same on every pre-B cell) and the signal transduction molecule, CD79 (formerly known as IgαIgβ) in a complex known as the pre-B receptor (pre-BCR), this represents the large pre-B cell stage (Figure 8). At this juncture, if the pre-BCR binds antigen, phosphorylation of the Immunoreceptor Tyrosine Activation Motifs (ITAMs) within the cytoplasmic tails of CD79 results, subsequently initiating a cytoplasmic signalling cascade that immediately halts recombination of the H chain and activates B cells to proliferate into a clone of cells all expressing the same μ chain (Figure 8). Following proliferation, the small pre-B cells formed begin combining their V and J segments on one Light (L) chain chromosome until the L chain is successfully rearranged and expressed on the cell surface with the μ chain (Figure 8). Next immature B cells undergo a process known as negative selection whereby cells that recognise self-antigen within the bone marrow die via apoptosis, this eliminates potentially harmful self-reactive B cells. B cells that do not recognise self-antigens are licensed to exit the bone marrow and become mature naive B cells expressing both membrane IgM and IgD (Figure 8).

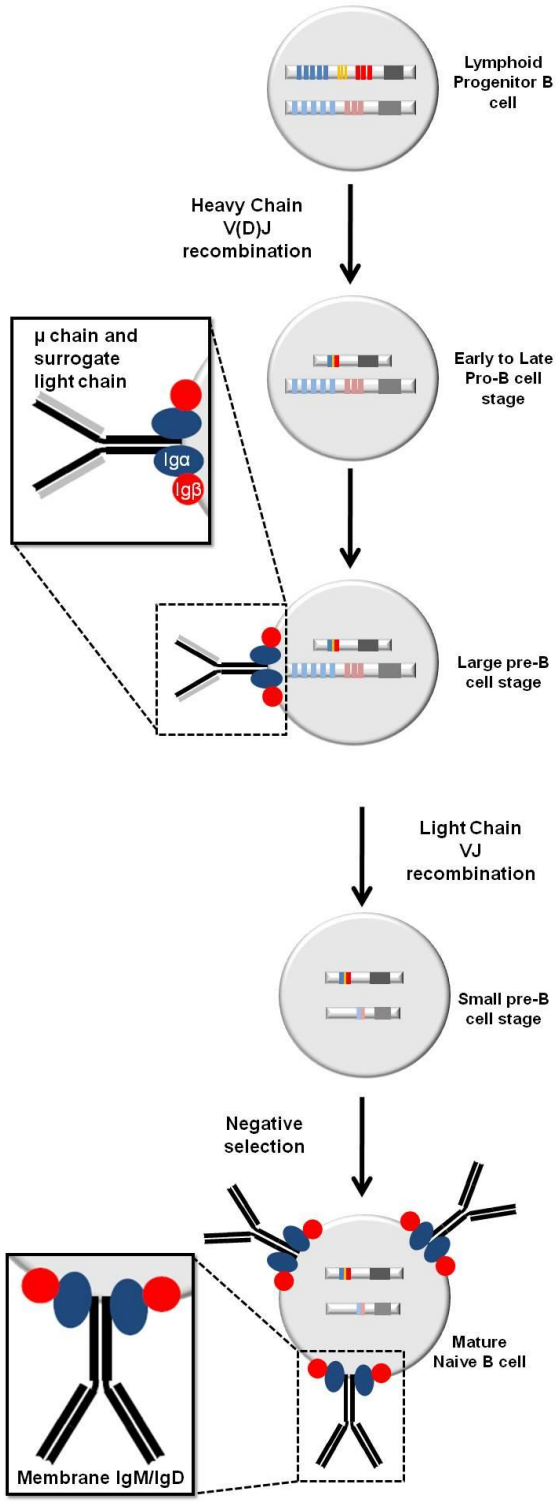

Figure 8. Stages in B cell development

With appropriate signals lymphoid progenitor B cells are stimulated to begin V(D)J recombination. In the first stage, D and J gene segments on the Heavy (H) chain chromosome

are recombined, the resulting cells are known as early pro-B cells. In late pro-B cell stage, cells begin expressing CD45 (B220) and MHCII and complete V(D)J rearrangement with the V segment joining to the combined D-J_H genes. After successful V(D)J recombination, cells begin to express pre-B receptor which, consists of membrane μ chains together with surrogate light chains and signal transduction molecules, IgαIgβ, this represents the large pre-B cell stage. Intracellular signalling resulting from binding of the pre-B receptor to antigen halts recombination of the H chain gene segments and stimulates cells to proliferate. Following clonal expansion, small pre-B cells begin combining their V and J segments on one Light (L) chain chromosome. If the L chain is successfully rearranged it will subsequently be expressed on the cell surface with the μ chain, this represents the immature B cell. Before exiting the bone marrow, immature B cells are negatively selected for their ability to bind self-antigen. B cells that do not recognise self-antigen are licensed to exit the bone marrow and are now referred to as mature naive B cells.

1.3.2.2.1 B cell activation

Mature naive B cells that have exited the bone marrow circulate continuously in the blood and lymph until they encounter antigen specific to their BCR. Antigen cross-linking of the BCR results in the recruitment and formation of multi-protein signalling complexes and consequently triggers a number of important signalling cascades (Figure 9)[71]. The culmination of the signalling cascades is the activation of a number of transcription factors and the transcription of various genes, including miR-155, responsible for B cell activation and enhanced expression of cell surface antigen presentation molecules[72].

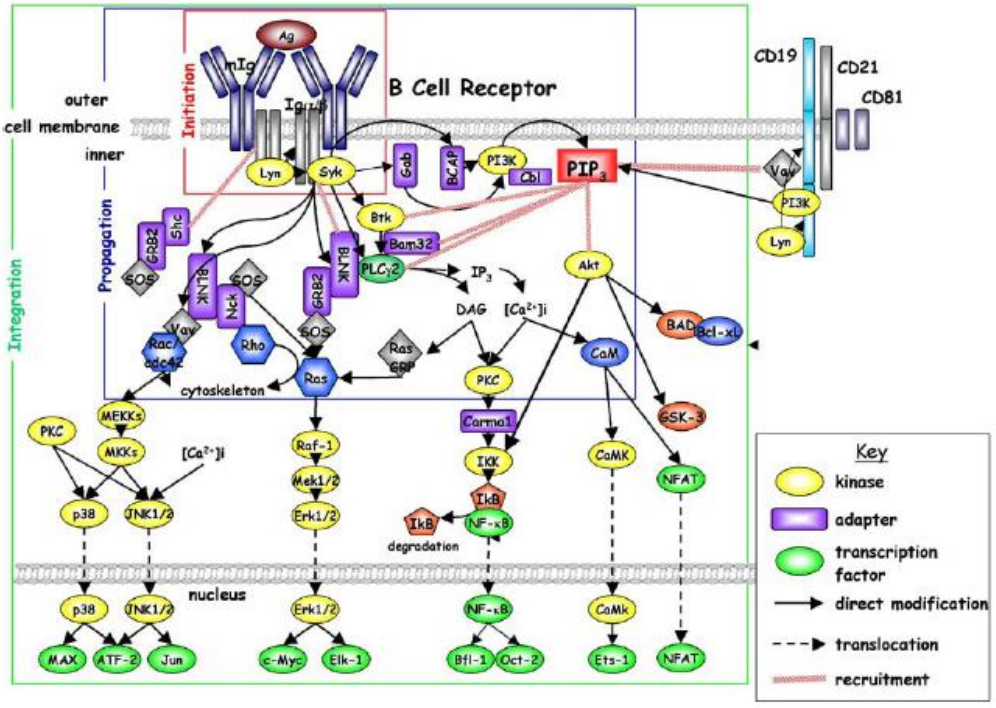

Figure 9. B cell antigen receptor signal transduction cascade

Signal transduction initiates at the cell membrane following ligand-induced aggregation of the membrane immunoglobulin (mIg) and associated signal transducing elements Igα and Igβ. Signals are then propagated by means of protein phosphorylation, modification, and interaction. The culmination of the signalling cascade is the regulation of transcription factor activation and gene expression. While not shown, accessory receptor molecules, such as CD19 or CD22, can influence BCR signalling at various levels in the signalling pathway[71].

During a T cell-dependent antibody response effector T_h cells recognise and bind antigen-MHCII on cognate B cells (Figure 10). Signals delivered through direct TCR-MHCII interaction greatly enhances B cell activation but also and more importantly stimulates B cells to traffic into the extrafollicular T cell rich regions of secondary lymphoid organs to begin differentiation into plasmablasts and plasma cells capable of secreting low affinity antibodies (Figure 10). Alternatively, several B cells relocate to primary follicles within the B cell zone and undergo exponential growth amongst a network of follicular dendritic cells (FDCs), this is known as the germinal centre reaction (Figure 10)[73-77].

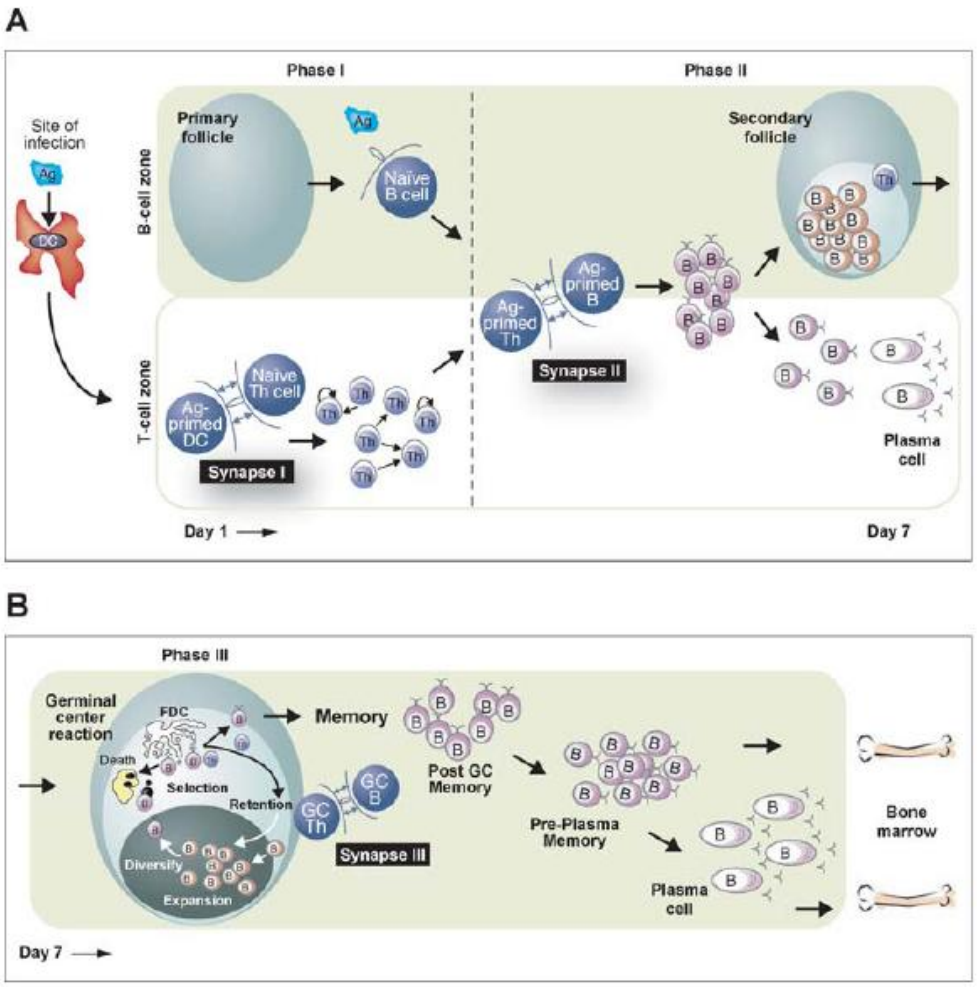

Figure 10. Th cell-regulated B cell memory development

(A) Schematic of the cellular interactions that proceed in the secondary lymphoid organs toward development of antigen-specific effector Th cells (Phase I) and effector B cells (Phase II). (B) A schematic of cellular activity in the GC cycle (Phase III) that leads to the development of antigen-specific memory B cell subsets. The three main cellular products of the GC are depicted[78].

Besides the MHCII-TCR interaction, several other receptor-ligand interactions are known to play an important role in T cell-dependent B cell activation (Figure 11)[72, 78]. CD40, an integral membrane protein constitutively expressed by B cells is known to play a pivotal role in governing humoral and cell-mediated immunity. Engagement of CD40 with CD40L (CD154) up-regulated on activated T_h cells can lead to B cell clonal expansion, germinal centre formation, isotype switching, affinity maturation and generation of

long-lived plasma cells[72, 78-80]. Furthermore, engagement of CD72, CD134 Ligand (CD134L), B cell activating factor (BAFF) and a proliferation-induced ligand (APRIL) as well as CD27 with their corresponding T cell ligands are all thought to be potentially important during humoral immune responses[72, 81]. Cell adhesion molecules, ICAM-1 (CD54) and LFA-1 (CD11a-CD18), have additionally been shown not only to enhance adhesiveness between B and T cells but directly transmit activation signals to B cells themselves,

B cell receptor	T cell ligand	B cell effector functions
Class II MHC	TCR; CD4	Cooperates with other activation signals to stimulate proliferation, differentiation and enhanced antigen presentation
CD11a–CD18/CD54	CD54/CD11a–CD18	Cell adhesion, enhanced antigen presentation and enhanced activation
CD72	CD100	Development of B-1 B cells, production of high-affinity IgG response and enhanced antigen presentation
CD40	CD154	Proliferation, differentiation, isotype switching, cytokine production, protection from apoptosis, and germinal center and memory response development
CD134L/OX40L	CD134/OX40	Stimulation and enhancement of IgG response
CD137L/4-1BBL	CD137/4-1BB	Stimulation of T cells through CD137
CD27	CD70	Differentiation into plasma cells
CD30/CD153	CD153/CD30	Inhibition of B cell responses, such as isotype switching and plasma cell differentiation
CD95/Fas	CD95L/FasL	Induction of programmed cell death (apoptosis)

Figure 11. B cell transmembrane receptors and T cell ligands involved in contact-dependent regulation of B cell activation[72].

contributing to enhanced B cell antigen-presentation and activation[72]. Furthermore, in addition to cell surface molecules which promote B cell activation there are a number of molecules which negatively regulate humoral immune responses. For example, CD30- CD153 interactions appear to inhibit isotype switching while B cells expressing CD95/Fas are more susceptible to CD95L-induced apoptosis in the absence of additional survival signals.

1.3.2.2.2 Germinal centres

Germinal centres (GCs) are regions within primary lymphoid follicles which support the generation of memory B cells and plasma cells capable of producing high affinity antibodies for a particular antigen[74, 82-98]. GCs only develop in response to immunogenic stimuli, this is highlighted in the fact that GCs are completely absent in germ-free animals[97]. In a T cell-dependent B cell response, GCs develop and persist during the first 3 weeks following immunization or exposure to antigen[77]. Naive B cells are activated within T cell-rich extrafollicular areas of secondary lymphoid organs before migrating to primary follicles to form germinal centres (Figure 10)[77]. However, before primary follicles are involved in antigenic responses they consist mainly of B cells re-circulating through a follicular dendritic cell (FDC) network. It has previously been shown that each follicle is colonized by an average of three B cell blasts, thus the resulting germinal centres are oligoclonal. Once within the follicle the aforementioned B blasts begin undergoing massive clonal expansion and somatic hypermutation (SHM) of their antibody variable region genes. Substantial clonal expansion of blasts displaces the small re-circulating follicular B cells to the outer edge of the FDC network where they form the follicular mantle (Figure 12). After approximately 3 days of rapid division, the B blasts are induced to move to one pole of the FDC network and differentiate into centroblasts ultimately forming the dark zone of the mature germinal centre (Figure 12)[76, 77]. It is known that the CXC chemokine receptor, CXCR4, which is highly expressed on centroblasts and, its ligand SDF-1 are required for repositioning of centroblasts to the dark zone[99, 100]. Proliferating centroblasts give rise to centrocytes which, are no longer in cell cycle and relocate to the light zone of the GC to interact with the network of FDCs (Figure 12)[101].

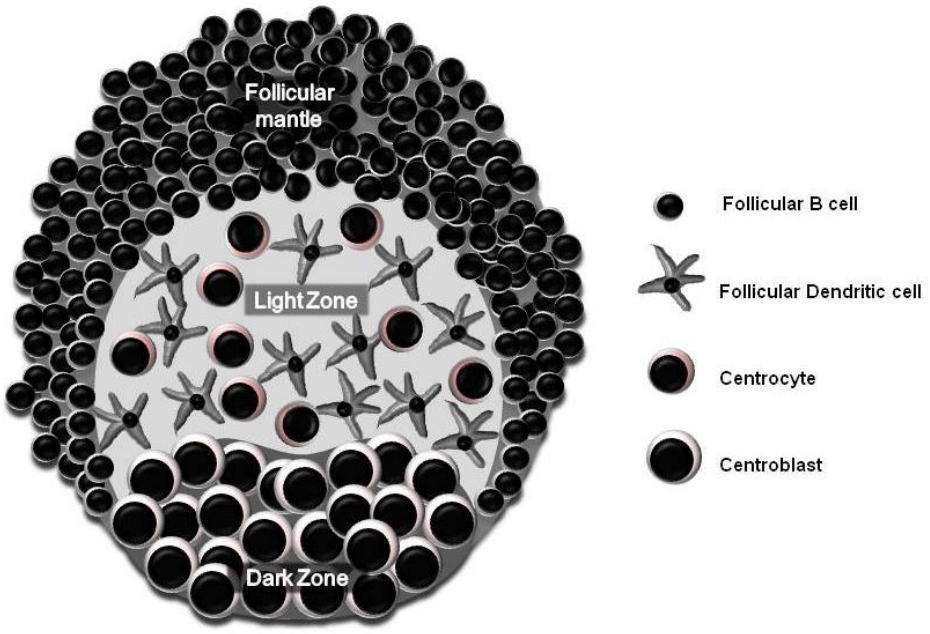

Figure 12. Schematic representation of germinal centre compartments

Small re-circulating follicular B cells displaced to the outer edge of the FDC network form the follicular mantle. The dark zone contains densely packed centroblasts, many of which are undergoing proliferation. The dark zone is adjacent to the T cell zone in the spleen and lymph nodes and the serosa in Peyer's patches. The light zone contains both non-dividing centrocytes and FDCs. In the spleen, the light zone is proximal to the marginal sinus while in the lymph nodes it is positioned close to the subcapsular sinus. Additionally, in the Peyer's patches, tonsils and appendix, the light zone is orientated toward the mucosal surface[101].

The FDCs in the light zone have the unique ability to capture and display large amounts of antigen in the form of immune complexes on their cell surface for periods of over a year[77, 101]. Centrocytes that have undergone successful SHM are selected on the basis of their affinity to bind such immune complexes. Based on the presence of tingible body macrophages within GCs it has been suggested that most B cells die during this selection process[101, 102]. Two possible models for GC B cell selection have been suggested; the first hypothesises that selection is based solely on adequate BCR signalling (Figure 13). B cells with no or very low affinity for antigen receive inadequate BCR signalling and rapidly die by apoptosis while those that express immunoglobulin (Ig) with increased affinity are able to survive and proliferate

with T cell help. In the second model, robust BCR signalling is necessary but, additional T cell help is required (Figure 13)[101]. Engagement of the BCR, ligation of CD40 and/or expression of *bcl-XL* or *bcl-2* inhibits apoptosis and promotes survival. It is important to note that in both models there is a strong positive selection for B cells that produce high affinity antibodies for the stimulating antigen.

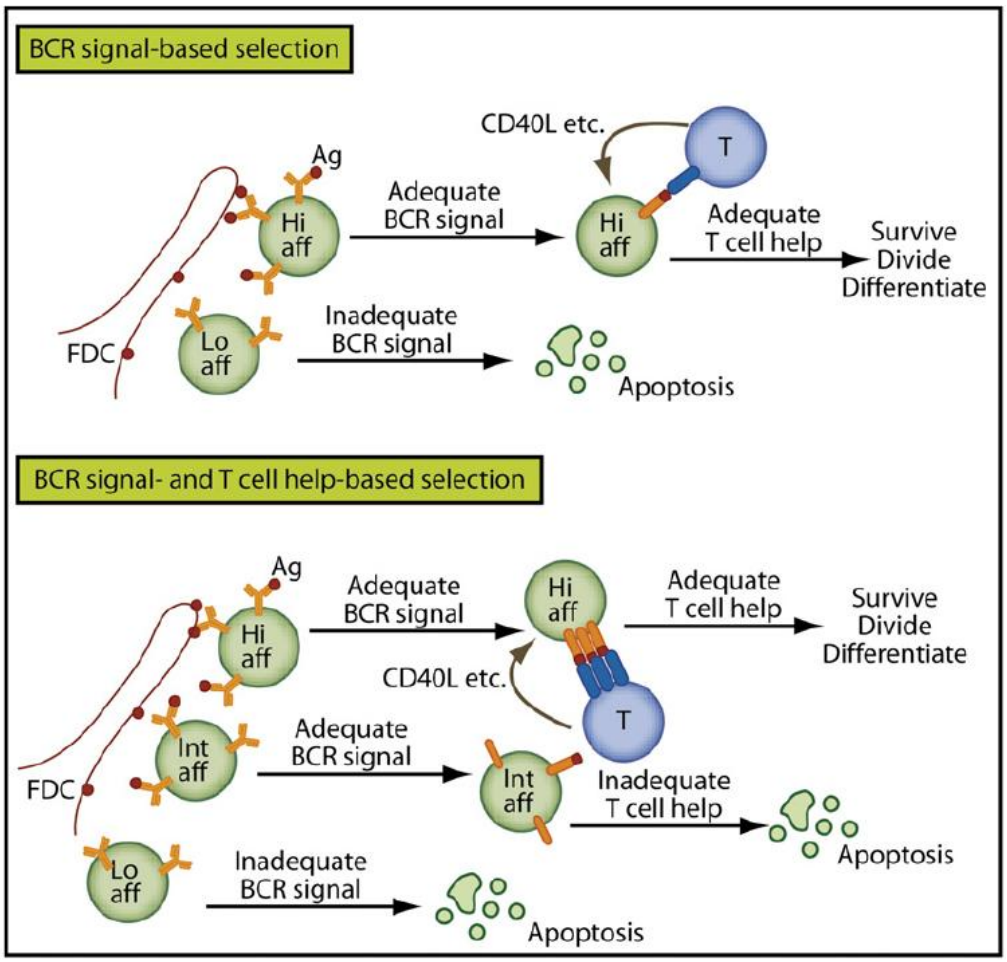

Figure 13. Two models of GC B cell Selection within the GC

In the first model, discrimination between cells of different affinity for the foreign antigen occurs solely at the level of different strengths of BCR signalling. In the second model, sufficient BCR engagement is still necessary, but further selection occurs because of competition for T cell help. B cells that have captured, processed, and presented more antigen as MHC-peptide complexes go on to receive T cell help at the expense of cells that have captured less antigen. “Hi Aff,” “Int Aff,” and “Lo Aff” refer to B cells with high-, intermediate-, and low-affinity BCRs, respectively[101].

1.3.2.2.3 Antibody Affinity maturation

During infection the average affinity of serum antibody to stimulating antigen has been shown to increase over time and has thus been termed affinity maturation. Affinity maturation is known to occur within germinal centres and involves both somatic hypermutation (SHM) and class switch recombination (CSR) (Figure 14). SHM involves the addition of point mutations into the variable- regions of heavy and light chain coding sequences. The mutation rate during SHM is extremely high, occurring approximately one million times faster than spontaneous mutations in other genes and as a result one mutation occurs per V-region coding sequence per cell generation. During CSR the default Ig constant region (Cμ) is exchanged with different constant regions such as Cγ, Cα, Cδ and Cε[83]. The RNA-editing deaminase, activation-induced cytidine deaminase (Aicda or AID) is both required and sufficient to induce SHM and CSR[103]. During SHM, aicda deaminates cytosine residues on the single stranded DNA (ssDNA) exposed in the non-transcribed strand of double stranded DNA (dsDNA) during transcription (Figure 14)[103-110]. Uracil·guanine (U·G) mismatches are recognised by the mismatch repair (MMR) complex and processed in a number of ways. Replication over the lesion by high fidelity polymerases results in a C→T transition on the newly synthesised strand (Figure 14). Alternatively U residues might be excised leading to the production of abasic sites that are unfaithfully repaired (Figure 14). For CSR to occur, aicda must generate double-stranded breaks within the donor switch constant μ region and the recipient donor switch region of another downstream constant exon (Figure 14). The donor switch regions are then joined together via non-homologous end joining creating a different antibody isotype and the constant exons between the two breaks are deleted (Figure 14)[103].

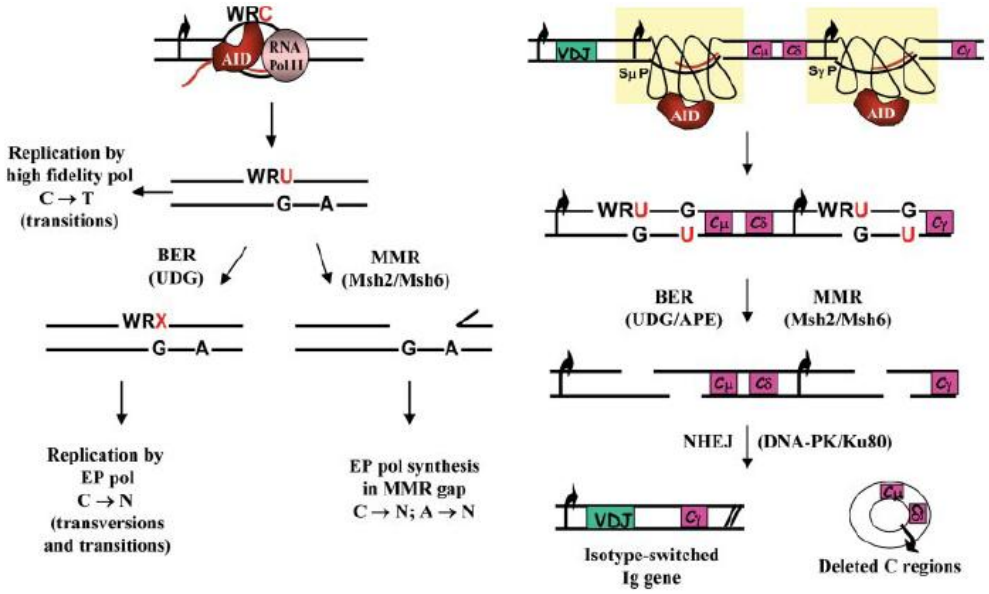

Figure 14. Processing the AID-induced U•G mismatches during SHM (left) and CSR (right)

AID deaminates C on ssDNA exposed during transcription in V and S regions. In the V region, U may be copied with high fidelity polymerases (*Pols*) giving rise to C→T transitions. Alternatively, uracil glycosylase (UDG) may remove U to create an abasic site, and if replicated over with error prone (EP) *Pols*, C→N transitions and transversions could be created. Mismatch repair (MMR) proteins could recognise the U·G mismatch and creates a large repair gap. If the gap is filled in by EP *pols*, C→N and A→N mutations could arise. AID-generated U in the donor switch (S) regions may be processed by basic excision repair (BER) or MMR enzymes to generate dsDNA breaks. The breaks are then repaired by non-homologous end-joining proteins giving rise to an isotype-switched Ig gene[103].

1.3.3 Lymphoid tissue and the Mucosal immune system

In vertebrates, the lymphatic system consists of a network of vessels transporting lymph fluid, circulating lymphocytes, leukocytes and APCs around the body. Most importantly, lymphatic vessels pass through lymphoid tissue whose main function is in the generation of immunity. Lymphoid tissue is rich in B and T lymphocytes as well as accessory cells such as macrophages and reticular cells. It is commonly classified according to the stage of lymphocyte development and maturation it participates in; for example, primary (central) lymphoid tissue such as the thymus and bone marrow are involved in the generation and early selection of lymphocytes, whilst the lymph nodes and lymphoid follicles within the tonsils, Peyer's patches, spleen, adenoids and skin constitute the secondary (peripheral) lymphoid tissues and are involved in the generation of immune responses.

Because secretory epithelia such as the gastrointestinal and respiratory tracts provide a portal into the body for pathogenic organisms, a large amount of lymphoid tissue is associated with mucosal surfaces[111]. The mucosa-associated lymphoid tissue (MALT) consists of organised lymphoid aggregates dispersed along the surfaces of all mucosal tissues. MALT is organized similarly to lymph nodes, with B cell follicles surrounded by interfollicular T cell areas and an efferent lymphatic system however, a striking difference is that there is no afferent lymph supply[112-114]. MALT is often subdivided depending upon the mucosal surface in which it resides; gut-associated lymphoid tissue (GALT), nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT).

GALT is comprised of patches of lymphoid tissue within the gastric mucosa of the small and large intestine, termed Peyer's patches (Figure 15)[115-119]. Peyer's patches are found in greatest density in the jejunum and are considered to be the main site for the development of IgA^+ B cells. In mice, there is a further collection of lymphoid tissue located near the blind end of

the caecum, called the caecal patch. Additionally, multiple small isolated lymphoid follicles (ILFs) have recently been identified on the anti-mesenteric wall of the mouse small intestine[120, 121]. ILFs are similar in structure and function to Peyer's patches with a follicle associated epithelium (FAE) over-laying aggregates of lymphocytes but distinctly lack T-cell rich interfollicular regions (Figure 15)[121-123]. It has been suggested that, in addition to the Peyer's patches, the ILFs may represent an important source of IgA^+ B cells.

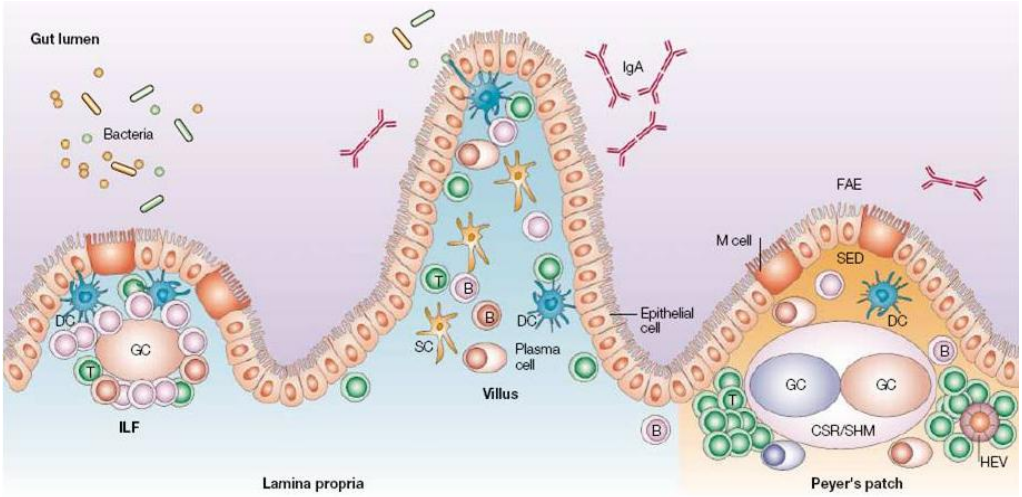

Figure 15. Gastric-associated lymphoid tissue (GALT)

Schematic representation of the gastric-associated lymphoid tissue (GALT), with organised lymphoid structures; Peyer's patches and isolated lymphoid follicles (ILFs) — and diffuse tissue of the epithelium and the lamina propria. Peyer's patches and ILFs are composed of a specialized follicle-associated epithelium (FAE) containing M cells, a subepithelial dome (SED) rich in dendritic cells (DCs), and B-cell follicle(s) that contain germinal centers (GCs), where follicular B cells efficiently undergo class-switch recombination (CSR) and somatic hypermutation (SHM). Migration of B cells into the mucosa takes place through high endothelial venules (HEVs), located in the interfollicular regions of Peyer's patches, which contain mostly T cells. The diffuse tissues of the lamina propria contain a large number of immunoglobulin A $(IgA)^+$ plasma cells, T and B cells, macrophages, dendritic cells (DCs) and stromal cells (SCs). IgA^+ B cells and plasma cells are shown in red, IgG^+ cells in blue and IgM^+ cells in pink[124].

1.3.3.1 Follicle-associated epithelium (FAE)

Simple columnar epithelial cells lining the small and large intestine are joined together by tight junctions which, render the epithelium impermeable to molecules and ions as well as pathogenic organisms and pathogen-derived antigens[125]. Yet in order to generate protective immune responses it is critical that cells of the immune system sample antigens from the gut lumen. To overcome this challenge the GALT is enclosed by a specialized epithelium dedicated to antigen sampling, termed the follicle-associated epithelium (FAE) (Figure 15 and 16)[126-146]. Interspersed within the FAE are unique, antigen sampling microfold (M) cells (Figure 16). M cells have several distinctive characteristics that allow them to be easily identified. These cells differ from normal enterocytes because they lack the typical enterocyte apical brush border. In its place they possess irregular, short, broad and poorly organised microfolds. M cells do not secrete mucus or digestive enzymes and the filamentous brush border glycocalyx (an extracellular polysaccharide layer found throughout the intestine adjacent to enterocytes) is either much thinner or completely absent, allowing them to readily sample antigen from the gut lumen. Notably, M cells have an extremely high capacity for transporting antigens through the epithelium, it has been documented that M-cell mediated endocytosis, translocation and exocytosis can take as little as 10 minutes[145, 146, 147]. Another unique feature of M cells is their intraepithelial 'pocket' formed by a large invagination of the basolateral membrane. Harboured within these 'pockets' are lymphoid cells and APCs such as macrophages which, phagocytose transcytosed antigen (Figure 16)[132]. Some bacteria however have adapted to exploit the use of M cells for invasion of the body. M cells, especially those clustered in the FAE of Peyer's patches are believed to be a major portal of entry for *Yersinia* and *Salmonella*[148].

It is also worth noting that antigen sampling in the mucosal epithelium of the oral cavity, pharynx, oesophagus, urethra and vagina can be carried out by lamina propria DCs directly projecting dendrites into the lumen[132, 149-151].

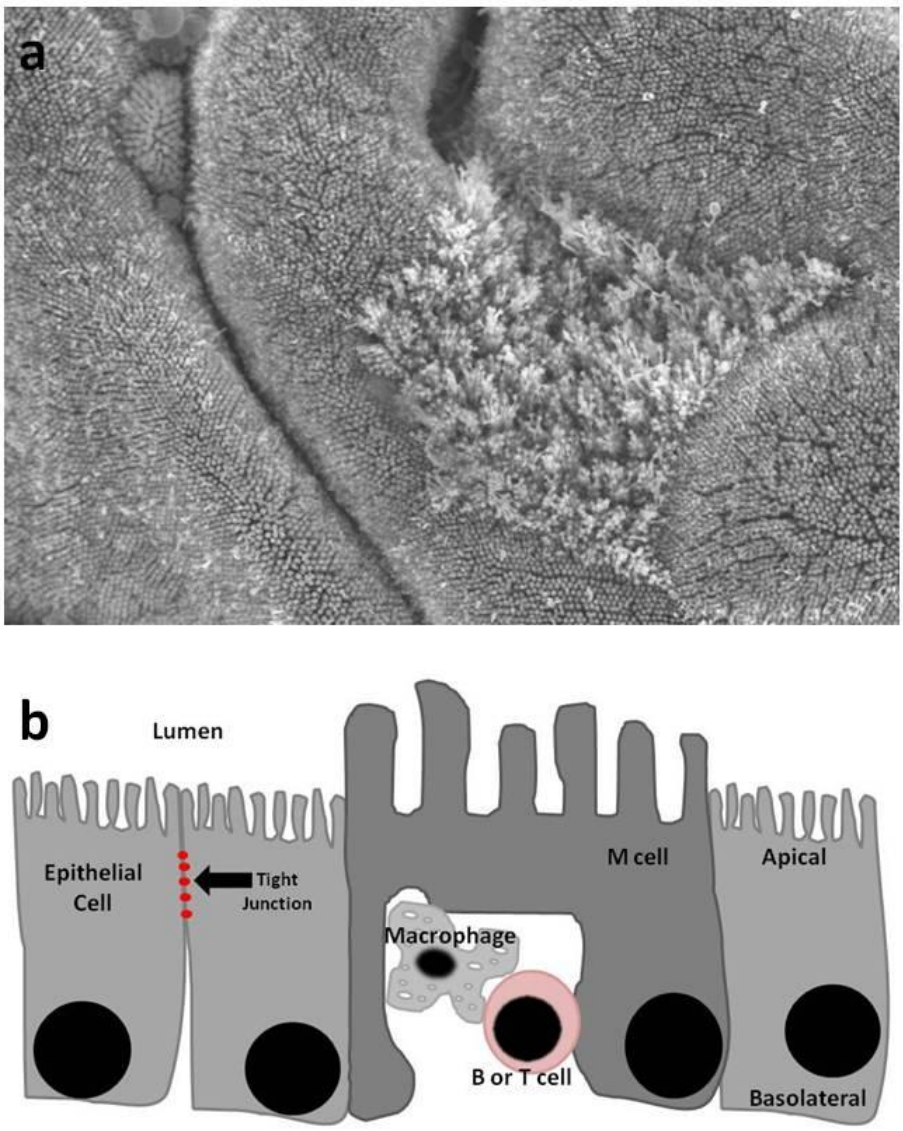

Figure 16. M cell

M cells are specialised residents of the FAE that mediate uptake and transcytosis of antigen from the intestinal lumen to APCs in the underlaying sub-epithelial dome (SED). (a) SEM of an Intestinal M cell, positioned within the FAE surrounded by enterocytes possessing uniform apical brush borders. The apical membrane of M cells is specially arranged into irregular, short and poorly organised microfolds instead of microvilli. Furthermore, M cells do not secrete mucus or digestive enzymes and the filamentous brush border glycocalyx (an extracellular polysaccharide layer found throughout the intestine attached to enterocytes) is much thinner or absent. (b) Schematic representation of an M cell within the FAE. Intestinal simple columnar epithelial cells (enterocytes) are joined together by tight junctions which prevent the passage of molecules, ions and pathogens through the spaces between cells. M

cells endocytose, translocate and exocytose antigen from the gut lumen to lymphoid cells and APCs positioned within an intraepithelial ‘pocket’.

1.3.3.2Mucosal immune system

There are two functional compartments associated with the GALT; inductive sites, where the immune response is initiated, and effector sites, where the immune response exerts its effects (Figure 17)[124]. At mucosal inductive sites, APCs phagocytose transcytosed pathogen-derived antigen and become activated, up-regulating their expression of MHCII and co-stimulatory molecule B7 (Figure 17). In addition they begin secreting cytokines such as IL-1, 6, 8, 12 and TNF-α which help to activate and recruit other cells of the immune system. Upon activation, APCs begin trafficking to secondary lymphoid tissues such as the Peyer’s patches/ILFs, spleen and mesenteric lymph nodes where they initiate a GC response and the development of committed antigen-specific B cells (Figure 17).

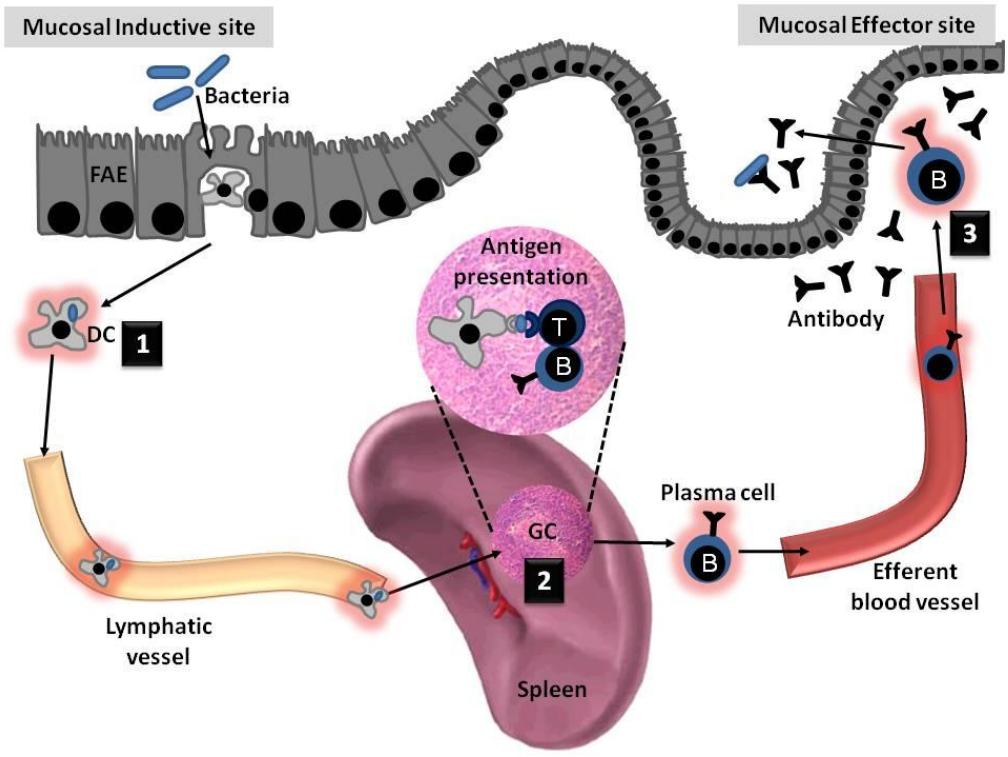

Figure 17. Function of GALT

Schematic representation of the function of GALT. M cells within the FAE sample antigen from the gut lumen and transport it through the epithelium to APCs, such as dendritic cells (DC), in the underlying sub-epithelial dome (SED). Upon binding immunogenic ligands, DCs are activated and begin up-regulating their expression of MHCII and co-stimulatory molecules B7 and CD40. (1) Activated DCs traffic via the lymphatic system to lymphoid follicles within secondary lymphoid organs; Peyer's patches, ILFs, spleen and mesenteric lymph nodes. (2) Once within the secondary lymphoid organs, APCs process the phagocytosed material and present derivative peptides complexed with MHCII to naive CD4 T cells. In turn, activated T cells stimulate differentiation and IgA class-switching in B cells. (3) IgA^+ B cells passage into the mucosa via efferent blood vessels and secreted IgA is transported across the epithelium, where it serves as a first line of defence against pathogens and for the maintenance of gut-flora homeostasis**.**

As discussed previously, the main function of GALT is in the development of IgA, an extremely important antibody that plays a critical role in protecting the mucosal surfaces from infection[124, 152]. IgA^+ B cells generated during germinal centre reactions in GALT enter the blood, and preferentially home to the gut lamina propria. Once within the lamina propria, IgA^+ B cells undergo terminal differentiation to become plasma cells, capable of secreting large volumes of IgA[124, 153]. Approximately 80% of all plasma cells are estimated to be located within the intestinal lamina propria[124]. Most of the IgA secreted is in the form of dimers, two IgA molecules linked at the constant regions of their heavy chains by a peptide known as the J chain[124, 132]. Mucosal epithelial cells express a polymeric immunoglobulin receptor (pIgR) on their basolateral membranes that recognize and bind dimeric IgA, triggering internalization and transport through the epithelial cells into the lumen[124, 132]. Secretory IgA serves as a first line of defence against mucosal pathogens; binding of IgA to luminal pathogens can prevent their attachment to the epithelium whilst IgA binding to pathogens positioned within the lamina propria is thought to promote their export back out into the lumen[132]. Furthermore, pathogens coated in IgA are either phagocytosed by macrophages or destroyed via antibody-dependent cell –mediated cytotoxicity (ADCC) mechanisms.

1.4 **Citrobacter rodentium**

1.4.1 History

C. rodentium, formerly known as *Citrobacter freundii* biotype 4280 is a non-invasive natural mouse pathogen that infects the distal colon of mice, potentially causing transmissible murine colonic hyperplasia (TMCH)[154]. *C. rodentium* was originally identified in the mid 1900s after a number of spontaneous disease outbreaks occurred in mouse colonies across the USA and Japan. All outbreaks were associated with thickening of the colon (colitis) and the bacteria isolated were either classified as atypical *C. freundii* strains or murine pathogenic *Escherichia coli* (MPEC). Eventually, all the atypical *C. freundii* and MPEC strains isolated during the outbreaks were grouped together under a new species name *C. rodentium*[155].

1.4.2 Attaching-Effacing enteric bacterial pathogens

C. rodentium is genetically related to *E. coli* and is a member of the Enterobacteriacae. *C. rodentium* shares a number of virulence factors and colonisation mechanisms with Enteropathogenic *E. coli* (EPEC) and Enterohaemorrhagic *E. coli* (EHEC). EPEC and EHEC are 'virulent' *E. coli* which, infect the gastrointestinal tract of humans. EPEC is the leading cause of infantile diarrhoea in developing countries whilst EHEC is the primary cause of haemorrhagic colitis and haemolytic uraemic syndrome (HUS), a potentially life threatening illness. *C. rodentium*, EPEC and EHEC all belong to a group of extracellular enteric pathogens which colonise the gastrointestinal mucosa by forming attaching and effacing (A/E) lesions (Figure 18). A/E lesions are characterised by localized destruction of the brush-border microvilli and intimate attachment of the bacteria with host epithelial cell plasma membranes (Figure 18). In addition, bacteria are able to subvert host cell signalling and induce the formation of actin-rich pedestal-like structures (Figure 18)[156-160].

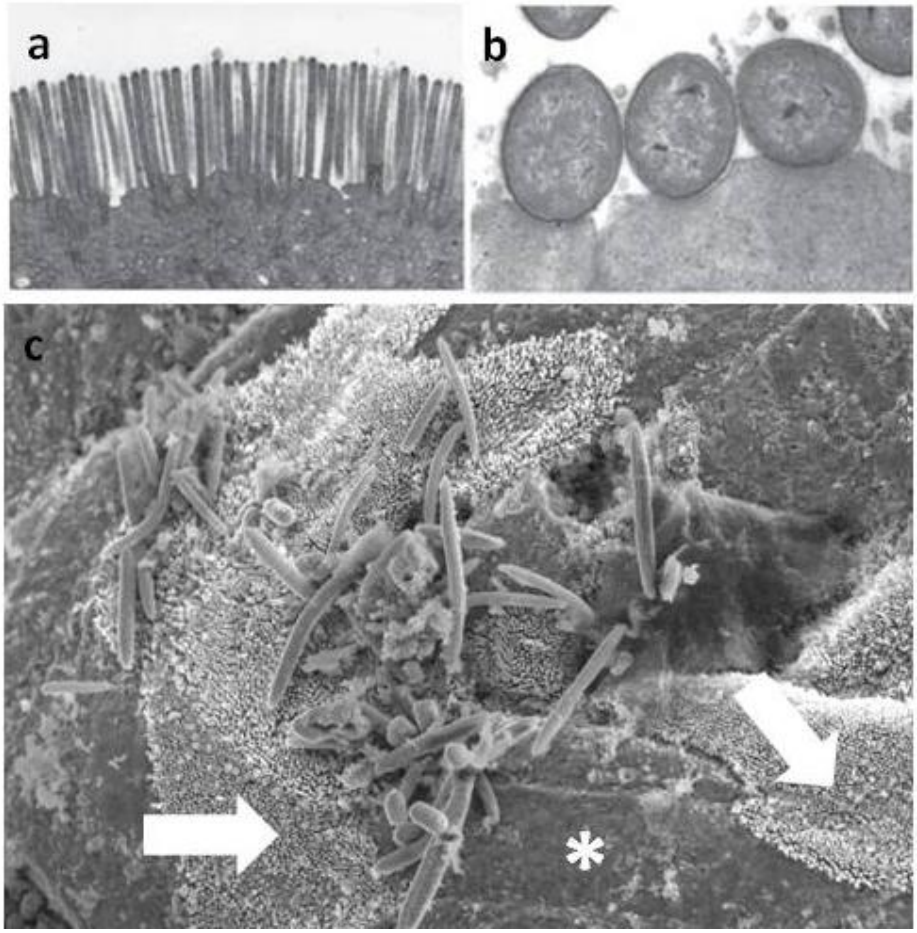

Figure 18. 'Attaching and Effacing' (A/E) lesions

Transmission electron micrographs showing normal brush border microvilli (a) and A/E lesions (b)[161]. Scanning electron micrograph (SEM) of A/E lesions in *C. rodentium* infected colon (c). A/E lesions are characterized by localized destruction (white asterisk) of brush border microvilli (white arrows indicate normal brush border microvilli) and intimate attachment of *C. rodentium* to the underlying apical cell membrane.

1.4.3 Attaching and Effacing (A/E) lesion formation

The genes required for attachment of *C. rodentium*, EPEC and EHEC to host cell plasma membranes and, subversion of host cell signalling during A/E lesion formation are primarily encoded on a horizontally acquired pathogenicity island, known as the locus of enterocyte effacement (LEE). The LEE of *C. rodentium* and those of EPEC and EHEC are not identical but are highly related in terms of overall genetic organization and gene function. The outer membrane adhesion molecule, intimin, and the translocated intimin

receptor (Tir) as well as a type III secretion system (T3SS) and effector proteins have been shown to be important virulence determinants and are encoded on the LEE. Initial attachment of bacteria to host cells requires plasmid encoded bundle forming pili (bfp) and LEE associated EspA filaments (Figure 19). Binding of bfp and EspA to host cell membranes stimulates the secretion of EspB and Tir directly into the host cells (Figure 19). Tir-intimin binding initiates a series of signalling cascades within host cells resulting in actin polymerisation and formation of actin-rich pedestal-like structures beneath the bacteria (Figure 19).

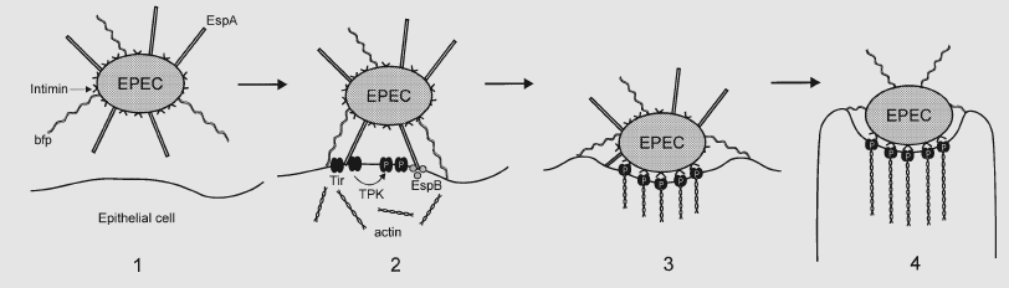

Figure 19. A model of EPEC interaction with epithelial cells

A model of EPEC interaction with epithelial cells. Growth of bacteria in tissue culture media results in expression of adhesins, bundle forming pili (bfp) and intimin and production of EspA filaments (stage 1). Initial attachment of EPEC via bfp and EspA filaments stimulates EspB and Tir translocation, and probably translocation of other, as yet to be identified, effector proteins into the host cell. This in turn leads to tyrosine protein kinase (TPK) activation, formation of the intimin receptor (Tir) and to actin rearrangements (stage 2). Intimin binds to Tir and polymerized actin accumulates beneath intimately attached bacteria; for this to happen, EspA filaments and other surface structures are eliminated from the region of intimate attachment (stage 3). Further actin polymerisation produces the mature A/E lesion in which all EspA filaments and intimin have been eliminated from the bacterial surface (stage 4)[162]

In contrast to our significant knowledge of how attaching-effacing enteric pathogens colonise epithelial cells *in vitro*, we know little about the host immune mechanisms employed to resolve infection. *C. rodentium* provides us with an extremely useful model for studying host-pathogen interactions *in*

vivo and under physiological conditions, with the ability to gain further insights through the manipulation of both host and pathogen[161].

1.4.4 *C. rodentium* infection in mice

The initial site of colonisation after oral gavage with *C. rodentium* is the caecal patch, a specialised lymphoid tissue in the caecum, although the site is actually dependent on how the bacteria are grown Colonisation has been documented to occur within a few hours of the mice receiving oral *C. rodentium*[163]. Within 2-4 days, bacteria spread from the caecal patch to the distal colon. Bacterial levels in the colon peak at around day 4-14 post inoculation (pi) dependent on the mouse strain used, with bacterial numbers reaching approximately 10^9 organisms[161, 164-166]. The infection is mainly restricted to the lumen of the intestine due to the non-invasive nature of the pathogen. Coincident with the peak of infection in the colon can be colonic hyperplasia, characterised by significant proliferation of the colonic epithelia, crypt hyperplasia and dilation and thickening of the colonic mucosa. *C. rodentium* is cleared systematically from the caecal patch first followed by the colon, with complete clearance occurring by days 21-28 pi.

1.4.5 Immune response to *C. rodentium*

Normal immunocompetent mice are able to mount a protective and potentially sterilising primary immune response towards *C. rodentium* with complete clearance of the infection normally occurring within 21-28 days pi. The mice that recover from infection are then highly resistant to further challenges with *C. rodentium*. Infection of the gastrointestinal epithelium elicits a very robust T_h1 biased response, there is a large influx of $CD4^+$ T cells into the colonic lamina propria which secrete copious amounts of the T_h1 cytokine IFN-γ[167, 168]. TNFα and IL-12, two extremely important type 1 cytokines are also upregulated within the colon. The adaptive immune system is critical for clearance. For example, $CD4^+$ T cells are known to be extremely important for the clearance of a *C. rodentium* infection as mice lacking $\alpha\beta$ T cells or CD4 cell subset develop a chronic infection accompanied by a high incidence of

mortality and morbidity (Figure 20)[169-171]. Mice depleted of $CD8^+$ T cells are as competent as control mice in their ability to eradicate a *C. rodentium* infection (Figure 20).

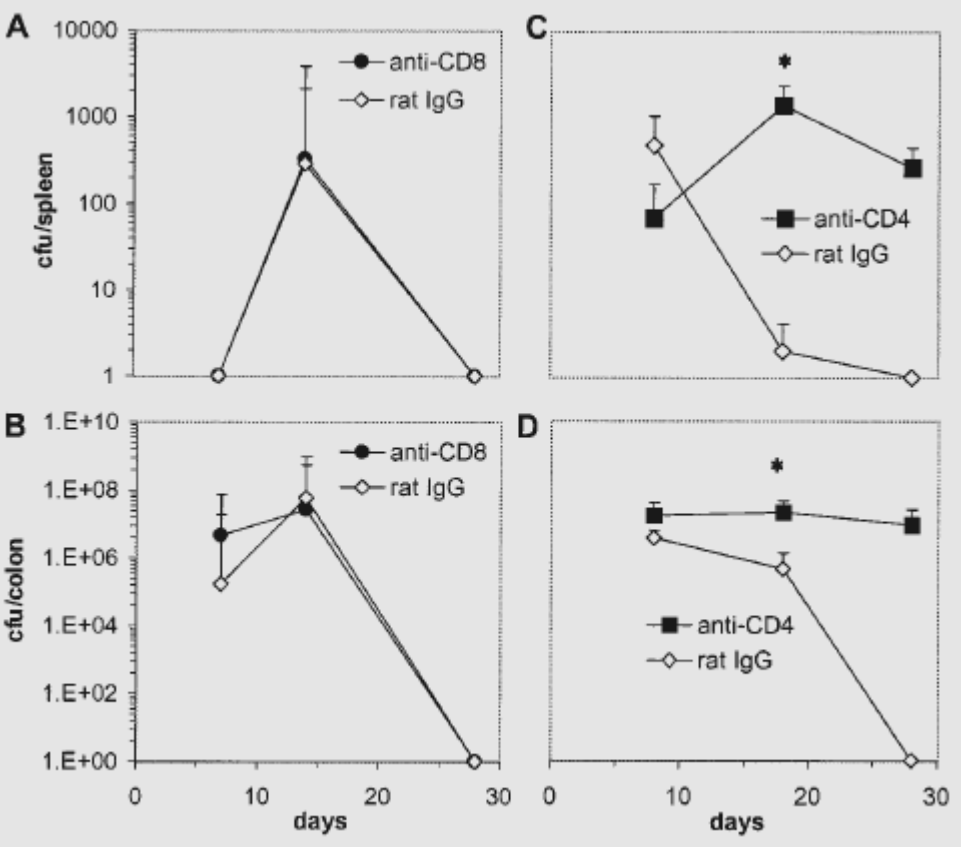

Figure 20. *C. rodentium* counts in mice depleted of CD4+ or CD8+ T cells

Viable counts of *C. rodentium* recovered from colons and spleens of mice depleted of $CD4^+$ or $CD8^+$ T cells. The data depict the mean number of *C. rodentium* CFU recovered from spleens (A) and colons (B) of C57Bl/6 mice (*n* = five per time point) infected orally with 109 CFU of *C. rodentium* and treated with the $CD8^+$-T-cell-depleting MAb YTS169 or the control antibody, rat IgG. Results from a parallel experiment show the number of *C. rodentium* CFU recovered from the spleens (C) and colons (D) of C57Bl/6 mice treated with the $CD4^+$-T-cell-depleting MAb GK1.5 or the control antibody, rat IgG. There were significantly more *C. rodentium* bacteria recovered from the spleen and colons of $CD4^+$-T-cell-depleted mice on day 18 post infection than from rat IgG-treated mice (asterisk, $P < 0.05$). On day 28 post infection, no viable *C. rodentium* bacteria could be recovered from C57Bl/6 mice treated with rat IgG. The data shown are from one of two experiments performed which gave similar results[171]

T cells alone are not able to provide sterilising immunity against *C. rodentium*; µMT mice lacking B cells also fail to clear infection (Figure 21)[171, 172].

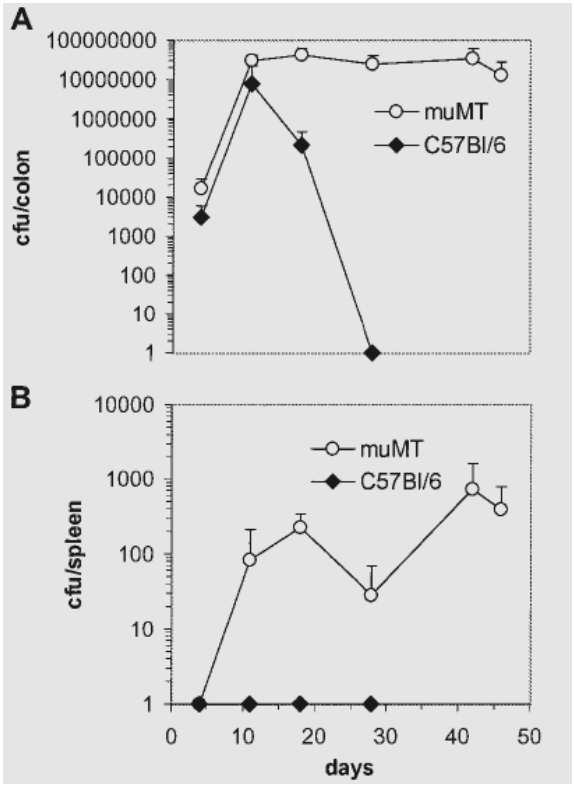

Figure 21. *C. rodentium* counts in µMT and control C57BL/6 mice.

Viable counts of *C. rodentium* recovered from colons and spleens of orally infected _MT and C57Bl/6 control mice. Mice were orally infected with 3×10^9 CFU of *C. rodentium.* The data depict the mean number (± standard deviation) of *C. rodentium* recovered from colons (A) and spleens (B) of muMT and C57Bl/6 mice. There were significantly more *C. rodentium* bacteria recovered from the colons of muMT mice on day 18 post infection than with control C57Bl/6 mice (asterisk, $P<0.05$). *C. rodentium* could not be recovered from C57Bl/6 mice beyond day 28. The data shown are from one of two experiments performed which gave similar results (Simmons et al,. 2003)

Surprisingly, although B cells are essential for clearance, secretory IgA or IgM antibodies are dispensable. Infections in mice lacking IgA or secretory IgM, pIgR or J chain proteins required for antibody secretion into the intestinal lumen showed normal clearance of *C. rodentium*. Transfer of serum

from mice that had cleared a *C. rodentium* infection to naive wild-type mice or pIgR deficient mice receiving oral *C. rodentium* however significantly reduced colonic bacterial numbers[172]. Removing the IgG antibody fraction from the sera before administration completely removed this effect. Thus T helper cell dependent serum antibody responses are required for sterilising immunity against a primary *C. rodentium* infection. Further evidence in support of this is provided by a study of the role of lymphotoxin $\alpha_1\beta_2$/lymphotoxin-β receptor signalling in *C. rodentium*-induced colitis (Figure 22). Inhibition of lymphotoxin $\alpha_1\beta_2$/lymphotoxin-β receptor interactions leads to an increase in *C. rodentium*-induced colitis and bacterial numbers in the colon as well as dissemination of *C. rodentium* to internal organs such as the liver and spleen (Figure 22 and 23)[173].

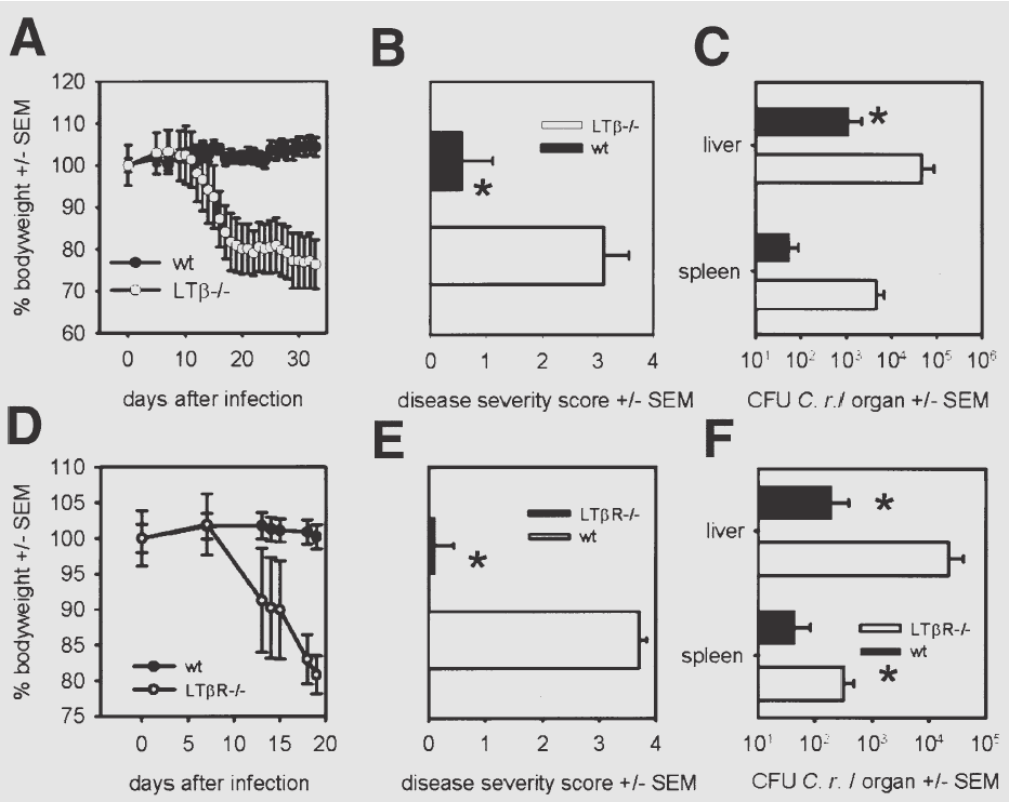

Figure 22. *C. rodentium*-induced colitis in LTβ-/- and LTβR-/- mice

Course of *C rodentium*–induced colitis in $LT\beta^{-/-}$ (*A–C*; *open bars* and *open circles*) and $LT\beta R^{-/-}$ (*D–F*; open bars and open circles) mice and the respective wt mice (filled bars and filled circles). Differences in body weight between $LT\beta^{-/-}$ and wt mice (*A*) were statistically

significant (P <.05) from day 17 after infection onward and between LTβR$^{-/-}$ and wt mice (*D*) on days 18–19 after infection. (*A*) Representative experiment out of 2 using 5 (LTβ$^{-/-}$) and 7 (wt) mice per group; (*D*) representative experiment out of 3 using 5–6 mice per group. The average histological disease severity score (*B*) represents pooled data from 2 experiments (wt, n =12; LTβ$^{-/-}$, n =10). (*E*) Pooled data from 2 experiments using 9–10 mice per group. Bacterial growth in liver and spleen organ homogenate cultures from dead LTβ$^{-/-}$ (*C*) and LTβR$^{-/-}$ (*F*) mice and control animals are shown. Bars indicate pooled data from 2 similar experiments: (*C*) wt, n=9; LTβ$^{-/-}$, n =8; (*F*) wt, n =6; LTβR$^{-/-}$, n=5. *P<.05, wt vs. $^{-/-}$. *C. r.*, *C rodentium*. [173]

Mice deficient in CD28 and CD40L develop polymicrobial sepsis, damage to the colon and internal organs and defects in bacterial clearance (Figure 23)[170]. CD28 is critical during a primary *C. rodentium* infection for the survival and proliferation of CD4+ T-helper cells, which not only have effector functions but also aid B cells in the development of protective antibody. CD40L is also involved in providing co-stimulatory effector functions needed to develop protective antibody responses. Summarised in the figure below are the cells, molecules and pathways identified in various studies that are involved in sterilising immunity against *C. rodentium* and have an impact on the pathology seen during infection (Figure 23).

Gene deleted mice who cannot clear infection	Gene deleted mice who clear infection	Gene deleted mice who show reduced pathology	Gene deleted mice who show enhanced pathology
• RAG-/- (lacking T and B cells) • TcRβ-/- (lacking αβ T cells) • CD4-/- mice • μMT (lacking B cells) • Igh-6^{tm1Cgn} (lacking B cells) • LTβR-/- (lacking the lymphotoxin-β receptor)	• TcRδ-/- (lacking γδ T cells) • CD8-/- • β7-/- (largely lacking mucosal lymphocytes) • pIgR-/- (lacking the polymeric immunoglobulin receptor on epithelial cells) • IgA-deficient • IgM-deficient • IgG3-deficient • J chain-deficient (lacking polymeric immunoglobulins) • mCRAMP-/- • MMP3-/-	• PAR2-/- (lacking proteinase activated receptor 2) • iNOS-/-	• TNFα-/- • IL-12 p40-/- • Interferon-γ -/- • LTβR-/- • μMT (lacking B cells) • MMP3-/-

Figure 23. Manipulation of the mouse host effect sterilising immunity, other antibacterial pathways and pathology[161].

1.5 *S.* Typhimurium

Salmonella enterica is a broad enterobacterial species that can cause invasive diseases in a number of animal species. Different members of *S. enterica* can be classified using antisera into serovars that can manifest different disease syndromes and exhibit different patterns of host restriction. For example, *Salmonella enterica* serovar Typhi (*S.* Typhi) is the causative agent of systemic typhoid fever. Although Typhoid fever has been virtually eradicated from the western world it still poses a major health problem in developing countries where water contamination with human faeces and urine is common and antibiotic treatment of infections is becoming increasingly difficult with the increasing emergence of resistant bacteria. In 2004, Crump *et al* estimated that there are more than 21 million cases of typhoid fever each year and approximately 216,510 deaths[174]. *S.* Typhi is human host restricted and consequently is avirulent in animals such as mice; this makes it extremely difficult to study the aetiology of typhoid fever in the laboratory. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes a typhoid-like enteric fever in susceptible mice with some similarities to that seen in humans infected with *Salmonella* Typhi. As a result *Salmonella* Typhimurium infection in mice is a widely used experimental model for studying typhoid fever in humans.

1.5.1 *S.* Typhimurium infection in mice

Salmonella naturally infects animals and humans via the oral route[175, 176]. Following oral infection of mice with either an attenuated strain or a sub-lethal dose of virulent *S.* Typhimurium, the resulting infection follows a regular time course made up of three relatively distinct phases[177]. During the first phase of infection, *Salmonella* begin colonizing and traversing the intestinal epithelium into the lymphoid follicles beneath (Figure 24)[175]. Experiments using murine intestinal ligated loop models have demonstrated that M cells within the FAE are an important portal of entry for invasive

Salmonella[175, 178]. Following passage through the intestinal epithelium, invading bacteria preferentially infect phagocytes such as dendritic cells and macrophages that inadvertently favour dissemination through the lymphatic system and blood stream to visceral organs such as the mesenteric lymph nodes, spleen and liver (Figure 24)[178].

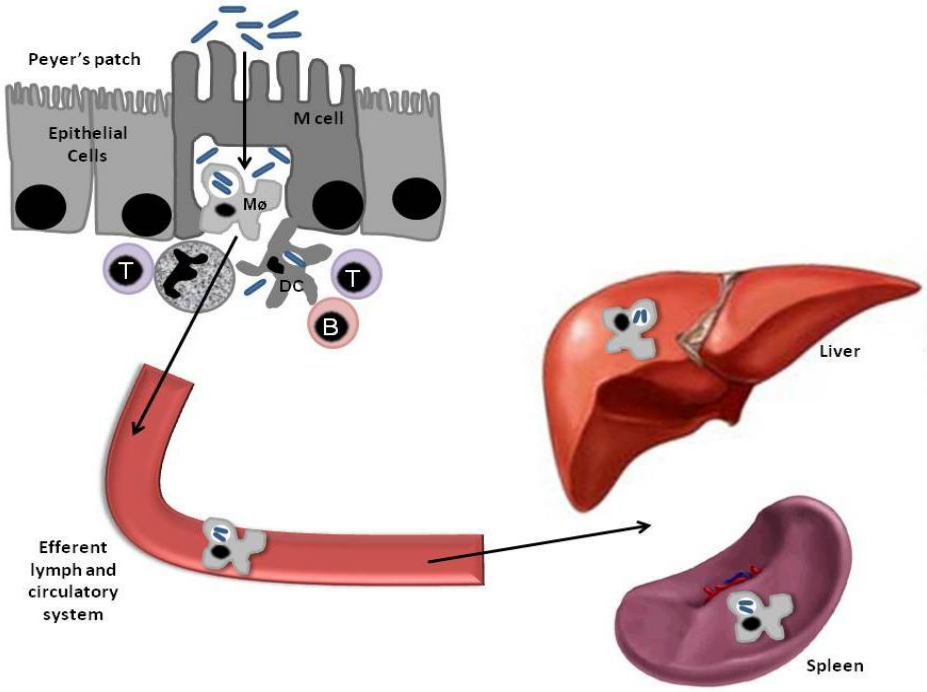

Figure 24. *S.* Typhimurium infection in mice

Schematic representation of *S.* Typhimurium infection in mice. Following oral ingestion of *S.* Typhimurium, bacteria enter the Peyer's patches of the intestinal tract mucosal surface by invading M cells. This is followed by inflammation and phagocytosis of bacteria by neutrophils, dendritic cells (DC) and macrophages (Mø) as well as recruitment of T and B cells. *Salmonella* disseminate through the lymphatics and blood stream to the mesenteric lymph nodes (mLNs) and to deeper tissues. Eventually bacteria are transported to the spleen and liver where they undergo exponential replication, intracellularly.

Bacteria are rapidly cleared from the blood by phagocytes within the liver and spleen, and a large fraction of bacteria are killed by these cells[178]. However, *Salmonella* has evolved a number of mechanisms to force entry into and

survive within the intracellular vacuoles of macrophages by preventing phago-lysosomal fusion and delaying vacuole acidification[179]. Viable and dividing *Salmonella* can be found within the un-fused vacuoles of macrophages[175]. Furthermore, previous experiments have shown that the growth state of *S.* Typhimurium rapidly changes after entry into macrophages[175]. Immediately after entry, bacteria can be killed by treatment with the antibiotics, chloramphenicol and ampicillin. However, if bacteria are allowed to reside within macrophages for greater than two hours prior to antibiotic treatment, some are no longer susceptible, although net growth is inhibited. This suggests that upon entry into macrophages, bacteria undergo a period of rapid growth and require protein synthesis for survival but as infection time increases, the bacteria adapt to their intracellular environment and switch from a rapidly growing state to a survival mode that does not require protein synthesis[175]. Bacteria that circumvent or escape destruction begin a period of intracellular replication, lasting several days[177, 178]. During this time, bacterial titres in the liver and spleen increase (Figure 25). Eventually, bacterial growth is restricted and a phase characterised by splenomegaly, general macrophage-mediated immune suppression and a plateau in bacterial numbers is observed (Figure 25)[178]. Conversely, in lethally infected mice, once bacterial titres reach a critical load threshold of approximately 10^8 bacteria per organ, the animal is no longer able to contain the infection and bacteraemia, endotoxic shock and rapid death ensue (Figure 25)[178]. Bacterial numbers in the liver and spleen begin to fall progressively during the ultimate phase of infection this, coincides with the generation of an acquired immune response capable of eliminating *S.* Typhimurium (Figure 25). Following eradication of bacteria, *Salmonella*-specific lymphocytes generated provide long lasting immunity against re-infection[178].

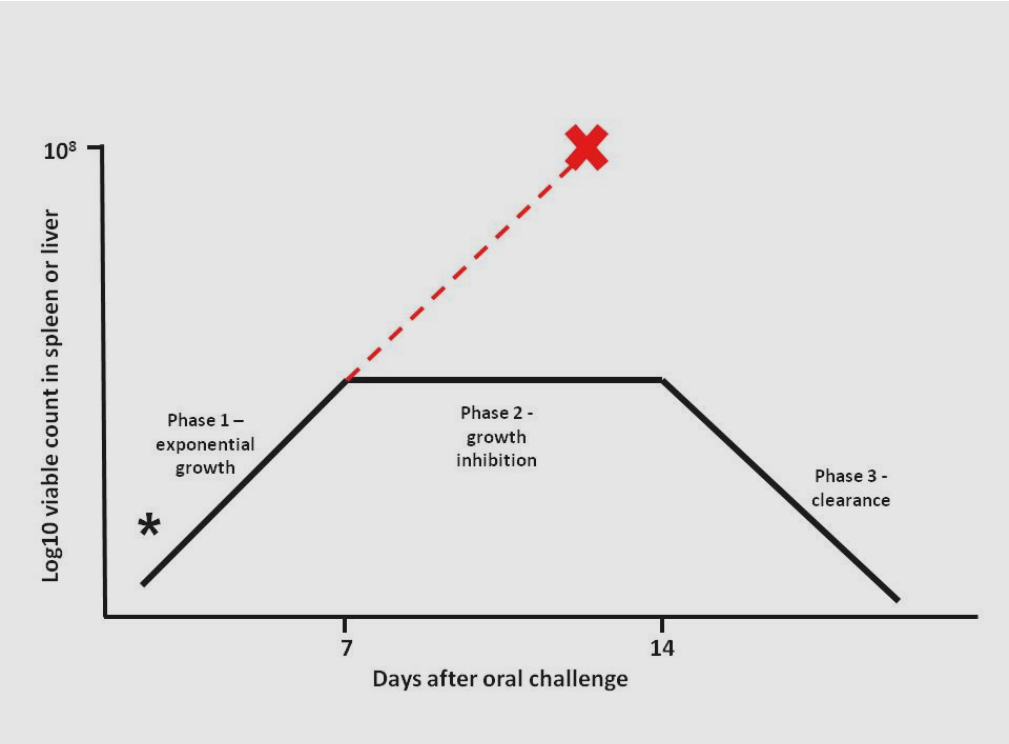

Figure 25. Pathogenesis of *S*. Typhimurium in mice

Schematic representation of the different phases of *S*. Typhimurium infection in mice. During *S*. Typhimurium infection, bacteria ingested orally colonise and breach the intestinal epithelium, and enter the lymphoid follicles beneath. From the follicles *S*. Typhimurium move into the mLNs, and from there bacteria spread via the lymphatic and circulatory systems to the spleen and liver. Bacteria then undergo a phase of intracellular replication lasting several days, during which bacterial titres in the liver and spleen increase. Lethally infected mice (red broken line) are unable to restrict bacterial growth and rapidly succumb to infection once bacterial titres reach a critical load of 10^8 bacteria. However, during non-fatal infection, mice restrict bacterial growth and a subsequent phase of infection characterised by a plateau level of bacterial load ensues. Depending on the mouse strain and the strain of S. Typhimurium used, this phase can last from one to several weeks. The final phase of infection is characterised by a progressive drop in bacterial titres until complete eradication of bacteria is achieved[178]. * A short period of initial kill of approximately a Log can occur following i.v. challenge.

1.5.2The innate immune response to *S*. Typhimurium

During the early phases of infection, the production of a number of cytokines and soluble factors as well as the recruitment of bone marrow derived macrophages and the development of organized granulomas have been shown to be critical for controlling *Salmonella* spread and growth in the reticuloendothelial system (Figure 26)[180].

Macrophages and neutrophilic granulocytes of the innate immune system are decisive for controlling the net growth of bacteria during the early phase (first week) of infection, with a large fraction of bacteria being killed by these cells[178]. The expression of a functional *Slc11a1* (formerly known as *Nramp1*) gene appears to be a key factor contributing to the efficiency by which macrophages kill *S*. Typhimurium[181]. *Slc11a1* encodes a proton/divalent-cation antiporter which is localised to the vacuolar membrane and controls the intracellular replication of *Salmonella* bacteria by depriving intravascular bacteria of divalent cations. Mice lacking a functional *Slc11a1* gene have a higher susceptibility to *Salmonella* infection[178].

Following infection, components of the bacterial cell wall such as lipopolysaccharide (LPS), DNA, flagella and certain lipoproteins activate Toll like receptors (TLRs) on host cells which in turn induces a robust inflammatory response within tissues, characterised by the production of T_h1-like cytokines IFN-γ, TNF-α and interleukins (IL)-1, 6, 12 and 18 as well as macrophage migration inhibitory factor and inducible nitric oxide synthase (iNOS) (Figure 26)[180]. IFN-γ has been shown to be vital for resistance against infections involving intracellular pathogens, especially *S*. Typhimurium, in part because it stimulates the bactericidal activity of macrophages, including the production of iNOS[180, 182]. Reactive nitrogen intermediates (RNIs) generated by iNOS expression inhibit the growth of phagocytosed *Salmonella*. Mice treated with anti-IFN-γ antibodies are impaired in their ability to clear a sub-lethal dose of virulent *S*. Typhimurium and succumb to infection 7-8 days post-inoculation (pi)[177, 183]. IL-12 and IL-18, secreted by activated macrophages act both independently and synergistically on NK and helper T

cells to induce the production of IFN-γ, which further activates the macrophages through a positive feedback loop. Additionally, IL-12 is important for the polarization of T helper cells toward the T_h1 response[59, 60, 178].

Mastroeni *et al* observed that the suppression of bacterial growth in the reticuloendothelial system coincided with the formation of granulomatous lesions in the liver and spleen and that administration of anti-TNF-α antibodies exacerbated the course of a *Salmonella* infection in both susceptible and resistant mouse strains by inhibiting their formation[184-186]. They also showed that treatment with anti- TNF-α antibodies well after the suppression of bacterial growth and formation of granulomatous lesions, prompted a relapse of the infection and a regression of already established granulomas suggesting that TNF-α is required throughout infection[177, 184-186].

It is important to note that T cells do not play a key role during the early phases of infection, as nude, αβ T cell knockout, and CD4 and CD8 T cell depleted mice are all capable of suppressing the growth of *Salmonella*. However, whilst the innate immune response is highly successful in controlling the initial growth of *S.* Typhimurium, it is insufficient for achieving full protective immunity. Effective control and eventual eradication of bacteria during the late phases of infection (3-4 weeks post-inoculation) and generation of protective immunity against subsequent infections requires the development of a *Salmonella*-specific lymphocyte response (Figure 26)[178].

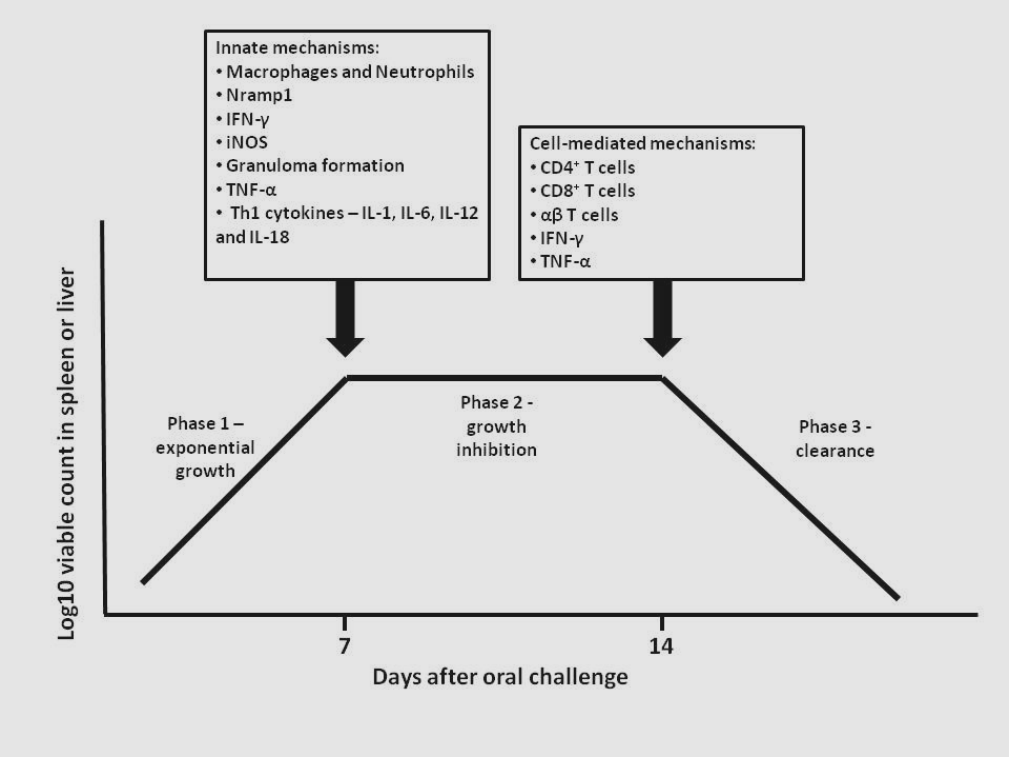

Figure 26. The immune response to *Salmonella.*

Schematic representation of the immune mechanisms required at various stages of *S.* Typhimurium infection in mice.

1.5.3 The adaptive immune response to *S*. Typhimurium

1.5.3.1 Role of T cells during the immune response against *S*. Typhimurium

Following intravenous challenge with *S*. Typhimurium, protective or acquired immunity is mainly T cell mediated, with the participation of both $CD4^+$ and $CD8^+$ subpopulations[182]. However, since a number of studies have shown that the depletion of $CD4^+$ T cells (as opposed to $CD8^+$ T cells) has a more profound effect on the control of primary *Salmonella* infection and on protection induced by vaccination with an attenuated strain of *S.* Typhimurium it would suggest that $CD4^+$ T cells are more important than $CD8^+$ T cells[178]. Additionally, αβ T cells appear to be more important than γδ T cells for protective immunity against *Salmonella* infection since mice on a

susceptible background and deficient in γδ T cells are able to control systemic infection with an attenuated strain of *S.* Typhimurium while mice containing defects in the αβ T cell receptor are not[178].

T cell-mediated protection most likely involves the production of cytokines, in particular IFN-γ, and through IFN-γ-independent mechanisms including the production of other macrophage-activating cytokines, cytotoxicity against infected host cells and provision of help for B cells[178]. For example, Th1 cells are capable of producing large amounts of IFN-γ and TNF-α which, as discussed previously are crucial for macrophage activation and granuloma formation, respectively. During infection with *S.* Typhimurium, $CD8^+$ T cells differentiate into CTLs which, may also play a key role in protection by liberating intracellular *S.* Typhimurium from infected macrophages[178]. Bacteria released during this process are more likely to be killed by activated phagocytes or by granulysin, an antibacterial molecule produced by the CTLs. Arguably, the most important function of T cells during the development of protective immunity is in the regulation of antigen-specific B cell activation and maturation which, will be discussed in greater detail later[178].

Infection of vaccinated mice with virulent *Salmonellae* has led to similar conclusions about the importance of IFN-γ-producing $CD4^+$ T cells. A number of groups have reported that there is a limited protective effect following vaccination with an attenuated strain of *Salmonella* if immunised mice are depleted of $CD4^+$ T cells just prior to challenge with a virulent strain. Additionally, the depletion of Th1-like cytokines such as IFN-γ, TNF-α and IL-12, using neutralising antibodies after vaccination, greatly exacerbates secondary infection. Thus, the activation of T_h1 cells is required not only for the defence against primary infection with *Salmonellae* but also for the vaccine-induced resolution of infection[187].

1.5.3.2 Role of B cells during the immune response against *S*. Typhimurium

Resolution of a primary infection in mice with attenuated *S*. Typhimurium is predominantly T cell mediated but is largely independent of B cells. In contrast, B cells have been shown to be essential for the expression of full protective immunity to virulent oral challenge. Mastroeni *et al* have used gene targeted B cell-deficient, innately susceptible mice on a C57BL/6 background (Igh-$6^{-/-}$) to investigate the role of B cells in protective immunity to *Salmonella* infection. They discovered that Igh-$6^{-/-}$ mice infected with a live, attenuated *aroA S*. Typhimurium vaccine strain were able to control and clear the inoculum from the reticuloendothelial system (Figure 27)[188]. However, unlike control C57BL/6 mice, Igh-$6^{-/-}$ mice challenged orally with virulent *S*. Typhimurium strain C5 four months after vaccination, were unable to control infection, suggesting that B cells are required for acquired resistance (Figure 27)[188]. It appears that the role of B cells in acquired resistance to *Salmonella* involves more than just the production of *Salmonella*-specific antibodies since passive transfer of large amounts of immune serum into immunized Igh-$6^{-/-}$ mice before challenge does not fully restore resistance[188]. Additionally, total splenocytes and purified $CD4^+$ T cells isolated from Igh-$6^{-/-}$ mice 4 months after vaccination showed a reduced ability to release the Th1-type cytokines IL-2 and IFN-γ upon re-stimulation in vitro with *S*. Typhimurium soluble extracts, but not ConA[188]. B cells have been shown to play an essential role *in vivo* to amplify the $CD4^+$ T cell response to antigen by providing co-stimulation[189]. Furthermore, the size of the memory $CD4^+$ T cell pool that develops is determined by the degree of B cell-dependent T cell expansion that occurs in the primary response thus, the absence of B cells results in a significant impairment of Th1-type immunological memory[189]. In fact, a recent study found that the frequency of memory $CD4^+$ T cells generated in μMT mice immunised with NP-KLH was ~ 30-fold lower than that in C57BL/6 mice[189].

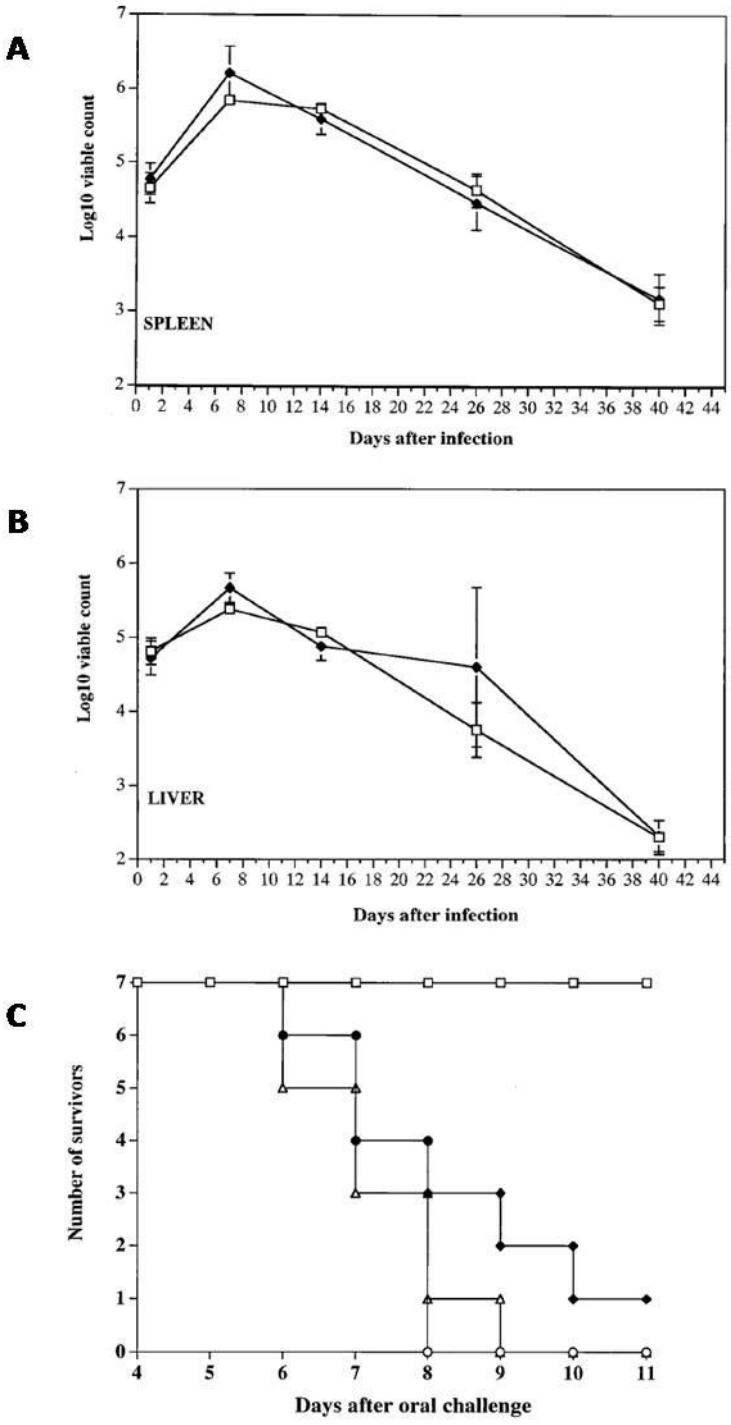

Figure 27. Igh-6$^{-/-}$ (B cell deficient) mice fail to mount solid acquired resistance to oral challenge with virulent *Salmonella* Typhimurium.

Igh-6$^{-/-}$ mice (closed diamonds) and C57BL/6 mice (open squares) were infected i.v. with 5 X 10^5 CFU of *S.* Typhimurium strain SL3261. Spleen (A) and liver (B) counts of viable bacteria were obtained thereafter. Results are means ± standard deviations from groups of four mice. Igh-6$^{-/-}$ mice (closed diamonds) and C57BL/6 mice (open squares) were immunized with *S.* Typhimurium SL3261 as for (A) and (B). Age-matched naive Igh-6$^{-/-}$ mice (closed triangles) and naive C57BL/6 mice (open circles) were used as unimmunized controls. Four months after vaccination, all mice were challenged orally with ca. 2.5 X 10^9 CFU of virulent *S.* Typhimurium strain C5 (C)[188].

1.6 **Hypothesis**

As discussed previously, recent studies have identified miR-155 as an extremely important miRNA of the immune system. To date miR-155 has been shown to be induced in cells of both the innate and adaptive immune system following antigenic stimulation[28, 29, 33, 34]. Furthermore, miR-155-deficient mice exhibit impairments in the ability of miR-155-deficient DCs to activate cognate T cells in addition to defective T and B cell immunity[31]. Therefore, we hypothesize that miR-155 is important for both the development of immunity and infection control.

1.7 **Aims of this thesis**

Relatively little is known about the role of miR-155 in protection against infection or in mucosal associated immunity. The aim of this study was to elucidate the role of miR-155 in controlling a mucosal *C. rodentium* infection and a systemic infection with *S.* Typhimurium, in the context of the overall immune response.