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Control of plasma cell generation
and population dynamics

Tom Slocombe

Thesis submitted for the degree of Doctor of Philosophy
The University of Edinburgh
2012
Declaration

I declare that this thesis has been composed by myself, describes my own work, and has not been submitted in any other application for a higher degree.

Tom Slocombe
2012
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Abstract

Plasma cells, the effector stage of the B cell compartment, secrete large amounts of antibody. These cells arise in two waves during T-dependent immune responses; an early wave (extrafollicular plasma cells) generate low-affinity antibodies that provide a first line of defence against invading pathogens. Later, plasma cells emerge from the germinal centre reaction and secrete high-affinity antibodies. These plasma cells have the capacity to migrate to the bone marrow, where they become established as long-lived, non-dividing plasma cells.

Here, I show that plasma cells found in the bone marrow of young (5-week-old) mice had a turnover comparable to that seen in the spleen. Long-lived plasma cells accumulated over the ensuing weeks until they came to dominate the bone marrow plasma cell compartment by 30-weeks of age. This accumulation required MHC II, CD40 and a normal B cell receptor repertoire, implying that these cells are generated during T-dependent immune responses.

Secondly, I determine the signalling pathways required to generate splenic extrafollicular plasma cell responses in the T-dependent response to sheep red blood cells (SRBC) and in bacterial infection with Salmonella. While T cell help, antigen recognition through the B cell receptor (BCR) and TLR signalling were required for maximal plasma cell responses to SRBC, in Salmonella infection TLR signalling was required for day 4 IgM plasma cell responses, whereas class-switched responses at day 8 required T cell help. The extrafollicular responses generated in Salmonella persisted for around 35 days, far greater than the 2-3 days seen following SRBC immunisation. This was likely due to both antigen persistence causing the generation of new plasma cells, and the induction of cellular populations that produced the plasma cell survival factor APRIL.

Thirdly, I document the failure of chronic immune responses to generate long-lived bone marrow plasma cells. This was accomplished by measuring the generation and survival of bone marrow plasma cells in models of rheumatoid arthritis (K/BxN mice), long-term infection with Salmonella, and a direct comparison between acute and chronic delivery of the T-dependent protein
antigen NP-KLH. In all cases, chronic immune responses generated few bone marrow plasma cells, ostensibly due to a failure to migrate to the organ.

Finally, I show the depletion of bone marrow plasma cell populations caused by inflammatory episodes. This was observed in *Salmonella* infection, *Schistosoma mansoni* infection and immunisation with protein antigen plus adjuvants. This depletion mediated a reduction of antigen-specific bone marrow plasma cell populations and serum antibody previously established by the secondary response to NP-KLH.
Abbreviations

AID – Activation-induced cytidine deaminase
APC – Antigen presenting cell
APRIL – A proliferation inducing ligand
*B. pertussis – Bordetella pertussis*
Bcl-6 – B cell lymphoma-6 transcription factor
BCMA – B cell maturation antigen
BCR – B cell receptor
BLIMP-1 - B lymphocyte maturation protein 1
BLYS – B lymphocyte stimulator
BrdU – Bromodeoxyuridine
BSA – Bovine serum albumin
CD – Cluster of differentiation
CFA – Complete Freud’s adjuvant
CFU – Colony-forming units
CSR – Class switch recombination
CXC (L) – C-X-C motif (ligand)
DNP – Dinitrophenyl
ELISA – Enzyme-linked immuno-sorbant assay
Fc – Fragment crystallizable
FcR – Fragment crystallizable receptor
FCS – Foetal calf serum
FDC – Follicular dendritic cell
FITC - Fluorescein
FSC – Forward scatter
G6PI – Glucose-6-phosphate isomerase
hi – High
ICAM-1 – Intracellular adhesion molecule 1
ICOS – Inducible co-stimulator
IFA – Incomplete Freud’s adjuvant
IFN-α/β/γ – Interferon-alpha/beta/gamma
*S. typhimurium, Salmonella or SL3261 – Salmonella enterica serovar*

Typhimurium (strain SL3261)
SIGN-R1 – SIGN-related gene 1
SRBC – Sheep red blood cells
SSC – Side scatter
TACI – Transmembrane activator and CAML-interactor
TCR – T cell receptor
TD – T-dependent
TGF-β - Transforming growth factor-beta
Th cell – T helper cell
TI – T-independent
TIRAP – Toll-interleukin 1 receptor domain containing adaptor protein
TLR – Toll-like receptor
TNF-α – Tumour necrosis factor-alpha
TRAM – TRIF-related adaptor molecule
TRIF – TIR-domain-containing adaptor-inducing interferon-β
Treg cell – Regulatory T cells
VCAM-1 – Vascular cell adhesion protein 1
VLA-4 – Very late antigen 4
WT – Wild type
XBP-1 - X-box binding protein 1
Chapter 1 - Introduction

The immune response

The immune response defends organisms from invading pathogens that would otherwise overrun the body. Many immune cell types exist, all with their own distinct roles in dealing with the multitude of infections that may be encountered by an organism in its environment. However, the interplay between these immune cells creates an extremely complex scenario that continues to challenge immunologists.

The immune system has two branches, originally thought of as distinct, but now known to be interrelated\(^1\,^2\). Innate immunity is the first arm to respond and contains many of the effector mechanisms required for clearing infections. Innate cells such as macrophages and granulocytes are able to phagocytose extracellular pathogens and kill infected cells through reactive nitrogen species\(^3\,^4\). Adaptive immunity takes longer to establish, but is critical for generating long-lasting, highly-specific immunity. This branch is able to enhance the efficiency of innate immune mechanisms through the production of antibodies that are of high affinity for a specific target, and the production of targeted cytokines that are able to further activate innate immune cells\(^5\). The end goal of the adaptive response is long-lasting, protective immunological memory that is able to rapidly produce a targeted, highly efficient response on subsequent encounters with a pathogen\(^5\,^6\).

One of the aims of immunological research is to produce vaccines that provide long-lasting, highly specific immunity against a particular pathogen, or to provide therapies or medication that can restore control to a dysregulated or misdirected immune response. This requires a thorough understanding of how the many aspects of the immune response function independently, and how they collaborate \textit{in vivo}. This chapter aims to provide a detailed background to the data presented in this thesis, concerning a number of aspects of plasma cell biology.
**Splenic structure**

The spleen, the largest secondary lymphoid organ in mammals, is composed of the red and white pulp (fig 1.3). The red pulp is an area in which blood flows from terminal arterioles into open sinusoids. Here, where blood flow is slower, macrophages scavenge and remove any dead or dying erythrocytes (or other particles found in the blood), which become entangled in fibres that filter the blood. CXCL12 is secreted by many stromal cells in the red pulp, and cells which reside there express the receptor CXCR4. Following filtration, blood exits the spleen via the collecting vein.

The white pulp, which is clustered around the central arteriole, is made up of T cell zones and B cell follicles. B and T cells, which are produced in the bone marrow and thymus respectively, migrate out of these organs early after formation and travel to secondary lymphoid organs including the spleen. Here CCR7 expressing T cells migrate to T zones, attracted by the CCR7-ligands CCL19 and CCL21, secreted by networks of fibroblast reticular cells. CXCR5 expressing ‘follicular’ B cells are attracted by CXCL13, secreted by networks of follicular dendritic cells (FDC). The defined structure of the white pulp allows for the efficient meeting of rare activated antigen-specific clones of B and T cells, as well as the rapid activation of T cells by antigen presenting dendritic cells.

Surrounding the white pulp is the marginal zone, where blood leaves the arterioles and enters the open sinuses. This is also the access point for cells and blood borne particulate antigen entering the spleen. Certain cell types reside in this area including two types of macrophage, marginal metallophilic macrophages and marginal zone macrophages. Marginal zone macrophages are located in the very outer ring of the marginal zone, closest to the red pulp. They are vital for the uptake of antigen as it enters the spleen, and express a number of receptors that allow this to be performed efficiently, such as SIGN-R1 (C-type lectin-specific intracellular adhesion molecule-grabbing non-integrin receptor) and the scavenger receptor MARCO (macrophage receptor with a collagenous structure). Marginal metallophilic macrophages reside in the inner ring of the marginal zone, adjacent to the white pulp. Their exact function
is unknown, although they may be important for type I interferon production during viral infections\textsuperscript{11}.

These two macrophage populations sandwich a subset of B cells (marginal zone B cells), which express low amounts of IgD and high amounts of IgM on their surface\textsuperscript{13-15}.

Through splenic architecture, the blood is constantly monitored for blood borne pathogens, and once encountered, adaptive immunity can be triggered in an efficient way despite the rarity of individual clones of T and B cells.

**Innate immune receptors**

Pattern recognition receptors (PRR) recognise conserved ‘pathogen associated molecular patterns’ or PAMPs that are generally evolutionarily ancient and therefore present on many species of pathogen\textsuperscript{3,16}.

Toll-like receptors (TLRs) are a major class of PRR, and are widely expressed in mammalian cells. Toll, a receptor identified in *Drosophila*, was found to recognise a cleaved form of the cytokine spaetzle, generated during fungal infection, and initiates inflammatory pathways\textsuperscript{17,18}. TLRs are the mammalian homologue and serve a similar function, although through the direct recognition of PAMPs\textsuperscript{1}. There are 11 known TLRs, many recognising different PAMPs, however all have related structures; a leucine-rich repeat extracellular domain and a toll-IL-1 receptor (TIR) intracellular domain\textsuperscript{19} (fig 1.2). TLR4, the first to be identified, binds bacterial lipopolysaccharide (LPS)\textsuperscript{20,21}, while TLR9 binds CpG motif-rich DNA sequences (found mainly in microbial DNA)\textsuperscript{22}, and TLR5 binds flagellin\textsuperscript{23}. On binding of ligands, the TIR domain associates with various adaptor proteins (exactly which depends on the receptor involved) such as MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain containing adaptor protein), TRAM (TRI-F-related adaptor molecule) and TRIF (toll-like receptor adaptor molecule 1)\textsuperscript{24}. This begins signalling cascades, through the I\(\kappa\)c (inhibitor of \(\kappa\)B kinase), MAP kinase (mitogen activated protein kinases) or IRF (interferon regulatory factor 4) pathways leading to activation.
Chapter 1 - Introduction

of transcription factors such as NFκB1 and 2 (nuclear factor-κB) and AP-1 (activator protein 1)\textsuperscript{24}.

Signalling cascades are complex; adaptor molecules often collaborate to induce many differential effects through the recruitment of distinct downstream signalling molecules (for a basic summary, see fig 1.2). For example, in TLR4 signalling, MyD88 and TIRAP signalling induces NFκB activation. This leads to the production of inflammatory cytokines such as IL-1, IL-6 and TNF-α, which produce inflammation\textsuperscript{19,24}. In addition, stimulated cells are activated, gaining enhanced phagocytic abilities, production of inducible nitric oxide species and the up-regulation of cell signalling molecules such as MHC II and CD80/86\textsuperscript{4,19,25}. TLR4 also signals through TRAM and TRIF, which leads to the production of type I interferons; potent stimulators of antiviral responses amongst other effects (discussed later)\textsuperscript{26}. However, it can be noted that, with the exception of TLR3 (which causes the production of type I interferons in response to double stranded RNA by signalling through TRIF alone) and TLR4, all other TLRs require MyD88 to signal\textsuperscript{19,24}.

TLRs are positioned in locations that enhance the likelihood of detecting their respective ligands. TLRs that detect extracellular ligands are present on the surface of cells, such as TLR4 (LPS), TLR5 (flagellin), and TLR1, 2 and 6 (bacterial lipopeptides)\textsuperscript{3,24,27}. Those that detect viral ligands, and bacterial ligands that are only exposed once a pathogenic cell is broken down, are located on the membranes of endosomal compartments such as TLR3 (double stranded RNA), TLR7, 8 (single stranded RNA) and TLR9 (CpG rich DNA)\textsuperscript{28}. This also sequesters these receptors away from any possible contact with self-DNA and RNA, which could potentially bind (despite preferentially binding of microbial DNA and RNA, mammalian nucleic acids are capable of binding to, and activating, these receptors)\textsuperscript{19,29}.

TLRs are tightly regulated, especially in the gut where commensal bacteria are common. Regulatory signalling molecules such as IRF4 and SHP (small
heterodimer partner) compete with inflammatory signalling molecules (such as IRF5 and IRF7) for access to the adaptor molecules associated with TLRs\textsuperscript{24,26,30}.

**Expression of TLRs**

TLRs are widely expressed in mammals; both in immune and non-immune cell types. Often, signalling in non-immune cells leads to the recruitment of innate immune cells through altered cytokine, chemokine and adhesion molecule expression\textsuperscript{19,28}. In mice, many antigen presenting cells (APC) as well as B cells express all known TLRs. Expression patterns vary in different subsets of cells; for example, marginal zone and B1 cells express more TLR2, 6 and 7 than follicular B cells\textsuperscript{31}. Dendritic cells express all TLRs, while plasmacytoid dendritic cells do not express TLR2, 3, 4 or 5 but signal through TLR7 and 9\textsuperscript{32}.

**Other PRRs and innate receptors**

Numerous other PRRs exist and are abundant, especially in APC. NOD-like receptors (NLRs) are a family of PRRs similar to TLRs. Some NLRs activate inflammatory signals through the NFκB and MAP kinase pathways much like TLRs (although signalling through RIP2 (receptor interacting protein 2) rather than MyD88 etc); others generate inflammasomes, leading to caspase-1 activation and the production of IL-1β and IL-18\textsuperscript{33}. These receptors are present in the cytoplasm and are important for recognising intracellular bacteria and viruses. Similar to NOD-like receptors are RIG-like receptors (RLR) and RNA-helicases, which are cytoplasmic PRRs able to trigger innate immune responses\textsuperscript{33}.

In addition to these, other surface receptors exist. Mannose receptors (present mainly on macrophages and dendritic cells) recognise repeated mannose units on the surface of bacteria, and through the activation of the complement cascade, form the membrane attack complex that is able to form transmembrane channels through the lipid bilayer of pathogenic cells\textsuperscript{34}. Scavenger receptors are a family of PRRs that are expressed mainly on macrophages. Although diverse in their specificities, the best studied (SR A-I
and SR A-II) recognise negatively charged low-density lipoproteins on the surface of bacterial cells. They also mediate the clearance of apoptotic cells.

**Fc receptors**

Other mechanisms exist for activating innate cells, which do not rely on the recognition of molecular patterns. An example of such is Fc receptors that bind to the Fc region of antibody molecules. These are expressed on the surface of many innate cell types, and must be cross-linked before signalling is induced. This requires that multiple antibody molecules have bound to a single pathogen or antigen molecule to form an immune complex. Generally, Fc receptors recognise class-switched IgG or IgE antibodies. As pre-existing natural antibody is non-class-switched IgM, an active B cell response against a pathogen is required for efficient activation through Fc receptors. Effects of cross-linking Fc receptors depend on the class of receptor, and the cell type it is expressed on. There are 4 classes of Fcγ receptor alone, three providing activating signals (FcγRI, FcγRII, and FcγRIII) that associate with activatory ITAM (immunoreceptor tyrosine-based activation motif) motifs and signal through the ERK (extracellular signal-related kinase), p38 MAP kinase, Jnk (c-JUN NH2-terminal kinase) pathway. One receptor provides inhibitory signals (FcγRIIb) by association with an inhibitory ITIM (immunoreceptor tyrosine-based inhibitory motif) motif.

Activatory Fc receptors on phagocytic cells initiate phagocytosis and the secretion of inflammatory cytokines, as well as the release of reactive oxygen species. Neutrophils, eosinophils and NK cells can also lyse antibody-bound cells through antibody-dependent cell-mediated cytotoxicity. On dendritic cells, Fc receptor cross-linking causes the presentation of phagocytosed particles to T cells on MHC II. Meanwhile, inhibitory receptors tend to cause a modulation of these effects; the activation state of these cells depends on the balance of inhibitory and activatory signals they receive. Interestingly, B cells express inhibitory Fc receptors, and if cross-linked with the BCR, this acts to inhibit proliferation and activation, whereas cross-linking of Fc receptors alone causes apoptosis (this effect is also seen in plasma cells, which express FcγRIIb).
**PRR signalling initiates adaptive immunity**

As stated above, PRR signalling leads to many pro-inflammatory effects. However, the extent to which signalling through innate PRRs controls adaptive immunity has only become apparent relatively recently. While it may seem counterintuitive that a primitive system with little specificity is required to direct one with fine specificity, it is now clear that the innate system controls the type of adaptive response that is generated. This is achieved both by signalling through different TLRs, leading to differential inflammatory cytokine production from APC, but also through intrinsic signalling in B cells, which directs antibody class-switching, germinal centre formation and plasma cell formation. The effects of TLR signalling on B cells will be covered in detail later, what follows is a description of the effects of innate signals on innate cells, and how innate signals cause the priming of the adaptive immune response.

Dendritic cells are ‘professional’ APC; their primary function is to survey the peripheral tissues for pathogens and present these to CD4 T cells, which express an antigen-specific receptor that must recognise antigen presented to it on an MHC II molecule.

It should be noted that while dendritic cells are the only professional APC, other cells exist that are capable of presenting antigen to CD4 T cells, notably macrophages and B cells. Immature dendritic cells in the periphery express many PRRs and other innate receptors and are primed to recognise pathogens. When they encounter such a pathogen, signals through PRRs or other receptors such as Fc and scavenger receptors lead to the ingestion of the invader into endosomal compartments. Here it is broken down and delivered to the endoplasmic reticulum where it is loaded onto MHC II molecules and presented to CD4 T cells. Co-stimulatory molecules such as CD80, CD86 and CD40 are up regulated and the dendritic cell migrates to the T cell zone of secondary lymphoid organs.

This is similar to the activation of T cells by macrophages, which are also able to phagocytose and present antigen. B cells however, are not capable of...
internalising antigen via innate receptors, and PRR stimulation does not cause the uptake of antigen. Instead, antigens must be specifically recognised by the BCR, (discussed below) before endocytosis occurs. This means that, early in the response to T-dependent (TD) antigens (when antigen-specific B cells are at low frequency), B cells are not a major contributor to T cell priming. However, they have been shown to be important at later phases, especially during the generation of memory T cells, and likely play a larger role in recall responses.

**T cell polarisation by cytokines**

Depending on the type of pathogen encountered, APCs will secrete different inflammatory cytokines that polarize naïve CD4 T cells in different ways. For example, IL-12 is secreted when APCs encounter pathogens such as intracellular bacteria and viruses, and induces CD4 T cells to adopt a Th1 phenotype. This leads to T cells expressing the transcription factor Tbet (T box expressed in T cells) and secreting IFN-γ, a potent activator of many innate immune cells such as macrophages and dendritic cells themselves. For Th2 pathogens, such as helminths infections, IL-4 from several cellular sources is the main driving cytokine for T cell polarisation, although the cognate interactions with APC are still vital. This leads to the expression of the transcription factor GATA3 (GATA binding protein 3) and the secretion of IL-4, 5 and 13 from T cells, which causes alternate activation of macrophages and the activation of basophils, mast cells and eosinophils.

Additional T cell subsets have been discovered in more recent years. Th17 cells can be generated through a combination of TGF-β (from many sources) and IL-6 or IL-21 (from APCs). These cells are generated in response to extracellular bacteria, express the transcription factor RORγT (retinoic acid receptor-related orphan receptor gamma T), and produce the highly inflammatory cytokine IL-17, as well as IL-21. IL-17 functions to attract neutrophils (which kill pathogens through phagocytosis and the release of anti-microbial molecules such as cathepsins and defensins in granules) and also to activate many cell types to produce inflammatory cytokines such as TNF-α and IL-6.
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Th9 cells may arise from unpolarised T cells responding to TGF-β and IL-4, or differentiate from Th2 polarised cells stimulated with TGF-β. These cells secrete IL-9 and are involved in pathogen clearance, although it is not known whether they are a truly distinct lineage\textsuperscript{47}.

CD4 T cells are capable of enhancing innate immune cell function, and also play a role in directing TD B cell responses, as the cytokines they secrete during interactions with B cells control the outcome of B cell activation, as will be discussed below.

**B cell responses**

**B cell receptors**

B cells express both a B cell receptor (BCR), and PRR\textsuperscript{31}. The BCR is a membrane-bound immunoglobulin molecule expressed on the surface of B cells, which is able to signal through the association with CD79α and β heterodimers as well as co-receptors such as CD19 and CD22\textsuperscript{50}. Ligation of the BCR causes the association of protein tyrosine kinases such as Syk (spleen tyrosine kinase) and Lyn (V-yes-1 Yamaguchi sarcoma viral related oncogene homolog), and the phosphorylation of ITAM motifs on the intracellular region of CD79a and b. This leads to further activation of Syk and also Btk (Bruton’s tyrosine kinase).

Through a complex signalling pathway (reviewed by Niiro et al, 2002\textsuperscript{50}) this leads to the activation of NF-κB and NFAT (nuclear factor of activated T cells) via p38 MAPK, ERK, Jnk, Iκκ and Akt (protein kinase B) activation\textsuperscript{50}.

Mature, follicular B cells co-express IgM and IgD on their surface\textsuperscript{50}. While the function of IgD remains unclear\textsuperscript{51,52}, surface IgM on follicular B cells is a highly specific receptor, allowing for the recognition of a single antigen\textsuperscript{50}. To allow for the multitude of possible antigens, each B cell clone expresses a different B cell receptor, meaning that any single specificity of B cell is particularly rare before antigen exposure. On other B cell subsets, such as B1 cells, the BCR is limited to specificities that recognise conserved pathogenic regions (much like PRRs), such as glycolipids and lipoproteins\textsuperscript{53,54}. While cross-linking of the BCR on
immature B cells causes apoptosis\textsuperscript{55}, mature B cells respond by differentiation to plasma cells, the production of cytokines and proliferation\textsuperscript{50}.

**TLR signalling in B cells**

B cells are known to express the TLRs 1-9 in mice. Their expression at protein level has been shown to vary between subsets of B cells\textsuperscript{31}. Stimulation has three effects, depending on the subset. Follicular B cells, when stimulated through TLRs alone, produce cytokine; notably IL-6 and IL-10 when stimulated by LPS\textsuperscript{31}. Interestingly, when differing combinations of TLRs stimulate B cells simultaneously, they secrete other cytokines (such as IFN-\gamma when stimulated through TLRs 2, 4 and 9)\textsuperscript{31}. Secondly, B cells stimulated through TLRs upregulate T cell stimulatory and co-stimulatory molecules such as MHC II, CD80 and CD86. Combined, this allows B cells activated by TLRs to function as capable APC and programmers of the CD4 T cell response\textsuperscript{31,44,56}. Thirdly, signals through TLRs drive B cells to differentiate to plasma cells. In B1 and marginal zone B cells, TLR signals alone are sufficient to drive differentiation, at least \textit{in vitro}\textsuperscript{57,58}. Follicular B cells require other signals to efficiently differentiate to plasma cells, however they undergo modest differentiation to plasma cells when stimulated \textit{in vitro}\textsuperscript{59}. TLR signalling, however, is critical to the generation of successful germinal centre responses\textsuperscript{60}, and can control antibody class switching in activated B cells, as will be discussed below.

**Antigen presentation by B cells**

Activated follicular B cells process and present antigen that is bound by their surface BCR. The BCR, with bound antigen is internalised into vesicles, where changes in pH mediate the release of bound antigen\textsuperscript{41,61}. This is then broken down into peptides by the proteases cathepsin B, D and L (which are also activated by the acidic pH in the endosomal compartment)\textsuperscript{61,62}. These vesicles fuse with MHC II containing vesicles (called the MIIC compartment). The peptides are loaded onto MHC II by HLA-DM, also present in the vesicles (firstly, the MHC II molecule has the invariant chain ‘Ii’ and stabilising peptide ‘CLIP’ degraded and removed by cathepsin L and HLA-DR respectively)\textsuperscript{9}. Once this is
accomplished, the MHC II:peptide complex is transported to the cell surface where it is presented to CD4 T cells.

**TD activation of B cells**

IgD+ B2 B cells found in the follicles (hereafter referred to as “follicular B cells”) are recirculating cells. After around a day in any particular follicle, they migrate into the blood stream towards other secondary lymphoid organs. Entering via the high endothelial venule (lymph node) or central arteriole (spleen) into the marginal zone, they then migrate to the T zone (fig 1.3)\(^6\). From here they migrate to the follicle where again they will rest for around a day\(^6\). When antigen is encountered, this migration pattern changes. Due to the up-regulation of CCR7 and the down-regulation of CXCR5, follicular B cells migrate directly to the T zone\(^6\). Antigen-specific CD4 T cells (that have been previously activated by APC) and activated B cells presenting peptide on MHC II molecules engage in cognate interactions. This involves the T cell engaging the MHC II:peptide complex with its T cell receptor (TCR) and CD4, followed by the engagement of accessory signalling molecules such as, CD40L:CD40, CD28:CD80/CD86\(^9,66,67\). Further downstream signalling occurs including the inducible T cell co-stimulator (ICOS):B7H\(^68,69\). These signals function to further activate the T cell and induce the differentiation of B cells to either plasma cells or germinal centre B cells (discussed later).

**Plasma cell responses**

Plasma cells differentiate from activated B cells. They secrete large amounts of antibody, which is critical for the clearance of many pathogens through both their ability to opsonise invading organisms and by mediating the activation of innate cells and complement pathways.

**T cell independent (TI) activation**

**B1 cells**

Certain subsets of B cells, including B1 and marginal zone B cells do not require T cell help to become activated (fig 1.1)\(^54,57\). This however, often requires
signals through PRRs, or certain types of highly repetitive antigens capable of extensively cross-linking the BCRs expressed on these more ‘innate’ B cells. B1 cells are a self-renewing population that are found in large numbers in the peritoneal cavity, but also are seen in the secondary lymphoid organs. They can be further subdivided into B1a cells (which are CD5+) and B1b cells (which are CD5-). B1 cells express restricted repertoires of BCR\(^70\). B1a cells are responsible for the production of ‘natural antibody’; IgM antibodies, which are often of broad specificity, found in the serum of naïve (or even germ free) mice. This includes antibodies that bind to conserved regions on pathogens with both low and high affinity\(^71,72\). B1b cells are thought to rapidly produce antibodies of broad specificity on stimulation through BCR or TLRs\(^71\).

**Marginal zone B cells**

Marginal zone B cells are located in a prime position to recognise blood-borne pathogens or antigen, and are able to sample the blood as it filters into the spleen\(^14,57,73\). Marginal zone B cells rapidly differentiate to plasma cells following encounter of TI antigens, primarily secreting low affinity IgM\(^57\). These cell types have a lower threshold of activation than follicular B cells, due to their high expression of complement receptor CD21, which when stimulated in combination with the BCR significantly lowers the threshold of activation\(^74\). In addition, they differentiate to plasma cells more readily following stimulation with LPS *in vitro*\(^59\). Although these cells do not express a restricted repertoire like B1 cells, their repertoire is enriched for certain specificities recognising conserved domains\(^75\). These cells are seemingly capable of taking part in (or at least enhancing) early TI responses through their skewing towards ‘innate-like’ BCR, their propensity to respond to polyclonal stimulation through TLRs, and their ability to concentrate TI antigens. In addition, marginal zone B cells take part in TD responses through the mechanisms described below.

The plasma cells generated during TI responses are generally short-lived and often secrete antibody that, while antigen specific, is of relatively low affinity compared to antibody secreted by plasma cells emerging from germinal centres. However, that they arise rapidly following infection or injection of TI antigen
has been shown to be critical for protection as they slow the early spread of infection\textsuperscript{71,76}.

**TD B cell activation**

For follicular B cells, T cell help is a requirement for efficient activation \textit{in vivo} (fig 1.1)\textsuperscript{59,77}. Once activated, follicular B cells provide two waves of plasma cells. An early wave contributes to the extrafollicular plasma cell response, whereas the later phase provides high affinity plasma cells that often migrate to the bone marrow\textsuperscript{78}.

The ‘choice’ between differentiation to germinal centre B cell or plasma cell is mediated by the affinity of the responding B cell’s BCR to the antigen. B cells binding antigen with relatively high affinity differentiate to plasma cells, while relatively low affinity mediates the differentiation to germinal centre B cell\textsuperscript{79-81}. It should be stressed that these cells, although of lower affinity, must still be able to bind the antigen and generate a strong enough signal to cause cellular activation.

**TD activation of marginal zone B cells**

Marginal zone B cells can also participate in TD responses. These cells express both MHC II and CD1d (an invariant, MHC-like molecule that binds lipid antigens). Lipid bound to CD1d is recognised by invariant TCR-expressing natural killer T cells (iNKT). Interestingly, the cognate interactions between iNKT cells and marginal zone B cells leads to the generation of early class-switched plasma cells, while IL-21 secretion leads to enhanced responses from follicular B cells, as will be described later\textsuperscript{82,83}.

**Extrafollicular plasma cell responses**

Early plasma cell responses are critical for providing antibody that can slow the spread of invading pathogens. As mentioned above, B cell clones expressing BCRs of relatively high affinity are recruited to the extrafollicular plasma cell response. In TD responses, following T cell help at the border of the T zone, activated follicular B cells initially migrate from the T zone to the follicle and
divide for around 2 days (fig 1.3)\textsuperscript{81}. Following this, many B cells differentiate to plasmablasts, mediating an upregulation of the chemokine receptor CXCR4 and down-regulation of CXCR5\textsuperscript{84}. This causes a migration from the follicle to the red pulp (where stromal cells secrete CXCL12)\textsuperscript{78,84,85}. These plasmablasts are capable of cellular division and migration, express MHC II and CD138 and also secrete antibody. Plasmablasts are capable of exponential growth, and the number of plasma cells produced is limited only by the number of B cells recruited into the response\textsuperscript{86}. Each plasmablast undergoes at least 5 divisions, over the course of around two days before maturation into non-dividing plasma cells occurs\textsuperscript{81,86}. In contrast, immunisation with the TI type 2 antigen NP-Ficoll produces a continuous B cell and plasmablast response from B1b cells that produces new NP-specific plasma cells for months after immunisation\textsuperscript{87}. The explanation for the observed differences between TI and TD responses are unknown. As plasma cells mature from plasmablasts, they migrate further out into the red pulp where they associate with myeloid cells and stromal cells (fig 1.5)\textsuperscript{88,89}. Most extrafollicular plasma cells are short-lived, surviving for just a few days\textsuperscript{78,86}. However, as will be described later, through their association with the aforementioned myeloid cells, their lifespan can be increased\textsuperscript{88}. In addition, some of these plasma cells can survive in the secondary lymphoid organs for long periods\textsuperscript{86}.

**Transcription factors**

Plasma cells express a phenotype that is indicative of their progression away from B cells towards terminal differentiation. This includes the down-regulation of many B cell surface markers, such as CD19, B220, MHC II, and the BCR\textsuperscript{90}. These phenotypic changes are brought about by a change in gene expression, caused by the upregulation of the master transcription factor ‘B lymphocyte maturation protein 1’ (BLIMP-1)\textsuperscript{91,92}. This is suppressed in naïve B cells by ‘B cell lymphoma 6’ Bcl-6, however on differentiation to plasma cells a decrease in Bcl-6 mediates an increase in BLIMP-1\textsuperscript{93}. This is a key step in the differentiation to plasma cells, and the resulting decrease in c-Myc (V-myc myelocytomatosis
viral oncogene homolog) causes the cell to exit cell cycle and undergo terminal differentiation\textsuperscript{94}. Similarly, IRF-4 further suppresses Bcl-6\textsuperscript{95,96}.

BLIMP-1 also suppresses ‘paired box protein 5’ (PAX-5), a transcription factor that is central to B cell development and the maintenance of the B cell phenotype, as it controls the expression of many B cell-related proteins, including BCR associated signalling proteins such as CD19 and CD80\textsuperscript{91}. It is therefore unsurprising that many such proteins gradually decline on the surface of plasma cells. The down-regulation of PAX-5 also leads to increased levels of another transcription factor, ‘X-box binding protein 1’ (XBP-1) that is required for commitment to the plasma cell lineage\textsuperscript{97}. This transcription factor brings about the unfolded protein response (UPR), a dramatic expansion of the endoplasmic reticulum and golgi apparatus that allows for the production and secretion of vast amounts of antibody\textsuperscript{98}. This response (usually a response to cellular stress) is brought about as a natural part of differentiation to plasma cells\textsuperscript{99}.

The changes in transcriptional profiles of B cells as they differentiate to plasma cells mediate a shift from cells with numerous functions to highly specialised, antibody secreting cells.

**Germinal centre responses**

Germinal centre responses occur during TD immune responses and are a mechanism by which high affinity B cell clones are generated\textsuperscript{100}. Following engagement with activated CD4 T cells in the T zone, activated follicular B cells proliferate for 1-2 days before either migrating to the red pulp and plasma cell differentiation, or migrating back into the B cell follicle to establish a germinal centre\textsuperscript{78}. Those that adopt this fate increase their expression of CXCR5 and migrate to networks of CXCL13-secreting FDC located in the B cell follicle\textsuperscript{101}. Here, they begin the processes of rapid division and somatic hypermutation as centroblasts in the dark zones of the germinal centre\textsuperscript{102}. Centroblasts increase their expression of Bcl-6, which suppresses the plasma cell phenotype, and encourages rapid cellular division\textsuperscript{103,104}. Through the Bcl-6 mediated
suppression of p53 and the DNA damage sensor ATR (ataxia telangiectasia and RAD3 related), mutations in DNA are allowed to arise without apoptosis occurring\textsuperscript{103,105}.

Simultaneously, centroblasts up regulate the enzyme ‘activation-induced cytosine deaminase’ (AID)\textsuperscript{100}. This mediates mutations in the variable region of genes encoding the BCR (somatic hypermutation).

Centroblasts then migrate to the light zone of the germinal centre, and re-express the BCR, now termed centrocytes. During this time class-switch recombination (CSR) is initiated, switching the isotype of immunoglobulin from IgM to IgG, IgA or IgE by moving the variable (VDJ) region to associate with a different constant (CH) region\textsuperscript{106}. In the light zone, they ‘test’ their newly mutated BCR against antigen deposits on the surface of FDCs\textsuperscript{100} (antigen, captured on the surface of these cells by the high expression of Fc receptors and complement receptors, amalgamates into antigen deposits known as iccosomes\textsuperscript{107}). Centrocytes which recognise the antigen through their BCR are provided with short-term survival signals by FDC\textsuperscript{100,108} (cross-linking of the BCR causes the transient up-regulation of the anti-apoptotic factor Bcl-2\textsuperscript{109}). It is still debated as to whether iccosomes are required for this process; mice with no detectable iccosomes still form germinal centres\textsuperscript{110}, however, without FDCs, both germinal centres and follicles rapidly dissolve\textsuperscript{101}. Further rounds of mutation may occur before centrocytes engage in interactions with T follicular helper cells (Tfh). Tfh are a subset of CD4 T cells activated during T cell:B cell interactions that express Bcl-6, ICOS, PD-1 and CXCR5 and migrate into the follicle during TD immune responses\textsuperscript{111}. They require ICOS expression for their generation and maintenance, and are necessary for germinal centre reactions\textsuperscript{112,113}. Interactions with germinal centre B cells, once again through MHC II:peptide complexes recognised by the TCR, CD40:CD40L, ICOS:B7H and further downstream interactions, mediate long term survival signals\textsuperscript{114} (again through Bcl-2 expression\textsuperscript{109}).
As well as mediating the survival of cells exiting the germinal centre, Tfh may also play a role in maintaining germinal centres, as injecting anti-CD40L following the establishment of germinal centre responses (and therefore blocking their interaction with B cells) causes their rapid abrogation\textsuperscript{115}.

Two cell types emerge from the germinal centre: B memory cells and long-lived plasma cells (described in detail later)\textsuperscript{102}. What causes the decision between differentiation to B memory cell or plasma cell is still poorly understood. Phan et al showed that higher affinity cells are recruited to the plasma cell compartment\textsuperscript{116}. Additionally, the decision may even be a stochastic event as, \textit{in vitro}, uniform populations of B cells appear to respond differently to the same signals\textsuperscript{117-119}. The authors of these studies suggested that at each division, there is a probability of each B cell differentiating to B memory cell or plasma cell, which may or may not be influenced by extrinsic or genetic factors\textsuperscript{117}.

Studies have however noted that early in a germinal centre reaction, most exiting cells are B memory cells, whereas later on, more long-lived plasma cells are generated\textsuperscript{120}, and that the memory cell compartment is far less mutated than the long-lived plasma cell compartment\textsuperscript{121}. Whether the microenvironment changes during this time, or it is advantageous to generate long-lived plasma cells of very high affinity (and memory cells of lower affinity) is unknown.

B memory cells themselves, following the resolution of the immune response, are transcriptionally similar to naïve B cells\textsuperscript{117,122}. On restimulation, however, they rapidly produce both high-affinity class switched plasma cells and germinal centres. Memory B cells are fairly long-lived and capable of self-renewal\textsuperscript{123}; while many reside in the splenic marginal zone, some recirculate through the blood stream\textsuperscript{124}. Although phenotypically similar to virgin B cells, many memory B cells express a class switched BCR. On re-exposure to antigen they respond rapidly by forming high-affinity plasma cells and the initiation of further germinal centre reactions\textsuperscript{125}. 
Factors influencing germinal centre formation

In addition to cognate signals supplied by Tfh cells, other signals can influence germinal centre formation and outcome. TLR stimulation plays an important role in germinal centre formation. It has long been known that adjuvant can enhance antigen-specific antibody responses, and some form of ‘danger signal’ is required to initiate TD responses. However, it was only relatively recent work that directly showed that TLR signalling is a requirement for optimal germinal centre formation and antigen-specific antibody in TD responses.

Follicular B cells stimulated by antigen and T cell help become activated, however TLR stimulation often enhances activation, and controls the nature of the response. This is intuitive, as the nature of the immune response that is required is indicated by the PAMPs present on a pathogen.

Cytokine environment too, plays a role in germinal centre development. IL-21 and IL-6 are important for the generation of Tfh; the absence of IL-21 mediates the loss of germinal centres (in acute LCMV infection), while the loss of IL-6 and IL-21 mediates a large drop in antigen-specific IgG. The loss of IL-6 mediates altered expression of co-stimulatory molecules and reduced synthesis of the complement pathway component C3d, both implicated in reduced IgG. IL-21 has also been shown to be effective at skewing the output of the germinal centre from memory B cells to plasma cells, as has IL-10, at least in human tonsillar B cells cultured with an FDC-like cell line. IL-4 has been shown to inhibit the germinal centre response in secondary responses to ovalbumin in complete Freund’s adjuvant. This creates a complicated picture of germinal centre reactions, which can be influenced by both TLR signalling, cytokine environment and contact-dependent signals.

Class-switching is controlled by TLR signals and cytokine environment

In much the same way that dendritic cells (and B cells) prime T cell responses differentially depending on the type pathogen they encounter, B cell antibody class-switching is controlled by TLR stimulation. It has been known for many years that Th1 responses generate IgG2 isotypes, whereas Th2 responses
generate IgG1, G3 and IgE responses\textsuperscript{132-134}. Originally, this was thought to be entirely mediated by the hallmark cytokines of these responses (IFN-γ and IL-4 respectively) from activated CD4 T cells. However, Pasare and Medzhitov showed that IgG2 switching, and to a lesser extent IgG1 switching, was dependent on B cell intrinsic TLR signalling through MyD88 following immunisation with OVA-LPS in incomplete Freund’s adjuvant\textsuperscript{39}. Similarly, data from our own lab has shown that B cell intrinsic signalling through MyD88 mediates the switch to IgG2c in \textit{Salmonella} infection. When chimeras in which B cells failed to express MyD88 were infected, switching to other isotypes occurred (notably IgG1)\textsuperscript{56}. So, while T helper associated cytokines can direct and enhance class switching, TLR ligands are also powerful drivers of switching to appropriate isotypes.

**Non-germinal centre class switching**

Class-switching does not only occur in the germinal centre, and also not only TD responses\textsuperscript{135}. While it is true that IgM is the main isotype of immunoglobulin produced during TI responses, it is by no means the only isotype. IgA switching, for example, is seen rapidly in cultures of peritoneal B1 cells stimulated with LPS, BLYS (B lymphocyte stimulator, also called ‘BAFF’) and TGF-β\textsuperscript{136}.

TI class switching is enhanced by numerous soluble factors (fig 1.4). The TNF family members BLYS and APRIL (‘A proliferation inducing ligand’) are two of these\textsuperscript{137,138}. These factors bind to receptors expressed on B cells such as TACI (‘transmembrane activator and CAML-interactor’), BCMA (‘B cell maturation antigen’) and (for BLYS) BAFF-R (‘B cell activating factor receptor’)\textsuperscript{139,140}. These glycoproteins are expressed as both transmembrane proteins on the surface of many innate cells, and also in soluble form from many cell types including monocytes, dendritic cells and activated B cells\textsuperscript{88,141-143}. They play roles in many areas of B cell biology: BLYS signalling is essential in the later stages of B cell development and survival of mature B cells\textsuperscript{144,145}, the regulation of B cell homeostasis\textsuperscript{146}, and plasma cell generation and survival\textsuperscript{88,147,148}. Signals through TACI on activated B cells also induce class switching to IgA or IgG isotypes, by recruiting MyD88, which activates NF-κB and subsequently AID\textsuperscript{149}. 

It is known that dendritic cells, following stimulation with IFN-γ, CD40L or (more potently) IFN-α, upregulate APRIL and BLyS\(^\text{138}\). This is especially true of plasmacytoid dendritic cells stimulated with IFN-α\(^\text{150}\). This facilitates class switching to IgG, IgA or (when IL-4 is present) IgE\(^\text{138}\). Many other cell types present in the red pulp of the spleen, including monocytes, macrophages, eosinophils, neutrophils and others produce these factors\(^\text{88,143,151,152}\).

Epithelial cells, too, have been implicated in the release of factors that initiate class switch recombination following TLR ligation at mucus membranes in a mechanism that may be distinct from those described above\(^\text{153}\).

Mechanisms that act on germinal centre B cells in influencing class switching are also important in non-germinal centre class switching. B cell intrinsic TLR stimulation during activation causes class switch recombination to occur, such as stimulation through TLR9 leading to switching to IgG2a\(^\text{154-156}\). In humans at least, this is enhanced by IL-10\(^\text{155}\).

T cell help, too, is an important factor for class switching in TD extrafollicular plasma cell responses. Toellner et al and Marshall et al showed that class switching occurs before the differentiation of activated B cells to plasmablasts, and is likely induced during interactions of activated T cells and B cells in the T zone\(^\text{157,158}\). During this phase, it is likely that TLR signals, cytokines and further signals from BLyS and APRIL control the class of antibody that is switched to.

In addition, Tfh-like cells in the red pulp have been shown to induce class switch recombination through their secretion of IL-21, and expression of CD40L in autoimmune mice\(^\text{159}\). Whether these T cells are important, or even induced, in responses to exogenous antigen is not yet known.

**Help For B cells in plasma cell responses**

While TI plasma cell responses may require only signals through the TLR or BCR, and TD responses require signals through the BCR and T cell help, there are also soluble factors that can enhance the differentiation of B cells to plasma cells (fig 1.4).
It has been known for some time that the cytokine IL-21, from activated CD4 T cells, can support and enhance class-switched antibody responses. IL-21/-mice exhibit reduced numbers of IgG plasma cells. Interestingly, IL-21 signalling must be in the context of BCR signalling, as naïve B cells which encounter IL-21 are induced to die by apoptosis. Although IL-6 was originally thought to induce a similar enhancement in plasma cell generation, it was found to be through IL-6-mediated increases in IL-21, although IL-6 does act as a survival factor for plasma cells. In extrafollicular and germinal centre responses, IL-21 is provided by Tfh-like and Tfh cells respectively during T cell:B cell interactions, which may be dependent on ICOS signalling.

In addition, IL-21 plays an important role in germinal centre formation, B memory cell formation and long-lived antibody responses. In seeming contradiction, it is capable of causing the expression of Bcl-6 or BLIMP-1 in germinal centre B cells that would lead to memory B cell or plasma cell fates respectively. It is likely that context is of great importance to the outcome of IL-21 signalling in B cells. In humans, IL-21 in synergy with BlyS causes the differentiation of marginal zone memory B cells to plasma cells, although it is unknown whether BlyS affects the decision between memory B cell or plasma cell fates in the germinal centre.

IL-6, secreted by CD11c+CD8+ myeloid dendritic cells in the secondary lymphoid organs mediate the support of plasmablast populations. It was found that when large numbers of B cells were recruited into antibody responses, they outgrew the available support from dendritic cells, and only those in tight association survived. Increased numbers of plasma cell associated dendritic cells (through the injection of anti-CD40, which causes their proliferation) led to the generation of greater numbers of plasma cells due to the support of increased levels of dividing plasmablasts for up to 2 days.

The TNF-α family members APRIL and BlyS are known to support the differentiation of plasma cells, however this is context dependent. Signals through BAFF-R in synergy with BCR and CD40 signalling leads to increased...
antibody responses\textsuperscript{177}. However, signalling through TACI, lead to repression of BLIMP-1 and reduced antibody levels in TD responses\textsuperscript{175}. In contrast, mice deficient in TACI have hyper-responsive B cells which proliferate and differentiate into plasma cells, causing SLE-like disorders and cancers\textsuperscript{146}. The precise roles of these factors are still debated, as \textit{in vitro} data and human studies have provided confounding evidence\textsuperscript{140,178}.

IFN-$\alpha$ is a cytokine that has been shown to enhance the production of plasma cells\textsuperscript{179}. Although whether this is a direct effect is unknown; it may be a result of stimulation of dendritic cells and the generation of increased levels of other cytokines and soluble factors, including APRIL and BlyS\textsuperscript{138,179}. It is also possible that IFN-$\alpha$ acts directly on B cells to induce their differentiation to plasma cells. This has been shown by human \textit{in vitro} data where IFN-$\alpha$ secreting plasmacytoid dendritic cells, cultured in combination with B cells activated with anti-CD40 and IL-6 caused an enhanced differentiation to plasmablasts\textsuperscript{180}. Mathian et al have shown that IFN-$\alpha$ causes early onset of SLE in lupus-prone (New Zealand Black x New Zealand White)F1 mice due to the early and enhanced production of short-lived plasma cells\textsuperscript{181,182}. This was not sufficient to cause the induction of plasma cells in non-autoimmune mice, and may be due to increased levels of BlyS in the context of autoimmune activatory signals\textsuperscript{181}.

Finally, CD4 T cells provide cognate signals to B cells, which cause differentiation to plasma cells as previously described. In extrafollicular plasma cell responses, two populations of cells have been described; Tfhl-like cells that express Bcl-6 and localise to the T-B border or similar cells that localise to the red pulp, require ICOS for their generation\textsuperscript{159,183}. Whether these are two distinct populations or one and the same is unknown. However, they both enhance extrafollicular plasma cell responses through the provision of CD40L and IL-21\textsuperscript{159,183}. In the germinal centre, Tfhl provide these signals. The role of ICOS during plasma cell development is less well defined.
Models of humoral memory and plasma cell lifespan

Antibody levels following vaccination or infection are maintained for many years. There are a number of models of how this is accomplished. Firstly, antigen could be retained in the body, causing the periodic restimulation of memory B cells and the generation of new plasma cells capable of maintaining antibody levels to prior infections or vaccinations\textsuperscript{184,185}. This idea initially was popular as it was thought that all plasma cells were short-lived, and that B memory cells required antigen for their maintenance\textsuperscript{184,186}. Similarly, antigen has been shown to be retained in B cell follicles for long periods after a single immunisation\textsuperscript{187}. Secondly, Bernasconi et al showed that in vitro stimulation of human B memory cells with TLR agonists lead to their differentiation to plasma cells\textsuperscript{188}. This data implied that memory B cells were induced, by polyclonal stimulation, to generate new plasma cells specific for prior infections during times of inflammation, which could ‘top up’ waning plasma cell populations and maintain serum antibody levels. Finally, the discovery of long-lived plasma cells, and strong evidence that suggests no requirement for B memory cells or antigen for their maintenance has caused a shift in opinion\textsuperscript{189,190}.

Long-lived plasma cells maintain serum antibody

Early studies utilising thymidine labelling and retention of that label to measure the turnover and lifespan of plasma cells indicated that mature plasma cells were non-dividing, and had a short half-life of around 8-12 hours\textsuperscript{186}. These studies, however, measured the lifespan of plasma cells generated at early time points following immunisation. When studies were conducted looking at the lifespan of plasma cells later in the response, it was found that some plasma cells survived for far longer\textsuperscript{191}. Later, Ho et al showed that plasma cells have variable lifespan, some short-lived (with a projected lifespan of less than 3 days) and others long-lived (in this study, a lifespan in excess of 3 weeks)\textsuperscript{192}. This was dependent on site rather than antibody class (with the exception of IgM plasma cells, which were all short-lived), and plasma cells in the spleen and lymph nodes were found to be mainly short-lived, whereas those in the bone marrow were found to be mainly long-lived\textsuperscript{192}. 
In the late 1990s, Manz et al showed that many plasma cells generated in the secondary response to ovalbumin migrated to the bone marrow and persisted, without cellular division for 90 days\(^\text{189}\). Similarly, Slifka et al tracked the survival of LCMV-specific plasma cells and antibody levels in mice following the depletion of B memory cells (through irradiation), and the transfer of LCMV-specific plasma cells to naïve hosts. In both models it was found that LCMV-specific plasma cells survived for long periods without further input from the peripheral B cell pool: with a half-life of 94 days in the bone marrow, and 172 days in the spleen\(^\text{193}\). These long-lived plasma cells were found in both the spleen and bone marrow, although far more in the latter\(^\text{193}\).

Further studies by Manz et al suggested that antigen was not required for the maintenance of long-lived plasma cells, and that long-lived plasma cells were unresponsive to restimulation with antigen, unlike memory B cells, which rapidly provided new antigen-specific plasma cells\(^\text{194}\).

Bernasconi et al showed that human B memory cells expressed TLRs constitutively, and could be induced to differentiate to plasma cells when stimulated through these receptors \textit{in vitro}\(^\text{188,195}\). This data suggested that, even in the absence of antigen, B memory cells could periodically be stimulated in a polyclonal manner and ‘top up’ plasma cell populations specific for prior infections that would otherwise gradually wane.

In agreement, Slifka’s data suggested that the periodic restimulation of memory B cells may contribute to the maintenance of antibody levels, as when memory B cells were depleted by irradiation, numbers of long-lived plasma cells fell compared to levels in control (non-irradiated) mice\(^\text{193}\). These initial studies by Slifka et al were criticised, as the sub-lethal irradiation used to deplete B memory cells was not B cell specific, and may have affected other populations in the bone marrow which supported plasma cell survival\(^\text{190}\). Therefore Ahuja et al used anti-CD20 to deplete B cells (but not CD20- plasma cells) from mice that had been previously immunised with NP-CGG. These data showed that numbers of NP-specific plasma cells did not fall in the bone marrow 16-weeks after B cell
depletion (antibody levels were not shown). Similar studies by DiLillo et al showed that following B cell depletion with anti-CD20, previously established populations of long-lived bone marrow plasma cells were not affected. Interestingly, they showed that when bone marrow plasma cells were depleted by the blockade of the intergrins LFA-1 and VLA-4 (described below), they were replenished if B memory cells were present, but not if B memory cells were depleted by co-injection of anti-CD20. So, although B memory cells are not required for the maintenance of plasma cells under homeostatic conditions, they may play a role in maintaining these populations under certain circumstances. This, however, is unlikely to be mediated by polyclonal restimulation of memory B cells through TLRs, as Richard et al demonstrated that TLR ligands alone were not sufficient to induce B memory cells to differentiate to plasma cells *in vivo*. For this, antigen was an absolute requirement.

**Short-lived and long-lived plasma cells**

Short-lived plasma cells are produced in large numbers during extrafollicular responses and tend to reside in the red pulp of the spleen, or the medullary chords of the lymph nodes. The majority of plasma cells that home to the bone marrow following primary immunisation with NP-KLH have undergone somatic hypermutation and therefore are high affinity cells emerging from germinal centres. These cells are produced in relatively low numbers following primary immunisation with protein antigen, but far greater numbers following secondary immunisation.

**Plasma cell migration**

What determines whether plasma cells migrate to the bone marrow or stay in the secondary lymphoid organs is not fully understood. Both extrafollicular plasma cells and those emerging from germinal centres down regulate CXCR5 and CCR7 (expressed by follicular B cells), and up regulate CXCR4, mediating the migration towards the chemokine CXCL12 from stromal cells. This attracts extrafollicular plasma cells towards the red pulp or medullary chords.
(alternatively this may simply be due to randomized linear movements\textsuperscript{202}), whereas long-lived plasma cells migrate to the bone marrow via the blood. Interestingly, CXCR4\textsuperscript{-/-} mice show fewer plasma cells in the secondary lymphoid organs and bone marrow, but more in the blood\textsuperscript{84,85}. Plasma cell entry into the blood stream may be regulated by the expression of ‘sphingosine-1-phosphate receptor 1’ S1P\textsubscript{1}\textsuperscript{203}. Plasma cell migration towards CXCL12, and the expression of S1P\textsubscript{1} was found to be controlled by XBP-1, although how this mediates differential migration of extrafollicular and long-lived plasma cells (when expressed in both) is still unclear\textsuperscript{99}.

Other chemokines, too, may be involved in plasma cell migration; CXCR3 (and its ligands CXCL9, CXCL10 and CXCL11) mediate the migration of plasma cells to inflamed sites\textsuperscript{84,201}. CCR9 expression by IgA plasma cells causes attraction to the intestine, where its ligand CCL25 is expressed\textsuperscript{84,204}.

Plasma cells also express numerous adhesion molecules, including the intergrins very late antigen 4 (VLA-4) and leukocyte-function associated molecule 1 (LFA-1) which adhere to vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) respectively\textsuperscript{205}, expressed throughout the red pulp of the spleen\textsuperscript{206}. These ligands are also expressed constitutively in the bone marrow\textsuperscript{207,208}, and blockade of these molecules has been shown to deplete bone marrow plasma cells\textsuperscript{196}. Fibronectin, another ligand for VLA-4 is also expressed in the splenic red pulp\textsuperscript{84}. The role of these factors in migration to the bone marrow is still unclear.

Selectins may play a role in plasma cell homing; plasma cells upregulate P-selectin glycoprotein ligand 1 (PSGL1), which binds both E and P selectins. This mediates binding to the endothelium and is expressed constitutively by bone marrow endothelial cells\textsuperscript{209,210}. IgG plasma cells undergo rolling interactions when cultured with E selectin\textsuperscript{205}. CD22, a member of the siglec family (immunoglobulin-like lectins) that binds sialic acid, may be important for migration to the bone marrow. In mature B cells this functions as a negative regulator of BCR signalling, and is essential for homing to the bone marrow.
CD22−/− mice however also exhibit reduced numbers of bone marrow plasma cells94,211.

Plasma cells lose the ability to migrate towards chemokines during maturation201. This occurs once they have reached either the red pulp or medullary chords202, or entered the bone marrow, and despite their continued expression of CXCR4201. Following this, it has been proposed that this receptor plays a role in plasma cell survival166. It is unknown what mediates the loss of migratory capacity in mature plasma cells.

**Plasma cell survival niches**

Plasma cells that home to the bone marrow are not inherently long-lived. They must be supported by a constant supply of survival factors (fig 1.5). These are available constitutively in the bone marrow in limited quantities, and therefore the bone marrow plasma cell pool is of finite size166. Similarly, survival factors are available at low levels in the secondary lymphoid organs during homeostatic conditions, and some of these are drastically increased during times of inflammation78,88,137. This allows for the long-term survival of small numbers of plasma cells during homeostasis, and the short-term survival of large numbers of plasma cells during inflammatory conditions. While much work has been done recently on determining the survival factors required to support plasma cells, the relative importance of each factor in each organ is still largely unknown.

**Survival factors**

Many survival factors have been previously mentioned, as they are also involved in other aspects of plasma cell biology.

APRIL is a key survival factor for plasma cells in both the bone marrow and inflamed organs. On differentiation to plasma cells, BAFF-R is down regulated and TACI expression remains similar to B cells. BCMA, which has high affinity for APRIL140, is up regulated88. APRIL is also able to bind the plasma cell surface marker CD138, although it is unknown what the effects of this are212. BCMA enhances the survival of bone marrow plasma cells when cultured *in vitro*213.
That APRIL is secreted by many cells of the innate immune system\textsuperscript{88,151}, as well as activated B cells\textsuperscript{141}, and VCAM-1 expressing bone marrow stromal cells\textsuperscript{214} means that it is rich in both the bone marrow, and in sites of inflammation. While BlyS may mediate plasma cell survival in inflamed sites\textsuperscript{215}, it is not thought to be important for survival of bone marrow plasma cells\textsuperscript{148}.

IL-6 was first shown to support plasma cell survival by Cassese et al; when cultured \textit{in vitro} plasma cells survived around 3 days longer when IL-6 was present\textsuperscript{166}. In the bone marrow, this IL-6 may come from stromal cells, as when plasma cells were cultured with bone marrow stromal cells, they mediated the survival of plasma cells for around 4 weeks\textsuperscript{216}. Moreover, IL-6\textsuperscript{-/-} stromal cells had greatly reduced capacity to support plasma cell survival\textsuperscript{216}. This also may be dependent on contact-based signals, as the addition of anti-VLA-4 to cultures also disrupted stromal cell-mediated survival\textsuperscript{216}. Similarly, data from Winter and Chu showed that megakaryocytes\textsuperscript{217} and eosinophils\textsuperscript{151} are both potent IL-6 producers in the bone marrow and mediate the survival of long-lived plasma cells (although both also produce APRIL). The role of IL-6 \textit{in vivo}, however, has come into question. While early studies showed that excess IL-6 led to increased numbers of IgG1 plasma cells in multiple organs\textsuperscript{218}, Cassese et al showed that immunisation with ovalbumin in IL-6 sufficient or deficient mice lead to no appreciable difference in numbers of OVA-specific plasma cells or levels of specific antibody after 21 weeks\textsuperscript{166}. More recently, data from MacLennan’s group showed that IL-6 is provided by CD11c\textsuperscript{+}, CD8\textsuperscript{-} dendritic cells in the inflamed lymph node, and that plasmablasts, but not mature plasma cells, associate closely with these\textsuperscript{78,88,137,172}. While the precise role of IL-6 still remains unclear, it may support plasmablast growth in the inflamed secondary lymphoid organs, and also act as a survival factor for long-lived plasma cells in the bone marrow.

While APRIL and IL-6 are the two main survival factors that have currently been identified, limited data is also available for certain other factors. IL-5 was also shown by Cassese et al to support plasma cell survival \textit{in vitro} for up to three days. However, when supernatants from IL-5\textsuperscript{-/-} bone marrow cultures were
used to culture plasma cells, they were equally sufficient at supporting plasma cell survival as supernatants from cultures of wild type bone marrow. Cassese et al also examined TNF-α and, *in vitro*, it was found to provide survival signals to plasma cells. *In vivo* effects have not yet been explored. Finally, CXCL12 (usually associated with migration of plasma cells) may also mediate survival of bone marrow plasma cells. This chemokine is expressed by stromal cells in the bone marrow, and many plasma cells are found to closely associate with these cells. However, studies utilising CXCL12 blocking antibodies showed that plasma cell survival in *in vitro* cultures with bone marrow stromal cells was not affected by the lack of CXCR4 signalling. Similarly, plasma cells in mice with a CXCR4 deficiency in the B cell compartment were found to populate the bone marrow and survive in normal numbers 90 days after secondary immunisation with NP-KLH, although their migration there was delayed. This highlights the apparent redundancy between many factors, which *in vitro* are all capable of extending plasma cell survival but *in vivo* may be of limited importance.

**Contact based signals**

As well as soluble factors that enhance plasma cell survival, contact based signals may be important (fig 1.4 and 1.5). Firstly VLA-4:VCAM-1 and LFA-1:ICAM-1 are important adhesion molecules known to mediate the retention of long-lived plasma cells in the bone marrow. Injection of blocking antibodies causes a dramatic depletion of bone marrow plasma cells. Plasma cells are known to associate closely with VCAM-1 expressing stromal cells in the bone marrow and areas rich in ICAM-1 and VCAM-1 in the spleen and lymph node. Blocking antibodies to VLA-4 caused the inhibition of antibody from plasma cells cultured with bone marrow stromal cells. However, the plasma cell intrinsic signalling pathways and anti-apoptotic factors that may be generated by these interactions are not known. Indeed, these close associations may be required simply to provide a constant supply of other survival factors.

CD44, another adhesion glycoprotein, is expressed on the surface of plasma cells. Its major ligand *in vivo* is hyaluronic acid (a component of the
extracellular matrix produced by stromal cells) and it is associated with trafficking cells to the bone marrow.\textsuperscript{223} In\textit{ vitro} cultures of plasma cells with antibody capable of cross-linking CD44, or with hyaluronic acid, showed significant enhancement in plasma cell survival over 5 days, which was further enhanced by the addition of IL-6.\textsuperscript{166} Interestingly, interactions between CD44 expressing myeloma cells and the extracellular matrix causes the secretion of IL-6 from stromal cells in the bone marrow.\textsuperscript{224} So, plasma cells may induce the secretion of survival factors from bone marrow stromal cells.

Finally, CD28, a co-receptor associated with TCR signalling is expressed on all plasma cells.\textsuperscript{225} This molecule, however, is only functional in long-lived plasma cells, and ligation of CD28 by CD80 or CD86 on bone marrow dendritic cells was found to be important for maintaining plasma cell populations in the bone marrow.\textsuperscript{226} Interestingly, ligation of CD80/86 by CD28 has also been shown to mediate the secretion of IL-6 by dendritic cells\textsuperscript{227}, and indeed, Rozanski et al showed that in\textit{ vitro}, plasma cells were able to cause the secretion of IL-6 by these cells.\textsuperscript{226} In addition, the culture of plasma cells with wild type or IL-6\textsuperscript{-/-} bone marrow dendritic cells showed that, while dendritic cells were sufficient to mediate survival of plasma cells for up to 30 days, IL-6 was required to mediate the secretion of IgG.

\textbf{Survival niches in the bone marrow}

Many of the cell types associated with plasma cell survival have been previously mentioned, however, they will be summarised here. It should be stressed that their characterisation is incomplete; many cell types have been indicated to be essential for the maintenance of plasma cell populations, but it is still not entirely clear whether soluble factors (from any cellular source) or specific contact-dependent signals are required for survival, and whether the signals described above act cumulatively or redundantly (fig 1.4 and 1.5).

Eosinophils were identified by Chu et al as necessary for the maintenance of bone marrow plasma cell populations.\textsuperscript{151} These Gr1\textsuperscript{int} F4/80\textsuperscript{hi} CD11b\textsuperscript{int} cells expressed both APRIL and IL-6 and were capable of supporting plasma cells in
vitro\textsuperscript{151}. Similarly, bone marrow plasma cells \textit{in vivo} were closely associated with eosinophils and the depletion of these cells caused the apoptosis of bone marrow plasma cells\textsuperscript{151}.

In a similar set of experiments, Winter et al showed that platelet forming, CD41+ megakaryocytes also secreted both APRIL and IL-6 and associated closely with OVA-specific plasma cells in the bone marrow following immunisation\textsuperscript{217}. Mice deficient in megakaryocytes (thrombopoietin receptor knockouts) had reduced numbers of antigen-specific bone marrow plasma cells following OVA immunisation, whereas the injection of thrombopoietin (inducing increased numbers of megakaryocytes) caused the accumulation of greater numbers.

Stromal cells, as mentioned above, have been shown to support plasma cell survival \textit{in vitro}. Again, a high percentage (around 90\%) of bone marrow plasma cells form tight associations with CXCL12-secreting bone marrow stromal cells\textsuperscript{219} and express VCAM-1\textsuperscript{214}. These cells however, express only low amounts of APRIL\textsuperscript{148}. It may be that plasma cells require a complex niche, made up of stromal cells with which they are able to form tight associations through VLA-4:VCAM-1, and the expression of CXCL12. Other cells such as megakaryocytes, eosinophils and others may then provide other necessary survival factors such as APRIL and IL-6.

\textbf{Survival niches in inflamed organs}

It is thought that short-lived and long-lived plasma cells are distinct populations. This may be true, however recent work has shown that many plasma cells can exhibit enhanced survival \textit{in vivo} when the supply of survival factors is great enough, as seen under inflammatory conditions in secondary lymphoid organs and non-immune organs\textsuperscript{78,88,228,229}. The survival factors required in peripheral organs are much the same as those in the bone marrow, although so far only the effects of APRIL and IL-6 have been characterised\textsuperscript{88}.

In the inflamed lymph node and spleen, the majority of IL-6 is provided by dendritic cells, which localise to and proliferate in the medullary chords and red
pulp, in close proximity to the bridging channels and perivascular areas (where plasmablasts emerge from the follicle)\textsuperscript{78,81,88,172}. As described previously, IL-6 at this site supports the continued expansion of dividing plasmablasts.

CD11b\textsuperscript{hi}, F4/80\textsuperscript{hi}, Gr1\textsuperscript{hi} monocytes and macrophages rapidly influx into the secondary lymphoid organs under inflammatory conditions, colonising the medullary chords of the lymph node and red pulp of the spleen\textsuperscript{78,88}. These cells produce high amounts of APRIL and are closely associated with mature plasma cells\textsuperscript{88}. Macrophages are present in the red pulp in lower numbers during homeostatic conditions\textsuperscript{230,231}, explaining the survival of low numbers of plasma cells for long periods in the secondary lymphoid organs\textsuperscript{193}.

Similarly, neutrophils\textsuperscript{88,143} and basophils\textsuperscript{232} have been identified as cells capable of supporting the survival of plasma cells in mice and humans, through their secretion of APRIL and IL-6 respectively\textsuperscript{143,232}.

All of these cellular populations are also present in the bone marrow, and their roles in that organ are yet to be determined. That innate cells produce many of the survival factors required for plasma cell survival is intriguing; their influx to secondary lymphoid organs (and non-immune sites) during inflammatory conditions allows for the temporary support of large numbers of plasma cells. These cells provide large amounts of antibody until the inflammation is resolved, at which point survival factors return to low levels, allowing for the survival of only very few plasma cells at these sites. By this stage, however, germinal centre formation has occurred in TD responses and high affinity plasma cells that migrate to the bone marrow have been produced. In this organ, levels of survival factors remain at a constant level, allowing for the long-term survival of any plasma cells that are able to enter a survival niche there. These plasma cells are then able to maintain serum antibody levels for months to years.

**Effects of survival factors**

While many of the survival factors required for the support of plasma cells have been determined, less is known about their downstream actions. While in B
cells, Bcl-2 (‘B cell lymphoma 2’) and Bcl-xL (‘B cell lymphoma extra large’) are common anti-apoptotic factors\textsuperscript{2,33} induced by survival signals such as CD40:CD40L and IL-10 during germinal centre reactions\textsuperscript{109,234,235}, and by BlyS signalling\textsuperscript{236-240}. Relatively little work has been done on the anti-apoptotic factors induced in plasma cells by survival factors. However data from multiple myeloma patients and cell lines (a human condition where malignant plasma cells accumulate to high numbers in the bone marrow) has shown that another anti-apoptotic factor, MCL-1 (‘Myeloid leukaemia cell differentiation protein 1’), and Bcl-xL are both important to the survival of neoplastic plasma cells\textsuperscript{241,242}. Interestingly, onset of multiple myeloma is associated with increased levels of IL-6\textsuperscript{243,244}, and the increased levels of MCL-1 and BC-xL seen in bone marrow plasma cells are IL-6 dependent\textsuperscript{241}. In mice, O’Connor et al showed that signalling through BCMA on bone marrow plasma cells induced MCL-1 expression\textsuperscript{213}. In addition, IL-6 may have additional survival effects on myeloma cells that is independent of MCL-1 or Bc-xL, including the STAT-3 dependent activation of the micro-RNA miR-21\textsuperscript{244}. The effects of IL-6 on normal human or mouse bone marrow plasma cells’ expression of MCL-1 and Bc-xL remains to be seen.

**Limited survival niches lead to competition**

The bone marrow plasma cell compartment, like other memory pools, is of limited size. A finite amount of survival factors means that only a limited number of plasma cells can be maintained in the bone marrow compartment\textsuperscript{89,166,185,245,246}. Although levels of these factors increase during inflammatory episodes in other organs, once inflammation is resolved, these levels once again decline\textsuperscript{88}. This mediates competition between previously established plasma cells, and newly generated plasma cells. In the human bone marrow, this is thought to be around 0.1-1 percent of total cells\textsuperscript{247}. Estimates from Radbruch et al are that the human bone marrow is capable of supporting around 1000 specificities of plasma cells in substantial enough numbers to provide effective serum antibody levels\textsuperscript{248}. 
Chapter 1 - Introduction

It has been observed in human patients vaccinated with tetanus toxoid that mature plasma cells which are not specific for the immunising antigen can be detected entering the blood stream at the same time as the newly generated tetanus toxoid-specific plasmablasts enter the bone marrow compartment\textsuperscript{249}. It is speculated that these are pre-existing plasma cells (established in previous immune responses) that have been displaced from their bone marrow survival niches\textsuperscript{248,249}. However, further estimations from Radbruch et al state that after almost 700 subsequent immune responses, serum antibody levels to a particular immunisation would only have declined to around 50 percent of its original levels\textsuperscript{248}. This is supported by data from Amanna et al, who show that anti-viral antibody responses in humans had half-lives ranging from 50 to 200 years, whereas those to tetanus and diphtheria were of 11 and 19 years respectively\textsuperscript{250}.

The precise mechanism mediating competition between plasma cells is unknown, however it is thought that the ability of newly generated plasmablasts to migrate towards plasma cell niches may confer an advantage over previously established, non-migratory plasma cells\textsuperscript{249}. There may be additional mechanisms; plasma cells express the death receptor FcγRIIb which, when cross-linked by IgG immune complexes, causes their apoptosis\textsuperscript{38}. It could be that increased levels of immune complexes during immune responses causes the death of certain bone marrow plasma cells, creating space for the entry of new plasma cells, although this is yet to be shown.

**Plasma cells in autoimmunity**

There is evidence that not all plasma cells are equally sufficient at obtaining access to survival factors or plasma cell niches. Rituximab is an anti-human CD20 antibody used to deplete B cells in patients suffering from certain autoimmune diseases. This treatment, originally designed to treat B cell lymphomas, is now often used to reduce levels of circulating autoantibodies by the depletion of CD20+ peripheral B cells. CD20- short-lived autoantibody-secreting plasma cells that are generated continuously by B cells activated by an unlimited supply of self-antigen would no longer be generated in the absence of
a B cell compartment\textsuperscript{251-258}. It was found that in rheumatoid arthritis patients, autoantibody levels fell while antibodies to prior vaccinations and infections were maintained by long-lived plasma cells\textsuperscript{253}.

For other autoimmune conditions, too, this was the case, as documented by Ferraro et al who showed a dramatic drop in the levels of autoantibodies specific for protinase-3 in patients with active vasculitis that received rituximab therapy\textsuperscript{259}. Conversely, little decline was found in levels of antibodies specific for external antigens (either TD or TI). The decline in autoantibody, however, occurred over a 5-month period. This suggests that while autoantibody-secreting cells are able to persist in inflamed organs, they do not enter the bone marrow compartment, and die as inflammation is resolved\textsuperscript{259}.

In agreement with this data, studies by Huang et al showed that, in K/BxN mice (a murine model of rheumatoid arthritis, described below), which generate significant splenic populations of plasma cells secreting antibody specific for the ubiquitously expressed enzyme glucose-6-phosphate-isomerase (G6PI)\textsuperscript{260}, the vast majority of G6PI-specific plasma cells were short-lived and resided in the spleen and lymph nodes\textsuperscript{261}. When these mice co-expressed mouse and human CD20, and were treated with rituximab, B cells were rapidly depleted and G6PI-specific plasma cells and antibody levels fell over the course of 120 days\textsuperscript{261}.

While effective in certain autoimmune diseases, sufferers of SLE did not comprehensively exhibit reduced autoantibody titres\textsuperscript{251-253}. SLE patients exhibit plasma cells secreting autoantibodies specific for self-DNA and self-RNA\textsuperscript{262}. While self-DNA specific antibody levels fall following treatment with rituximab (and therefore DNA-specific plasma cells are likely to be short-lived), anti-RNA antibodies do not fall, meaning that self-RNA specific plasma cells are likely to be long-lived\textsuperscript{262}. The TLR ligands involved in the initiation of these autoimmune conditions are, as described previously, recognised by TLR7 (RNA) and TLR9 (double stranded DNA). TLR7, when used as an adjuvant, has been shown to be a potent inducer of persistent antibody responses\textsuperscript{263}. Additionally, treatment of SLE patients with belimumab, which blocks the binding of BlyS to BAFF-R and
TACI has shown more efficient reductions in anti-RNA antibodies\textsuperscript{264}. Trials with atacicept, an APRIL-blocking antibody, are ongoing\textsuperscript{265}.

Studies in mice have shown similar, although not identical, results. Studies from Rudolf Manz’s lab using the (NZBxNZW)F1 model of SLE showed that plasma cells were greatly expanded in the spleens and kidneys of these mice. Around 40 percent of these were non-dividing and resistant to treatment with cyclophosphamide\textsuperscript{228,229,266}. Of the anti-DNA component of the plasma cell response, only around 20 percent were in the long-lived, non-dividing compartment, as determined by BrdU labelling\textsuperscript{228}.

These results paint a complex picture of the ability of certain plasma cells, especially those generated during autoimmune disease, to become long-lived. The reasons for this will require further experimentation.

**Plasma cells as APC**

Despite their down-regulation of antigen presenting molecules such as MHC II, CD80 and CD86, Pelletier et al recently showed that plasma cells, were capable of presenting antigen to, and activating, CD4 T cells \textit{in vivo}\textsuperscript{267}. However, CD4 T cells activated by plasma cells did not express Bcl-6 or differentiate to Tfh cells, likely due to a lack of IL-21 production by plasma cells\textsuperscript{267}. Mice lacking plasma cells also had increased numbers of Tfh\textsuperscript{267}. This may provide a negative feedback loop that limits humoral immunity. Whether plasma cells play a role in recall responses or not is unknown.

**Models used throughout this thesis**

**Salmonella**

In this project we have used infection with the aro-A\textsuperscript{-} attenuated strain of \textit{Salmonella enterica} serovar \textit{Typhimurium} (also called SL3261). This strain is commonly used as a live-attenuated vaccine strain, and confers protection to future infections with virulent strains\textsuperscript{268}. \textit{Salmonella} is a Gram-negative intracellular bacterium that infects humans orally. From here they enter the Peyer’s patches via the type III secretion system, and through the mesenteric
lymph nodes to the blood stream, after which they rapidly become systemic\textsuperscript{269,270}. The bacteria reside and multiply mainly in macrophage populations.

Here, mice have been infected with \textit{Salmonella} intravenously. While this is not the route of infection seen in natural infections, there are a number of justifications for using this method. Firstly, here we have simply used \textit{Salmonella} as a model of bacterial infection, and would hope that any observations seen here may apply to infection with other similar bacteria. Secondly, as the bacteria rapidly become systemic, infecting intravenously should not provide wildly differing results to oral infection. Thirdly, our own experiments have shown that oral infection provides a more variable dose that can be controlled more tightly by intravenous injection (D. Gray unpublished observations).

Infection with live \textit{Salmonella} is initially controlled by macrophage populations\textsuperscript{271}. However, systemic infection generates a potent Th1 response and the cytokines produced (IFN-γ, TNF-α and IL-12) are all crucial to controlling infection\textsuperscript{272-274}. Similarly, TLR4 signalling is key in controlling bacterial loads, as C3HeJ mice (which cannot respond to LPS) are susceptible to infection\textsuperscript{275}; this is thought to be due to a lack of TLR4-dependent cytokine production\textsuperscript{276}. TLR2 is also is important later in the infection\textsuperscript{277}. Similarly, MyD88\textsuperscript{-/-} mice exhibit significantly higher bacterial loads due to reduced IL-12 and Th1 cytokines\textsuperscript{44,278,279}.

B cells are not necessary for the primary responses to \textit{Salmonella} but are required for protection in secondary infections\textsuperscript{44,280}. This is surprising, as the plasma cell response in the spleens of infected mice is considerable. TI B1b cells contribute to the early plasma cells response and secrete antibody specific for outer membrane protein porins (OMP) C, D and F, which is capable of impairing infection\textsuperscript{76}. A TD plasma cell response also occurs in the spleen, which is mainly class-switched to IgG2c, but does not arise from germinal centres\textsuperscript{281}. B cell intrinsic signals through MyD88 are required for the switch to IgG2c, and
without these switching to IgG1 occurs\textsuperscript{56}. This response persists for around 5-weeks without germinal centres, of which formation is considerably delayed in \textit{Salmonella} infected mice. This delay in germinal centres would appear to correlate to bacterial load: when bacterial loads were decreased prematurely by treating with antibiotic, germinal centres were found to arise earlier\textsuperscript{281}. Although not necessary for protection, antibody from these plasma cells does impede the invasion of macrophages by \textit{Salmonella} bacteria, and reduces bacterial loads in the blood\textsuperscript{281}. Interestingly, \textit{Salmonella}-specific antibody levels remains low in infected mice until around 4-5 weeks after infection, as plasma cell numbers decline and germinal centres appear\textsuperscript{56}, and high affinity \textit{Salmonella}-specific antibody remains almost undetectable until this point\textsuperscript{281}. Further experiments are required to clarify the specificity of the large numbers of plasma cells produced during the extrafollicular plasma cell response to \textit{Salmonella}.

\textbf{K/BxN mice}

K/BxN mice are a cross between KRN-C57Bl/6 mice (which express a TCR transgenic for a bovine ribonuclease (RNase 42-56) presented by the I-A\textsuperscript{k} MHC II molecule, and the autoimmune-prone non-obese diabetic (NOD) mice. This led to the unexpected finding that the KRN TCR recognised the ubiquitously expressed enzyme glucose-6-phosphate isomerase (G6PI) on the NOD-derived A\textsuperscript{Ag7} MHC II molecule\textsuperscript{282}. A proportion of T cells expressing the KRN TCR escape tolerance-induced death and go on to mediate severe chronic arthritic symptoms by around 3 weeks of age, characterised by swollen joints and high levels of anti-G6PI in the serum\textsuperscript{260,282}. While initiation of disease was found to be dependent on the KRN TCR, autoantibodies were found to mediate disease thereafter, and transfer to wild type, B cell deficient, or lymphocyte deficient mice caused the onset of acute arthritis within 24 hours, lasting 15-30 days\textsuperscript{283}. This was found to be dependent on immune complex-dependent pathways, such as the activation of the alternative complement pathway\textsuperscript{284,285}. This mouse strain has commonly been used as a model of rheumatoid arthritis, and to clarify the mechanisms behind autoantibody-induced arthritis\textsuperscript{282}. 
Recently, as described earlier, Huang et al used K/BxN mice co-expressing human and mouse CD20 to show that in this model of autoantibody-induced arthritis, autoantibody is secreted by short-lived plasma cells, which can be depleted using rituximab treatment, mediating a decline in anti-G6PI antibody levels\textsuperscript{261}.

**5-bromo-2-deoxyuridine**

5-bromo-2-deoxyuridine (BrdU) is a synthetic thymidine analogue that can be used to label dividing cells either in vitro or in vivo. Dividing cells incorporate BrdU into their DNA as it is synthesised, in place of thymidine\textsuperscript{286}. In these experiments, BrdU has been used in vivo. Here it can be injected into the peritoneal cavity, or added to the drinking water. Importantly, once BrdU is removed from the water (or injections stop), it is rapidly removed from the body (presumably as it is rapidly used by dividing cells), but once incorporated into the DNA, BrdU remains stable for long periods\textsuperscript{287}. However, if further cellular division occurs once BrdU provision has been stopped, detectable levels of BrdU are rapidly lost in just 2-3 divisions\textsuperscript{86,288}.

BrdU has been used for many years to detect the lifespan and turnover of plasma cells\textsuperscript{287}. This can be done in two ways: firstly a sustained period of BrdU injection or feeding (here termed a BrdU ‘pulse’) before the mouse is culled (for example, for 10 days) allows the differentiation between non-dividing, long-lived plasma cells (which do not incorporate the label) and dividing plasmablasts or short-lived plasma cells, which have both turned over during the pulse\textsuperscript{228,229}. A second technique involves giving the label for a defined pulse to label plasma cells generated during this time (for example, following immunisation), followed by a ‘chase’ period where BrdU is not provided\textsuperscript{189,287}. When mice are culled, cells that contain the label must have been generated during the defined pulse, as those that were generated before or after will be BrdU-. The fact that mature plasma cells do not divide means that in this way their lifespan can be tracked over long periods\textsuperscript{189}.
Hapten-carrier proteins

Haptens are chemically defined molecules that are capable of binding to certain antibodies, but by themselves cannot illicit an immune response. For this reason they are conjugated to ‘carrier’ proteins that can initiate either TD (e.g. keyhole limpet hemocyanin (KLH), ovalbumin (OVA), hen egg lysosyme (HEL)) or TI-2 (e.g. ficoll) responses. The advantage to hapten-carrier immunisations is that hapten-specific antibody responses can be easily measured, as the hapten provides a defined epitope, unlike the carrier protein, which is broken down to form many epitopes. Similarly, high frequencies of hapten-specific plasma cells are induced following immunisation. In this study, we have used three hapten-carrier proteins: 4-hydroxy-3-nitrophenyl conjugated to KLH (NP-KLH), 2,4-dinitrophenyl conjugated to OVA (DNP-OVA) and fluorescein conjugated to OVA (FITC-OVA).

Sheep red blood cell immunisation

The response to sheep red blood cells in mice is entirely TD and generates large populations of extrafollicular plasma cells by day 5. At the same time, germinal centres are apparent. The rapid TD response seen is equivalent to that in mice with increased availability of T cell help (generated by priming with a carrier protein, followed by boosting with a hapten conjugated to the same carrier). The response is associated with large numbers of extrafollicular plasma cells that persist for just 2-3 days, however the germinal centre response has been reported to persist for up to 240 days.
Figure 1.1. T-Independent and T-dependent B cell responses
Figure 1.2. Toll-like receptor signalling in the mouse. Depicts TLRs 1-9, their ligands, locations, signalling adaptor proteins and outcome of ligation.
Figure 1.3. Splenic structure and TD responses.
The spleen is separated into white and red pulp; divided by the marginal zone. Naïve circulating T and B cells enter the spleen via the central arteriole and migrate through the marginal zone to the T or B cell zones. Activated T and B cells meet at the border of these zones, and B cells either form germinal centres or migrate to the red pulp and differentiate to plasmablasts and later, plasma cells. These, respectively, are supported by IL-6 from dendritic cells and APRIL from monocytes. Plasma cells emerging from the germinal centre migrate to the bone marrow.
Figure 1.4. Stages of plasma cell development and requirements for progression and survival
Figure 1.5. The plasma cell survival niche in secondary lymphoid organs and bone marrow.
Aims of this PhD

The aims of this PhD were to:

1. Develop models to identify and measure the turnover and lifespan of plasma cells

2. Determine the requirements for the generation of extrafollicular plasma cells in *Salmonella* infection compared to the TD response to SRBC

3. Determine whether the lifespan of plasma cells is affected by the chronicity of antigen delivery

4. Determine the effects of infection and inflammation on previously established bone marrow plasma cell populations and circulating antibody
Chapter 2 - Materials and Methods

Mice and license
C57Bl/6, MyD88−/−, TRIF−/−, MyD88/TRIF−/−, TLR 4−/−, ICOS−/−, Aβ−/−, MD4−/−, CD1d−/−, CD40−/− (a kind gift from Andrew MacDonald, University of Edinburgh), IFNα/βR−/− (a kind gift from Caetano Reis e Sousa, London Research Institute) mice (all on C57Bl/6 backgrounds) were bred and maintained in specific pathogen free conditions at the School of Biological Sciences Animal Facility at the University of Edinburgh. Mice were aged 6-10 weeks at the start of experiments, unless otherwise stated.

K/BxN260 mice were a kind gift from Mohini Gray at the University of Edinburgh Queen’s Medical Research Unit. These mice are the F1 progeny of NOD and KRIN mice and were aged 30 days before use. Arthritis development was determined by scoring joint swelling.

Experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act 1986. This license was approved locally by the University of Edinburgh Ethical Review Committee.

Protein conjugations
Proteins used are detailed in each experiment. For most immunisation experiments, hapten-carrier conjugates are used (DNP-OVA, NP-KLH or FITC-OVA)

2,4-Dinitrophenyl (DNP) was coupled to ovalbumin from chicken egg white (OVA) (Sigma) by reacting 32µl dinitrofluorobenzene (DNFB) (Sigma, D1529-10ML) per mg OVA (Sigma, A5503) in an equal volume of 0.1M borate buffer (pH 8.4) for 30 minutes at 37°C with regular mixing. To remove free hapten molecules, the resulting solution was transferred to 10,000MW cut off dialysis tubing and dialysed against 5 changes of PBS (3 litres per change) with constant stirring. For the first 3 changes of PBS, dialysis was at room temperature for 2 hours. For the final 2 changes of PBS, dialysis was at 4°C overnight. The
conjugate was then concentrated to 5mg/ml (assuming no loss of protein) using vivaspin-6 centrifugal ultrafiltration tubes (Sartorius-Stedim Biotech) following manufacturer’s instructions.

For NP-KLH conjugation, 4-hydroxy-3-nitrophenylacetic acid active ester (NP-OSu) (Biosearch technologies, N-1010-100) was dissolved in dimethyl sulphoxide (DMSO) (Sigma, 472301) at 100mg/ml. 100µl of this was added to 25mg keyhole limpet hemocyanin (KLH) (Calbiochem, 374811) in an equal volume of 0.1M borate buffer (pH 8.4) for 30 minutes at 37°C before dialysis and concentration adjustment as above.

FITC-OVA was bought from Biosearch Technologies (F-1511-10) and suspended in sterile PBS at 1mg/ml.

To detect hapten-specific antibody, enzyme-linked immunosorbent assay (ELISA) plates were coated with the relevant hapten, conjugated to bovine serum albumin (BSA). Conjugation was performed as follows:

NP-OSu (Biosearch technologies) was conjugated to BSA (Sigma, A7906) by reacting 100µl of NP-OSu (dissolved in DMSO at 100mg/ml) to 50mg BSA (dissolved in 0.1M borate buffer, pH 8.4) for 30 minutes at 37°C before dialysis and concentration adjustment as above.

For detection of Hen Egg Lysosome (HEL)-specific plasma cells (and B cells) by FACS, HEL (Sigma, L6876) was biotinylated using the Pierce ‘EZ-Link Sulfo-NHS-LC-Biotin’ kit (21435). HEL at 1mg/ml was reacted with 75µl of 10mM Sulpho-NHS-LC-Biotin (2.2mg in 400µl of ultrapure water) per mg of HEL. This was left on ice for 2 hours with regular mixing before dialysis as before. The conjugate was used from a stock solution (stored at -80°C) at 3.5mg/ml as determined by BCA protein assay (Pierce, 23225).

**Primary immunisations**

Sub-Cutaneous immunisations were administered to the hind flanks (100µl to each). 100µg protein and 10µg LPS (Invivogen, LPS-SM) were suspended in an
emulsion of incomplete Freund's adjuvant (IFA) (Sigma, F5506) and PBS (1:1). This was sonicated on ice, until a thick solution was obtained.

For intraperitoneal immunisations, proteins were alum-precipitated (briefly; protein at 5mg/ml was mixed 1:1 with 9% aluminium potassium sulphate in PBS. Solutions were neutralised by the addition of sterile sodium hydroxide. After 1 hour at room temperature, solutions were washed three times in PBS and resuspended at 5mg/ml). 100µg alum-precipitated protein was injected with 2.5x10^8 killed *Bordetella pertussis* (BD, 225851) in 200µl PBS.

Secondary immunisations were performed 5 weeks after primary. Unless otherwise stated, 100µg soluble protein (in 200µl PBS) was injected intravenously (I.V.) into lateral tail veins. For ‘chronically stimulated’ mice, 20x5µg doses every 3 days over a 60-day period (also I.V. in PBS). In some experiments, secondary immunisations were given I.P. (either with soluble protein or alum-precipitated with *B. pertussis* as described above).

**Sheep red blood cell immunisations**

Heparinised blood from sheep housed indoors was purchased from the Moredun Research Institute (University of Edinburgh). The blood cells were washed three times in PBS and used within 8 weeks. Mice received 5x10^8 SRBC (diluted in PBS for a total volume of 200µl) I.V.

**Salmonella enterica serovar Typhimurium infection**

*Salmonella* vaccine strain SL3261 was grown in Luria-Bertani (LB) broth (Difco labs) at 37°C for 16 hours. Cultures were diluted in sterile PBS so as to give approximately 1x10^6 colony forming units (CFU) per 200µl. This was injected I.V. into tail veins. Doses were plated out onto LB agar plates in serial dilution to determine infectious dose for each experiment. To determine the bacterial load per organ in infected animals, organs were removed and total organ weight was determined. A section of organ was removed and weighed, before mashing between sterile gauze into a known volume of PBS. This was diluted and plated into 5ml of molten LB agar at a range of concentrations in duplicate. LB agar was allowed to set, and cultures were incubated at 37°C for 24 hours and CFU
were counted. From this, total CFU per organ (or per gram of organ) was calculated.

**Schistosoma mansoni infections**

*Biomphalaria glabrata* snails infected with *S. mansoni* were obtained from F. Lewis (Biomedical Research Institute, Rockville, MD). Mice were infected percutaneously with 20, 40, 80 or 120 cercariae.

**BrdU labelling**

5-Bromo-2′-deoxyuridine (BrdU) (Sigma, B5002) was either injected I.P. daily (2mg per mouse per day) in 200µl PBS, or (for pulses over 3 days) given in the drinking water at 0.8mg/ml (protected from light and changed every 3 days). BrdU incorporation from the two methods was tested and found to be comparable.

**Histology**

Spleens were removed and cut into pieces. A central piece was suspended in embedding medium and rapidly frozen by placing on dry ice, followed by storage at -70°C. Tissue sections were cut at 5-6µm thicknesses and mounted onto slides. These were dried for 1 hour at room temperature and fixed in acetone for 10 minutes before storage at -20°C. Sections were thawed, washed in PBS for 5 minutes and blocked with PBS+0.5% BSA (Sigma) for a further 20 minutes. Subsequently, sections were stained with a combination of antibodies listed in table 2.2 for 2 hours before washing in PBS three times (for 20 minutes per wash). For biotinylated antibodies, a second staining step with streptavidin-conjugated Alexa Fluor 350 (Invitrogen) for 1 hour before washing in PBS as before. Cover slips were mounted using the mounting agent Mowiol (Clariant, Frankfurt, Germany) with the anti-fading agent 1,4-Diazabicyclooctane (DABCO) (Sigma, D27802) (at 25mg/ml). Slides were viewed on a fluorescent microscope (Olympus BX50) and images captured using Openlab software (Improvision, MA, USA).
**Medium**

Medium used throughout was Iscove’s Modified Dolbecco’s Medium (IMDM) supplemented with 5% FCS, 1% penicillin-streptomycin (Gibco, UK), 0.1% 2-mercaptoethanol (BDH laboratory supplies, Poole, UK). Hereafter, this shall be referred to simply as medium.

**Tissue preparation**

Spleens were removed and single cell suspensions produced by mashing between gauze squares into medium. Femurs were removed and flushed with 3ml medium (3ml per femur). Erythrocytes were lysed by suspension in red blood lysis buffer (Sigma) for 3 minutes. Cells were washed and re-suspended in medium, and counted by dilution in 0.1% trypan blue solution (Sigma).

**FACS staining**

2-5x10^6 cells, prepared as described above, were transferred to FACS tubes and washed three times by centrifugation, aspiration of supernatants, and re-suspension in FACS buffer (PBS supplemented with 4% FCS and 0.05% sodium azide). For experiments using the ‘Aqua Live/Dead kit’ (Invitrogen), cells were first washed in PBS and stained with 20µl of the kit (diluted in PBS) for 15 minutes at 4°C, followed by washing in FACS buffer. Cells were routinely blocked with 50µl of 1% FcR block (2.4G2 supernatant, produced in house), 2% mouse serum (Calbiochem, NS03L) and 2% rat serum (Invitrogen, 01-9601) for 20 minutes. Cells were washed and stained with 50µl of antibodies against surface markers, diluted in FACS buffer, for 30 minutes at 4°C before washing 3 times in FACS buffer (see table 2.1 for a complete list of antibodies used, and their respective dilutions etc). If biotinylated antibodies were used, a second staining step was performed where streptavidin-conjugated fluorochrome of the required colour was added, diluted in FACS buffer, for 30 minutes at 4°C, followed by a further 3 washes.

**Intracellular staining**

Where intracellular markers were of interest, cells were suspended overnight in the eBioscience FoxP3 fixation/permeabilisation kit (00-5521-00) at 4°C,
followed by washing 3 times with permeabilisation buffer (eBioscience, 00-8333-00) (hereafter referred to as 'PB'). Cells were then suspended in 50µl of antibodies against intracellular markers, diluted in PB, for 30 minutes at 4°C, followed by washing 3 times in PB and suspension in FACS buffer (see table 2.1 for a complete list of antibodies used).

**BrdU staining**

For BrdU staining, cells were blocked, surface stained, and fixed/permeabalised as described above before treatment with 1mg of DNase I (Sigma, DN25), dissolved in PB, for 1 hour at 37°C. Cells were washed in PB and incubated with 50µl of a 1:20 dilution of anti-BrdU-FITC (BD) for 30 minutes at room temperature. Finally, cells were washed in PB and resuspended in FACS buffer.

FACS samples were acquired on a FACS Canto or LSRII (BD) and analysed using FlowJo (Treestar, USA).

**Antibody ELISA**

To measure anti-NP antibodies, anti-FITC-OVA antibodies, anti-*Salmonella* antibodies and anti-SRBC antibodies, 96-well Nunc-maxisorp plates (Fischer Scientific) were coated in NP-BSA, FITC-OVA, or a crude sonicate of *Salmonella* or SRBC respectively (all at 10µg/ml, diluted in PBS) and incubated overnight at 4°C. Serum was separated from whole blood by centrifugation for 10 minutes at 13,000g in a micro-centrifuge (this was stored at -20°C until required). Plates were washed twice in PBS+0.1% tween, and blocked for 2 hours at room temperature with 100µl PBS+1% BSA per well. Plates were again washed twice with PBS+0.1% tween before serum was added, in duplicate, at a pre-determined top dilution, and 2-fold dilutions made down the plate (in PBS+1%BSA). A positive control ('standard') was also added to each plate. After a 2-hour incubation, plates were washed three times in PBS+0.1% tween and alkaline phosphotase-labelled detection antibodies (specific for IgM (1020-04), IgG1 (1070-04), IgG2b (1090-04), IgG2c (1079-04) or IgG3 (1100-04) – all from Southern Biotech) were added for 1 hour, diluted in PBS (at 1:2000, 1:2000, 1:2500, 1:1000 and 1:1000 respectively). Plates were again washed 3 times in
PBS+0.1% tween and 50µl of P-Nitrophenyl phosphate (Sigma, N1891) dissolved in Diethanolamide buffer at 1mg/ml. Plates were read at 405nm using Labsystems Multiskan plus and analysed using Graphpad Prism. Titres are the dilution at which samples reached half of the maximal optical density (OD) of the standard for each plate. Limits of detection are the top dilution for each sample.

**Statistics**
Throughout the study, students’ t-test, one-way or two-way ANOVA were used to determine statistical significance Where required, for one-way ANOVA, the Dunnet post-test or Bonferroni post-test were used. For two-way ANOVA the Bonferroni post-test was used. Tests were performed on Graphpad Prism software (Graphpad Software Inc., San Diego, CA). P values are represented as asterisks (where ‘*’ represents p=0.01 to p=0.05, ‘**’ represents p=0.001 to p=0.01, ‘***’ represents p<0.001 and ‘NS’ represents not significant (p>0.05)).

**Contributions by others**
*Schistosoma mansoni* infections and BrdU labelling of mice presented in figure 6.2 were performed by Alex Phythian-Adams and Stefano Casserta. Dissection of animals and tissue preparation was a combined effort.

*Salmonella* infections and dissection of mice shown in figure 4.3a and 4.4a were performed by Sheila Brown. Histology shown in figure 4.4a was also performed by Sheila Brown.

Data shown in figure 4.11 was in collaboration with Marta Trueb.

DNP-OVA used in this project was conjugated by Sheila Brown.
Table 2.1. Antibodies used for flow cytometry.
## Table 2.2. Antibodies used for immunohistology.

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Chapter 3 – Plasma cell identification, measurement of lifespan and long-lived plasma cell accumulation in the bone marrow of naïve mice

Introduction

Plasma cells are focused on synthesising and secreting antibody, and although originating from B cells, lose many of the functions associated with their precursors. This results from reduced expression of many receptors and signalling molecules expressed on the cell surface of B cells. A secondary effect of this is that there are fewer markers with which to identify the cells using flow cytometry. CD138 is one marker that is up regulated on the surface of plasma cells and is frequently used for their identification.

Here we observe that CD138 is not expressed on all plasma cells; during *Salmonella* infection we show that many antibody-secreting cells express only low levels of CD138, comparable to that of B cells. We demonstrate that labelling with intracellular Ig is a more accurate method of identifying antibody-secreting plasma cells.

The lifespan of plasma cells is key to long-lasting humoral immunity. The constant production of antibody undoubtedly puts plasma cells under high levels of stress, resulting in the rapid death of many such cells. However, some plasma cells are able to persist for months or even years without cellular division. Although it is unknown why some plasma cells are able to survive while others are not, it is clear that the provision of certain survival factors, such as APRIL and IL-6, is vital. The bone marrow is known to be a major site where these factors are enriched under homeostatic conditions.

Plasma cell responses occur in two phases following infection or immunisation. In the first few days, extrafollicular plasma cells arise; these plasma cells secrete antibody of low affinity and are thought to survive for only a few days. Secondly, in TD responses, plasma cells are generated during the germinal centre reaction. These plasma cells arise from B cell clones that have undergone
affinity maturation through hypermutation and selection, and they secrete antibody of high affinity. These plasma cells migrate to the bone marrow, where they survive for long periods without division or re-stimulation.

We document the accumulation of a population of long-lived plasma cells in the bone marrow of naïve mice over time. This population was found to contribute to serum antibody levels, and required T cell help, as well as both BCR and TLR signals for its generation.

Results

Identification of plasma cells through intracellular immunoglobulin
CD138 is the prototypical plasma cell marker. We immunised C57Bl/6 mice with alum precipitated NP-KLH with killed Bordetella pertussis I.P. and looked at the peak of the plasma cell response in the spleen (at day 8). Some mice were boosted 4-weeks after the primary response with soluble NP-KLH I.V. By day 8 following primary immunisation, a substantial population of CD19int, CD138hi cells were induced in the spleen (2.25% ±0.34 of total cells) (fig 3.1a). However, this was far smaller than the response seen at day 5 following secondary immunisation, where CD138hi cells accounted for 12.51% ±1.16 of total spleen cells (fig 3.1a). Mice primed at the same time as boosted mice, but not receiving secondary immunisation contained low frequencies of plasma cells in the spleen (0.60% ±0.11 of total spleen cells, data not shown). Due to greater populations, we chose to characterise splenic plasma cell responses following secondary immunisation with NP-KLH. Co-staining for both surface and intracellular immunoglobulin showed that CD138hi cells express low levels of surface immunoglobulin (BCR) and high levels of cytoplasmic immunoglobulin (secreted antibody) when compared to CD138lo, CD19hi B cells; thus confirming their identity as antibody-secreting plasma cells (fig 3.1b). Low staining of CD19hi B cells when stained for intracellular Igκ likely represents Igκ on the surface of B cells (i.e. the BCR). A population of BCRlo CD19hi cells could also be seen (fig 3.1b); this is likely to represent lambda light chain expressing B
Chapter 3 – Accumulation of bone marrow plasma cells

cells (around 10 percent of murine B cells), as anti-kappa light chain (Igκ) was used for detection.

Plasma cells were also identified in the bone marrow, however, populations were not found to expand dramatically at day 5 following secondary immunisation with NP-KLH (fig 3.1c). This will be looked at in more detail in chapter 5.

Interestingly, in certain situations, such as infection with Salmonella, not all intracellular Ig-containing cells were found to express high levels of CD138. Figure 3.1d shows that in a time course of infection, a population of CD138^{hi}, CD19^{int} cells was generated at day 8 (fig 3.1d top row). However, intracellular Ig staining revealed a far larger population (fig 3.1d middle row). Further disparity between the populations was revealed by the two stains at later time points, when over 50 percent of plasma cells did not express high levels of CD138. A comparison of the two stains shows that CD138 staining does not reveal all antibody secreting cells (fig 3.1d bottom row), and in fact, many of the cells revealed by CD138 staining in naïve mice, and at later points in the time course, did not express high levels of intracellular Ig.

To ensure that we analysed total plasma cell populations in all experiments, intracellular Ig, in conjunction with CD138, was subsequently used to identify plasma cells.

**Kinetics of plasma cell maturation in the secondary response to NP-KLH**

In figure 3.1, we developed a more sensitive technique for identifying plasma cells. In order to further our understanding of phenotypic changes to plasma cell populations as they matured, we examined the phenotype of plasma cells through a time course following secondary NP-KLH immunisation.

Plasma cells in the spleens of naïve mice were found to express high levels of intracellular Ig and CD138. They retained high levels of MHC II, but expressed only intermediate levels of CD19. Interestingly, their forward and side scatter was relatively low, suggesting an immature state. This was similar in the bone
Chapter 3 – Accumulation of bone marrow plasma cells

marrow, as was their expression of CD19 and intracellular Ig. However, bone marrow plasma cells expressed higher CD138 and lower MHC II and had higher forward and side scatter than their splenic counterparts (fig 3.2).

Plasma cells arising in the spleen at day 5 of the secondary response to NP-KLH retained many of the surface proteins expressed on B cells (fig 3.2). CD19 and MHC II expression were found to be similar to those of splenic B cells (with CD19 being higher than plasma cells found in the naïve spleen), however CD138 was up regulated, as was intracellular Ig. No increase in forward or side scatter was observed.

At day 9, splenic plasma cells had adopted a more mature phenotype, with low CD19 and MHC II expression and high forward and side scatter. Intracellular Ig was further increased while CD138 expression was decreased. At day 45, MHC II and CD19 were further decreased, while intracellular Ig, forward and side scatter were maintained. Interestingly, CD138 levels had recovered to levels seen at day 4 and in naïve mice.

Plasma cells in the bone marrow did not change as rapidly as those in the spleen. This is in agreement with data from figure 3.1, which shows that frequencies of plasma cells here were not overtly affected at day 4 of secondary immunisation. However, it can be seen that MHC II expression and CD19 were rapidly and progressively down regulated throughout the time course. Intracellular Ig production was similar to that of splenic plasma cells, but peaked at higher levels at days 9 and 45.

**Measurement of plasma cell turnover using BrdU labelling**

In figure 3.2, we saw that plasma cells down regulate MHC II as they mature. Plasma cells are known to be short or long-lived, but few phenotypic differences have been reported which can be used to distinguish between these two plasma cell populations. We next looked at whether mature plasma cells are long-lived cells with slow turnover, or short-lived with rapid turnover. We assessed the turnover of plasma cells through BrdU labelling. Mice were continuously fed BrdU in their drinking water for 10-day periods. Cells that divided in this period
would incorporate BrdU into their DNA, and this was detected through staining with anti-BrdU antibodies. Dividing plasma cells were termed ‘short-lived’ plasma cells; those that did not divide in a 10-day period were termed ‘long-lived’ plasma cells. Following BrdU labelling of naïve 6-week old C57Bl/6 mice, turnover of total (intracellular Igκ+) plasma cell populations was assessed under homeostatic conditions. It was found that 42.24% (±2.57) of plasma cells in the spleen had divided in this time, and slightly higher percentages (53.86% (±2.00)) in the bone marrow (fig 3.3a).

We next examined whether mature, MHC II<sub>lo</sub> plasma cells were dividing at a different rate to immature, MHC II<sub>hi</sub> plasma cells. It was found that the spleen contained a larger fraction of MHC II<sub>lo</sub> plasma cells than the bone marrow (47.06% (±4.07) and 41.54% (±2.73) respectively) (fig 3.3b). In both spleen and bone marrow, we observed a statistically significant difference in turnover of MHC II<sub>hi</sub> and MHC II<sub>lo</sub> plasma cells, with 28.40% (±3.69) and 38.64% (±2.60) of mature MHC II<sub>lo</sub> plasma cells turning over in the spleen and bone marrow respectively. In contrast to this, 54.12% (±3.47) and 65.82% (±1.71) of immature, MHC II<sub>hi</sub> plasma cells turned over during a 10-day period in the spleen and bone marrow respectively (fig 3.3b and c).

**(Plasma cells accumulate in the bone marrow over time and contribute to serum antibody levels**

Although we had developed a model to distinguish short and long-lived plasma cells, our data was not complimentary to that published in the literature. The frequency of bone marrow plasma cells that we had observed in 6-week old C57Bl/6 mice was 0.081% (±0.013) (fig 3.1c). This was towards the lower end of those published in the literature<sup>38,247</sup>. Likewise, we had observed that the turnover of plasma cells was more rapid in the bone marrow than the spleen (fig 3.3a), contradictory to reports suggesting that long-lived plasma cells primarily reside in the bone marrow<sup>193</sup>. We therefore hypothesised that long-lived plasma cells may take time to accumulate in the bone marrow and therefore were not present in high numbers in 6-week old mice. This could be due to increased support for long-lived plasma cells in the bone marrow of older
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mice (through increased availability of survival factors), or the fact that only
certain plasma cells (generated during germinal centre reactions) are able to
become long-lived and therefore are not present in high numbers in young mice
that have undergone few such responses.

We first tested whether plasma cells increased over time in the spleen and bone
marrow. C57Bl/6 mice from age-matched litters were aged to 5, 15 and 30-
weeks after birth before examination of the spleen and bone marrow for plasma
cell frequency and number. As shown in figure 3.4a, frequencies of total (Igκ),
IgM and IgG plasma cells increased in mice as they aged. In the spleen,
frequencies of both total and IgG plasma cells increased significantly between
the age of 5 and 30-weeks. In the bone marrow the increase was greater, with
total, IgM and IgG plasma cells increasing significantly by 30-weeks of age. Igκ
plasma cells in these mice reached frequencies 0.76% (± 0.08) of total bone
marrow cells.

When absolute numbers of plasma cells were calculated, 30 week old mice had
not accumulated significantly higher numbers of plasma cells in the spleen over
this period. However, in the bone marrow numbers of total, IgM and IgG plasma
cells had increased significantly compared to 5 week old mice (fig 3.4b). This
10-fold increase occurred predominantly between the 15 and 30-week time
points.

To determine whether the accumulation of bone marrow plasma cells
significantly affected serum antibody levels, the serum of 5, 15 and 30 week old
naïve C57Bl/6 mice (from figure 3.4) was evaluated for total IgM, IgA, IgG1,
IgG2b, IgG2c and IgG3. Many of the antibody classes increased significantly over
time. IgM increased by week 30 and IgA by week 15. IgG2b, IgG2c and IgG3 had
significantly increased by week 15 and continued to increase until week 30.
Interestingly IgG1 did not appear to increase throughout the time course (fig
3.5).
Most plasma cells accumulating in the bone marrow of naïve C57Bl/6 mice are long-lived

As stated above, we had observed that the turnover of plasma cells in the bone marrow of young mice was surprisingly high (fig 3.3), as this is cited as the major organ in which long-lived plasma cells reside\textsuperscript{189,193,306}. To determine whether the accumulation of plasma cells seen in the bone marrow (fig 3.4) was due to an increase in short or long-lived plasma cells, 5 or 30-week old C57Bl/6 mice were given BrdU in the drinking water for a 10-day period, and plasma cell populations in the spleen and bone marrow were assessed for BrdU incorporation. Figure 3.6a shows example plasma cell gating and representative FACS plots of BrdU incorporation and MHC II expression on total plasma cells in the spleen and bone marrow of 5 and 30-week old mice. From these plots, it is clear that frequencies of plasma cells increased both in the spleen and bone marrow between the two time points (fig 3.6a top row). In fact, splenic plasma cells increased 2.4 fold, whereas bone marrow plasma cells increased 8-fold. 10-day BrdU pulses given to 5 or 30-week old mice revealed that in both the spleen and bone marrow, the proportion of plasma cells dividing during this time was lower in 30-week old mice. Turnover was higher in the bone marrow than the spleen in 5-week old mice, but this was reversed in 30-week old mice (fig 3.6a middle row). While the proportion of plasma cells expressing MHC II in the spleen did not change between the two time points, a higher proportion of plasma cells were MHC II\textsuperscript{lo} in the bone marrow of 30-week old mice compared to 5-week old mice (fig 3.6a bottom row).

In this experiment, splenic Igκ and IgG plasma cells had significantly increased in terms of frequency and number between the two time points (although IgM plasma cell numbers were not significantly different, the trend was also towards an increase) (fig 3.6b and c). Bone marrow plasma cells had increased as seen in earlier experiments, and both frequencies and numbers of Igκ, IgM and IgG plasma cells were significantly increased in 30-week old mice compared to 5-week-old mice (fig 3.6b and c).
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The BrdU incorporation of these plasma cells was assessed. In the spleen, the turnover of Igκ plasma cells in 30-week old mice was lower than that of 5-week old mice (42.24% (±2.57) in 5-week old mice, 29.86% (±3.71) in 30-week old mice). Little difference in the turnover of IgM or IgG plasma cells was observed between the two groups (fig 3.6d).

In the bone marrow, the turnover of total, IgM and IgG plasma cells was significantly lower in 30-week old mice compared to 5-week old mice (for total: 53.86% (±2.00) in 5-week old mice, 10.71% (±1.11) in 30-week old mice) (fig 3.6d).

When numbers of either BrdU+ or BrdU- plasma cells were calculated, there was no significant increase in total, IgM or IgG plasma cells in the spleen for either dividing or non-dividing plasma cells. In the bone marrow, no significant increase in dividing plasma cells was seen, but there was a significant increase in non-dividing plasma cells (especially total and IgG) (fig 3.6e).

Similarly, when plasma cells were examined for MHC II expression, there was no significant difference in the proportion of MHC IIlo plasma cells in the spleens of 5 or 30-week old mice. In the bone marrow however, there was a significant increase in the proportion of mature MHC IIlo plasma cells (fig 3.6f). Figure 3.6g shows that there was a small but statistically significant increase in MHC IIhi plasma cells in both organs (with the exception of IgM plasma cells, which did not increase significantly). While this was similar for MHC IIlo plasma cells in the spleen, the bone marrow compartment showed a large and highly significant increase in total and IgG MHC IIlo plasma cells (and a smaller increase in IgM plasma cells).

Niche providing cells in young and aged mice

It has so far been shown that mature, long-lived plasma cells accumulate in the bone marrow of mice over time. We now investigated whether cells known to support plasma cell survival also increased in the bone marrow of mice aged. Frequencies of total APRIL-producing cells in either the spleen or bone marrow of 5 and 30-week old mice did not differ significantly (data not shown).
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However, a population of cells which produced 10-fold higher levels of APRIL than other cell types (including the various ‘niche-providing cells’ cited in the literature) were identified and are hereafter referred to as APRIL\textsuperscript{hi} cells. These cells, along with CD11c\textsuperscript{+} dendritic cells; CD41\textsuperscript{+} megakaryocytes; CD11b\textsuperscript{+} Gr1\textsuperscript{int} F4/80\textsuperscript{+} monocytes and macrophages; CD11b\textsuperscript{int} Gr1\textsuperscript{int} F4/80\textsuperscript{+} eosinophils were quantified.

In the spleen, only monocytes/macrophages increased significantly in terms of numbers (and not frequency). In the bone marrow, it was found that APRIL\textsuperscript{hi} cells and dendritic cells increased significantly in terms of both frequency and number (fig 3.7).

**Long-lived plasma cell accumulation in the bone marrow of naïve mice requires signals through the BCR and T cell interactions through MHC II and CD40**

To determine whether TLR signalling, T cell-B cell interactions or antigen recognition through the BCR were important for the accumulation of long-lived bone marrow plasma cells, we aged naïve wild type, MyD88/TRIF\textsuperscript{-/-}, A\textbeta\textsuperscript{-/-}, CD40\textsuperscript{-/-} and MD4 mice, in which all B cells express a BCR that recognises HEL.

Firstly, we assessed splenic and bone marrow plasma cells in wild type and knockout mice aged 5-7 weeks. It was found that at this age, most strains had approximately comparable numbers of plasma cells in both organs, although MD4 mice had fewer plasma cells in the spleen (fig 3.8a). The frequency of plasma cells was higher in A\textbeta\textsuperscript{-/-} spleens due to a reduction in certain other cell types; absolute numbers of plasma cells in the spleens of these mice were comparable to those of wild type mice.

Next, wild type and genetically modified mice were aged to 30 weeks, before assessment of spleen and bone marrow for accumulation of long-lived plasma cells. All strains of mice had similar frequencies and numbers of plasma cells in the spleen. Wild type mice had accumulated plasma cells in the bone marrow, while A\textbeta\textsuperscript{-/-}, CD40\textsuperscript{-/-} and MD4 mice had not. MyD88/TRIF\textsuperscript{-/-} mice had significantly greater variance than wild type mice (p=0.0016) and therefore any difference was not significant. A\textbeta\textsuperscript{-/-}, CD40\textsuperscript{-/-} and MD4 mice all had significantly
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lower frequencies and numbers of bone marrow plasma cells compared to wild type mice.

To determine if reduced numbers of plasma cells in the bone marrow of these mice was due to a lack of accumulation of long-lived plasma cells, we next gave aged genetically modified mice 10-day BrdU pulses and assessed turnover of splenic and bone marrow plasma cells. Figure 3.9a shows that in the spleen, only CD40−/− mice had lower turnover than wild type mice, and differences were small. In the bone marrow however, wild type mice showed significantly lower turnover than the genetically modified strains (except for MD4 mice) suggesting that they have more long-lived plasma cells.

When numbers of BrdU+, short-lived plasma cells were calculated, there was little difference between any of the strains in either spleen or bone marrow (only Aβ+/− had significantly fewer short-lived plasma cells in the bone marrow) (fig 3.9b). Interestingly in the spleen, MyD88/TRIF−/− mice had significantly fewer long-lived plasma cells than wild type mice, whereas in the bone marrow they were the only group to have no significant difference in long-lived plasma cells. All other strains had significantly fewer long-lived plasma cells than wild type mice. Taken together, these data show that many of these strains fail to accumulate long-lived plasma cells in the bone marrow over time.

To investigate whether knockout mice do not accumulate bone marrow plasma cells due to reduced ‘niche-providing-cells’, we stained for markers revealing a number of cell populations known to support plasma cell survival. APRILhl cells were significantly decreased in the spleens of Aβ+/−, and also in the bone marrow of both Aβ+/− and MyD88/TRIF−/− mice (fig 3.10). Only MyD88/TRIF−/− had fewer dendritic cells in the bone marrow compartment. There were no significant differences in numbers of eosinophils or megakaryocytes in the spleen or bone marrow of any of the strains used.
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Figure 3.1

A

Naive  Primary D8  Secondary D5

CD138

CD19

B

Intracellular Ig  Surface Ig

% Max

CD19+, CD138-  CD19int, CD138+

C

Naive  Immunised

Spleen

CD138

Bone Marrow

Int IgG

Figure 3.1 continues overleaf
Figure 3.1 Plasma cells in the spleen and bone marrow can be identified through intracellular antibody and CD138.

(A) FACS profiles showing that a CD138\textsuperscript{hi} population of CD19\textsuperscript{int} cells expands in the spleen by day 8 following primary immunisation with alum-NP-KLH and by day 5 following secondary immunisation with soluble NP-KLH. (B) Splenic CD19\textsuperscript{hi}, CD138\textsuperscript{lo} B cells (blue) and CD19\textsuperscript{int}, CD138\textsuperscript{hi} plasma cells (red) from NP-KLH boosted mice were co-stained for intracellular (left) and surface (right) Ig (using anti-mouse heavy and light chain). (C) Plasma cells in the spleen (top) and bone marrow (bottom) of naïve (left) and day 5 NP-KLH boosted mice (right) identified through intracellular IgG and CD138 expression. (D) Splenic plasma cells from Salmonella infected mice through a time course of infection, identified through either CD138/CD19 staining (top) or CD138/Intracellular Ig\kappa staining (middle), and an overlay whereby cells identified through CD138/CD19 staining are shown in red. FACS plots representative of 4-5 mice. Results representative of at least 5 independent experiments.
Figure 3.2. Plasma cells mature following secondary immunisation with NP-KLH. (A) Expression of intracellular IgG, CD138, MHC II, CD19 as well as forward and side scatter of IgG plasma cells from the spleen (solid line) and bone marrow (dashed line) of mice 0, 5, 9 and 45 days following secondary immunisation with NP-KLH. Points on graph=mean of 4 mice, error bars=standard deviation. Results are representative of at least 3 independent experiments.
Figure 3.3

Figure 3.3 continues overleaf
Figure 3.3 cont.

Figure 3.3. Turnover of plasma cells in naïve C57Bl/6 mice given BrdU for 10-days. (A) Turnover of total (Igκ) plasma cell populations in spleen and bone marrow of naïve C57Bl/6 mice. (B) Percentage of plasma cells that are MHC II<sup>hi</sup> or MHC II<sup>lo</sup> in the spleens and bone marrow of mice from (A) (top row), and corresponding BrdU incorporation of MHC II<sup>hi</sup> or MHC II<sup>lo</sup> plasma cells (bottom row as indicated). FACS plots are representative of 5 mice. (C) Summary graphs of data from (B) showing a significant difference in the turnover of MHC II<sup>hi</sup> or MHC II<sup>lo</sup> plasma cells in both spleen and bone marrow. Points on graph=1 mouse, bar=mean. Results are representative of at least 3 independent experiments.
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Figure 3.4

A

Spleen

Bone Marrow

% Spleen

% Bone Marrow

Age (Weeks)

Age (Weeks)

Igκ

IgM

IgG

Figure 3.4 continues overleaf
Figure 3.4 cont.

Figure 3.4. Plasma cells accumulate in the bone marrow of naïve mice over time. (A) Frequency of total (Igκ), IgM or IgG plasma cells in the spleen (left) or bone marrow (right) of naïve C57Bl/6 mice aged to 5, 15 or 30 weeks. Cells were identified by staining with intracellular antibody against the relevant class and subsequent flow cytometry. (B) Numbers of total, IgM or IgG plasma cells per spleen (left) or per 100mg femur (right) of mice from (A). Points on graph=1 mouse, bar=mean. Results representative of 2 independent experiments.
Figure 3.5. Total antibody in the serum of naïve C57Bl/6 mice aged 5, 15 or 30 weeks.

Total IgM, A, G1, G2b, G2c and G3 titres in serum taken from naïve C57Bl/6 mice aged to 5 (square), 15 (triangle) or 30 (inverted triangle) weeks. Titres were calculated by the dilution at which samples reached half of the maximal optical density of the standard for each plate. Standard was pooled sera from three 15-week old naïve C57Bl/6 mice. Points on graph=1 mouse, bars=mean. Results representative of 2 independent experiments.
Figure 3.6

A

**Spleen**

- 5 Weeks
- 30 Weeks

**Bone Marrow**

- 5 Weeks
- 30 Weeks

B

**Plasma Cell Frequencies**

- Spleen
- Bone Marrow

C

**Plasma Cell Numbers**

Figure 3.6 continues overleaf
D

5 weeks
30 weeks

Percentage BrdU+

Spleen

Bone Marrow

% PC BrdU+

N.S.

N.S.

N.S.

N.S.

***

**

***

N.S.

N.S.

N.S.

N.S.

Number BrdU+/

BrdU+

BrdU−

Number per Spleen

Number per 100mg Femur

Number per 100mg Bone Marrow

Igκ

IgM

IgG

N.S.

N.S.

N.S.

N.S.

***

**

***

Figure 3.6 continues overleaf
Chapter 3 – Accumulation of bone marrow plasma cells

Figure 3.6 cont.

(A) FACS plots of frequencies of total (Igκ) plasma cells (top), BrdU incorporation (middle) and MHC II expression (bottom) on plasma cells from spleen (left) and bone marrow (right) of 5 and 30-week old C57Bl/6 mice. (B) Frequency and (C) numbers of total, IgM and IgG plasma cells in spleens and bone marrow of mice in (A). (D) Percentage BrdU incorporation of total, IgM and IgG plasma cells in spleens and bone marrow of mice in (A) following a 10-day BrdU pulse. (E) Numbers of BrdU+ and BrdU- plasma cells (total, IgM and IgG) per spleen or 100mg femur of mice in (A). (F) Percentage of plasma cells that are MHC IIlo in mice from (A). (G) Numbers of MHC IIhi and MHC IIlo plasma cells (total, IgM or IgG) per spleen or 100mg femur of mice in (A). FACS plots are representative of 5 mice, bars on graph=mean of 5 mice, error bars=standard deviation. Results are representative of 2 independent experiments.
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**Figure 3.7**

**Spleen Frequency**
![Spleen Frequency Graph]

**Bone Marrow Frequency**
![Bone Marrow Frequency Graph]

**Spleen Number**
![Spleen Number Graph]

**Bone Marrow Number**
![Bone Marrow Number Graph]

Figure 3.7. Frequency and numbers of ‘niche providing cells types’ in the spleen and bone marrow of naïve 5 or 30-week old C57Bl/6 mice. APRIL<sup>hi</sup> cells identified through high levels of intracellular APRIL protein. Dendritic cells were CD11c<sup>hi</sup>, Megakaryocytes were CD41<sup>hi</sup>, Monocytes/Macrophages were CD11b<sup>hi</sup>, F4/80<sup>hi</sup>, eosinophils were CD11b<sup>int</sup>, F4/80<sup>hi</sup>. Each bar is the average value seen in 5 mice. Bars=mean of 5 mice, error bars=standard deviation. Results are representative of 2 independent experiments.
Fig 3.8. Plasma cell accumulation in knockout strains of mice. (A) Total (lgx) plasma cell frequencies (top) and numbers (bottom) in the spleen (left) and bone marrow (right) of 5-7 week old wild type (square), MyD88/TRIF−/− (triangle), Aβ−/− (inverted triangle), CD40−/− (diamond) and MD4 (circle) mice. (B) As for (A) but with mice aged 30-32 weeks. Points on graph=1 mouse, bars=means. Graphs show results from a single experiment.
Figure 3.9. BrdU incorporation by plasma cells in 30-week old wild type and knockout mice during a 10-day pulse. (A) Percentage of total (lgx) plasma cells incorporating BrdU in the spleen (left) or bone marrow (right) of 30-week old wild type (square), MyD88/TRIF⁻/⁻ (triangle), Aβ⁻/⁻ (inverted triangle), CD40⁻/⁻ (diamond) or MD4 (circle) mice. (B) Number of BrdU⁺ (top) or BrdU⁻ (bottom) plasma cells per spleen or per 100mg femur of mice from (A). Points on graph=1 mouse, bars=means. Graphs show results from a single experiment.
Figure 3.10. Failure of plasma cell accumulation in the bone marrow of knockout mice is unlikely to be caused by an absence of APRIL producing cells. Numbers of ‘niche providing cells’ in the spleen (left) and bone marrow (right) of aged wild type (square), MyD88/TRIF⁻/⁻ (triangle), Aβ⁻⁻ (inverted triangle), CD40⁻⁻ (diamond) and MD4 (circle) mice. APRIL= APRIL<sup>hi</sup> cells, DC= CD11c<sup>hi</sup> cells, Megakaryocytes= CD41<sup>hi</sup> cells, Eosinophils= CD11b<sup>int</sup>, Gr1<sup>int</sup>, F4/80<sup>hi</sup> cells. Points on graph=1 mouse, bars=means. Graphs show results from a single experiment.
Discussion

Plasma cells are end stage B cells that secrete large amounts of antibody and do not divide. Their lifespan can be short, around 3 days, or long, a half-life of months to years\(^{193}\). Long-lived plasma cells are a key part of humoral immunity and are the mechanism by which circulating antibody is maintained without antigen re-exposure or retention\(^{189,307}\).

Here we have developed methods for identifying plasma cells, and the differentiation of short-lived and long-lived plasma cells using BrdU labelling. We document the accumulation of long-lived plasma cells in the bone marrow over time in naïve mice and determine the likely requirements for this to occur.

Identifying plasma cells using intracellular antibody

We detected large populations of CD138\(^{hi}\) cells in the spleen 5 days after secondary immunisation with NP-KLH (fig 3.1a). These cells had high levels of intracellular Ig (fig 3.1b). However, by day 10, CD138 was expressed only at low levels on intracellular Ig\(^{hi}\) cells (fig 3.2). Similarly, at early time points during *Salmonella* infection CD138\(^{hi}\) cells could be detected, many of which were intracellular Ig\(^{hi}\) (fig 3.1d). This was not the case at later time points, where intracellular Ig\(^{hi}\) cells did not express high levels of CD138. This is a reported phenomenon in other infection models such as *Trypanosoma cruzi*, where CD138\(^{lo}\) plasma cells are reported in both the peritoneum and spleen following infection\(^{308,309}\). Many plasma cells in humans do not express CD138, however in contrast to the data shown here (where early plasma cells express the marker, while more mature plasma cells do not), CD138\(^{hi}\) plasma cells in humans express a more mature phenotype than CD138\(^{lo}\) plasma cells\(^{310}\). Intriguingly, plasma cells seen 45 days after secondary immunisation with NP-KLH once again expressed high levels of CD138, suggesting that both early plasmablasts and highly mature plasma cells express the marker, but an intermediate stage of low CD138 expression occurs on plasma cells in mice.

Plasma cells are known to downregulate many other surface markers as they mature (such as MHC II, B220, CD19 and the BCR\(^{245}\)), and this was observed
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Here. These markers, in contrast to CD138, remained low at day 45 after secondary NP-KLH immunisation. CD138 is predominantly used as a phenotypic marker for plasma cells, and its function is not clear, although it may play a role in cellular adhesion and trafficking to the bone marrow. Further experiments would be required to determine why some plasma cells do not express high levels of CD138, and whether this affects their entry to the bone marrow compartment.

Based on the markers analysed in this experiment, we could not detect any phenotypic difference between splenic and bone marrow plasma cells in 6-week old naïve mice (fig 3.2). However, immunisation appeared to affect these populations in different ways. The rapid generation of splenic plasmablasts expressing high levels of CD19, MHC II and CD138 has been previously reported. These cells subsequently matured, expressing lower amounts of MHC II and CD19 while increasing the amount of antibody they produced by day 9. It is likely that these cells no longer divide, characteristic of mature plasma cells.

In the bone marrow changes were not so rapid; reflecting the fact that plasma cells are produced in secondary lymphoid organs and must migrate there. There was only a modest increase in plasma cell frequency in the bone marrow after secondary immunisation with NP-KLH (fig 3.1c) despite the dramatic increases seen in the spleen. This supports the idea that the bone marrow plasma cell pool is of limited size and, like many memory pools, the influx of new cells comes at the expense of older, pre-established cells. Plasma cells entering the bone marrow at day 5 expressed intermediate levels of MHC II, CD19 and relatively low levels of intracellular Ig (fig 3.2), but continued to mature thereafter. In fact, bone marrow plasma cells were found to produce more antibody than their splenic counterparts. This is likely due to the increased expansion of intracellular machinery for producing and secreting antibody such as the endoplasmic reticulum and Golgi apparatus. The expansion of these are controlled by the transcription factor XBP-1, downstream of the master transcription factor for plasma cells, BLIMP-1. Why bone marrow
plasma cells have an enhanced ability to secrete antibody is unknown, but may be mediated by signals enriched in the bone marrow\textsuperscript{166}.

**Turnover of plasma cells using BrdU labelling**

It was surprising that all bone marrow plasma cells had an altered phenotype 10 days after secondary immunisation with NP-KLH (fig 3.2). This population is reported to be a pool of long-lived, non-dividing ‘memory’ plasma cells, maintaining serum antibody levels to a range of different specificities (i.e. from previous immune responses)\textsuperscript{78,193,194,307}. It would be expected that only newly generated plasma cells coming into the bone marrow would express immature phenotypes, whereas pre-established plasma cells, generated in prior immune responses, would maintain mature phenotypes. BrdU labelling experiments showed that bone marrow plasma cells turned over at a similar rate to those in the spleen (tending towards higher turnover in the bone marrow) (fig 3.3a). Similarly, the fact that the bone marrow also contained a higher proportion of MHC II\textsuperscript{hi} plasma cells than the spleen suggested that the bone marrow of these 6 to 8-week old naïve mice did not contain high numbers of long-lived plasma cells.

This data confirmed that MHC II\textsuperscript{hi} plasma cells were mainly immature, dividing plasmablasts, while MHC II\textsuperscript{lo} plasma cells were mainly non-dividing plasma cells (fig 3.3b and c), however some MHC II\textsuperscript{lo} plasma cells were seen to divide in both spleen and bone marrow. Pelletier et al have shown that plasma cells are capable antigen presenting cells\textsuperscript{267}. It is possible that MHC II is actively maintained on the surface of some long-lived, non-dividing plasma cells, or can be up regulated through unknown signals. CD4 memory T cells also reside in the bone marrow, where (similar to long-lived plasma cells) they occupy survival niches, and slow their rate of division and gene expression, allowing for long-term survival\textsuperscript{312,313}. It is an attractive proposition that bone marrow plasma cells play a role in the activation of resting CD4 memory T cells, mediated by periodic increases in (or maintenance of) MHC II. These ideas will be explored further in Chapter 6.
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Plasma cells accumulate in the bone marrow over time

Through the course of our experiments we observed that young naïve (5-8 week old) mice had fewer plasma cells in the bone marrow than older (15-30 week old) naïve mice. To quantify this, we performed experiments looking at frequencies and numbers of plasma cells through a 30-week time course (fig 3.4a and b). Indeed, mice were found to accumulate significantly higher frequencies and numbers of plasma cells in the bone marrow over time. These plasma cells were of a mature, MHC II\textsuperscript{lo} phenotype and turned over slowly (fig 3.6d). This was not an inherent change in the lifespan of plasma cells generated in these mice as turnover of splenic plasma cells was not significantly altered (fig 3.6d).

We found that both IgM and IgG plasma cells accumulated in the bone marrow, with IgM plasma cells, surprisingly, increasing to higher frequencies and numbers than IgG plasma cells (fig 3.4a and b). IgM plasma cells turned over at a similar rate to IgG plasma cells (fig 3.6d), contrary to the idea that only class-switched plasma cells can become long-lived\textsuperscript{69}. In fact, long-lived IgM plasma cells were found in greater numbers than IgG plasma cells in both the bone marrow and spleens of these mice. This does not necessarily contradict reports claiming that the majority of long-lived plasma cells are class switched following a TD immune response\textsuperscript{248}. IgG plasma cells are somewhat under-represented in the naïve mouse’s repertoire; the majority of plasma cells generated in the spleen are IgM (fig 3.4b and 3.6c). This may mean that, while class switched plasma cells are more proficient at accessing survival niches, by simple ‘weight of numbers’ IgM plasma cells come to occupy the majority of niches in naïve mice. It is certainly true that IgM plasma cells are not impaired in their ability to migrate to the bone marrow compartment, as they outnumber class switched plasma cells here as well (fig 3.4b and 3.6c). It is known that long-lived IgM plasma cells are generated to bacterial infection with \textit{Ehrlichia muris}\textsuperscript{314}, parasitic infection with \textit{Plasmodium chabaudi chabaudi}\textsuperscript{315} and a polysaccharide antigen from \textit{Streptococcus pneumoniae}\textsuperscript{316}. 


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Despite accumulation of long-lived IgM plasma cells in the bone marrow, many of these were MHC II^hi. This is similar to data from figure 3.3, where many non-dividing plasma cells in the spleen (and to a lesser extent the bone marrow) of 6 to 8-week old naïve mice expressed MHC II. Likewise, Racine et al showed that many IgM plasma cells accumulating in the bone marrow following infection with *E. muris* continued to express B cell markers^{314}. In that paper this was interpreted as an accumulation of short-lived plasmablasts, however our data suggests that these MHC II^hi IgM secreting cells are predominantly long-lived. This would make sense, as their data show that IgM cells persisted for long periods after *E. muris* was cleared by antibiotic treatment; it would therefore be surprising if short-lived plasmablasts continued to be generated.

That total antibody in the serum of the mice we analysed increased with age supports the idea that long-lived bone marrow plasma cells are responsible for maintaining serum levels to previously encountered antigens (fig 3.5)^{189,193,317}. Why different antibody classes increased with different kinetics is unknown.

The plasma cells producing this antibody are likely generated in response to environmental antigens and gut microflora. The elevated levels of some classes rather than others may simply reflect the type of immune response generated to encountered antigens. It would be interesting to test whether antibody levels, and bone marrow plasma cell numbers increased in germ-free mice, which would encounter few antigens. It is known that these mice have reduced circulating IgG, but similar levels of IgM^{72,318}.

**Aged mice have increased levels of plasma cell survival factors in the bone marrow**

It is thought that mainly post-germinal centre, class-switched plasma cells migrate to the bone marrow where they persist for long periods, as mice deficient in germinal centres generate reduced numbers of long-lived bone marrow plasma cells following immunisation^{319}. Also, plasma cells that persist for long periods require the support of survival factors such as APRIL, IL-6, IL-5 and others that are secreted from a number of sources. Chu et al observed that in mice with a reduced number of eosinophils, bone marrow plasma cells were
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reduced\textsuperscript{151}, while Winter et al used mice with an absence of megakaryocyte populations and saw a similar reduction in bone marrow plasma cells\textsuperscript{217}. It has been shown by Mohr et al (and confirmed by our own data in chapter 4) that monocyte and macrophage populations produce APRIL and support plasma cell survival in the spleen, although this has not been shown to be important in the bone marrow\textsuperscript{88}. Similarly, dendritic cells are known to produce both APRIL and IL-6, and are thought to be important in supporting the early stages of plasma cell responses, although their role in the bone marrow (if any) is still unclear\textsuperscript{88,137}.

We therefore looked at frequencies and numbers of relevant cell types (all which stained positive for intracellular APRIL, see figure 4.10 for a detailed analysis of APRIL secreting cells), as well as a population of cells that secreted very high amounts of APRIL (‘APRIL\textsuperscript{hi} cells’), but were not labelled with any antibodies against common leukocyte surface markers (CD11b, Gr1, F4/80, B220, CD19, CD4, CD3, CD8, CD11c, NK1.1, IgM, IgG, CD138 and the endothelial marker VCAM-1 were all tested, data not shown).

We saw little statistically significant accumulation of niche-providing cells in the spleen or bone marrow in terms of frequency or number between 5 and 30-week-old naïve mice (fig 3.7). In the bone marrow, ‘APRIL\textsuperscript{hi} cells’ significantly increased, as did dendritic cells, but this was only around a 2-fold increase. As plasma cells increased around 10-fold over the same time period, it is unlikely that this is the sole reason for the accumulation of plasma cells. Although we have not evaluated certain other plasma cell survival factors (such as IL-6), the cell types analysed in this experiment cover the majority of those thought to contribute to plasma cell survival, and the lack of a substantial increase in their numbers indicates that an increase in survival factors is not the primary reason for plasma cell accumulation in naïve mice.
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Accumulation of long-lived plasma cells in the bone marrow of strains of knockout mice with defects in germinal centre responses

Long-lived bone marrow plasma cells have been shown to arise mainly from the germinal centre response\textsuperscript{87}. To determine the factors required for the accumulation of bone marrow plasma cells over time, we utilised a number of strains of knockout mice deficient in either TLR signalling (MyD88/TRIF\textsuperscript{-/-}), T cell activation and therefore T cell help for B cells (Aβ\textsuperscript{-/-} and CD40\textsuperscript{-/-}), and BCR signalling (MD4 mice in which all B cells express a BCR specific for hen egg lysosyme). All of these mice are impaired in germinal centre formation, as T cell help and BCR signalling are required, and TLR signalling has been shown to enhance germinal centre formation\textsuperscript{29,67,80,115,299}.

There were few differences in numbers of plasma cells in the spleen or bone marrow of wild type and knock out mice at 5 weeks of age (fig 3.8a). In fact, only MD4 mice had lower numbers than wild type mice in the spleen, likely relating to this strain’s deficiency in B1 cells\textsuperscript{300}. Aβ\textsuperscript{-/-} mice had higher frequencies of splenic plasma cells, but similar numbers to wild type mice. This is due to the smaller spleen size of this strain (data not shown). There was no deficiency in bone marrow plasma cells at this stage (fig 3.8a). This is likely due to the fact that even wild type mice have few long-lived plasma cells in the bone marrow by 5-weeks of age.

Despite generating similar numbers of plasma cells in the spleen, 30-week-old Aβ\textsuperscript{-/-}, CD40\textsuperscript{-/-} and MD4 mice did not accumulate long-lived plasma cells in the bone marrow (fig 3.8b). This implies that there is a requirement for T cell help, and BCR signalling for the accumulation of long-lived bone marrow plasma cells. This is in agreement with data suggesting that most long-lived plasma cells arise from germinal centre responses\textsuperscript{86}, which require both T cell help and recognition of antigen through the BCR.

It is perhaps surprising that all mouse strains contained similar numbers of splenic plasma cells at both time points. Many of these likely arise from B1 cell populations, which secrete ‘natural’ antibody in response to either BCR
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stimulation through weak recognition of self-antigen (B1a cells), or TLR stimulation and conserved pathogenic antigens recognised by a limited BCR repertoire (both B1a and B1b cells). MD4 mice have been shown to be deficient in B1 cell populations and this may explain their reduced (although not absent) splenic plasma cell numbers at 5-7 weeks of age. However, they were not significantly lower at the 30-week time point, suggesting that plasma cells present under homeostatic conditions (i.e. plasma cells found in the spleens of unimmunised mice) in the spleen do not all arise from B1 cells. MyD88/TRIF signalling is not essential for the generation of natural antibody from B1a cells (although is critical for that of B1b cells and does stimulate plasma cell differentiation from B1a cells). This may explain why MyD88/TRIF−/− mice have similar numbers of splenic plasma cells to wild type mice. B1a cells may differentiate readily to plasma cells in the naïve state, but these plasma cells are exclusively short-lived; in fact B1 cells do not enter germinal centre reactions. Therefore it is likely that plasma cell accumulation in wild type mice arises from the B2 compartment, responding to environmental antigens and gut microflora.

Few significant differences were seen in niche-providing cell populations in the spleens of 30-week old wild type or knockout mice, reflecting similar numbers and turnover of plasma cell populations at this site. In the bone marrow, lower proportions of APRIL hi cells and dendritic cells were seen in MyD88/TRIF−/− mice, and similarly lower proportions of APRIL hi cells were seen in Aβ−/− mice (fig 3.10). Without further characterisation of APRIL hi cells, it remains unclear why their frequencies were lower in these strains. It is not surprising that fewer dendritic cells were seen in MyD88/TRIF−/− mice as TLR signalling is essential for dendritic cell function and controls their differentiation from progenitor populations. These results seemed to have little bearing on plasma cell number and turnover in the bone marrow; Aβ−/− mice had similar number and turnover of plasma cells to CD40−/− and MD4 mice despite having lower frequencies of APRIL hi cells, while MyD88/TRIF−/− had higher numbers of long-lived plasma cells in the bone marrow, despite lower frequencies of both
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APRIL<sup>hi</sup> cells and dendritic cells. It is therefore likely that the deficiency in the accumulation of long-lived bone marrow plasma cells in these strains of mice is one of generation, and not provision of survival factors and niches.

**Conclusion**

In this chapter we have described methods for identifying plasma cells by staining for intracellular Ig, and shown that (at least in certain immune responses) this is a more appropriate marker, as not all antibody secreting cells express high levels of the conventional plasma cell marker CD138. Following this, we identified plasma cells in the bone marrow of 6 to 8-week old naïve mice, which appeared phenotypically similar to plasma cells found in the spleen. In contradiction to the literature, these plasma cells had a relatively rapid rate of turnover, as measured by BrdU incorporation. It was found that naïve mice accumulated long-lived plasma cells in the bone marrow over time, and this correlated with an increase in serum antibody levels. These plasma cells required signals through the BCR, and T cell help for their generation. Availability of plasma cell survival factors in the bone marrow did not substantially increase over time, and therefore was unlikely to play a major role in the accumulation of long-lived plasma cells.
Chapter 4 – Factors required for extrafollicular plasma cell responses to SRBC immunisation and *Salmonella* infection

Introduction

Plasma cell responses in the spleen can arise as part of either the early extrafollicular response or the later germinal centre response. Plasma cells derived from germinal centres are relatively well defined; they secrete high affinity antibody, are generally class-switched and have the capacity to travel to the bone marrow where they persist for long periods. Germinal centre responses take time however, first antigen-specific CD4 T cells must be primed by activated APC, followed by their encounter with activated antigen-specific B cells. This is followed by a period of proliferation by B cells before germinal centres are initiated at the centre of B cell follicles. FDCs are an important component of the germinal centre; they express many Fc and complement receptors on their surface and bind large amounts of antigen in the form of immune complexes and complement components bound to antigen.

During the germinal centre reaction, B cells that have undergone somatic hypermutation ‘test’ their B cell receptors against antigen bound to the surface of FDCs. If their receptors recognise the antigen, they are provided with short-term survival signals and either undergo further rounds of mutation, or exit the germinal centre and differentiate to B memory cells or long-lived plasma cells. This process takes a number of days, during which time mechanisms exist to provide antibody which is of low-affinity, but crucial to limiting the spread of infections during the early stages through opsonisation of bacteria, and activating innate cells through Fc receptor cross-linking and complement activation. This antibody is provided by plasma cells generated in extrafollicular sites.

It is known that extrafollicular plasma cell responses to TI antigen are generated through mechanisms such as TLR stimulation, and often arise from the B1 and marginal zone B cell compartments. Extrafollicular responses to TD antigens require T cell help, arise from follicular B cells, and are generally
slower to appear\textsuperscript{78,81}. However, no definitive study exists comparing the factors required for extrafollicular plasma cell responses in a purely T-dependent response (SRBC) with both the early and late stages of the extrafollicular responses to a complex bacterial infection with both TI and TD components (\textit{Salmonella} infection). Here, we utilise strains of mice with deficiencies in parts of the pathway vital for T cell-B cell interactions (\textit{Aβ}\textsuperscript{−/−}, \textit{CD40}\textsuperscript{−/−} and \textit{ICOS}\textsuperscript{−/−} mice), B cell receptor signalling (MD4 mice) or TLR signalling (MyD88/TRIF\textsuperscript{−/−} mice) to determine the relative contributions of these pathways to extrafollicular plasma cell responses.

\section*{Results}

\textbf{Requirements for extrafollicular plasma cell and germinal centre responses to TD antigen}

Mice were immunised with either SRBC or alum-precipitated NP-KLH with killed \textit{Bordetella pertussis}. At day 0 (pre-immunisation), 5, 8 and 14 spleens were harvested and histology sections prepared and stained with anti-IgM, IgG and PNA. At D0 plasma cells were seen at only low frequencies in the spleen, however, 5 days after immunisation with SRBC substantial plasma cell responses were observed. Plasma cells appeared grouped into large clusters in the red pulp and were often in close proximity to the bridging channels, which cut across the marginal zone and allow entry to the red pulp (fig 4.1a). These plasma cells were a mix of IgM and IgG, indicating that class switching had already occurred in these mice. Large germinal centres had already formed in the follicles, although at this stage there were few obvious light and dark zones (indicating that germinal centres were recently formed). By day 8 of this response, germinal centres had matured considerably and now had obvious light (patches of bright IgM and IgG staining where immune complexes are bound to Fc receptors on networks of FDCs) and dark zones. By this point in the response the frequency of plasma cells was considerably reduced, with remaining plasma cells either found at the bridging channels, or further out in the red pulp, often not in close proximity to the follicles. These plasma cells
Extrafollicular plasma cell responses were still arranged in clusters, but each cluster was far smaller than those seen at day 5. By day 14, the germinal centre response persisted, but the frequency of plasma cells had returned to levels seen in naïve mice.

NP-KLH immunised mice responded with different kinetics. At day 5 only small clusters of plasma cells were observed, located almost exclusively at the bridging channels between follicles. Many plasma cells were class switched at this time point. Germinal centres were not yet apparent, however, small patches of bright IgM and IgG staining could be seen at the centre of some follicles, indicating that FDC networks had accumulated immune complexes on their surfaces. By day 8, small germinal centres were seen in a minority of follicles. Clusters of extrafollicular plasma cells had formed; these were mainly located in close proximity to follicles and again were fairly evenly split between IgM and IgG plasma cells. By day 14, large germinal centres (often occupying much of the follicle) could be seen with well-developed dark and light zones. Large numbers of class switched plasma cells were now evident throughout the red pulp (although these were more concentrated nearer the follicles).

We were interested in dissecting the relative roles of T cell help, B cell receptor signalling and TLR signalling in the extrafollicular plasma cell response to SRBC. We immunised a number of strains of mice with deficiencies in TLR signalling (MyD88/TRIF−/− mice), T cell-B cell interactions and CD4 T cell activation (Aβ−/−, CD40−/− and ICOS−/− mice), and B cell receptor signalling (MD4 mice) with SRBC. At day 5 mice were culled and examined for plasma cells and germinal centre formation by FACS and histology.

As seen in figure 4.1, 4.2a and 4.2c, wild type mice responded at day 5 with simultaneous class switched extrafollicular plasma cell and germinal centre responses. Aβ−/− mice (and MD4 mice, which cannot class switch) exhibited a severe impairment in the IgG response; CD40−/− and ICOS−/− mice were also impaired in the class-switched plasma cell response, although less so than Aβ−/− mice. Interestingly, MyD88/TRIF−/− mice were also significantly impaired in their ability to mount a class-switched plasma cell and germinal centre
response, despite there being no obvious PAMP component to the immunisation (fig 4.2a, b and c). Aβ−/−, CD40−/− and ICOS−/− mice and MD4 mice also failed to mount germinal centre responses (fig 4.2b). Although IgM plasma cells are not a major component of the response to SRBC, they were not significantly impaired in any of the knockout strains (fig 4.2c).

**Delay of germinal centre formation and enduring extrafollicular plasma cell response following *Salmonella* infection**

Mice receiving 1x10⁶ CFU of *Salmonella* undergo a long-term infection, taking 60-70 days to clear. During this time, mice exhibit significant splenomegaly, with spleens expanding up to ten times their size in naïve mice. Previous experiments by Cunningham et al²⁸¹, and our own lab (unpublished) had shown that during the first 25 days of infection (when germinal centres usually peak in standard hapten-carrier immunisation protocols), there was no formation of germinal centres. We therefore extended this time course to look for germinal centres throughout the duration of infection. Germinal centres are best visualised through microscopy and therefore this technique was utilised. As shown in figure 4.3a, infected mice displayed considerable disruption of splenic architecture. By day 25, follicles were reduced in size and often T cell zones and follicles were often intermixed. This continued until day 43 of infection when normal splenic architecture began to return. Not until day 53 were germinal centres apparent, and B cell follicles and T cell zones returned to their normal sizes and appeared more distinct. By day 77, spleens had regained normal architecture.

Plasma cell frequencies were examined over a 65-day time course of infection. By day 8, frequencies of both IgM and IgG plasma cells had increased substantially compared to naïve mice (fig 4.3b). IgG plasma cells had increased to 3.59% (±0.68) of the spleen, and this corresponded to 1.31x10⁷ cells (±4.22x10⁶); this represents a 163-fold increase over naïve mice. Similarly, IgM plasma cells peaked at 5.81x10⁶ cells (±5.84x10⁵), representing around a 16-fold increase over naïve mice. Plasma cell numbers then gradually declined until day 65, when frequencies and numbers were equivalent to those in naïve mice.
Chapter 4 – Extrafollicular plasma cell responses

These kinetics were comparable to bacterial loads in the spleen, although these peaked slightly later, at day 18 (fig 4.3b).

We stained for FDC networks using anti-CD35 (complement receptor 1), which is expressed on the surface of these cells. Figure 4.4 shows that in the naïve spleen, FDC networks can be seen in the centre of B cell follicles. Following infection, these rapidly disappeared, only to re-establish later, by day 53 of infection. This coincides with the emergence of germinal centres, as shown in figure 4.3.

We tested whether the delayed appearance of germinal centres was an inherent property of the Salmonella bacterium, or whether it required live bacterial infection. Mice were immunised with 1x10^8 heat killed Salmonella (SL3261) or 1x10^8 heat killed B. pertussis. We chose this control as prior experiments had shown that, when immunised with B. pertussis, mice developed germinal centres with typical timing (i.e. appearing early in the second week (around day 7), and persisting for around 2-weeks (to day 21) before gradually dissipating (see fig 4.1)). We saw that, 12-days following immunisation with heat killed Salmonella and B. pertussis, mice generated germinal centre responses, although B. pertussis induced a substantially greater response (7.74% (±0.77) of B cells following B. pertussis and 2.65% (±0.52) of B cells following Salmonella) (fig 4.4b and c). At day 55 following immunisation with either species of heat killed bacteria (i.e. when germinal centres were seen to form following infection with live Salmonella) germinal centres had declined to background levels.

**Requirements for early (day 4) extrafollicular plasma cell responses to Salmonella infection**

We determined the factors required for the early (day 4) plasma cell response to Salmonella by infection of various knockout strains of mice with deficiencies in TLR signalling (MyD88/TRIF^-/- mice), T cell-B cell interactions/CD4 T cell activation (Aβ^-/-, CD40^-/- and ICOS^-/- mice) and B cell receptor signalling (MD4 mice). At day 4, spleens were taken and analysed by histology and FACS for plasma cell induction.
We found that the response at this early time point was dominated by non-class-switched IgM plasma cells (fig 4.5a and b). In wild type mice, 0.332\% (±0.048) of the spleen had differentiated to IgM plasma cells (1.13x10^6 (±1.67x10^5) cells). This was unaffected in Aβ−/−, CD40−/− and ICOS−/− mice, however MyD88/TRIF−/− and MD4 mice were severely impaired with significantly lower frequencies and numbers. IgG plasma cells do not play a major role at this stage of the response, but wild type mice had 5.12x10^4 (±7.35x10^3) class switched plasma cells. Frequencies of IgG plasma cells in all genetically modified strains were significantly affected, although differences in numbers did not reach statistical significance apart from in MD4 mice. Bacterial loads were also significantly higher in MyD88/TRIF−/− mice, but not in other strains of mice (fig 4.5c).

We had shown in figure 4.5 that early extrafollicular responses to *Salmonella* consisted largely of IgM plasma cells. These did not require T cell help, but TLR signalling was essential for their formation. To further characterise where these TI plasma cells arose from, we used mice deficient in either MyD88 (and therefore impaired in TLR signalling), or CD1d (these mice lack the marginal zone B cell compartment). We compared IgM plasma cell formation in these mice at day 1, 4 and 8 of *Salmonella* infection. As can be seen in figure 4.6a, thick marginal zones could be seen in naive wild type mice, surrounding the white pulp. These cells were identified through their expression of high levels of IgM, and lack of expression of IgD. Similarly, MyD88−/− mice had marginal zones, but these were absent in CD1d−/− mice. As early as day 1 after infection with *Salmonella*, marginal zones rapidly disappeared from the spleens of wild type mice. This was not the case in MyD88−/− mice, which maintained marginal zones at day 1, and even at day 4 (albeit by this stage, marginal zones had thinned considerably).

Wild type mice mounted a TI IgM plasma cell response at day 4, but this was absent in both MyD88−/− and CD1d−/− mice (fig 4.6a and b). Wild type mice also mounted class switched IgG plasma cell responses at day 8 of infection, which will be examined further in the subsequent figure (fig 4.8). While spleens of
MyD88−/− mice contained significantly fewer class switched plasma cells, differences were relatively small compared to wild type mice. CD1d−/− mice exhibited a far greater impairment (fig 4.6b).

**Requirements for late (day 8) extrafollicular plasma cell responses to Salmonella infection**

To examine the factors required for the class switched plasma cell response seen in figure 4.7, wild type or knockout mice (MyD88/TRIF−/−, Aβ−/−, CD40−/−, ICOS−/− and MD4) were infected with *Salmonella*, and spleens harvested at day 8. These were analysed for plasma cell formation by FACS analysis and histology. By day 8 of infection, wild type mice made a substantial class-switched IgG plasma cell response (fig 4.7a and c). Interestingly, at this stage MyD88/TRIF−/− mice made an equivalent response, in contrast to day 4. Aβ−/−, CD40−/− and ICOS−/− mice were all significantly impaired, although Aβ−/− mice exhibited the greatest deficiency.

At this stage of the response, although IgG plasma cells dominate in wild type mice, IgM plasma cells are present at similar frequencies to those seen at day 4 (fig 4.5c and 4.7c). Due to continued expansion of the spleen, total numbers of IgM plasma cells are higher than those seen at day 4. Interestingly however, by day 8 none of the knockout strains (including MD4 mice) were significantly impaired in terms of numbers of IgM plasma cells. MD4 mice, which only recognise Hen Egg Lysosyme (HEL) through the BCR, produced equivalent numbers of IgM plasma cells to wild type mice. The specificity of these plasma cells was tested via intracellular staining with fluorescently labelled HEL (fig 4.7b). While few IgM plasma cells in wild type mice bound HEL (1.73% ± 0.33), the majority of IgM plasma cells in MD4 mice bound the protein (91.8% ± 1.02). We presume these plasma cells in MD4 mice have been generated in a BCR-independent, TLR-dependent manner.

At day 8, as at day 4, MyD88/TRIF−/− mice were the only strain of mice to have significantly higher bacterial loads than wild type mice (fig 4.6d).
**Salmonella**-specific antibody levels remain low throughout the first 8 days of the response, and do not correlate to bacterial load

*Salmonella* specific serum antibody titres of the mice in figure 4.7 were quantified by ELISA (fig 4.8a). While all mouse strains had produced equivalent numbers of IgM plasma cells at day 8 of the response (fig 4.7c), interestingly Aβ−/− mice failed to accumulate *Salmonella* specific IgM in the serum. MD4 mice also did not produce *Salmonella*-specific antibody, confirming that the plasma cells seen in figure 4.7c were not *Salmonella* specific. While CD40−/−, ICOS−/− and MyD88/TRIF−/− mice made IgM responses, these were still significantly lower than those seen in wild type mice (table 4.1). Interestingly, MyD88/TRIF−/− mice made significantly higher IgG1 responses than all other mouse strains (fig 4.8a and table 4.1). No strains made any detectable IgG2b at this time point, and only wild type mice were able to produce IgG2c. There was little detectable IgG3 in any mouse strain, and no significant differences between strains were seen.

It can be seen in figure 4.8b that class-switched *Salmonella*-specific antibody responses do not peak until late in the response, while *Salmonella*-specific IgM levels peak by day 8, but remain low throughout the course of infection.

At day 4 of infection MyD88/TRIF−/− mice exhibited lower numbers of plasma cells and higher bacterial loads than wild type mice (fig 4.5b and c). By day 8, their bacterial loads remained high despite plasma cell numbers being equivalent to wild type mice. Their *Salmonella*-specific IgM antibody levels, while reduced compared to wild type mice, were equivalent to CD40−/− and ICOS−/− mice, which had normal bacterial loads (fig 4.7c and d and fig 4.8a).

Innate cells are known to be of importance in the response to *Salmonella* infection, as the bacteria invade macrophage populations that must be activated to mediate clearance. Therefore we looked at CD11b+ cells in wild type and knockout mice at day 8 of infection to determine whether there was a deficiency of innate cell infiltration into the spleen in MyD88/TRIF−/− mice. Indeed, MyD88/TRIF−/− mice had significantly fewer CD11b+ cells in the spleen. While
Aβ−/− mice also had a significant reduction in numbers, this was not as great as the decrease seen in MyD88/TRIF−/− mice (fig 4.8c).

**Extended survival of extrafollicular plasma cell populations in the response to Salmonella infection likely reflects an increase in the survival factor APRIL from multiple sources**

Extrafollicular plasma cell responses persist for long periods in *Salmonella* infection, maintaining high numbers until around day 35 (fig 4.3b). We tested whether these plasma cells were rapidly dividing short-lived plasma cells, or whether a single cohort of plasma cells, produced early in the response, persisted for the duration of the response. *Salmonella* infected mice were given 4-day BrdU pulses at various time points, and the percentage and numbers of plasma cells incorporating BrdU was determined. In naïve mice, 32.70% (±5.16) of splenic plasma cells incorporated BrdU during a 4-day pulse (fig 4.9a). During days 1-4 of infection, this rapidly increased to 66.75% (±4.04) and further increased to 81.58% (±2.37) during days 5-8 (fig 4.9a and b). When mice were pulsed at days 14-17, turnover had slowed considerably; 23.10% (±3.92) of plasma cells incorporated BrdU. Mice were also pulsed later in infection, at days 51-54, when 13.34% (±4.33) of plasma cells divided. Interestingly, the turnover of IgM plasma cells at this time point was even lower, with 3.19% (±0.69) dividing.

Although turnover of plasma cells slowed throughout the time course, considerable numbers of plasma cells were found to be still dividing at days 14-17, as total plasma cell numbers are very high at this time point (fig 4.9b).

To conclusively show that plasma cells were not all generated at early time points following infection, and were able to persist until the resolution of infection, we gave mice BrdU for 4-day pulses at days 1-4 or 5-8 of infection. The survival of IgM and IgG plasma cells incorporating BrdU during these times was tracked out for 16 days (fig 4.9c). Despite 66.75% (±4.04) of IgG plasma cells incorporating BrdU during days 1-4 and 81.58% (±2.37) at days 5-8, only 12.66% (±2.13) and 16.93% (±4.86) contained the label 16 days later. Similarly,
68.30% (±4.27) and 70.95% (±8.25) of IgM plasma cells incorporated BrdU at days 1-4 and 5-9 respectively, however only 8.48% (±1.10) and 14.33% (±1.09) contained the label 16-days later. This data implies a slow but continuous turnover of splenic plasma cells during *Salmonella* infection, with a half-life of 4.0 days for IgM plasma cells generated at day 1-4 of infection and 6.4 days for IgM plasma cells generated at day 5-8. IgG plasma cells generated at days 1-4 had a half-life of 6.1 days, while those generated at days 5-8 had a half-life of 6.6 days.

In figure 4.9, we showed that extrafollicular plasma cell responses in *Salmonella* infection persist, but that plasma cells are not continuously produced at the very high rate seen in the first 8 days of infection. We investigated whether the plasma cell survival factor APRIL, or certain ‘niche-providing cells’ were induced in the infected spleen.

Firstly, we looked at APRIL induction in the spleen of infected mice. It can be seen in figure 4.10a that a population of intracellular APRIL\textsuperscript{hi} cells were induced on infection. This population increased from less than 0.24% (±0.05) of the spleen in naïve mice to over 12.06% (±1.63) of the spleen in infected mice (figure 4.10a and c). Previous reports identified a number of cells that produce high amounts of APRIL. These included monocytes and macrophages, which express intermediate amounts of the granulocyte marker Gr1. However, Gr1\textsuperscript{int} cells produced only intermediate amounts of APRIL. By gating on Gr1\textsuperscript{int} cells (figure 4.10b-top row) and examining for F4/80 and CD11b expression (further markers of monocyte/macrophage populations), it was shown that Gr1\textsuperscript{int} cells are a heterogeneous population. In naïve mice, few of these cells express CD11b or F4/80, however this increases as infection progresses. Despite the induction of monocyte/macrophage populations in the spleen, these cells did not produce high amounts of APRIL, as APRIL\textsuperscript{hi} cells at no point expressed CD11b, F4/80, or Gr1 (fig 4.10a and b-bottom row).

We further examined a number of ‘niche-providing cells’ for their expression of APRIL in either naïve or day-16 infected mice (fig 4.10d, e and f).
Megakaryocytes (CD41+) were induced to produce relatively high amounts of APRIL in infection (MFI $2.94 \times 10^3 \pm 1.81 \times 10^2$), albeit lower than ‘APRIL$^{hi}$ cells’ ($3.18 \times 10^4 \pm 4.45 \times 10^2$). Their frequency also remained low in the infected spleen (0.255% ± 0.06).

CD11c+, CD11b$^{lo}$ dendritic cells expanded moderately (from 0.224% (±0.015) to 0.354% (±0.024)) but their production of APRIL significantly decreased following infection ($3.68 \times 10^3 \pm 6.15 \times 10^1$ in naïve mice to $2.93 \times 10^3 \pm 1.14 \times 10^2$ in infected mice) (fig 4.10f). CD11c+, CD11b$^{hi}$ dendritic cells expanded to a far greater extent on infection (from 0.607% (±0.015) to 2.56% (±0.037)) and also significantly increased their APRIL production (from $2.35 \times 10^3 \pm 5.43 \times 10^1$ to $3.79 \times 10^3 \pm 8.1 \times 10^1$).

Eosinophils (CD11b$^{int}$, F4/80$^{hi}$, Gr1$^{int}$) and monocyte/macrophage (CD11b$^{hi}$, F4/80$^{int}$, Gr1$^{int}$) populations expanded greatly on infection, as did neutrophils (CD11b$^{hi}$, F4/80$^{lo}$, Gr1$^{hi}$) (Eosinophils; 0.02% ± 0.00, to 1.16% ± 0.11, Monocytes/macrophages; 1.31% ± 0.08 to 10.51% ± 0.80, Neutrophils; 4.40% ± 0.74 to 7.58% ± 1.45) While monocytes/macrophages and eosinophils also increased their APRIL production on infection, neutrophils did not (Eosinophils; 4.59 x 10$^3 \pm 1.50 \times 10^2$, Monocytes/macrophages; 2.61 x 10$^3 \pm 6.98 \times 10^1$, Neutrophils; 1.76 x 10$^3 \pm 3.24 \times 10^1$ to 1.87 x 10$^3 \pm 4.41 \times 10^1$) (fig 4.10d and f).

APRIL$^{hi}$ cells increased greatly in terms of both number and frequency ($6.84 \times 10^4 \pm 5.08 \times 10^3$ to $4.22 \times 10^7 \pm 2.64 \times 10^6$; and 0.15% ± 0.04 to 7.27% ± 0.32), but did not further up regulate APRIL production on infection (fig 4.10e and f). These results imply that the infected spleen is rich in APRIL, as many APRIL secreting cells expand and up regulate its production on infection.

In figure 4.1, we had shown that extrafollicular plasma cell responses generated following SRBC immunisation were short lived, surviving just 2-3 days. Therefore we next looked at whether APRIL producing cell populations were induced following SRBC immunisation. At day 3, 7 and 14 following SRBC immunisation (or Salmonella infection) we looked at the frequencies of APRIL$^{hi}$ cells, monocytes and macrophages and dendritic cells in the spleen (fig 4.11a).
Chapter 4 – Extrafollicular plasma cell responses

We saw little change in the frequency of any of these three populations in the spleens of SRBC immunised mice, while *Salmonella* infected mice generated far greater populations of all three.

To further show that APRIL induction can enhance the lifespan of splenic plasma cell responses, NP-KLH primed mice were boosted with either soluble NP-KLH or alum-precipitated NP-KLH with killed *B. pertussis* (referred to as alum-NP-KLH). While soluble NP-KLH induced a transient increase in the frequencies of APRIL producing cells, this rapidly returned to naïve levels (fig 4.11b). The decline of APRIL producing cells in alum-NP-KLH boosted mice was slower, with significantly higher frequencies at day 4, day 8 and day 20 (although by this point frequencies were low in both immunisation protocols). NP-specific plasma cells were detected through intracellular staining with fluorescently labelled NP. Although there was no difference in the frequency of splenic NP-specific IgG plasma cells induced by either immunisation at day 4, those mice boosted with alum-NP-KLH maintained high frequencies of NP-specific plasma cells at day 8, unlike soluble NP-KLH boosted mice. At day 20, alum-NP-KLH boosted mice maintained higher frequencies of splenic NP-specific plasma cells, although differences were not statistically significant.
Figure 4.1. Speed and longevity of extrafollicular plasma cell response depends on antigen. Histology from mice immunised with SRBC or alum-NP-KLH with *Bordetella pertussis* at day 0, 5, 8 and 14 stained with PNA (green), IgM (red) and IgG (blue). T=T cell zone, BC= Bridging channel, R=Red pulp, F=B cell follicle, G=Germininal centre, P=extra follicular plasma cells, L=Light zone, D=Dark zone. Pictures representative of spleens from 5 mice.
Chapter 4 – Extrafollicular plasma cell responses

Figure 4.2

A

WT

My/TRIF−/−

Aβ−/−

CD40−/−

ICOS−/−

MD4

PNA IgM IgG

B

Frequency

% of B Cells

Number

Number per Spleen

GT B Cells

Figure 4.2 continues overleaf
Figure 4.2 cont.

C

Frequency

Number

**

N.S. ** N.S. N.S. N.S.

0.00 0.25 0.50 0.75

% of Spleen Cells

IgM Plasma Cells

**

N.S. N.S. N.S. N.S.

1.0x10^3

Number per Spleen

IgG Plasma Cells

**

N.S. N.S. N.S. N.S.

1.0x10^7

Number per Spleen

Figure 4.2. Extrafollicular plasma cell and germinal centre responses to SRBC immunisation require both T cell help and signals through MyD88/TRIF and the B cell receptor. (A) Spleen sections from wild type, MyD88/TRIF^−/−, Aβ^−/−, CD40^−/−, ICOS^−/− and MD4 mice 5 days after SRBC immunisation, stained with PNA (green), IgM (red) and IgG (blue). (B) Percentage of B cells and total number of germinal centre B cells in spleens of mice from (A): wild type (filled square), MyD88/TRIF^−/− (triangle), Aβ^−/− (inverted triangle), CD40^−/− (diamond), ICOS^−/− (circle) and MD4 (open square) mice. (C) Frequency and number of IgM and IgG plasma cells in spleens of mice from (A). Pictures representative of spleens from 4-5 mice. Points on graphs=1 mouse, bars=mean. WT, MyD88/TRIF^−/− & MD4 data representative of 2 experiments, Aβ^−/−, CD40^−/−, ICOS^−/− is data from a single experiment.
Figure 4.3

Figure 4.3 continues overleaf
Figure 4.3 Cont. A Cont. D53 D77

B Plasma Cell Frequency

Plasma Cell Number

C Bacterial Loads

Figure 4.3. Germinal centre formation is delayed in Salmonella infection, however extrafollicular plasma cell responses persist for long periods. (A) Spleen sections showing germinal centre formation in Salmonella infected mice. Sections are stained with PNA (green), IgM (red) and CD4 (blue). (B) Frequency and number of IgM (dashed line) and IgG plasma cells (solid line) in spleens of Salmonella infected mice. (C) Bacterial loads in spleens of mice from (B). Pictures representative of spleens from 4 mice at each time point. Points on graph=5 mice, bars=standard deviation. Results representative of at least 5 experiments.
Figure 4.4 continues overleaf
Figure 4.4 cont.

Figure 4.4. Networks of follicular dendritic cells in B cell follicles are depleted in the spleens of *Salmonella* infected mice. (A) Mice were infected with *Salmonella* and spleens sections stained for IgM (B cells), CD4 (T cells) and CD35 (Follicular dendritic cells) at day 0 (naïve mice), day 8, day 25 and day 53. (B) FACS plots gated on CD19<sup>th</sup> B cells from spleens of naïve mice or mice immunised with heat killed *Salmonella* (SL3261) or heat killed *B. pertussis*. Gate shows PNA+ IgD- germinal centre B cells at day 12. (C) Summary graph of mice from (B) showing days 12 and 60 post immunisation. Pictures and FACS plots representative of spleens from 4 mice. Points on graphs= mean of 5 mice, bars=standard deviation. Results representative of 2 independent experiments.
Chapter 4 – Extrafollicular plasma cell responses

Figure 4.5

- WT
- My/TRIF⁻/⁻
- Aβ⁻/⁻
- CD40⁻/⁻
- ICOS⁻/⁻
- MD4

PNA IgM IgG

Figure 4.5 continues overleaf
Figure 4.5. Extracellular plasma cell responses in the spleens of wild type and knockout strains of mice at day 4 of *Salmonella* infection. 
(A) Histology from spleens of wild type, MyD88/TRIF−/−, Aβ−/−, CD40−/−, ICOS−/− and MD4 mice at day 4 of *Salmonella* infection showing IgM (red) and IgG (blue). M2=Marginal zone B cells. (B) Frequencies and numbers of plasma cells in spleens of mice from (A) as determined by FACS analysis (wild type (filled square), MyD88/TRIF−/− (triangle), Aβ−/− (inverted triangle), CD40−/− (diamond), ICOS−/− (circle) and MD4 (open square)). (C) Bacterial loads in spleens of mice from (A). Points on graph=1 mouse, bars=means. Pictures representative of 5 spleens. Results representative of 2 experiments.
Fig 4.6. Plasma cells at day 4 of *Salmonella* infection arise primarily from marginal zone B cells and are MyD88 dependent.

(A) Spleen sections from wild type, MyD88−/− and CD1d−/− mice stained for IgM (red) IgD (green) and IgG (blue) at day 0 (naïve), 1, 4 and 8 of *Salmonella* infection. White arrows indicate marginal zones. (B) Frequency and number of IgM and IgG plasma cells as identified by flow cytometry in spleens of wild type (black), MyD88−/− (red) and CD1d−/− (blue) mice. Pictures and points on graphs represent results from 5 mice. Error bars=standard deviation. Results representative of 2 independent experiments.
Chapter 4 – Extrafollicular plasma cell responses

Figure 4.7

A

WT

My/TRIF−/−

Aβ−/−

CD40−/−

ICOS−/−

MD4

PNA IgM IgG

B

% of Max

Intracellular HEL

Figure 4.7 continues overleaf
Figure 4.7 Plasma cell production in wild type and knockout mice at D8 of *Salmonella* infection. (A) Histology from spleens of *Salmonella* infected wild type and knockout mice at 8 of infection showing extrafollicular plasma cell formation (IgM in red, IgG in blue). Pictures representative of 5 spleens per groups. (B) FACS profile (representative of 5 mice per group) gated on IgM plasma cells from spleens of infected wild type (red) or MD4 (blue) mice showing intracellular staining with labeled HEL. Numbers indicate percentage of IgM plasma cells which bind HEL. (C) Frequencies and numbers of plasma cells in spleens of wild type (filled square), MyD88/TRIF^−/−^ (triangle), Aβ^−/−^ (inverted triangle), CD40^−/−^ (diamond), ICOS^−/−^ (circle) and MD4 (open square) mice from (A) as determined by FACS analysis. (D) Bacterial loads in spleens of mice from (A). Points on graph=1 mouse, bars=mean. Results are representative of 2 experiments.
Chapter 4 – Extrafollicular plasma cell responses

**Figure 4.8**

A

**IgM**

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B

**IgM**

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**IgG**

**Figure 4.8 continues overleaf**
Figure 4.8. *Salmonella*-specific antibody titres in the serum of infected mice remains low despite considerable extrafollicular plasma cell responses in the spleen. (A) *Salmonella*-specific serum antibody titres at day 0 (naive) and day 8 of infected wild type (green), MyD88/TRIF−/− (blue), Aβ−/− (Purple), CD40−/− (red), ICOS−/− (yellow) and MD4 (black). Points on graph=5 mice, error bars=standard deviation. For statistics see table 4.1. (B) *Salmonella*-specific IgM and IgG titres in the serum of infected mice over a 65 day time course. Points on graph=5 mice, error bars=standard deviation (C) Frequencies and numbers of CD11b expressing cells in the spleens of wild type (filled square), MyD88/TRIF−/− (triangle), Aβ−/− (inverted triangle), CD40−/− (diamond), ICOS−/− (circle) and MD4 (open square) mice at day 8 of *Salmonella* infection. Points on graph=1 mouse, bars=mean. Results representative of 1-5 experiments.

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Table 4.1. Statistics comparing data in figure 4.8a. One-way ANOVA comparing *Salmonella*-specific antibody titres in wild type mice at day 8 of infection to titres found in MyD88/TRIF−/−, Aβ−/−, CD40−/−, ICOS−/− and MD4 mice at the same time point.
Chapter 4 – Extrafollicular plasma cell responses

**Figure 4.9**

A

| % of Max |  
|--------|--------|--------|--------|--------|--------|--------|
| D0     | D5     | D9     | D18    | D55    | ISO    |
| 28.2   | 56.3   | 89.3   | 17.1   | 11     | 0.191  |

B

**Frequency of Total Plasma Cells**

**Plasma Cell Turnover (Percentage)**

- IgM
- IgG

**Number of Plasma Cells**

**Number of Dividing Plasma Cells**

Figure 4.9 continues overleaf
Figure 4.9 Cont.

Figure 4.9. BrdU incorporation of extrafollicular plasma cells in the spleen of *Salmonella* infected mice throughout a time course of infection. (A) FACS profiles of BrdU incorporation of splenic IgG plasma cells at the stated time points following infection with *Salmonella*. (B) Frequency of IgM (dashed line) and IgG (solid line) plasma cells in the spleen of *Salmonella* infected mice (top left); total number of plasma cells in the spleen (bottom left); percentage of splenic plasma cells incorporating BrdU in a 4-day pulse given prior to the indicated time point (top right); numbers of splenic plasma cells incorporating BrdU in a 4-day pulse given prior to the indicated time point (bottom left). (C) Percentage of IgM (left) or IgG (right) plasma cells retaining BrdU at 1 or 20 days following a 4-day BrdU pulse at days 1-4 or 5-8 of *Salmonella* infection. FACS profiles and points on graphs representative of 5 mice per time point. Error bars=standard deviation. All results representative of 2-3 independent experiments.
Chapter 4 – Extrafollicular plasma cell responses

Figure 4.10

A

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B

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C

Figure 4.10 continues overleaf
Figure 4.10 cont.

Figure 4.10 continues overleaf
Figure 4.10. APRIL producing cells in *Salmonella* infected mice.
(A) FACS plots showing induction of Gr1<sup>int</sup> APRIL<sup>hi</sup> cells is in the spleen following *Salmonella* infection (representative of 5 mice). (B) F4/80 and CD11b expression on APRIL<sup>int</sup> Gr1<sup>int</sup> cells (top row) and APRIL<sup>hi</sup> cells (bottom row). (C) Frequency and number of APRIL<sup>hi</sup> cells in the spleen through a time course of *Salmonella* infection (points on graph=5 mice, error bars=standard deviation). (D) FACS profiles of the major populations of ‘niche-providing cells’ (megakaryocytes, CD11c<sup>-</sup> CD11b<sup>-</sup> and CD11c<sup>+</sup> CD11b<sup>hi</sup> dendritic cells, eosinophils, monocytes and macrophages and neutrophils) and their respective APRIL production in naïve (red) and day-16 infected (blue) mice (representative of 5 mice). For comparison, APRIL<sup>hi</sup> cells are shown in green, and isotype control (grey filled). (E) Frequency and number of ‘niche-providing cells’ in the spleens of naïve and D16 infected mice. (F) MFI of APRIL stain in the populations from (E). Bars representative of 5 mice per group, error bars=standard deviation. Results representative of at least 3 experiments.
Chapter 4 – Extrafollicular plasma cell responses

Figure 4.11

A

APRIL\textsuperscript{H1}  

\begin{align*}
\text{% of Spleen Cells} & \quad \text{Day} \\
0 & \quad 0 \quad 5 \quad 10 \quad 15 \\
\text{ns} & \quad *** & \quad *** \\
\end{align*}

Monocytes/Macrophages

\begin{align*}
\text{% of Spleen Cells} & \quad \text{Day} \\
0 & \quad 0 \quad 5 \quad 10 \quad 15 \\
* & \quad *** & \quad *** & \quad *** \\
\end{align*}

Dendritic Cells

\begin{align*}
\text{% of Spleen Cells} & \quad \text{Day} \\
0 & \quad 0 \quad 5 \quad 10 \quad 15 \\
\text{ns} & \quad *** & \quad *** \\
\end{align*}

SRBC

\begin{align*}
\rightarrow & \quad \text{NP-KLH + Adjuvant} \\
\rightarrow & \quad \text{KP-KLH - Adjuvant} \\
\end{align*}

B

NP-IgG Plasma Cells

\begin{align*}
\text{% of Spleen Cells} & \quad \text{Day} \\
0 & \quad 0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 20 \quad 24 \\
\text{N.S.} & \quad *** & \quad N.S. \\
\end{align*}

APRIL\textsuperscript{H1} Cells

\begin{align*}
\text{% of Spleen Cells} & \quad \text{Day} \\
0 & \quad 0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 20 \quad 24 \\
*** & \quad *** & \quad * \\
\end{align*}

Fig 4.11. APRIL and APRIL secreting cells support extrafollicular plasma cell responses  
(A) Graphs showing frequency of APRIL\textsuperscript{H1} cells (top left), monocytes/macrophages (top right) and  
dendritic cells (bottom left) in the spleens of naive mice and mice 3, 7 and 14 days  
following SRBC immunisation or Salmonella infection. (B) Graphs showing frequencies of NP- 
specific IgG plasma cells and APRIL\textsuperscript{H1} cells in the spleens of mice boosted with either ‘soluble  
NP-KLH’ or ‘alum-NP-KLH and B. pertussis’ at days 0 (not boosted), 4, 8 and 20. Points on  
graphs=5 mice, error bars=standard deviation. Results represent a single experiment.
Chapter 4 – Extrafollicular plasma cell responses

**Discussion**

Extrafollicular plasma cell responses arise in the first few days following immunisation or infection, and provide early antibodies that can opsonise infecting organisms, activate complement pathways and activate innate cells through cross-linking of Fc receptors. This allows time for plasma cells that secrete high affinity antibody to be generated in germinal centre reactions. Extrafollicular plasma cells are short-lived cells, and do not migrate to the bone marrow. However, it has been shown that during inflammatory episodes, survival factors such as APRIL and IL-6 are induced and support the survival of these cells for extended periods.

The data presented here examine the requirements for extrafollicular plasma cell responses in complex bacterial infection and in the response to SRBC. In addition, we examine how the lifespan of extrafollicular plasma cells can be extended by the provision of survival factors.

**Rapid extrafollicular plasma cell responses and germinal centre responses following SRBC immunisation**

We observed striking differences between the timing of extrafollicular plasma cell responses and germinal centre responses following primary immunisation with alum-precipitated NP-KLH with killed *B. pertussis* or SRBC.

Splenic plasma cells (both IgG and IgM) peaked 5-days after immunisation with SRBC, whereas this peak was not until day 8 in NP-KLH immunised mice. Similarly, germinal centres formed at least 3 days earlier in SRBC immunised mice. We hypothesised that this accelerated response is likely to be the result of rapid T cell priming, although it is unclear why this is the case. The kinetics were similar to those seen in mice with availability of increased levels of T cell help, as observed by Sze et al who saw more rapid production of NP-specific plasma cells in NP-CGG (NP-Chicken gamma globulin (CGG)) immunised mice when they were first primed mice with the carrier protein (CGG), to increase the frequency of antigen-specific CD4 T cells. The kinetics are also similar to those
seen during secondary responses to hapten-carrier protein antigens such as NP-KLH (see fig 3.1), although in this situation memory cells abound.

Mice deficient in the expression of genes relating to T cell activation and T cell-B cell interactions (Aβ⁻/⁻, CD40⁻/⁻ and ICOS⁻/⁻) were deficient in germinal centre and class-switched extrafollicular plasma cell formation following SRBC immunisation. T cell help is an absolute requirement for germinal centres, and MHC II and CD40 are crucial for their interactions with B cells²⁹⁹,³²⁸. Interestingly, ICOS was found to be of absolute importance to germinal centre formation, whereas Dong et al had previously reported that ICOS⁻/⁻ mice immunised with SRBC exhibit germinal centres of reduced size in primary immunisation, and complete absence in secondary responses⁶⁸. This may relate to differences in dose (which is unspecified in this article).

Aβ⁻/⁻ mice were completely impaired in making class switched plasma cell responses, demonstrating the complete requirement for T cell help for this process in SRBC immunised mice. It is likely that the impairment seen here is due to a failure of T cell priming. CD40⁻/⁻ mice and ICOS⁻/⁻ mice were progressively less impaired; while MHC II was crucial for class switching, the co-stimulatory stages of T cell interactions were less important. Interestingly, ICOS⁻/⁻ mice had slightly enhanced IgM plasma cell responses. This may be due to commitment to a plasma cell fate, but failure to class switch. It is known that ICOS is required for the generation of a population of CD4 T cells that help class switching in extrafollicular sites through IL-21 production in a mouse model of lupus, however the impairment of T cell activation is not absolute in ICOS⁻/⁻ mice¹⁵⁹. It is not known whether these cells play a role in extrafollicular responses to exogenous antigens.

MyD88/TRIF⁻/⁻ mice were entirely deficient in germinal centre production (fig 4.2a and b). Although it is widely accepted that B cell signaling through TLRs enhances germinal centre formation and antibody responses³⁹, this result is surprising as SRBCs contain no obvious PAMP. MyD88 is also required for signaling through the IL-1 receptor, and this can enhance TD antibody
responses and germinal centre formation by up-regulating CD40 and OX40 on CD4 T cells\textsuperscript{295,329}. Similarly, Meyer-Bahlburg et al have shown that B cell-intrinsic MyD88 signaling is of particular importance to germinal centre B cells, which express more of this key adaptor molecule\textsuperscript{60}. That MyD88/TRIF\textsuperscript{-/-} mice were impaired in making class-switched plasma cell responses was again surprising. This may be due to the observed deficiency in germinal centre formation, and therefore reduced numbers of class-switched plasma cells. Similarly, defects in TI methods of class switching may account for this deficit, such as reduced APRIL or BLYS from dendritic cells (and other sources), which are able to induce class switching through binding to TACI and BCMA on activated B cells\textsuperscript{138}. In addition, signalling through TACI has been shown to require MyD88\textsuperscript{149}. Further experiments, such as the immunisation of chimeras in which B cells alone were deficient in MyD88, could help to determine whether these mice failed to generate class-switched plasma cells due to an intrinsic defect in B cell stimulation or due to reduced factors produced by other cells, requiring MyD88 signaling.

HEL-specific B cells in MD4 mice did not respond in terms of germinal centre formation or extrafollicular plasma cell formation. Antigen recognition through the BCR is required for entry into germinal centres\textsuperscript{330}. These mice are incapable of class switching, and therefore could not produce IgG plasma cell responses. **Germinal centre formation is delayed in Salmonella infected mice**

Following infection with *Salmonella*, we observed striking disruption of splenic architecture (fig 4.3a). B cell follicles were reduced in size and spatially distinct, separated by expanded red pulp. This may explain the delay in germinal centre formation following infection, as splenic architecture is important for the efficient meeting of rare, activated antigen-specific B and CD4 T cell clones\textsuperscript{115}. Similarly, we observed a depletion of FDC networks from B cell follicles (fig 4.4a), which are crucial to the establishment of successful germinal centre responses\textsuperscript{326,331}. Indeed, the disruption of B cell follicles and depletion of FDC networks may be linked, as Wang et al recently showed that FDCs maintain B cell follicular structure\textsuperscript{101}. Germinal centres formed at day 53, when splenic
architecture and FDC networks were comparable to naïve spleens (fig 4.3a and 4.4a). This also corresponded to low bacterial loads (fig 4.3c). Data from Cunningham et al suggests that high and persistent bacterial loads in the spleen impeded germinal centre formation during *Salmonella* infection, as when infected mice were treated with antibiotic (to reduce bacterial loads), germinal centres formed sooner. Our own data support this, as immunisation with killed *Salmonella* bacteria (which are likely to be rapidly cleared) generates germinal centres by day 12, and not at day 55 (fig 4.4b).

**Factors required for the extrafollicular plasma cell response to *Salmonella***

Despite delayed germinal centre formation, plasma cell responses in the spleens of infected mice were maintained for the duration of infection (fig 4.3b). Infection of wild type mice revealed a biphasic plasma cell response. At day 4, plasma cells were mainly IgM (fig 4.5a and b), whereas by day 8 plasma cells were mainly IgG (fig 4.7a and c).

Numbers of IgM plasma cells at day 4 were not significantly affected in mouse strains where CD4 T cell activation, and interactions with B cells, are impaired (Aβ−/−, CD40−/− and ICOS−/− mice). Similarly, MyD88/TRIF−/− mice were significantly impaired in making IgM plasma cell responses at day 4. This is strong evidence that IgM plasma cells seen at this time point are TI, requiring TLR signalling for their generation. TLR signalling alone, however, was seemingly not sufficient to generate early IgM plasma cells as MD4 mice failed to respond, indicating a role for B cell receptor signalling.

Surprisingly, MD4 mice responded at day 8 by the generation of IgM plasma cells in equivalent numbers to wild type mice (fig 4.7a and c). These plasma cells were specific for HEL (fig 4.7b). This implies that BCR signalling (and antigen specificity) is irrelevant for the generation of IgM plasma cells at day 8. That MyD88/TRIF−/− mice also were not deficient in IgM plasma cells by day 8 leads to a number of possible explanations of how these plasma cells arise. Firstly, B cells at day 4 may require signals through both TLR and BCR for efficient differentiation to IgM plasma cells. If antigen cannot be recognised
through the B cell receptor, their differentiation may be delayed. Factors, such as BlyS, IL-6 (via IL-21) and IFN-α have all been shown to enhance differentiation of B cells to plasma cells\textsuperscript{165,175,176,181} and are likely to be present at high levels in the inflamed spleen. It is possible that these signals alone, or combined with TLR signalling are sufficient to induce the differentiation of B cells to plasma cells in a polyclonal manner. Secondly, MD4 mice are deficient in B1 cell populations\textsuperscript{300}. As these are known to be important in the first few days of infection\textsuperscript{76}, this may explain the lack of IgM plasma cells seen at day 4 in these mice. Thirdly, many PRRs other than TLRs exist that do not signal through MyD88 or TRIF (such as NOD receptors). Signalling through these receptors may lead to the differentiation of B cells to plasma cells by day 8 of infection.

This leads to the probability that, in other mouse strains too, IgM plasma cells seen at day 4 or day 8 may not be \textit{Salmonella}-specific. Indeed, antigen-specific IgM antibody levels were reduced in Aβ\textsuperscript{−/−} and MD4 mice, but not MyD88/TRIF\textsuperscript{−/−} mice. Despite the fact that MHC II was not required to generate the large number of IgM plasma cells seen in the first 8 days of infection, it is required for the generation of \textit{Salmonella}-specific IgM. This implies a role for T cells in the generation of \textit{Salmonella}-specific IgM.

The observation that MyD88/TRIF\textsuperscript{−/−} mice retained marginal zone B cells at day 4 of infection, and that MyD88\textsuperscript{−/−} mice had similar deficiencies in day 4 IgM plasma cell responses as CD1d\textsuperscript{−/−} mice (which lack marginal zone B cells) may indicate that many IgM plasma cells seen at this time point arise from marginal zone B cells stimulated through TLRs. It is thought that marginal zone B cells are early responders; they rapidly differentiate into plasma cells, requiring TLR and BCR signals following injection NP-Ficoll\textsuperscript{57}. However, Gil Cruz et al have shown that B1b cells also provide early IgM plasma cells in \textit{Salmonella} infection (and \textit{Salmonella}-specific antibody)\textsuperscript{76}. Chimeras in which B cell deficient μMT mice are reconstituted with a mix of CD1d\textsuperscript{−/−} and DS-RED bone marrow would create mice in which marginal zone B cells expressed the DS-RED protein (DS-RED mice express the red fluorescent protein variant ‘DS-RED’ under the control of the chicken beta actin promoter coupled with the cytomegalovirus immediate
early enhancer, producing ubiquitous expression of DS-RED in all tissues). If early IgM plasma cells arise from the marginal zone compartment following *Salmonella* infection, they would express the DS-RED protein, while most follicular B cells would not. This system could be used to more definitively identify the source of these plasma cells.

IgG plasma cells, arising at day 8, required MHC II signalling, as Aβ−/− mice were entirely impaired in their generation. CD40−/− and ICOS−/− mice were also impaired in the generation of class switched plasma cells, although the deficiency was far smaller than in Aβ−/− mice (fig 4.5b and 4.7c). Indeed, ICOS−/− mice generated 100-fold greater numbers. This shows an absolute requirement for MHC II signalling in the generation of class switched plasma cells in *Salmonella* infection, but not co-stimulatory signals. Class switching of B cells is not necessarily mediated through interactions with CD4 T cells. Litinskiy showed that APRIL or BLyS, in combination with IL-10 or TGF-β induces switching to IgG and IgA138. In addition, direct signalling through TLRs expressed on B cells can initiate class-switching155. These mechanisms can bypass the requirement for CD40 signalling for class switching149. In *Salmonella* infection, however, MHC II signalling appears to be required. Whether this is B cell intrinsic MHC II, or that on other cell populations is unknown.

It is likely that IgG plasma cells are mainly antigen-specific, as MHC II (and therefore the activation of antigen-specific CD4 T cells) was required for their generation. In agreement with this, only wild type and MyD88/TRIF−/− mice were able to generate *Salmonella*-specific antibody by day 8 (albeit MyD88/TRIF−/− mice switching to the Th2-associated isotype IgG1). However, the slow accumulation of *Salmonella*-specific IgG in the serum of wild type mice (first appearing at day 20 and not peaking until day 65), despite large numbers of IgG plasma cells in the spleen from day 8, does not support this idea (fig 4.7 and 8b). This could be tested by the infection of SW-HEL mice, which contain a high proportion of HEL-specific B cells and, unlike MD4 B cells, are able to class switch. If many HEL-specific IgG plasma cells were seen at day 8, it would be
strong evidence that even many IgG plasma cells are not antigen-specific during the early stages of *Salmonella* infection.

Finally, CD1d−/− mice failed to respond efficiently at day 8. This may imply that either all plasma cells seen in the first 8 days of *Salmonella* infection arise from the marginal zone B cell compartment, or that a deficiency in NKT cells (seen in this strain of mice301) impairs plasma cell formation and class-switching. The contribution of these cells may be of great importance, as they recognise lipid antigens presented on CD1d, and have been shown to directly activate CD1d-expressing marginal zone B cells through cognate interactions82, and to enhance activation of other (CD1d-) B cell subsets through the secretion of IL-2183. The contribution of marginal zone and follicular B cells, and the importance of NKT cells for their differentiation to plasma cells could be further dissected by the transfer of sorted follicular or marginal zone B cells, with or without NKT cells to RAG−/− (T and B cell deficient) hosts, followed by infection with *Salmonella*. If B cell subsets were taken from different strains, such as wild type, MD4 and MyD88/TRIF−/− mice, this would allow for a greater understanding of the requirements for plasma cell generation in *Salmonella* infection.

**Extrafollicular plasma cells are generated throughout *Salmonella* infection and are supported by survival factors**

Many plasma cells were generated during the first 8-days of *Salmonella* infection (fig 4.5 and 4.7), and high numbers of plasma cells could be detected in the spleen throughout the course of infection, declining gradually over time (fig 4.3b). Using BrdU-pulses we determined that plasma cells generated in the first 8 days were not maintained throughout infection, and in fact they turned over gradually. That turnover was high during the first 8 days is unsurprising; the number of plasma cells in the spleen rose dramatically during this time, and therefore BrdU incorporation was high (fig 4.9a and b). Following the generation of these cells, turnover dropped dramatically. This, however, did not reflect long-term survival of these plasma cells as only few survived for more than 16 days (fig 4.9c). Instead, we observed a continuous (but not rapid) turnover of plasma cells with a half-life of around 6-days. This is in stark
contrast to the lifespan of plasma cells generated following SRBC immunisation, which peaked at day 5, but had mostly died by day 8 (fig 4.1).

As shown by Mohr et al, populations of plasma cells in the lymph nodes can be supported by APRIL from numerous sources following immunisation with alum-precipitated NP-OVA. APRIL in the lymph node comes predominantly from CD11b, GR1, F4/80 monocytes and macrophages. In the spleens of Salmonella infected mice, we found numerous APRIL producing populations, of which monocytes and macrophages were just one. Gr1 cells were heterogeneous, but uniformly expressed intermediate amounts of APRIL. APRIL cells (as described in chapter 3) were dramatically expanded in the infected spleen and likely provided much of the APRIL found in the organ. Here we have not quantified APRIL, nor have we quantified the proximity of the many APRIL producing cell populations to plasma cells. However, it is clear from figures 4.10a, d and e that the inflamed spleen is rich in APRIL and that many cell types that are known to support plasma cell survival expand considerably.

That SRBC immunised mice did not increase in APRIL cells, monocytes/macrophages or dendritic cells (fig 4.11a) likely means that the spleens in these mice are not rich in APRIL. This may explain why plasma cells generated during this immunisation survive for just 2-3 days. Similarly, secondary immunisation with NP-KLH and adjuvant allowed NP-specific IgG plasma cells to persist for greater periods through the generation of APRIL cells (fig 4.11b).

APRIL is likely not the only plasma cell survival factor in the inflamed spleen, and here we have not looked at the generation of other survival factors such as IL-6, TNF-α, IL-5 and CXCL12, which are thought to be of importance in the bone marrow. The role of many of these cytokines in the spleen is unknown, although IL-6 is known to support plasmablast survival. Similarly, the proximity of plasma cells to APRIL-producing cells (which could be evaluated by histology) could be important in determining which cell types are critical to the survival of plasma cells.
Chapter 4 – Extrafollicular plasma cell responses

While this data is correlative, together with previously published reports on the importance of APRIL in maintaining splenic plasma cell responses, we conclude that APRIL from numerous sources contributes to the maintenance of splenic plasma cell responses. These populations are induced in inflammatory conditions, although the precise signals required are as yet unknown.

The advantages to supporting plasma cells during inflammation are unknown, but it seems likely that the continuous generation of extrafollicular plasma cells that survive for just 2-3 days would require a high level of resources. To extend the lifespan of these cells through the provision of survival factors may relieve some of the burden of maintaining high antibody levels before the generation of high affinity long-lived plasma cells that travel to the bone marrow.

Conclusion

These data show that, following SRBC immunisation, short-lived extrafollicular responses are induced. This response is TD, requiring MHC II, CD40, ICOS and BCR signalling. Following infection with Salmonella, mice generate an early IgM response that is TI, requires TLR signalling and may arise (at least in part) from the marginal zone B cell compartment. By day 8, mice generate a class switched response, which requires MHC II signalling but is less impaired in CD40 or ICOS deficient mice. Antigen-specific antibody responses are entirely MHC II dependent in this infection, and the possibility exists that many of the early plasma cells seen in Salmonella infection are induced non-specifically, as MD4 mice make a significant plasma cell response by day 8. Turnover of extrafollicular plasma cells in Salmonella infected spleens is relatively slow, with a half-life of around 6-days, unlike those that arise during SRBC immunisation which die within 2-3 days. This is likely due to the dramatic increase in availability of survival factors in the infected spleen.
Chapter 5 – Chronic antigen supply impairs the establishment of long-lived bone marrow plasma cell populations

Introduction

Certain plasma cells, especially those arising from germinal centres during TD immune responses, travel to the bone marrow, where they are supported by survival factors and persist for long periods of time\textsuperscript{147,148,151,166,189}. Unlike the extrafollicular plasma cells discussed in chapter 4 (which could be supported for a few days), these plasma cells can survive for months or even years\textsuperscript{88,189,193,194}. This is likely a result of the constant availability of survival factors such as APRIL and IL-6 in the bone marrow under homeostatic conditions, but may also result from inherent differences between ‘short’ and ‘long’ lived plasma cells\textsuperscript{192}. Similarly, the factors that control a plasma cell’s migration from its site of generation to the bone marrow is also poorly understood; CXCL12 and its receptor CXCR4 are thought to play a role\textsuperscript{166}, but this chemokine also controls plasma cell migration from the follicle to the red pulp of the spleen\textsuperscript{78}. Further (as yet unknown) levels of control likely exist.

Long-lived plasma cells are generated in large numbers following secondary immunisation with TD antigens\textsuperscript{249}. They are also generated from the second week onwards during primary responses to such antigens, although in lower numbers. These plasma cells have been shown to persist for long periods without the need for further input from activated B cells or antigenic stimulation\textsuperscript{194,196}. Experiments utilising irradiation (which depletes B cells, but not long-lived plasma cells) showed that bone marrow plasma cells generated by prior immunisation persist for long periods even when peripheral B cells are entirely depleted, and corresponding antibody levels are hardly affected\textsuperscript{193}. These results were confirmed by efficient B cell specific depletion using anti-CD20\textsuperscript{196}. As discussed earlier, long-lived plasma cells do not require cellular division, which likely accounts for their ability to persist despite B cell depletion\textsuperscript{189}.
Interestingly, however, the persistence of pre-existing antibody levels following B cell depletion does not always occur. It was found that antibody levels to autoimmune conditions such as rheumatoid arthritis and (in some cases at least) SLE, characterised by high levels of circulating anti-self antibodies, fell following injection of anti-CD20. These diseases are the result of TD anti-self responses, which lead to the spontaneous generation of germinal centres and anti-self plasma cells (and antibody). This led to the use of human anti-CD20 (rituximab) as a treatment for such diseases, which has proved effective especially in rheumatoid arthritis.

The implication of these data is that antibody produced during certain autoimmune disease is generated exclusively by short-lived plasma cells. That short-lived plasma cells are major contributors is not entirely surprising; the unlimited availability of auto-antigen in these diseases would lead to a constant generation of new plasma cells from activated B cells, not all of which could survive in the bone marrow. However, there appears to be no phenotypic reason why some of these plasma cells could not access bone marrow survival ‘niches’ and survive for long periods, even following B cell depletion.

As there is no known phenotypic difference between plasma cells generated during autoimmune disease and infectious disease (or immunisation with exogenous antigen), we hypothesised chronic immune responses (whether against self or exogenous antigen) exclusively generate short-lived plasma cells, whereas acute immune responses generate both short and long-lived plasma cells.

In the present chapter, this hypothesis has been tested by measuring the turnover and lifespan of plasma cells generated during the immune response to a number of acute and chronic inflammatory conditions (protein antigen, autoimmunity and bacterial infection).
Chapter 5 – Long-lived plasma cells in acute and chronic responses

Results

Lifespan of plasma cells following primary and secondary immunisation with the TD protein antigen DNP-OVA

We sought to develop a system whereby the lifespan of plasma cells generated during a defined time (i.e. following immunisation) could be tracked. To accomplish this, we took advantage of the fact that antibody secreting cells divide rapidly during their formation (as plasmablasts), but stop division upon maturation into plasma cells. By providing a sustained ‘pulse’ of BrdU, all plasmablasts generated during that pulse are labelled. Those that mature to non-dividing plasma cells during this time retain BrdU in their DNA until cell death occurs. BrdU is rapidly lost from cells that continue to divide after the ‘pulse’ has ended. Therefore, long-lived plasma cells generated during a BrdU pulse can be detected, as they will maintain BrdU for long periods after the pulse has finished.

Figure 5.1a shows a schematic of the experimental design. Mice were immunised sub-cutaneously with DNP-OVA (suspended in incomplete Freund’s adjuvant with 10ug LPS) at day 1. Either ‘early’ (days 4-6) or ‘late’ (days 12-14) following immunisation, mice were injected with 2mg BrdU into the intraperitoneal cavity at 24 hour intervals; labelling plasma cells generated during the extrafollicular and germinal centre phases respectively. A control group received a 3-day BrdU pulse, but was not immunised. Finally, a forth group was boosted intravenously with 100ug soluble DNP-OVA (at day 35 after primary immunisations). This was followed with a 3-day BrdU pulse at days 38-40. Mice were then culled at 7 and 22 days following BrdU.

Figure 5.1b shows representative FACS plots (summarised in fig 5.1d) of BrdU retention by splenic plasma cells either 7 days (middle row) or 22 days (bottom row) after BrdU pulses in the various groups (for plasma cell gating, see top row). In all immunised groups just 6.93% (±1.21), 7.34% (±0.47) and 7.81% (±0.77) of splenic plasma cells in early, late or boosted mice respectively retained BrdU 7-days after pulsing. After 22 days, 1.52% (±0.12), 1.93% (±0.59)
and 3.01% (±1.08) of splenic plasma cells retained BrdU. There was no significant difference between any of the immunised groups. Naïve mice had a lower proportion of labelled splenic plasma cells at both 7 after pulsing (3.07% ±0.59), with only very low proportions of labelled plasma cells surviving to 22 days after pulsing (0.025% ±0.015).

11.14% (±1.59) of bone marrow plasma cells in primed mice pulsed 'late' (day 12-14) retained BrdU 7 days after pulsing, however few of these (1.66% ±0.19) survived for 22 days (fig 5.1c and 5.1d, left). This was similar for primed mice pulsed ‘early’ (days 4-6) and naïve mice, which generated few plasma cells that survived for 22 days after pulsing (6.94% ±0.44 to 0.71 ±0.04). Boosted mice however, generated detectable populations of plasma cells that retained their BrdU label. This population did not decline between days 7 and 22 following BrdU and therefore is, by definition, long-lived (4.43% ±0.61 to 4.25% ±0.89).

**Generation of stable, antigen-specific populations of bone marrow plasma cells following secondary immunisation with the TD protein antigen NP-KLH**

The data shown in figure 5.1 confirmed the data of others; following secondary immunisation, populations of long-lived plasma cells could be detected in the bone marrow. We sought to enhance this system, with the aim of detecting the lifespan of antigen-specific plasma cells following immunisation. We therefore modified our protocol, and instead used the TD protein antigen NP-KLH, as it is possible to detect NP-specific plasma cells on a flow cytometer by intracellular staining with fluorescently labelled NP (we had little success in using labelled DNP to detect DNP-specific plasma cells). Similarly, we found that larger splenic plasma cell responses were obtained through primary immunisation with alum-precipitated NP-KLH with killed *Bordetella pertussis* via intraperitoneal injection.

Initially, we examined the kinetics of NP-specific plasma cell generation following primary and secondary immunisation with NP-KLH. Figure 5.2a shows representative FACS plots from spleen and bone marrow of naïve mice (left) and NP-KLH primed mice at day 5, 9 and 45, gated on IgG plasma cells.
Despite substantial splenic plasma cell generation (see fig 3.1a) in primed mice, only low proportions of these bound intracellular NP (fig 5.2a and c). Similarly, in the bone marrow, few NP-specific plasma cells were seen. 45 days following immunisation, around 3.57\% (±0.31) splenic and 6.17\% (±0.52) bone marrow plasma cells were NP-specific.

In contrast to this, following secondary immunisation, a rapid generation of NP-specific plasma cells could be seen in the spleen by day 5 (fig 5.2b and c). Initially, these were immature, MHC II\textsuperscript{hi} plasma cells but rapidly matured into MHC II\textsuperscript{lo} plasma cells by day 9. NP-specific plasma cells appeared in the bone marrow rapidly and came to dominate the bone marrow plasma cell compartment just 9 days following secondary immunisation (41.10\% ±7.97). Plasma cells generated during the secondary immune response to NP-KLH continued to dominate the bone marrow plasma cell compartment to day 45, and numbers were also maintained (figures 5.2b, c and d). In the spleen, numbers of NP-specific plasma cells declined rapidly between day 9 and 25 (4.87x10\textsuperscript{6} ±1.53x10\textsuperscript{6} to 1.87x10\textsuperscript{5} ±7.92x10\textsuperscript{4}; fig 5.2d) and continued to decline at a slower rate until day 45 (1.09x10\textsuperscript{5} ±2.99x10\textsuperscript{4}). This represented a decline in the total plasma cell population, as proportionally NP-specific plasma cells continued to dominate the splenic plasma cell pool.

NP-specific antibody levels (IgM and the IgG isotypes) were measured throughout the time course of primary and secondary immunisations (fig 5.2e). As would be expected, NP-specific IgM was rapidly generated following primary immunisation (levels rose by day 5, but continued to climb until day 25), whereas NP-specific IgG was not generated until day 9. This too peaked at day 25 and was maintained until the end of the study (day 45). Kinetics were similar for all IgG isotypes. By day 9 following secondary immunisation, antibody levels rose to around a log fold higher than at the peak of the primary response. This was accompanied by a slow decline throughout the remainder of the time course, with levels remaining higher than mice only receiving primary immunisation.
NP-specific plasma cells in the spleen and bone marrow of mice 25 days after secondary immunisation with NP-KLH are non-dividing, long-lived plasma cells

To confirm that the NP-specific plasma cells seen in the bone marrow of NP-KLH boosted mice were long-lived, NP-KLH boosted mice were provided with BrdU for a 10-day period (at days 16-25 after boost). Long-lived plasma cells (which survive for long periods without division) would not incorporate BrdU, whereas short-lived plasma cells (which require constant replenishment from the activated B cell pool every few days, involving cellular division) would incorporate BrdU. Figure 5.3a shows representative FACS plots gated on IgG plasma cells from boosted mice or primed controls (which were primed at the same time as boosted mice, but received no secondary immunisation) showing BrdU incorporation of NP-specific or non-NP-specific plasma cells in the spleen and bone marrow. NP-specific plasma cells, at this stage following secondary immunisation, had only a very low turnover. It can be seen in figure 5.3b that NP-specific plasma cells in both spleen and bone marrow were primarily long-lived, with just 6.26% (±2.40) and 7.01% (±2.54) incorporating BrdU in the spleen and bone marrow respectively in a 10-day pulse.

Long-lived bone marrow plasma cell generation is impaired in mice receiving a continuous ‘chronic’ supply of NP-KLH compared to those receiving a single ‘acute’ dose

The above figures confirmed that an acute secondary immunisation with NP-KLH generated long-lived bone marrow plasma cells. We next sought to directly compare the generation of long-lived plasma cells when antigen was delivered in a single acute dose, or a continuous supply over a long period. We chose to use a secondary immunisation system, as the above figures show it produces a higher proportion of antigen-specific bone marrow plasma cells; as these cells are relatively rare, this was an important consideration to produce more reliable data.

A schematic of the experiment is shown in figure 5.4a. Briefly, mice were primed with alum-precipitated NP-KLH and B. pertussis and rested for 5 weeks. The first group of mice received no secondary immunisation (primed controls),
Chapter 5 – Long-lived plasma cells in acute and chronic responses

A second group of mice received a single dose of 100μg soluble NP-KLH intravenously (acute group), while a third group received 20 doses of 5μg soluble NP-KLH (for a total of 100μg) intravenously at 3-day intervals over a 60-day period (chronic group). At three time points, separate groups of mice were given a 10-day pulse of BrdU and culled immediately afterwards. This allowed us to assess the number of NP-specific plasma cells in the spleen and bone marrow, and whether NP-specific plasma cells were short or long-lived. BrdU was given at day 1-10 (day 1 was the day when the acute group received their secondary immunisation, and when the chronic group's series of immunisations began), day 51-60 (day 60 was when the chronic group's series of immunisations ended) and day 111-120.

It can be seen in figure 5.4b that at day 10, the acute group had high numbers of NP-specific plasma cells and, as in figure 5.3, had established substantial populations of NP-specific plasma cells in the bone marrow. Figure 5.4c shows that the majority (70.85% ±6.31) of NP-specific plasma cells in the spleen of acutely immunised group had incorporated BrdU in the first 10-days following immunisation. Similarly in the bone marrow 56.98% (±4.79) of NP-specific plasma cells had incorporated BrdU. Numbers of NP-specific plasma cells in the spleen had declined dramatically by day 60 and, although turnover had decreased from the day 10 time point (to 39.45% ±2.76). The dramatic drop in dividing NP-specific plasma cells can be seen in figure 5.4d. These trends were continued out to day 120, although by this time point turnover of NP-specific plasma cells in the spleen had decreased further to 20.17% (±3.90). In the bone marrow at day 60, NP-specific plasma cells had declined only very slightly, populations remained stable until the end of the experiment. Turnover of NP-specific plasma cells in the bone marrow was also low, 21.08% (±2.47) at day 60, and 5.91% (±1.03) by day 120. These data, in agreement with that from earlier figures, suggests that acute secondary immunisation with NP-KLH generates long-lived bone marrow plasma cell populations.

The chronic group had generated significantly fewer NP-specific splenic plasma cells by day 10 compared to mice in the acute group (fig 5.4b). This was not
surprising, as they had received only a sixth of their complete dose at this point of the experiment. Similarly, they had significantly fewer NP-specific plasma cells in the bone marrow. Turnover of NP-specific plasma cells was high in spleen and bone marrow (71.52% ± 2.74) and 45.63% ± 3.85 respectively). At day 60, when chronically stimulated mice had received the full dose of NP-KLH, the number of splenic plasma cells remained similar to that seen at day 10, as would be expected. In fact, figure 5.4c and d show that a similar proportion and number of NP-specific plasma cells incorporated BrdU when pulsed at days 1-10 or days 50-60, showing that NP-specific plasma cells were continuously generated throughout the 60-day period of immunisations. Remarkably, despite this fact, populations of NP-specific plasma cells in the bone marrow had not increased since day 10. BrdU incorporation of NP-specific plasma cells in the bone marrow fell considerably by day 60 (22.13% ± 2.19) and was comparable to turnover of NP-specific plasma cells in the bone marrow of acutely immunised mice. Figure 5.4d confirms that only very few ‘new’ NP-specific plasma cells were generated during days 51-60. As immunisations in these mice had now stopped, we looked at day 120 to see if the NP-specific plasma cells that were seen in chronically stimulated mice would persist without further antigenic stimulation. Indeed, numbers at day 120 were similar to those at earlier time points, while the percentage of these plasma cells incorporating BrdU had dropped to 7.12% ± 1.30. Figure 5.4d shows that very few new NP-specific plasma cells were generated in the spleen or bone marrow of these mice between days 111-120.

These data indicate that, despite continuous generation of new splenic plasma cells in chronically stimulated mice, few bone marrow plasma cells were formed compared to acutely stimulated mice. The NP-specific plasma cells that were formed appeared to be generated primarily in the first 10-days of the response, and were able to persist after NP-KLH immunisations had ceased.
NP-specific antibody levels following acute or chronic secondary immunisation with NP-KLH

To evaluate whether chronic provision of NP-KLH leads to reduced NP-specific antibody levels, or antibody levels that fall once antigen provision has stopped, we assessed the serum of the mice described above for NP-specific antibody (fig 5.5). Acutely immunised mice generated antibody similarly to mice evaluated previously. NP-specific IgM and IgG (of all isotypes) levels peaked at day 10 following secondary immunisation and gradually declined thereafter. Generally this was a fairly linear decline to day 120, when the experiment finished. During that period, levels fell by around 10-fold. This was similar to the decline seen in primed controls, in which levels fell at a similar rate (albeit starting from around a log-fold lower).

Chronically stimulated mice received antigen for the first 60-days of the study, and therefore it was not surprising that levels of most of the classes assessed here remained constant during that period (although levels to IgG2b and IgG3 fell during this period). Following this, when antigen was no longer provided to these mice, levels uniformly fell. In many cases (IgM, IgG1 and IgG2b) levels fell slightly faster than those of the acute group. However, IgG2b fell slower, while IgG3 fell at approximately the same rate in the two groups. Only for IgM and IgG1 were the differences between the acute and chronic groups significantly different by day 120.

Autoimmune K/BxN mice continuously generate large splenic plasma cell populations, but few long-lived bone marrow plasma cells

The data above suggest that while acute immunisation generates both short and long-lived plasma cells, chronic antigenic stimulation with NP-KLH generates mainly short-lived splenic plasma cells and few long-lived bone marrow plasma cells. As this very property is utilised to treat autoimmune patients via depletion of B cells, we were interested in testing the principle in autoimmune mice. K/BxN mice are the F1 progeny of NOD mice and TCR transgenic KRN mice. By chance T cells in these mice recognise the ubiquitous glycolytic enzyme glucose-6-phosphate isomerase (G6PI) in the context of the I-Ag7 MHC II molecule (the
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only MHC II molecule expressed by NOD mice). These mice go on to spontaneously develop a TD, antibody mediated autoimmune disease characterised by swollen joints, germinal centres, splenic plasma cells and high levels of anti-G6PI. This has made these mice a commonly used model of rheumatoid arthritis; a disease where the use of rituximab to deplete B cells (and thereby reduce levels of auto-antibody secreted by short-lived plasma cells) is a widespread one. At our facility, these mice are bred as heterozygotes, which generates 50% of mice that develop arthritis and 50% that do not.

We first compared the spleens of non-arthritic and arthritic K/BxN mice (hereafter referred to as non-arthritic and arthritic mice) for plasma cell generation. Figure 5.6a (top row) shows that arthritic mice had considerably larger splenic plasma cell populations compared to non-arthritic controls (0.41% (±0.02) in arthritic mice, 0.11% (±0.02) in non-arthritic). Figure 5.6b confirms that arthritic mice had significantly higher numbers of plasma cells per spleen compared to non-arthritic control mice (1.93x10⁶ (±1.90x10⁵) in arthritic mice and 1.13x10⁵ (±1.07x10⁴) in non-arthritic). By pulsing mice with BrdU for 3-days, it was revealed that plasma cells in the spleens of arthritic mice turned over slightly faster than those in control mice (40.79% (±3.06) in arthritic mice, 28.69% (±1.74) in non-arthritic mice). We next performed a BrdU pulse-chase experiment, where age matched arthritic and non-arthritic mice were given a 3-day BrdU pulse following the onset of disease (at days 21-24 after birth). Immediately after BrdU pulsing (day 1), some mice were culled and analysed for BrdU labelled plasma cells. Following this, a ‘chase’ period ensued, whereby mice were culled and analysed for surviving BrdU labelled plasma cells at day 8, 18 and 28 after BrdU pulsing. Plasma cells retaining the label are long-lived by definition.

Immediately after BrdU pulsing, arthritic mice contained a higher proportion of labelled plasma cells than non-arthritic control mice, and this corresponded to 5-fold higher numbers (fig 5.6d and e, left graphs). Conversely, non-arthritic mice had higher proportions of labelled plasma cells in the bone marrow, corresponding to significantly higher numbers (fig 5.6d and e, right).
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Interestingly, just 8 days after pulsing, labelled plasma cells had entirely decayed in both groups. This suggests that, although arthritic mice generate significantly higher numbers of splenic plasma cells in a 3-day period, very few of these travel to the bone marrow (as fewer BrdU labelled plasma cells were seen in the bone marrow of arthritic mice than control mice). Similarly, although 28.06% (±3.69) of bone marrow plasma cells were labelled in arthritic mice, few of these plasma cells survived for more than a few days (1.13% (±0.39) 7-days after BrdU pulsing, and 0.47% (±0.08) 21-days after BrdU); and no more than survived in non-arthritic control mice. This is strongly indicative that arthritic K/BxN mice do not generate long-lived plasma cells.

Few long-lived bone marrow plasma cells are generated during the peak of *Salmonella* infection

In the previous data, we observed that chronic antigen supply leads to decreased long-lived bone marrow plasma cell formation. This has been shown using a protein immunisation system, and in an autoimmune setting. We next infected mice with *Salmonella*. This model has been described elsewhere (see chapter 4), but briefly is a long-term bacterial infection, taking around 65 days to clear. During this time, large numbers of splenic plasma cells are produced. We now used a similar BrdU pulse-chase system to determine whether any of these plasma cells were travelling to the bone marrow and surviving for long periods of time.

Firstly, we infected mice with *Salmonella* and examined the spleen and bone marrow for plasma cells over a time course of 65 days. Plasma cells in the spleen peaked at day 8 and declined gradually thereafter until the resolution of infection at day 65. Interestingly, as plasma cell generation peaked in the spleen, there was a significant decrease in plasma cells in the bone marrow (fig 5.7a). Bone marrow plasma cells gradually recovered by day 65.

Secondly, we infected mice and gave 4-day BrdU pulses at various times following infection. Mice were culled immediately after BrdU and the proportion and number of BrdU labelled plasma cells in the spleen and bone
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marrow was calculated. Figure 5.7b shows that, early following infection,
turnover of plasma cells in both the spleen and bone marrow is high (66.75% (+4.04) and 81.58% (±2.37) in the spleen during days 1-4 and 5-8, and 40.93% (+1.39) and 51.75% (±4.75) in the bone marrow during days 1-4 and 5-8 respectively). The number of plasma cells produced in the spleen maps closely
to the bacterial loads of the organ (fig 5.7c). There was, however, a sharp
decline in new plasma cell entry to the bone marrow after day 8, which
persisted until bacteria loads were extremely low at day 55 (fig 5.7b and c).
These data imply that early following infection, even when antigen is in constant
supply, some plasma cells enter the bone marrow. Following this, few plasma
cells enter the bone marrow until bacterial loads fall.

Finally, we performed a BrdU pulse-chase experiment, whereby mice were
infected with *Salmonella* and given 3-day BrdU pulses at either days 5-8 (early)
or days 25-28 (late). Mice were culled 7 or 28 days after BrdU pulses and spleen
and bone marrow examined for BrdU labelled plasma cells. Mice pulsed early or
late following infection rapidly lost BrdU labelled plasma cells in both the spleen
and bone marrow (fig 5.7d). In the early group, just 3.07% (±0.59) of splenic
plasma cells retained the BrdU label 7 days after pulsing and no plasma cells
retained the label 28 days after pulsing (0.00% ±0.00). Similarly, in the bone
marrow, only 2.48% (±0.54) and 0.52% (±0.32) retained the label at 8 and 28
days after pulsing respectively. Results were similar for mice pulsed later, with
few plasma cells surviving in the spleen. However 28 days after labelling, 1.57% (+0.22) of bone marrow plasma cells retained BrdU in this group. These data
indicate that, despite plasma cell entry into the bone marrow early on following
infection, these plasma cells are relatively short-lived. Although fewer plasma
cells enter the bone marrow at later time points, these cells do have an
increased capacity to become long-lived. It could be speculated that plasma cells
labelled around day 55 (when antigenic loads are relatively low) may persist for
longer periods.

Figure 5.7e shows that, despite failure of plasma cells in infected mice to enter
the bone marrow, they express similar levels of CXCR4 to plasma cells from the
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spleens of mice 5 days after boosting with NP-KLH (MFI= 1.46x10^3 ±6.05x10^1 in NP-boosted mice, 1.64x10^3 ±7.02x10^1 at day 20 of infection). Interestingly, bone marrow plasma cells from infected mice express significantly higher levels of CXCR4 (MFI= 1.38x10^3 ±4.51x10^1 in NP-boosted mice, 1.73x10^3 ±4.57x10^1 at day 20 of infection).
Figure 5.1

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Figure 5.1 continues overleaf
Figure 5.1 Cont.

Figure 5.1. Long lived bone marrow plasma cell formation following primary and secondary immunisation with the protein antigen DNP-OVA. (A) Schematic showing experimental design. (B) FACS plots showing plasma cell gating (top row) and BrdU labeling of splenic plasma cells either 7 or 21 days after a 3-day BrdU pulse. (C) as for (B), but bone marrow plasma cells. (D) Summary graphs of data in (B) and (C), dotted line: naive, dashed line: DNP-OVA-immunised, early pulsed, solid line: DNP-OVA-immunised, late pulsed; dotted/dashed line: DNP-OVA primed and boosted mice. FACS plots and points on graph representative of 4 mice per group. Error bars=standard deviation. Results representative of 2 independent experiments.
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**Figure 5.2**

A

**Naive**

Day 5

**Primary**

Day 5

Day 9

Day 45

Spleen

Bone Marrow

Int NP

Int NP

MHC II

MHC II

B

**Secondary**

Day 5

Day 9

Day 45

Spleen

Bone Marrow

MHC II

Int NP

Int NP

Figure 5.2 continues overleaf
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Figure 5.2 Cont.

C

Spleen Percentage PC

Bone Marrow Percentage PC

D

Spleen Number NP-PC

Bone Marrow Number NP-PC

Figure 5.2 continues overleaf
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Figure 5.2 Cont.

E

IgM

\[ \text{Titre} \]

\[ 1.0 \times 10^1 \]

\[ 1.0 \times 10^2 \]

\[ 1.0 \times 10^3 \]

\[ 1.0 \times 10^4 \]

\[ 1.0 \times 10^5 \]

Day

- ■ Primary
- ▲ Secondary

IgG1

IgG2b

IgG2c

IgG3

\[ \text{Titre} \]

\[ 1.0 \times 10^1 \]

\[ 1.0 \times 10^2 \]

\[ 1.0 \times 10^3 \]

\[ 1.0 \times 10^4 \]

\[ 1.0 \times 10^5 \]

Day

Day

Day

Day

Figure 5.2. NP-specific plasma cells persist for long periods following secondary immunisation with NP-KLH, maintaining high titres of NP-specific antibody. 
(A) FACS plots gated on IgG plasma cells showing MHC II expression and NP-specificity in spleens and bone marrow of naïve and NP-KLH primed mice. (B) As for (A), but following secondary immunisation with NP-KLH. (C) Percentage of IgG (solid) or IgM (dashed) plasma cells that were NP-specific in the spleens and bone marrow of NP-KLH primed (grey) and boosted (black) mice. (D) as for (C) but numbers of NP-specific plasma cells per organ. (E) NP-specific antibody titres in the serum of NP primed (dashed) or boosted (solid) mice. Points on all graphs represent 4-5 mice, error bars=standard deviation. Results representative of at least 3 independent experiments.
Figure 5.3. NP-Specific plasma cells found in the spleen and bone marrow of mice 25 days after secondary immunisation with NP-KLH are non-dividing, long lived plasma cells. (A) FACS plots showing BrdU incorporation of NP-specific IgG plasma cells in the spleen and bone marrow of NP-KLH primed or primed and boosted mice, given BrdU for 10-days at day 16-25 following respective immunisation. (B) Summary graph showing percentage of BrdU+ NP-specific plasma cells in the spleen (white) and bone marrow (black) of boosted mice described above. FACS plots and bars on graph representative of 4-5 mice per group. Error bars=standard deviation. Results representative of 3 independent experiments.
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Figure 5.4

A

All groups primed 100µg NP-KLH

Acute Group boosted 100µg NP-KLH (day 1)

Time Point 1
day 10

Time Point 2
day 60

Time Point 3
day 120

5 Weeks

Chronic Group boosted with 20 doses of 5µg NP-KLH at 3 day intervals (days 1-60)

10 day BrdU pulse

Cull Time Point

B

Spleen Number NP-PC

Bone Marrow Number NP-PC

Number per Spleen

Number per 100mg Femur

Antigen to Chronic Group

Days After Initial Boost

Antigen to Chronic Group

Days After Initial Boost

*** ns ns

*** *** **

Figure 5.4 continues overleaf
Figure 5.4 Cont.

C

Spleen % NP-PC BrdU+

Bone Marrow % NP-PC BrdU+

% NP-PC

Days After Initial Boost

BrdU Labeling

Periods

Days After Initial Boost

D

Spleen Number NP-PC BrdU+

Bone Marrow Number NP-PC BrdU+

Number per Spleen

Number per 100mg Femur

Days After Initial Boost

BrdU Labeling

Periods

Figure 5.4. Mice immunised with NP-KLH over an extended period (‘chronic NP-KLH’) generate fewer NP-specific long lived bone marrow plasma cells than mice immunised with NP-KLH in an acute fashion. (A) Schematic showing experimental design. (B) Numbers of NP-specific plasma cells in the spleen and bone marrow of mice primed (dotted), primed and acutely boosted (solid) or primed and chronically boosted (dashed) with NP-KLH. (C) Percentage of NP-specific IgG plasma cells in spleen or bone marrow of mice from (B) incorporating BrdU during a 10-day pulse before culling. (D) As for (C), but showing numbers of BrdU+ NP-specific IgG plasma cells per organ. Points on graphs=5-6 mice per group, error bars=standard deviation. Results represent a single experiment.
Figure 5.5. NP-specific antibody titres fall at similar rates in mice boosted with NP-KLH in an acute or chronic fashion.

NP-specific antibody titres from serum of mice primed (dotted), primed and acutely boosted (solid) or primed and chronically boosted (dashed) with NP-KLH. Dashed line underneath graphs indicates the duration of antigen provision to chronically boosted mice. Points on graphs=5-6 mice per group, error bars=standard deviation. Results represent a single experiment.
Figure 5.6. K/BxN mice fail to generate long lived bone marrow plasma cells. (A) Turnover of CD138^hi cells in spleens of arthritic and non-arthritic K/BxN mice given BrdU for 3-days. (B) Total numbers of plasma cells in the spleen and bone marrow of mice from (A). (C) Percentage of CD138^hi cells that incorporate and retain BrdU following a 10-day pulse in the spleen and bone marrow of K/BxN mice. (D) Numbers of BrdU+ plasma cells in the spleen and bone marrow of mice from (C). Points on graphs=5-8 mice per group, error bars=standard deviation. Results are representative of 3 independent experiments.
Figure 5.7

A  Number PC per Spleen

Number per Spleen

1.0x10^4
1.0x10^5
1.0x10^1
1.0x10^1

Day post Infection

B  % PC BrdU+

% PC BrdU+

Day post Infection

C  Bacterial Load per Gram of Spleen

CFU per Gram Spleen

Day post Infection

D  % Splenic PC BrdU+ % Bone Marrow PC BrdU+

% PC BrdU+

Days after BrdU

Figure 5.7 continues overleaf
Figure 5.7 Cont.

Figure 5.7. Mice infected with *Salmonella* generate large splenic plasma cell populations throughout the course of infection, but few of these survive in the bone marrow. (A) Number of plasma cells in the spleen and bone marrow of *Salmonella* infected mice through a time course of infection. (B) BrdU incorporation of splenic or bone marrow plasma cells given 4-day BrdU pulses at various times post-infection (IgM: dashed line, IgG: solid line). (C) Bacterial loads in the spleens of mice from (B). Points on graphs=5-7 mice per group, error bars=standard deviation. Results representative of at least 5 independent experiments (D) Percentage of plasma cells in the spleen or bone marrow of naive (dashed) or *Salmonella* infected mice that remained BrdU+ either 7 or 28 days after a 3-day BrdU pulse at days 5-8 (triangle) or 25-28 (inverted triangle). Points on graphs=3 mice per group, error bars=standard deviation. Results representative of 2 independent experiments. (E) FACS plots (top) (representative of 5 mice per group) and summary graphs (bottom) showing CXCR4 expression of splenic or bone marrow plasma cells from mice immunised and boosted with NP-KLH (at day-5 after boost) or infected with *Salmonella* (at day 16 post-infection). Points on graphs=5 mice per group, error bars=standard deviation. Results represent a single experiment.
Discussion

In this chapter we show that during chronic inflammatory conditions, few long-lived bone marrow plasma cells are formed. We use protein immunisation, and models of autoimmune conditions and long-term bacterial infection to confirm these findings.

**Acute secondary immunisation with protein antigen generates detectable long-lived plasma cells**

It is well established in the literature that long-lived bone marrow plasma cells are formed following immunisation with protein antigen\(^ {193,303}\). Additionally, it is known that greater numbers of these plasma cells are generated by secondary immunisation\(^ {189}\). However, as discussed previously, there are no known phenotypic markers by which to distinguish long-lived plasma cells. We therefore developed a model by which we could track the lifespan of plasma cells generated during a defined period. A sustained pulse of BrdU given to mice following immunisation, labels any dividing plasmablasts. As these mature into non-dividing plasma cells, they retain BrdU for an indefinite period. Therefore survival of plasma cells generated during the BrdU pulse can be evaluated at later time points by their retention of the label.

We tested this method using mice that were primed or boosted with DNP-OVA. Mice given BrdU at days 4-6 following primary immunisation maintained few plasma cells in the spleen or bone marrow (fig 5.1b and c), likely due to the fact that mainly extrafollicular plasma cells are formed during this time, and these are not thought to become long-lived\(^ {199}\). That few surviving plasma cells were seen from day 12-14 (during the germinal centre phase) of primary immunisation likely reflects data from other groups which states that far more long-lived plasma cells are formed after secondary immunisation\(^ {189,193,194}\). Indeed, we could identify detectable surviving plasma cells in the bone marrow, but not the spleen, of boosted mice, in agreement with data that suggests that the bone marrow is the major site of long-lived plasma cell retention\(^ {193,194,317}\).
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Our own data confirmed that, when using a system allowing for the detection of antigen-specific (NP-specific) plasma cells, primary immunisation generates only relatively few NP-specific plasma cells in comparison to secondary immunisation (fig 5.2b and c). Additionally, far fewer of these travelled to the bone marrow. Following secondary immunisation, conversely, almost half of all bone marrow plasma cells were NP-specific, and these persisted in the bone marrow throughout the course of the experiment. These likely provided much of the NP-specific serum antibody, which exhibited only a very slow decline (fig 5.2e) (unlike NP-specific plasma cells in the spleen, which declined more rapidly (fig 5.2d)).

It can be seen in figure 5.3 that by day 15 following secondary immunisation NP-specific plasma cells in the spleen and bone marrow are long-lived, non-dividing cells. However, there is an important distinction between these. While the spleen is able to support non-dividing plasma cells during periods of inflammation, when survival factors are readily available in the organ (see chapter 4), these survival factors decline as inflammation resolves, and this causes a gradual decline in the number of non-dividing plasma cells which the spleen can support (fig 5.2d)\(^88\). The bone marrow, however, provides a constant level of plasma cell survival factors, even after inflammation has resolved, and therefore is able to support the survival of plasma cells for truly long periods\(^151,189,217\). Therefore, for generating long term, high levels of antibody, entry into the bone marrow is critical.

**Chronic secondary immunisation with NP-KLH generates few bone marrow plasma cells**

By providing NP-KLH in a chronic fashion, we saw that NP-specific plasma cells were produced continuously in the spleen throughout the 60-day period of antigen administration (fig 5.4c and d, left). Despite this fact, few NP-specific plasma cells entered the bone marrow (fig 5.4b, right). It is likely that the vast majority of NP-specific plasma cells that were able to enter the bone marrow did so in the first 10 days of antigen administration, as it can be seen that few newly generated (BrdU labelled) NP-specific plasma cells were present in the
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bone marrow during the final 10 days of antigen administration (fig 5.4d, right). In fact, the BrdU incorporation of NP-specific plasma cells was not different to that seen in the acutely immunised group (fig 5.4c, left).

Why this may be is unknown, however it would seem reasonable that mechanisms exist to stop the bone marrow plasma cell compartment from becoming overrun by plasma cells of a single specificity. This compartment is of limited size, and does not expand greatly following immunisation (fig 3.1c). In much the same way as other ‘memory’ pools are regulated, newly generated cells enter the pool and compete with older cells (generated during previous immune responses) for a limited supply of survival factors. Therefore, if a constant stream of long-lived plasma cells of a single specificity were produced (during chronic immune responses), they would eventually come to dominate the pool, and antibody levels to prior infections would fall. Our data suggests that there is a finite window during which long-lived plasma cells are able to enter the bone marrow during chronic inflammation (i.e. the first 10 days during secondary responses), and once past, despite the continued generation of plasma cells, they do not migrate to the bone marrow.

In this experiment, we saw that NP-specific antibody levels were maintained in both the acute and chronic group, even following the termination of administration of antigen (fig 5.5). Antibody levels declined in both groups at similar rates, although were significantly lower in the chronic group. This likely reflects the generation of fewer NP-specific bone marrow plasma cells in the chronic group. However, the plasma cells that did enter the bone marrow appear to be as proficient as those generated by acute immunisation at surviving and secreting antibody. This is in contrast to reports from human and mouse studies of chronic autoimmune disease treated with rituximab or anti-mouse CD20 respectively, where autoantibody levels were reported to drop far more rapidly than anti-vaccine antibodies. This would suggest a more complete failure of plasma cells to enter the bone marrow compartment.
Autoimmune K/BxN mice generate few long-lived bone marrow plasma cells

Autoimmune K/BxN mice generated large numbers of splenic plasma cells during a 3-day BrdU pulse (fig 5.6a). Turnover was significantly higher than that seen in non-arthritic control mice (fig 5.6c), but was not rapid (40.79% (±3.06) in arthritic mice, 28.69% (±1.74) in non-arthritic mice). This was similar to data from Salmonella infected mice, where populations of plasma cells in the spleen turned over with a half-life of over 6 days (fig 4.9). In agreement with our data, Huang et al showed a similar turnover of plasma cells in the spleen261. This moderate turnover likely results from an increased availability of plasma cell survival factors present in the inflamed spleen of these mice. Indeed, plasma cells generated during chronic autoimmune conditions have no impairment in their ability to persist in inflamed organs provided there are sufficient survival factors; Hoyer et al used NZB/W mice (a model of SLE) to show that plasma cells in the inflamed spleen and kidney were not rapidly dividing, and could survive for significant periods without division228. Similarly, Ferraro et al hypothesised that the relatively slow decline of autoantibody levels following depletion of B cells is due to the persistence of plasma cells in survival niches in inflamed organs, which gradually diminish259.

Despite this, arthritic mice generated large numbers of plasma cells during a 3-day period (fig 5.6d, left), far larger than the numbers generated in non-arthritic control mice. This indicates that, while some plasma cells may be able to persist for relatively long periods in inflamed organs, constant availability of antigen in chronic conditions still drives the continuous generation of new plasma cells. In agreement with data from mice chronically stimulated with NP-KLH, plasma cells generated in the spleen of arthritic mice did not travel to the bone marrow or survive for long periods. In fact, fewer labelled plasma cells were seen in the bone marrow of arthritic mice than non-arthritic controls, and only background numbers of labelled plasma cells survived for 7-days or more (fig 5.6d, right).

Data from Huang et al, showing that treating K/BxN mice co-expressing mouse and human CD20 with rituximab depleted G6PI-specific plasma cells in the spleen and lymph nodes, and caused a decline of anti-G6PI antibody levels261.
Our data compliments theirs, by showing that plasma cells produced in the spleen of arthritic mice fail to accumulate in the bone marrow, and this is likely the reason why treatment with anti-CD20 antibodies is effective for autoimmune conditions, but does not diminish antibody levels to prior vaccination (or infection).

*Salmonella* infection generates early plasma cells that travel to the bone marrow but do not survive

*Salmonella* is a long term infection, during which bacterial loads peak in the first 10 days followed by a gradual decline, taking around 65-70 days to clear. We saw a depletion of bone marrow plasma cells in the first 20 days, followed by a gradual recovery (fig 5.7a). Despite a decrease in numbers of bone marrow plasma cells during this time, newly generated plasma cells did enter the bone marrow in the first 10-days of infection (as determined by BrdU labelling, fig 5.7b, right). Indeed, this may itself mediate the observed loss of bone marrow plasma cells, as plasma cells generated at days 7-10 of infection likely displace pre-established long-lived plasma cells, but do not survive for more than a few days (fig 5.7d, right).

When BrdU-pulsed at days 15-18, few new plasma cells entered the bone marrow of infected mice (fig 5.7b, right). This is similar to data from chronic NP-KLH immunisations, where plasma cells entered the bone marrow in the first 10-days of antigen administration, but not after. In that experiment, NP-specific plasma cells survived for long periods; this likely reflects the fact that these were generated as part of a secondary response, which rapidly generates long-lived plasma cells, whereas those generated during the first week of *Salmonella* infection are extrafollicular plasma cells, which do not become long-lived. The very fact that they enter the bone marrow is surprising, and the reason for their entry is unknown.

Plasma cells generated during days 28-31 (i.e. the chronic phase of *Salmonella* infection) did not survive in the bone marrow for long periods (fig 5.7d, right). However, data from this model is confounded by the fact that germinal centres
Long-lived plasma cells in acute and chronic responses do not form in *Salmonella* infection until day 50-55 (fig 4.3a), so these plasma cells are still extrafollicular. It would therefore be interesting to see if plasma cells generated around the time of germinal centre formation do migrate to the bone marrow and become long-lived. Our data indicates that a second ‘wave’ of plasma cells is generated and enters the bone marrow at days 51-55 (fig 5.7b, right), although whether these survive for long periods would require further experimentation.

Despite the failure of plasma cells from day 20 of infected mice to enter the bone marrow, they expressed similar levels of CXCR4 to plasma cells generated at day 5 following secondary NP-KLH immunisation (fig 5.7f). This chemokine receptor is thought to mediate migration to the bone marrow in response to CXCL12 from bone marrow stromal cells\(^{166}\). Similar expression of the chemokine receptor does not necessarily mean similar responsiveness to CXCL12, as K/BxN mice that lacked T regulatory cells generated plasma cells that failed to migrate in response to CXCL12, despite similar expression of CXCR4\(^{333}\). Additionally, following establishment of plasma cells in the bone marrow, plasma cells lose the ability to migrate towards the chemokine, but maintain expression of CXCR4; it is thought to be required for their survival and may also provide signals which mediate plasma cell survival in the bone marrow\(^{166}\). Intriguingly, it is known that CXCL12 from bone marrow stromal cells is reduced during inflammation (mediated by increased TNF-α and IL-1β)\(^{334}\). It is therefore likely that continued high levels of these cytokines during chronic inflammation lead to a decrease in CXCL12 from bone marrow cells, and this may mediate a reduction in migration of plasma cells from secondary lymphoid organs to the bone marrow, or their survival once established there.

**Conclusion**

Our data strongly indicates that, while acute immunisation with protein antigen (especially secondary immunisation) generates plasma cells that travel to the bone marrow where they persist without division for long periods. Chronic immunisation, autoimmunity, and the ‘chronic-phase’ of long-term bacterial infection fail to generate plasma cells that become established in the bone
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marrow. Therefore, once inflammation is resolved, only low antibody levels are maintained. Plasma cells appear to enter the bone marrow during a defined window early following secondary immunisation. This may be a control mechanism, to stop plasma cells of a single specificity overrunning the bone marrow plasma cell pool.

Interestingly, there is no difference in the ability of plasma cells to receive survival signals, as plasma cells may survive in the spleen (or other inflamed organs) during times of inflammation. It is therefore likely that migration of plasma cells is altered by responsiveness of plasma cells to CXCL12, or a lack of CXCL12 secretion from bone marrow cells. This could be tested using a migration assay of plasma cells from the spleens of mice undergoing long-term inflammation, compared to those arising following acute secondary immunisation with NP-KLH.
Chapter 6 – Bone marrow plasma cells are depleted during infection mediating establishment of new bone marrow plasma cells

Introduction

Bone marrow plasma cells maintain serum antibody levels to prior infections for long periods without the need for division, replenishment from the peripheral B cell pool or division. They are supported by survival factors secreted by cells that provide a survival niche. These niches are limited in number; only a finite number of bone marrow plasma cells can be maintained. Therefore, newly generated bone marrow plasma cells must compete with pre-established ones for access to these niches. In this chapter we look at a possible mechanism of competition, by which plasma cells are depleted from the bone marrow during bacterial and helminth infection, as well as the intriguing finding that established bone marrow plasma cells increase their expression of MHC II during infection.

Results

Bone marrow plasma cells are depleted during Salmonella infection

Figure 6.1a (top row) and 6.1b shows that during the course of Salmonella infection, large numbers of plasma cells were produced in the spleen, peaking at day 8 \(1.52 \times 10^7 \pm 2.07 \times 10^6\). However, the bone marrow plasma cell compartment was significantly reduced in terms of frequency (naïve, 0.173% ±0.006; day 16, 0.032% ±0.005) and number per femur \((44.25 \times 10^4 \pm 5.19 \times 10^3\) in naïve mice; \(4.03 \times 10^3 \pm 1.01 \times 10^3\) at day 16 of infection) (fig 6.1a bottom row and 6.1b left). B cells, too, were significantly depleted from the bone marrow, reduced from \(5.35 \times 10^6 \pm 6.89 \times 10^5\) per femur of naïve mice to \(3.66 \times 10^5 \pm 4.13 \times 10^4\) at day 16 (fig 6.1c). Figure 6.1e shows that two populations of B cells exist in the spleen, with differential expression of surface antibody (i.e. the BCR). Those expressing little BCR (bottom gate) were also found to express higher levels of CD138 (fig 6.1f, left) and thus likely to be pre B cells and immature B cells, whereas those expressing high levels of BCR expressed low
levels of CD138 and were likely to be mature B cells. Although both populations were depleted from the bone marrow, pre and immature B cells were almost entirely absent from the infected organ (12.28 ±0.82 in naïve mice to 0.14 ±0.02 at day 16 of infection; an 87.7-fold decrease), whereas mature B cells were reduced 5.7-fold (10.64% ±0.68 in naïve mice to 1.87% ±0.22 at day 16 of infection) (fig 6.1e and data not shown).

Although a statistically significant reduction in the frequency of B cells also occurred in the spleen, absolute numbers in the organ remained constant throughout infection (fig 6.1c), reflecting the large changes in cellularity and influx of other cell populations to the inflamed spleen.

Cells expressing CD11b increased dramatically from 14.57% (±0.959) in the spleen of naïve mice, to 39.10% (±1.99) at day 16 of infection (fig 6.1d, left). Similarly, an increase in frequency was seen in the bone marrow, where CD11b^{hi} cells came to dominate the organ during infection (increasing from 57.69% (±1.34) in the bone marrow of naïve mice to 82.24% (±0.53) at day 16 of infection) (fig 6.1d, right).

**Long-lived bone marrow plasma cells are depleted during chronic infection with Schistosoma mansoni**

To test whether other types of infection also depleted bone marrow plasma cells, we implanted mice with various doses of *Schistosoma mansoni* cercariae. 8-weeks post implantation, mice were culled and spleen and bone marrow examined for plasma cells. Similar to infection with *Salmonella, Schistosoma mansoni* infection generated significant populations of plasma cells in the spleen (fig 6.2a, left), while reducing the proportion and number of plasma cells found in the bone marrow in a dose dependent fashion (fig 6.2a, right). Plasma cells were reduced from 0.284% (±0.045) in the bone marrow of naïve mice, to 0.083% (±0.013) in the bone marrow of mice receiving 160 cercariae. This equated to 6.3x10^{4} (±5.7x10^{3}) per femur of naïve mice, and 2.4x10^{4} (±5.4x10^{3}) per femur of mice receiving 160 cercariae, on average a 2.6-fold reduction.
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To determine whether this was a reduction of short- or long-lived plasma cells in the bone marrow, mice were given an 8-day BrdU pulse from days 49-56 of infection and culled immediately afterward. It was found that, while there was little difference in the number of short-lived (BrdU+) plasma cells in the bone marrow (fig 6.2b, left), the number of long-lived plasma cells was significantly reduced in the groups receiving higher numbers of cercariae (fig 6.2, right).

**Eosinophils are depleted from the bone marrow during *Salmonella* infection**

As similar results were seen for infection with both *Salmonella* and *Schistosoma mansoni*, we now focussed our experiments on the *Salmonella* model. We hypothesised that the depletion of bone marrow plasma cells seen during infection may be due to the egress of certain cell types from the bone marrow that are known to support plasma cell survival. Figure 6.3a shows the frequency of eosinophils (blue gate), Gr1\textsuperscript{int} cells (red gate) and Gr1\textsuperscript{hi} cells (green gate) in the bone marrow of naïve or day 16 infected mice, and their relative expression of APRIL (fig 6.3b). It can be seen that eosinophils are depleted during infection (from 3.94% ±0.22 in the bone marrow of naïve mice to 0.89% ±0.16 at day 16 of infection) (fig 6.3a and c). These cells, although producing relatively low amounts of APRIL (fig 6.3b), have been shown to be vital to the support of bone marrow plasma cell populations\textsuperscript{151}. Other cell types that have been shown to support plasma cell survival, or produce plasma cell survival factors by our own data and that of others, such as dendritic cells\textsuperscript{88}, Gr1\textsuperscript{hi} cells (monocytes and macrophages)\textsuperscript{88} and Gr1\textsuperscript{int} cells (a major source of APRIL in the inflamed spleen, see fig 4.10a) are not depleted, and significantly increase during infection (fig 6.3c). APRIL\textsuperscript{hi} cells were not found to significantly change in frequency during infection, whereas megakaryocytes\textsuperscript{217} were slightly reduced (fig 6.3c). These data show that, while eosinophils were significantly reduced, other cell populations that produce plasma cell survival factors are increased or unaffected by infection.
Mice deficient in signalling components of the TLR pathway are not resistant to depletion of bone marrow plasma cells

We hypothesised that TLR signalling may mediate the depletion of plasma cells from the bone marrow, either through direct signals or indirectly, through the release of inflammatory cytokines from various cell populations that may cause bone marrow plasma cells to leave the bone marrow or die. However, on infection, MyD88\(^{-/-}\), TRIF\(^{-/-}\) and TLR4\(^{-/-}\) mice showed a similar phenotype to wild type mice. Figure 6.4a shows that wild type and knockout mouse strains exhibited similar splenomegaly and a similar (or more severe) drop in cellularity in the bone marrow. All three strains of knockout mice showed significantly elevated bacterial loads compared to wild type mice in both the spleen and bone marrow (fig 6.4b). However, higher numbers of bacteria did not correspond to elevated numbers of plasma cells in the spleen. In fact, while TRIF\(^{-/-}\) and TLR4\(^{-/-}\) mice had similar numbers of plasma cells in the spleen to wild type mice at day 16 of infection, MyD88\(^{-/-}\) mice had significantly fewer (wild type 1.86x10\(^7\) ±7.43x10\(^6\); MyD88\(^{-/-}\) 6.16x10\(^6\) ±1.02x10\(^6\)) (fig 6.4c).

The knockout mouse strains used here experienced a greater fold-reduction of bone marrow plasma cells when comparing naïve and infected mice (fig 6.4e), and this may result from the elevated bacterial loads seen in the organ (per femur: wild type 8.75x10\(^3\) ±1.03x10\(^3\); Myd88\(^{-/-}\) 5.80x10\(^4\) ±2.96x10\(^3\); TRIF\(^{-/-}\) 3.88x10\(^4\) ±1.97x10\(^3\); TLR4\(^{-/-}\) 4.00x10\(^4\) ±7.67x10\(^3\)) (fig 6.4b). Only MyD88\(^{-/-}\) mice contained significantly fewer bone marrow plasma cells at day 16 of infection compared to wild type mice (wild type 4.58x10\(^4\) ±1.13x10\(^4\); MyD88\(^{-/-}\) 6.92x10\(^3\) ±1.01x10\(^3\)), although whether this was due to having fewer plasma cells in the bone marrow in the naïve state, or due to increased bacterial loads in the bone marrow during infection is unclear.

Pre-established NP-specific bone marrow plasma cells are depleted by *Salmonella* infection

As bone marrow plasma cells maintain serum antibody levels to prior infections, immunisations or vaccinations, the depletion of these cells could lead to reduced circulating antibody and protection. To test this theory, we primed
Chapter 6 – Effects of inflammation on bone marrow plasma cells and boosted mice with NP-KLH to establish long-lived NP-specific plasma cells in the bone marrow. 5-weeks after boosting, mice were infected with *Salmonella*, and the effect on NP-specific plasma cells was observed at day 16 of the infection.

As before, we observed a striking drop in cellularity in the bone marrow of infected mice \(4.57 \times 10^7 \pm 1.69 \times 10^6\) per 100mg femur of non-infected mice to \(2.77 \times 10^7 \pm 1.56 \times 10^6\) per 100mg femur of infected mice) (fig 6.5a). Interestingly, despite a significant decrease in the frequency of total IgG plasma cells in the bone marrow of mice infected with *Salmonella* only, mice that were immunised with NP-KLH and then infected ('NP+Salmonella') did not exhibit a significant decrease in bone marrow plasma cells (fig 6.5b, left). However, the frequency of NP-specific IgG plasma cells was significantly decreased in infected versus non-infected mice (fig 6.5b, right).

In terms of numbers (factoring in the drop in cellularity) total IgG plasma cells per 100mg femur decreased by 2.2-fold from \(2.14 \times 10^4 \pm 2.27 \times 10^3\) to \(9.70 \times 10^3\) \((\pm 1.06 \times 10^3)\) in infected mice. Mice that were not immunised with NP, but were infected were further depleted to \(5.45 \times 10^3\) \((\pm 2.20 \times 10^3)\), a 3.9-fold decrease (fig 6.5c, left).

NP-specific plasma cells also decreased 2.2-fold from \(4.48 \times 10^3\) \((\pm 5.9 \times 10^2)\) in non-infected mice to \(2.02 \times 10^3\) \((\pm 1.10 \times 10^2)\) in infected mice (fig 6.5d). As both the decrease in total and NP-specific IgG plasma cells was 2.2-fold, it was unsurprising that the proportion of IgG plasma cells in the bone marrow that was NP-specific remained the same in both non-infected and infected mice (fig 2.5c, right).

Interestingly, we observed that remaining NP-specific plasma cells in the bone marrow of infected mice had high expression of MHC II, with just 26.2\% \((\pm 4.44)\) being MHC II\(^{hi}\) in non-infected mice, and 67.2\% \((\pm 6.74)\) being MHC II\(^{hi}\) in infected mice (fig 6.5e). As there was no statistical difference between the number of MHC II\(^{hi}\) plasma cells (fig 6.5f, left), it is likely that MHC II\(^{lo}\) plasma cells are depleted during infection. It is also possible that all MHC II\(^{lo}\) plasma
cells are induced to upregulate MHC II on their surface. This will be explored in later figures. In the spleen, we saw a dramatic increase in the number of NP-specific plasma cells in infected mice (3.79x10^4 ± 9.73x10^3 in non-infected mice and 3.94x10^5 ±9.25x10^4 in infected mice) (fig 6.5g). Again, these were predominantly MHC II^hi in phenotype.

**NP-specific plasma cells in Salmonella infected mice are not dividing, and do not increase their expression of T cell co-stimulatory molecules**

As we observed an increased number of MHC II^hi NP-specific plasma cells in the spleen of infected mice, and an increase in proportion of NP-specific plasma cells that were MHC II^hi in the bone marrow, we determined whether these plasma cells were newly generated, dividing plasma cells, or were long-lived plasma cells that were induced to express high levels of MHC II. Similar to the previous experiment, we primed and boosted mice with NP-KLH, and 5-weeks later infected half with *Salmonella*. At days 6-8 and 14-16, groups of mice were given 48-hour BrdU pulses, and the proportion of NP-specific plasma cells incorporating BrdU was determined. As can be seen in figure 6.6a, there was no significant difference between the turnover of NP-specific plasma cells in the spleen and bone marrow of non-infected or infected mice.

As many NP-specific plasma cells expressed high levels of MHC II during infection, we hypothesised that they may also express high levels of T cell co-stimulatory molecules CD80, CD86 and CD40. Plasma cells in the spleens of infected mice at day 16 increased in CD80 and MHC II expression, similar to the increase seen in B cell populations, but they did not increase in CD86 expression (fig 6.6b). Bone marrow plasma cells, however, increased only in MHC II expression (fig 6.6b and c). Despite the fact these plasma cells appeared to adopt a more immature phenotype through the upregulation of MHC II, they expressed significantly reduced levels of B220, a phenotype usually associated with mature plasma cells (fig 6.6c).

We next determined the mechanism by which MHC II was up regulated on bone marrow plasma cells. We infected wild type, MyD88^−/−, TRIF^−/−, TLR4^−/− mice and
looked at the expression of MHC II on bone marrow plasma cells. While TRIF−/− and TLR4−/− mice both showed significantly elevated levels of MHC II on bone marrow plasma cells following infection, those in MyD88−/− mice did not (fig 6.6d). In fact, MHC II on bone marrow plasma cells of infected MyD88−/− mice was significantly lower than that of plasma cells from infected wild type mice.

**Following depletion, NP-specific plasma cells do not recover as infection resolves**

To see whether NP-specific plasma cells recovered as *Salmonella* infection resolved, we performed a time course experiment whereby mice primed and boosted with NP-KLH were infected with *Salmonella* and mice culled 16, 45 and 75 days later. During this time, the *Salmonella* bacteria were cleared by mice, as determined by splenic bacterial loads (fig 6.7a). As bacterial loads decreased over time, so did splenic plasma cell numbers (fig 6.7b, left). By day 45 of infection (when bacterial loads were low), total bone marrow plasma cells had recovered to numbers seen in non-infected controls; this was maintained at day 75, although no further increase was seen (fig 6.7b, right). The proportion of plasma cells in the spleen that were NP-specific was far lower in infected mice, due to the fact that large numbers of plasma cells of other specificities were generated during *Salmonella* infection (fig 6.7c, left). This however, still corresponded to an increase in NP-specific plasma cells in the spleen at day 16 of infection (from 3.79x10⁴ ±9.73x10³ in non-infected mice to 3.94x10⁵ ±9.25x10⁴), although these numbers were not maintained (fig 6.7d, left). In fact, infected mice contained fewer NP-specific plasma cells in the spleen at day 75 than non-infected mice (1.59x10³ ±2.76x10² in infected mice compared to 1.93x10⁴ ±8.31x10³ in non-infected mice). As plasma cell numbers in the bone marrow recovered, the proportion that were NP-specific fell, indicating that new plasma cells entering the bone marrow were not NP-specific (and were likely specific for *Salmonella* antigens). Numbers of NP-specific plasma cells remained significantly lower in the bone marrow of infected mice following the clearance of *Salmonella* infection (2.94x10³ ±9.85x10² in infected mice compared to 9.48x10³ ±2.22x10² in non-infected mice) (fig 6.7d, left).
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The elevated levels of MHC II seen on NP-specific plasma cells at day 16 of infection in both the spleen and bone marrow was not maintained to later time points, when MHC II expression was similar to NP-specific plasma cells in non-infected mice (fig 6.7e).

Numbers of APRIL\(^{hi}\) cells did not change significantly in the bone marrow throughout infection, although they were significantly elevated in the spleen at day 16. By day 45, numbers had fallen in the spleen and were not significantly different from non-infected controls (fig 6.7f).

We next looked at NP-specific antibody levels in non-infected versus infected mice over the same time course of infection. For all IgG isotypes, the infected group had significantly less circulating NP-specific antibody. This was true at all time points for most isotypes. Interestingly, following an initial drop in NP-specific IgM at day 16, this recovered and by day 75 was significantly higher than non-infected controls, although differences were not large (fig 6.8).

**Differences in Salmonella-mediated depletion of bone marrow plasma cells in naïve mice or mice pre-immunised with NP-KLH is not due to differences in plasma cell expression of Fc\(\gamma RIIb\)**

In figure 6.5b, it was noted that mice immunised and boosted with NP-KLH did not exhibit as full a depletion of bone marrow plasma cells as mice that had not received NP-KLH before *Salmonella* infection. We therefore hypothesised that there may be a difference in the expression of the plasma cell death receptor Fc\(\gamma RIIb\) on bone marrow plasma cells from naïve mice and those generated by immunisation with NP-KLH. NP-specific plasma cells in non-infected mice expressed significantly greater levels of Fc\(\gamma RIIb\) than non-NP-specific plasma cells in both the spleen and bone marrow (splenic NP-specific plasma cells: \(3.44\times10^2 \pm 1.4\times10^1\), splenic non-NP-specific: \(2.74\times10^2 \pm 6.46\times10^0\); bone marrow NP-specific: \(4.55\times10^2 \pm 1.66\times10^1\), bone marrow non-NP-specific \(3.61\times10^2 \pm 4.54\times10^0\) (fig 6.9a and b). Interestingly, at day 16 of infection, previously established NP-specific plasma cells further increased in Fc\(\gamma RIIb\) expression (this was significant in the spleen (\(p=0.0005\)), but not in the bone marrow
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(p=0.2367). Non-NP-specific plasma cells did not significantly increase in expression of the death receptor in either spleen or bone marrow (fig 6.9a and b).

**TNF-α injection mediates reduced bone marrow cellularity, but not a specific reduction in plasma cells**

Inflammation has been shown to reduce the expression of the chemokine CXCL12 from bone marrow cells\(^3\)\(^3\)\(^4\). This was, at least in part, mediated by the inflammatory cytokine TNF-α, which is produced in large amounts during infection with *Salmonella*\(^3\)\(^3\)\(^5\),\(^3\)\(^3\)\(^6\). In addition, CXCL12 is the chemokine thought to mediate the migration of plasma cells from secondary lymphoid organs to the bone marrow (and also possibly their retention or survival there)\(^8\)\(^5\),\(^1\)\(^9\)\(^4\),\(^2\)\(^0\). We therefore hypothesised that the depletion of bone marrow plasma cells seen in infection may be due to increased levels of TNF-α during infection. Mice were given 1μg of recombinant mouse TNF-α I.V. on days 1 and 2, and culled at day 5. Figure 6.10a shows that injection of TNF-α significantly reduced bone marrow cellularity (2.52x10\(^7\) ±7.54x10\(^5\) to 2.11x10\(^7\) ±5.03x10\(^5\), \(p=0.002\)). While bone marrow plasma cell numbers fell accordingly (4.75x10\(^4\) ±4.79x10\(^3\) to 3.27x10\(^4\) ±3.85x10\(^3\), \(p=0.043\)), there was no decrease in frequency within the compartment (fig 6.10b and d) (as was evident by day 16 of infection with *Salmonella*, fig 6.1). In agreement with data from Ueda et al, bone marrow B cell frequencies were reduced to a far greater extent (26.04% ±1.38 to 11.95% ±1.20, \(p<0.0001\)) (fig 6.10c)\(^3\)\(^3\)\(^4\). In contrast to these populations, CD11b+ cells increased in frequency (58.43% ±1.99 to 72.22% ±2.21, \(p=0.0027\)) (fig 6.10e). There was little change in numbers of most niche providing cells in the bone marrow following TNF-α injection, apart from APRIL\(^b\)\(^i\) cells, which fell significantly (5.39x10\(^5\) ±6.37x10\(^4\) to 1.49x10\(^5\) ±1.64x10\(^4\), \(p=0.0003\)).

**Adjuvant depletes previously established bone marrow plasma cells, and mediates a reduction in the establishment of newly generated plasma cells in the bone marrow**

It is thought that the bone marrow plasma cell pool is of limited size, and that newly generated plasma cells must compete with previously generated ones for
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Survival factors (or access to a survival niche)\textsuperscript{194,306}. It has been documented that, on vaccination with tetanus toxoid, mature plasma cells of other specificities could be seen entering the blood. It was hypothesised that these are likely to be 'old' plasma cells being pushed out of their survival niches by newly generated plasma cells\textsuperscript{249}. We sought to test whether the depletion of plasma cells seen during inflammatory episodes 'created space' for newly generated plasma cells. To test this we primed mice with two protein antigens; NP-KLH and FITC-OVA. 5-weeks later, mice were boosted with NP-KLH to generate a population of NP-specific plasma cells in the bone marrow. A further 5-weeks later mice received either no further immunisation (NP-only), a booster immunisation with soluble FITC-OVA (sol FITC-OVA), or a booster immunisation with alum-precipitated FITC-OVA with killed \textit{B. pertussis} (adjuvant/FITC-OVA).

Figure 6.11a shows that adjuvant/FITC-OVA boosted mice produced the greatest number of plasma cells in the spleen, and also exhibited a significantly reduced number of plasma cells in the bone marrow at day 5 (spleenic plasma cells: 2.98x10^6 ±1.26x10^5 in adjuvant/FITC-OVA mice compared to 1.07x10^5 ±1.73x10^4 in NP-only mice. Bone marrow plasma cells: 1.52x10^4 ±1.29x10^3 in adjuvant/FITC-OVA mice compared to 2.76x10^4 ±2.72x10^3 in NP-only mice). This depletion in bone marrow plasma cells significantly reduced numbers of the previously established NP-specific bone marrow plasma cells, and these continued to decline throughout the 30-day experiment (fig 6.11c). However, this did not mediate the establishment of increased numbers of newly generated FITC-OVA specific plasma cells. In fact, despite generating similar numbers in the spleen compared to sol FITC-OVA immunised mice at day 5 (2.26x10^5 ±9.05x10^4 in sol FITC-OVA and 1.85x10^5 ±2.7x10^4 in adjuvant/FITC-OVA), only very few of these cells became established in the bone marrow compartment by day 12 (1.03x10^4 ±1.51x10^3 in sol FITC-OVA and 7.23x10^2 ±6.65x10^1) (fig 6.11b). Interestingly, and despite the lack of entry of FITC-OVA-specific plasma cells to the bone marrow, total bone marrow plasma cell numbers had recovered by day 12 (fig 6.11a).
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Boosting with sol FITC-OVA generated similar numbers of FITC-OVA-specific plasma cells in the spleen to adjuvant/FITC-OVA (fig 6.11b). These entered the bone marrow by day 5 (5.82x10^3 ± 1.67x10^3), and peaked at day 12 (1.03x10^4 ± 1.51x10^3). Initially, no depletion in NP-specific plasma cells was seen, and total plasma cell numbers in the bone marrow increased (2.76x10^4 ± 2.73x10^3 in NP-only mice and 3.47x10^5 ± 2.57x10^3 in sol FITC-OVA mice at day 12) (fig 6.11a and c). However, by day 30, numbers of plasma cells in the bone marrow returned to levels seen in control mice (NP-only). This mediated a decline in both NP-specific and FITC-OVA-specific plasma cells, although numbers of both specificities remained significantly higher than in alum/FITC-OVA mice (NP-plasma cells: sol FITC-OVA 2.36x10^3 ± 9.6x10^1, adjuvant/FITC-OVA 2.84x10^2 ± 9.4x10^0. FITC-OVA-plasma cells: sol FITC-OVA 5.74x10^3 ± 2.52x10^2, adjuvant/FITC-OVA 1.39x10^3 ± 6.1x10^1).

Boosting with adjuvant/FITC-OVA generated far higher levels of FITC-OVA-specific IgM in the serum than sol FITC-OVA by day 12 (3.40x10^3 ± 0 in adjuvant/FITC-OVA mice and 4.16x10^2 ± 1.72x10^2 in sol FITC-OVA mice) (fig 6.11d). Interestingly, levels of NP-specific IgM also increased significantly by day 12 following adjuvant/FITC-OVA boosting (from 4.60x10^2 ± 5.36x10^1 in NP-only mice to 5.61x10^3 ± 8.11x10^2 in adjuvant/FITC-OVA mice) (fig 6.11e).

Boosting with soluble FITC-OVA did not increase FITC-OVA specific IgM, and reduced NP-specific IgM (FITC-OVA-specific: from 3.05x10^2 ± 0 in NP-only mice to 2.83x10^2 ± 0 in sol FITC-OVA mice at day 30. NP-specific: from 4.60x10^2 ± 5.36x10^1 in NP-only mice to 1.65x10^2 ± 2.68x10^1 in sol FITC-OVA mice at day 30).

NP-specific IgG was not initially affected by either immunisation, although by day 30 mice receiving adjuvant/FITC-OVA had reduced levels (from 8.67x10^3 ± 3.33x10^2 in NP-only mice to 4.02x10^3 ± 5.90x10^2 in adjuvant/FITC-OVA mice at day 30) (fig 6.11e). FITC-OVA-specific IgG peaked at day 12 in adjuvant/FITC-OVA boosted mice, and had declined by day 30 (4.28x10^4 ± 9.55x10^3 at day 12 to 1.54x10^4 ± 2.77x10^3 at day 30) (fig 6.11d). Mice receiving sol FITC-OVA
Chapter 6 – Effects of inflammation on bone marrow plasma cells maintained elevated levels of FITC-OVA-specific IgG between day 12 and day 30 (1.98x10^4 ± 2.44x10^3 at day 12 and 2.24x10^4 ± 3.10x10^3 at day 30).
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A

Naive  

Salmonella

0.154  

2.44

Spleen

0.164  

0.0326

Bone Marrow

CD138

Int Ig

B

Spleen PC Percentage

Bone Marrow PC Percentage

Percentage Spleen

Percentage Bone Marrow

Spleen PC Numbers

Bone Marrow PC Number

Number per Spleen

Number per Femur

C

Spleen % CD11b+

Bone Marrow % CD11b+

Percentage Spleen

Percentage Bone Marrow

Figure 6.1 continues overleaf
Chapter 6 – Effects of inflammation on bone marrow plasma cells

Figure 6.1 Cont.

D

Spleen % B Cell

Bone Marrow % B Cells

Spleen Number B Cells

Bone Marrow Number B Cells

Figure 6.1. Bone marrow plasma cells are depleted during *Salmonella* Infection.

A) Representative FACS plots from the spleen (top) and bone marrow (bottom) of naïve (left) and *Salmonella* infected mice (at day 16) gates show plasma cells identified through CD138 and intracellular Igx. (B) Summary graphs showing percentage (top) and number (bottom) of plasma cells in the spleen (left) and bone marrow (right) of through a time course of *Salmonella* infection. (C) Graphs showing percentage of cells expressing CD11b+ in the spleen and bone marrow a time course of infection. (D) Graphs showing the percentage (top) and number (bottom) of CD19+ B cells in the spleen (left) and bone marrow (right) of mice from (B). (E) FACS plots showing mature B cell (top gate) and pre/pro B cell (bottom gate) populations in the bone marrow of naïve (left) and day 16 infected (right) mice. (F) CD138 expression of populations from (E). Points on all graphs=5 mice per group. Error bars=standard deviation. FACS plots representative of 5 mice per group. Results representative of at least 5 independent experiments.
Figure 6.2

(A) Chronic infection with *Schistosoma mansoni* leads to depletion of bone marrow plasma cells in a dose dependent fashion. (A) Mice were infected percutaneously with 0 (naive), 20, 40, 80 or 160 *Schistosoma mansoni* cercariae and culled 56 days later. Graphs show percentage (top) and number (bottom) of plasma cells in the spleen (left) and bone marrow (right). (B) mice from (A) were given an 8-day continuous BrdU-pulse at day 49-56 after egg implantation, and culled immediately afterwards. Dividing (left) or non-dividing (right) plasma cells in the bone marrow were enumerated. Points on graphs=1 mouse, bars=mean. Results represent a single experiment.
Figure 6.3. Eosinophils and megakaryocytes are depleted from the bone marrow during *Salmonella* infection. (A) FACS plots showing eosinophils (blue), Gr1<sup>int</sup> (red) and Gr1<sup>hi</sup> (green) cell populations in the bone marrow of naive mice (left) and mice at day 16 of *Salmonella* infection. (B) APRIL expression of the populations gated in (A) as well as isotype control (grey filled). (C) Summary graph showing percentage of bone marrow that are APRIL<sup>hi</sup>, Gr1<sup>hi</sup>, Gr1<sup>int</sup>, dendritic cells, megakaryocytes and eosinophils in the bone marrow of naive (white) and day 16 *Salmonella* infected (black) mice. FACS plots representative of 5 mice. Bars on graphs=mean of 5 mice per group, error bars=standard deviation.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

**Figure 6.4**

**A**  
Spleen Cellularity  
Bone Marrow Cellularity

**B**  
Spleen Bacterial Loads  
Bone Marrow Bacterial Loads

**C**  
Spleen % Plasma Cells  
Spleen Number Plasma Cells

Figure 6.4 continues overleaf
Figure 6.4 Cont.

D  
Bone Marrow % Plasma Cells

Bone Marrow Number Plasma Cells

E  
Fold Decrease Bone Marrow Plasma Cells (%)

Fold Decrease Bone Marrow Plasma Cells (Number)

Figure 6.4. Mice deficient in MyD88, TRIF or TLR4 show similar depletion of bone marrow plasma cells to wild type mice following infection with *Salmonella*.  
(A) Cellularity of spleen (left) and bone marrow (right) of naïve (square) and infected (triangle) wild type (blue), MyD88−/− (red), TRIF−/− (green) and TLR4−/− (black) mice.  
(B) Bacterial loads in the spleen (left) and bone marrow (right) of mice from (A).  
(C) percentage (left) and number (right) of plasma cells in the spleens of mice from (A).  
Points on graphs=1 mouse, bars=standard deviation.  
(D) as (C) but for bone marrow.  
(D) Fold decrease in the mean percentage (left) or number (right) of plasma cells in the bone marrow of 5 mice (per group) from naïve to day 15 of *Salmonella* infection.
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Figure 6.5

A. Bone Marrow Cellularity

B. Bone Marrow % IgG PC

C. Bone Marrow Number IgG PC

D. Bone Marrow Number NP-PC

E. Bone Marrow % NP-PC MHC II<sup>hi</sup>

Figure 6.5 continues overleaf
Figure 6.5 Cont.

F  Bone Marrow  Bone Marrow
Number MHC II$^{hi}$ NP-PC  Number MHC II$^{low}$ NP-PC

![Graph](image)

G  Spleen  H  Spleen
Number NP-PC  % NP-PC MHC II$^{hi}$

![Graph](image)

Figure 6.5. NP-specific plasma cells are depleted by subsequent *Salmonella* infection. Mice were primed and boosted with NP-KLH at 5 week intervals. After a further 5-weeks, 1 group of mice was infected with *Salmonella* (white bar), one was given PBS (black bar). A third group was not primed or boosted with NP-KLH but was infected (checked bar). (A) Cellularity of 100mg of femur from mice at day 16 post-infection (or PBS). (B) Percentage of bone marrow that was IgG plasma cells (left) or NP-specific IgG plasma cells (right). (C) Number of IgG plasma cells per 100mg femur (left) and the percentage of these that were NP-specific (right). (D) Number of NP-specific plasma cells per 100mg femur. (E) Percentage of NP-specific IgG plasma cells that were MHC II$^{hi}$. (F) Number of MHC II$^{hi}$ (left) and MHC II$^{low}$ (right) IgG plasma cells per 100mg femur. (G) Number of NP-specific IgG plasma cells per spleen of mice from (A). (H) as for (E) but for splenic NP-specific plasma cells. Bars=mean of 5 mice, error bars=standard deviation. Results representative of 2 independent experiments.
Figure 6.6

A

Spleen % NP-PC BrdU+  

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<th>NP</th>
<th>NP + Salmonella</th>
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<td>10.0 ns</td>
</tr>
<tr>
<td>16</td>
<td>5.0 ns</td>
<td>7.5 ns</td>
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Bone Marrow % NP-PC BrdU+

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<thead>
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<th>Days after Infection</th>
<th>NP</th>
<th>NP + Salmonella</th>
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<tbody>
<tr>
<td>8</td>
<td>1.0 ns</td>
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<tr>
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B

Plasma Cells  

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B Cells

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<td></td>
</tr>
<tr>
<td>Bone Marrow</td>
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Figure 6.6 continues overleaf
Figure 6.6 Cont.

Figure 6.6. NP-specific plasma cells (PC) do not divide or up-regulate co-stimulatory molecules following infection with Salmonella, despite up-regulating MHC II. Mice immunised and boosted with NP-KLH were infected with Salmonella (white) or received PBS (black). At day 6-8 or 14-16, mice were given BrdU pulses. (A) BrdU incorporation of NP-specific PC in the spleen and bone marrow of mice described above. (B) CD80 (top left panel), CD86 (top right) and MHC II (bottom left) expression on splenic (top) and bone marrow (bottom) plasma cells (left) or B cells (right) from naïve (blue) or infected (red) mice. (C) MFI of CD80, CD86, MHC II, CD40 and B220 on PC from naïve (black) or infected (white) bone marrow PC. (D) Percentage of bone marrow PC that were MHC IIhi in naïve (solid) or infected (checked) wild type (blue), MyD88−/− (red), TRIF−/− (green) and TLR4−/− (black) mice. Bars on graphs=mean of 5 mice, error bars=standard deviation. FACS plots representative of 5 mice per group. Results represent a single experiment.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

Figure 6.7

A

Bacterial Loads

Day after Infection

B

Spleen Number IgG PC

Day after Infection

Bone Marrow Number IgG PC

Day after Infection

C

Spleen % PC NP-Specific

Day after Infection

Bone Marrow % PC NP-Specific

Day after Infection

D

Spleen Number NP-PC

Day after Infection

Bone Marrow Number NP-PC

Day after Infection

Figure 6.7 continues overleaf
Figure 6.7 Cont.

E

Spleen % NP-PC MHC II$^{hi}$

Bone Marrow % NP-PC MHC II$^{hi}$

F

Spleen Number APRIL$^{hi}$

Bone Marrow Number APRIL$^{hi}$

Figure 6.7. NP-Specific IgG plasma cells are depleted from the bone marrow and do not recover during the course of *Salmonella* infection.

(A) Bacterial loads in the spleens of *Salmonella* infected (dashed) and non-infected (solid) mice that had previously been primed (10 weeks prior) and boosted (5 weeks prior) with NP-KLH. (B) Number of total IgG plasma cells in the spleen (left) bone marrow (right) of mice from (A). (C) Proportion of IgG plasma cells that are NP-specific in spleen and bone marrow of mice from (A). (D) Number of NP-specific IgG plasma cells in the spleen and bone marrow of mice from (A). (E) Percentage of NP-specific IgG plasma cells that express MHC II in the spleens and bone marrow of mice from (A). (F) Number of APRIL$^{hi}$ cells in the spleen and bone marrow of mice from (A). Points on graph=mean of 5 mice, error bars=standard deviation. Results representative of 2 independent experiments.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

Figure 6.8

Figure 6.8. NP-specific antibody is reduced during infection with *Salmonella*. Mice primed and boosted with NP-KLH at 5-week intervals and rested for 5 further weeks were infected with *Salmonella* (dashed line) or received PBS (solid line). Graphs show NP-specific IgM, IgG1, IgG2b, IgG2c and IgG3 titres in the serum of mice at days 16, 45 and 75 post-infection (or PBS). Points on graph=mean of 5 mice, error bars=standard deviation. Results representative of 2 independent experiments.
**Figure 6.9**

A

![Graph showing cell numbers for Spleen and Bone Marrow](image)

**Spleen**

- **NP PC**
- **Non-NP PC**

**Bone Marrow**

- **NP PC**
- **Non-NP PC**

**Non-Infected**

**Salmonella Infected**

B

**Spleen MFI**

- **Non-Infected, Non-NP PC**
- **Non-Infected, NP PC**
- **Infected, Non-NP PC**
- **Infected, NP PC**

**Bone Marrow MFI**

- **Non-Infected, Non-NP PC**
- **Non-Infected, NP PC**
- **Infected, Non-NP PC**
- **Infected, NP PC**

Figure 6.9. Reduced depletion of bone marrow plasma cells in mice previously primed and boosted with NP-KLH is not due to lower expression of plasma cell death receptor FcγRIIb. Mice immunised and boosted with NP-KLH were infected with *Salmonella* or given PBS (Non-Infected). (A) shows relative expression of FcγRIIb by non-NP-specific (blue) or NP-specific (red) IgG plasma cells from the spleen (left) and bone marrow (right) of infected (bottom) or non-infected (top) mice. (B) summary graphs showing MFI of Non-NP-specific (square), and NP-specific (triangle) IgG plasma cells from the spleen (left) and bone marrow (right) of non-infected (blue) and infected (red) mice. Points on graph=1 mouse, bars=mean. Results represent a single experiment.
Figure 6.10. TNF-α reduces bone marrow cellularity, and plasma cell numbers. Mice were injected with 1µg of TNF-α i.v. on days 1 and 2, and culled at day 5. (A) cellularity of 1 femur of bone marrow. (B) Number of plasma cells per femur. (C) Frequency of CD19+ B cells. (D) Frequency of plasma cells. Points on graph=1 mouse, bars=mean. (E) Frequency of CD11b+ cells. (F) Number of 'niches providing cells' (APRILα, cells, Monocytes/Macrophages (CD11blo, Gr1lo, F4/80lo-hi), Eosinophils (CD11blow, Gr1int, F4/80hi), Dendritic cells (CD11c+) and Megakaryocytes (CD41+) per femur of naive mice (white) or mice receiving TNF-α mice (black). Bars on graph=mean of 5 mice, error bars=standard deviation. Results represent a single experiment.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

Figure 6.11

A

Spleen Total PC

Bone Marrow Total PC

B

Spleen FITC-OVA PC

Bone Marrow FITC-OVA PC

C

Spleen NP PC

Bone Marrow NP PC

1.0x10^3
1.0x10^4
0
5
Day
1.0x10^1
1.0x10^2

Figure 6.11 continues overleaf
Figure 6.11 Cont. 

D  

**FITC-OVA IgM**  

**FITC-OVA IgG**  

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E  

**NP IgM**  

**NP IgG**  

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<th>Group/Day</th>
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<tr>
<td>Sol FITC-OVA/Alum FITC-OVA</td>
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F  

**Prime with NP-KLH and FITC-OVA**  

**Boost with NP-KLH**  

Cull D5, D12, D30  

**Prime with NP-KLH and FITC-OVA**  

**Boost with NP-KLH**  

Boost with Alum-FITC-OVA + B. pertussis  

Cull D5, D12, D30  

Figure 6.11. Adjuvant depletes previously established bone marrow plasma cells (PC) and antibody, and reduces the influx of newly generated PC to the bone marrow. Mice were primed with 100µg alum-NP-KLH and FITC-OVA with 1*10⁶ B. pertussis. At 5-weeks, mice were boosted with 100µg NP-KLH. A further 5-weeks later mice were boosted with 100µg soluble FITC-OVA or alum-precipitated FITC-OVA with B. pertussis, or injected with PBS (NP-Only). (A) Total PC in spleens (left) and bone marrow (right) of mice described above. (B) Numbers of FITC-OVA specific PC in spleens and bone marrow of mice from (A). (C) Numbers of NP-specific PC in spleens and bone marrow of mice from (A). (D) FITC-OVA specific IgM (left) and IgG (right) in serum of mice from (A). (E) NP-specific IgM and IgG from serum of mice from (A). (F) Schematic of experimental design. Tables beneath graphs show results of statistical tests comparing values between groups at each time point. Points on graph = mean of 5 mice, error bars = standard deviation. Graphs show results from a single experiment.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

Discussion

In this chapter we show that bone marrow plasma cells, which maintain serum antibody levels to prior infections and immunisations, are depleted during inflammatory episodes. This causes a reduction in circulating antigen-specific serum. We look at the effects of this depletion on the establishment of newly generated plasma cells.

Infection depletes bone marrow plasma cells

The upkeep of antibody levels specific for prior infections in the serum is thought to be reliant on the maintenance of bone marrow plasma cell numbers\textsuperscript{317}. Unlike the majority of plasma cells in secondary lymphoid organs, bone marrow plasma cells persist for long periods, maintaining a first line of defence in case of reinfection. Under homeostatic conditions antigen-specific plasma cells in the bone marrow decline slowly, even when peripheral B cells are depleted, indicating their long lifespan and independence of further generation from the B cell pool for the maintenance of these populations\textsuperscript{189,193,196,306}.

*Salmonella* infection caused a dramatic depletion in bone marrow plasma cells (fig 6.1a and b), in part mediated by a drop in total bone marrow cellularity (fig 6.4a), but also a reduction in the frequency of bone marrow plasma cells within the compartment (fig 6.1b). This suggests a mechanism exists by which plasma cells are specifically induced to exit the bone marrow during infection, or that plasma cells are especially sensitive to changes in the cellular make-up of the compartment, as will be discussed later.

The infection of mice previously primed and boosted with NP-KLH revealed that previously generated populations of NP-specific plasma cells in the bone marrow were similarly depleted (fig 6.5c and d), and this negatively affected NP-specific antibody in the serum (fig 6.8). Despite total plasma cells returning to numbers seen in non-infected mice by day 45 following infection (fig 6.7b), NP-specific plasma cells failed to recover, even by day 75 (fig 6.7d), when bacteria was cleared from these mice (fig 6.8a). The recovery of plasma cells in
Chapter 6 – Effects of inflammation on bone marrow plasma cells

The bone marrow plasma cell pool, much like other memory pools, is of limited size; immunisation does not dramatically expand this population (see fig 3.1). Odendahl et al have shown that vaccination of human patients with tetanus toxoid (TT) mediates an increase in non TT-specific, mature plasma cells in the blood; speculated to be the egress of pre-established plasma cells caused by the influx of newly generated ones\textsuperscript{249}. Commonly, the literature cites the “pushing out” of old plasma cells (which are non-migratory) by the migratory new crop\textsuperscript{6,248,249}, although the precise mechanism behind this is unclear. Our data presented here may suggest that plasma cell competition is mediated by the depletion of previously established plasma cells by inflammatory episodes. This would appear to be a distinct event, occurring prior to the entry of newly generated plasma cells.

Similarly to Salmonella infection, Schistosoma mansoni (a chronic, Th2 infection) caused a depletion in bone marrow plasma cells that was found to be dose dependent (fig 6.2a). The bone marrow plasma cell pool consists of mainly long-lived mature plasma cells, but also a short-lived immature component (see chapter 3). It was seen that Schistosoma infection caused depletion of long-lived, and not of short-lived plasma cells (fig 6.2b). Similarly, the majority of remaining plasma cells seen in the bone marrow following Salmonella infection expressed high levels of MHC II. Mature plasma cells are non-migratory\textsuperscript{201}, and may be more susceptible to depletion than immature plasmablasts. To test this, a system using two different protein antigens (e.g. NP-KLH and FITC-OVA) could be used to establish long-lived plasma cells at different times (e.g. NP-KLH at day 1, then FITC-OVA at day 35) followed by infection with Salmonella shortly afterwards. It may be that the more mature, older, NP-specific plasma cells would undergo a greater depletion.
Salmonella infection causes an increase in previously established NP-specific plasma cells in the spleen, and an increase in MHC II expression on all NP-specific plasma cells

We saw that Salmonella infection caused an increase of NP-specific plasma cells in the spleen (fig 6.5g). These could potentially arise from two sources; firstly new NP-specific plasma cells may be generated in the spleen by the restimulation of memory B cells through polyclonal stimulation\textsuperscript{188}. However, this seems unlikely, as the NP-specific plasma cells had low turnover when labelled with BrdU at days 6-8 or days 14-16 (fig 6.6a). Similarly, it has been shown that, in vivo, polyclonal stimulation alone is not sufficient to cause the activation of memory B cells and their differentiation to plasma cells\textsuperscript{197}. Secondly, the reduction in NP-specific bone marrow plasma cells could represent a migration out of that organ to the secondary lymphoid organs. Indeed, it is known that B cell precursors in the bone marrow migrate to the spleen during inflammation due to reduced expression of CXCL12 from bone marrow cells\textsuperscript{334}. We also observed this depletion in CD138 expressing, BCR\textsuperscript{b} B cell precursor cells from the bone marrow during Salmonella infection (fig 6.1c, e and f)\textsuperscript{337}. It is possible that the same mechanism causes bone marrow plasma cells to migrate to the spleen. CXCL12 is the chemokine responsible for plasma cell migration from the secondary lymphoid organs to the bone marrow, and possibly plays a role in their close association with niche providing cells\textsuperscript{84,85}. However, the chemokine is also present in the splenic red pulp and other sites of extrafollicular plasma cell responses\textsuperscript{78}. It is possible that if CXCL12 is reduced from the bone marrow compartment during inflammation, plasma cells at this site are attracted to migrate back to the secondary lymphoid organs.

It has been shown that mature bone marrow plasma cells are non-migratory and fail to respond to CXCL12, despite the continued expression of its receptor CXCR4\textsuperscript{201}. However, on infection, there was a highly significant up-regulation of MHC II on NP-specific plasma cells remaining in the bone marrow, and those appearing in the spleen (fig 6.5e and h). Although these plasma cells did not divide, this adoption of a more immature phenotype may allow for these cells to
once again become migratory in response to CXCL12. This could be tested by assessing the migration of bone marrow plasma cells from NP-only or NP+Salmonella mice towards CXCL12.

There are other interesting connotations of the reversion to an immature, MHC II\textsuperscript{hi} phenotype by antigen-specific plasma cells during inflammation. Plasma cells have been shown to be capable APC, as discussed previously\textsuperscript{267}. While untested, it would be a reasonable assumption that plasma cells expressing high levels of MHC II would be more proficient at presenting antigen to CD4 T cells. As professional memory CD4 T cells are also thought to reside in the bone marrow\textsuperscript{212}, it may be that bone marrow plasma cells are in a prime location to activate these cells on re-exposure to antigen. This could be tested by pulsing sorted NP-specific bone marrow plasma cells from NP-only or NP+Salmonella mice with NP-KLH and assessing their ability to activate CD4 T cells from NP-KLH-immunised mice.

The up-regulation of MHC II on bone marrow plasma cells during Salmonella infection was seen to be dependent on MyD88 signalling (fig 6.6d). However, it would be interesting to know whether the increase was due to intrinsic MyD88 signalling in plasma cells, or an indirect pathway, for example from the induction of inflammatory cytokines from other cell populations. The use of chimeras in which only B cells were deficient in MyD88 could help to determine this. It is known that inflammatory cytokines, such as IFN-\textalpha can enhance immature plasma cell responses, and that IL-6 can support plasmablast responses\textsuperscript{88,182}, however whether these cytokines can cause mature plasma cells to revert to an immature phenotype (at least in certain aspects), and the potential effects of this remain untested. In addition, while plasma cells up regulated MHC II, co-stimulatory molecules involved in antigen presentation to CD4 T cells (CD80, CD86 and CD40) were not up regulated, at least in the bone marrow (fig 6.6b and c). How efficient bone marrow plasma cells are at antigen presentation remains to be seen.
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Neither the increase in MHC II seen on NP-specific plasma cells, nor the increased number of NP-specific plasma cells in the spleen persisted as, by day 45 of infection, these had returned to levels in non-infected mice (fig 6.7d and e). This corresponded to the decline in bacterial loads in the spleen by day 45 (fig 6.7a) and, although not measured here, the likely decrease in TLR stimulus and inflammatory cytokines. The decrease in splenic NP-specific plasma cells seen by day 75 of infection compared to non-infected controls (fig 6.7d) is likely due to the increased competition faced by NP-specific plasma cells for survival factors (large numbers of splenic plasma cells generated by Salmonella infection would likely decrease the availability of survival factors for NP-specific plasma cells).

**Mechanism of Bone Marrow Plasma Cell Depletion**

The mechanism by which plasma cells are depleted from the bone marrow is not yet clear, although there are a number of possibilities, some of which have been tested here.

Firstly, depletion caused a striking decrease in eosinophils from the bone marrow compartment (fig 6.3a and c). While in our own experiments, eosinophils in the bone marrow did not secrete large amounts of APRIL (fig 6.2b), Chu et al have shown that these cells are vital for the maintenance of bone marrow plasma cells\(^\text{151}\). Whether their depletion alone is sufficient to cause the observed reduction is unknown, but could be tested by the injection of an anti-siglec-F antibody, which causes the specific depletion of eosinophils\(^\text{151}\). Other changes to the bone marrow compartment occur during infection, such as a large increase in CD11b+ cells (fig 6.1c) and small increases in GR1\(^\text{hi}\) and Gr1\(^\text{int}\) cells (fig 6.3c). It is known that TLR signalling can cause the rapid egress of monocytes from the bone marrow into the blood stream via changes in gradients of CCR2\(^\text{1}\). In another mechanism, neutrophils exit the bone marrow during inflammation due to reduced CXCL12 from the bone marrow, and increased CXCL2 from endothelial cells\(^\text{338}\). This altered environment in the bone marrow compartment may reduce the availability of survival factors to plasma cells, by disrupting their tight association with niche providing cells, or altering
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the availability of secreted factors. Both of these are thought to be important for
the maintenance of bone marrow plasma cell populations\(^1\,^9\,^4\,^3\,^0\,^6\), although to test
this definitively a greater understanding of the exact nature of the bone marrow
plasma cell niche would be required.

Secondly, we thought that TLR stimulation may cause the depletion in bone
marrow plasma cell numbers. This could be through direct stimulation of
plasma cells, or through changes to the bone marrow plasma cell compartment
mediated by altered secretion of survival factors from niche providing cells. B
cells respond to TLR stimulation by the up-regulation of antigen presentation
machinery and cytokines\(^3\,^1\,^3\,^9\) as well as differentiation to plasma cells\(^5\,^8\,^5\,^9\).
However, effects of TLR stimulation on bone marrow plasma cells are unknown;
little literature exists looking at this subject, although it is known that human
tonsillar plasma cells express TLRs at different levels to human B cells, and that
stimulation with TLR ligands leads to enhanced antibody secretion\(^3\,^4\,^0\). Here we
saw that TLR stimulation did not appear to control plasma cell migration from
the bone marrow, as MyD88\(^/-\), TRIF\(^/-\) and TLR4\(^/-\) all exhibited a greater fold-
decrease in bone marrow plasma cell numbers (fig 6.4d and e). In fact, this was
likely due to increased bacterial loads in the bone marrow of these mice (fig
6.4b) due to their reduced capacity to control the infection\(^4\,^4\). This could be
better tested by the infection of mice with various doses of Salmonella, although
a dose dependent effect was already seen in Schistosoma mansoni infected mice.

If not the action of TLR ligands on plasma cells, the likelihood exists that an
inflammatory cytokine, or chemokine up regulated during inflammation, causes
the depletion of bone marrow plasma cells. As previously mentioned, Ueda et al
have shown that CXCL12 is reduced from the bone marrow during
inflammation, mediating a migration of early-stage B cells from bone marrow to
spleen\(^3\,^3\,^4\). This decrease in CXCL12 could be observed by a simple injection of
1ug of the inflammatory cytokine TNF-\(\alpha\). As CXCL12 controls plasma cell
migration to the bone marrow\(^8\,^5\), we performed similar experiments to see if
TNF-\(\alpha\) reduced bone marrow plasma cells. Despite observing a small, but
significant reduction in bone marrow plasma cells 3 days after the injection of
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TNF-α, this was primarily mediated by the change in cellularity observed in the bone marrow during inflammation (fig 6.10a and b). That no change in frequency of plasma cells within the bone marrow compartment was seen (fig 6.10d) implies that there may be multiple mechanisms that combine to generate the substantial depletion in bone marrow plasma cell numbers and frequencies seen during Salmonella infection. TNF-α appears to mediate the reduction in cellularity in the bone marrow, and this likely depletes plasma cells due to a reduction of plasma cell survival factors or plasma cell niches, or due to a reduction in CXCL12 from bone marrow cells. We did not, however, see a significant depletion in eosinophils or megakaryocytes following injection of TNF-α (fig 6.10f), two populations that are known to be important in maintaining bone marrow plasma cells, and that are depleted during Salmonella infection. It would be interesting to extend the provision of TNF-α from 2 days to 16 days; it may be that high levels of TNF-α over a sustained period (as is likely the case in Salmonella infection) would generate a greater depletion in bone marrow plasma cells.

It has been previously reported that subsequent ‘non-specific’ immunisation leads to a reduction of antigen-specific antibody levels to prior immunisations\(^3^8\). This was thought to be mediated by the apoptosis of bone marrow plasma cells caused by the cross-linking of the Fc receptor FcγRIIb. It was observed that autoimmune plasma cells in models of SLE were resistant to this mechanism of death, as they did not express the Fc receptor. We observed that mice that were primed and boosted with NP-KLH exhibited a reduced depletion in bone marrow plasma cells during Salmonella infection compared to those receiving Salmonella alone (fig 6.5c). We therefore hypothesised that the relatively recently generated NP-specific plasma cells may be somewhat resistant to Salmonella-mediated depletion, and this may be due to a reduced expression of FcγRIIb. However, as shown in figure 6.9, NP-specific plasma cells expressed greater levels of the receptor than non-NP-specific plasma cells in the same host, in both spleen and bone marrow (fig 6.9b). On infection, surviving NP-specific plasma cells in the spleen and bone marrow expressed elevated levels
Chapter 6 – Effects of inflammation on bone marrow plasma cells of this receptor. This implies that FcyRIIb may be induced on plasma cells in a similar way to MHC II; it would be interesting to know whether this occurred in MyD88-/ mice. It also implies that FcyRIIb does not control plasma cell death following infection with Salmonella, as plasma cells with higher expression of the receptor survive. Interestingly, non-NP-specific plasma cells in the spleens of infected mice expressed low levels of the receptor (fig 6.9b), this may help to explain their extended survival in the spleen despite the abundance of antibody that inevitably results from the high numbers of splenic plasma cells seen throughout the first 35 days of Salmonella infection.

That NP-specific plasma cells were depleted by the same amount as non-NP-specific plasma cells in the bone marrow of NP+ Salmonella mice means that there is no inherent difference in the capacities of these cells to survive during infection. The greater depletion of total bone marrow plasma cells in Salmonella-alone mice compared to NP+ Salmonella mice is therefore likely due to changes in the niche providing cells between these two groups; perhaps a more stable compartment of these cells is generated during the NP-KLH prime and boost regime, and these are therefore less affected by subsequent infection with Salmonella. A more detailed characterisation of these cells would be required to fully understand this difference.

**Competition between previously established and newly generated plasma cells in the presence or absence of adjuvant**

As we had witnessed the depletion of previously established bone marrow NP-specific plasma cells during Salmonella infection, followed by repopulation of the bone marrow with non-NP-specific plasma cells (which are likely to be specific for Salmonella antigens), we hypothesised that this depletion may ‘create space’ in the limited bone marrow plasma cell pool, and allow for the enhanced establishment of newly generated plasma cells entering from the secondary lymphoid organs. To test this, mice were primed with two protein antigens (NP-KLH and FITC-OVA), and subsequently boosted with soluble NP-KLH. 5-weeks later, mice were boosted with soluble, or alum-precipitated FITC-OVA + killed B. pertussis (for schematic, see fig 6.11f).
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The group receiving adjuvant underwent a reduction in both total and NP-specific bone marrow plasma cell numbers by day 5, similar to *Salmonella* infection (fig 6.11a and c). However, interestingly (and despite similar generation of FITC-OVA-specific plasma cells in the spleens of mice receiving soluble FITC-OVA or adjuvant/FITC-OVA, see fig 6.11b), mice receiving adjuvant failed to establish comparable populations of FITC-OVA-specific plasma cells in the bone marrow even by 30-days after boosting (fig 6.11b). This may be due to the idea, discussed earlier, that inflammation (or specifically, TNF-α) mediates a reduction in CXCL12 from bone marrow cells. This may play a dual role, firstly in depleting bone marrow plasma cells, but also by reducing the influx of antigen-specific plasma cells to the bone marrow, as CXCL12 attracts plasma cells to this site. As previously observed (see chapter 5), boosting with soluble protein antigen led to the rapid seeding of large numbers of antigen-specific plasma cells in the bone marrow (fig 6.11b). While boosting with soluble antigen is common practise among immunologists, and has been used extensively by groups examining the establishment of long-lived bone marrow plasma cells, including ourselves, the response seen is surprising. As shown in chapter 5, by 12-days following booster immunisation, NP-specific plasma cells came to dominate the bone marrow plasma cell compartment as a large ‘wave’ of plasma cells migrated to the organ. Many of these cells survived, and even 45 days after the secondary response, the frequency of NP-specific plasma cells within the bone marrow plasma cell compartment was around 50 percent. This raises two points of interest: firstly, although common practise in mouse experiments, booster vaccinations are often given with adjuvant. This may lead to the reduced establishment of bone marrow plasma cells generated during the recall response, and lower antibody levels. Secondly, if plasma cells generated during the recall response came to dominate the bone marrow plasma cell pool (as seen in these experiments), it may occur at the expense of plasma cells and antibody specific for other prior infections, and significant antibody levels could only be maintained against a few specificities.
Chapter 6 – Effects of inflammation on bone marrow plasma cells

It is possible that inflammation, in this case mediated by adjuvant (but in infections, mediated by TLR ligands present on many pathogens), reduces the entry of plasma cells to the bone marrow, in order to prevent a single specificity of plasma cell from dominating the compartment, and effectively reducing circulating antibody to prior immune responses. This would allow (as seen here) for high numbers of antigen-specific plasma cells to be generated in the spleen, creating large amounts of high affinity antibody. These plasma cells are also supported in the spleen, for at least 12 days, likely by the induction of APRIL producing cell populations (as discussed in chapter 4), as they survived in far greater numbers in mice receiving adjuvant/FITC-OVA to those receiving soluble FITC-OVA (fig 6.11b). However, rather than a mass-migration to the bone marrow, the vast majority of these plasma cells are allowed to die as inflammation resolves and plasma cell survival factors are reduced. Only relatively low numbers migrate to the bone marrow to become established as long-lived plasma cells. This may also be a means of allowing only the highest affinity plasma cells to migrate to the bone marrow, as these are generated during the latter stages of the immune response, when affinity maturation has been occurring for some time. Inflammatory signals, which are high at early stages of the immune response (and when the affinity of antibody secreted by plasma cells is relatively low), drastically reduce the entry of plasma cells to the bone marrow. As inflammation resolves, plasma cells of the very highest affinity (generated during the later stages of the response) are allowed to enter the bone marrow compartment. It would therefore be interesting to look at time points beyond the 30-days examined here, to see if only very high affinity FITC-OVA-specific plasma cells accumulated in the bone marrow of mice receiving adjuvant/FITC-OVA.

As can be seen in figure 6.11d, early FITC-OVA-specific IgG was not impaired by the reduced FITC-OVA-specific bone marrow plasma cells in mice receiving adjuvant. This is likely due to high numbers persisting in the spleen. By day 30, FITC-OVA-specific IgG levels had begun to decline in this group (and were already significantly lower than levels in mice receiving soluble FITC-OVA),
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although whether they continue to decline is not yet known. Experiments looking at later time points will be performed to determine this. IgG has a half-life of around 3-weeks, and so may take some time to decline even after the majority of splenic FITC-OVA-specific plasma cells die. As seen previously, mice receiving soluble FITC-OVA maintained FITC-OVA-specific IgG for at least 30 days, and previous experiments have shown that this persists for far longer (see fig 5.5).

Immunisation with soluble FITC-OVA also showed interesting points. While there was initially no depletion in the previously generated NP-specific plasma cells, despite the influx of large numbers of FITC-OVA-specific plasma cells, these did decline by day 30 (fig 6.11b and c). This reflects an increase in total bone marrow plasma cells at day 12 (due to new FITC-OVA-specific plasma cells entering the compartment). However these expanded numbers of plasma cells could not be supported, presumably due to the limited amounts of survival factors or niches available in the bone marrow, and declined to levels in control mice by day 30 (fig 6.11a). This mediated a similar decrease in both FITC-OVA-specific and NP-specific plasma cells (fig 6.11ba and c). This is likely due to competition between all bone marrow plasma cells for the available niches. While it is thought that newly generated plasma cells have an advantage over older, previously established plasma cells at accessing niches (due to their ability to migrate towards CXCL12\textsuperscript{85,201}), it is not known of the relative importance of secreted survival factors (such as APRIL and IL-6) and cognate interactions between plasma cells and niche-providing cells (data suggests that plasma cells associate closely with ‘niche-providing cells’ and may receive cognate signals through VLA-4, LFA-1, CD28 and other molecules\textsuperscript{151,194,217,226}).

**Conclusion**

Here we have documented a striking depletion of previously established bone marrow plasma cells during infection with *Salmonella* or *Schistosoma mansoni*. This reduced circulating antigen-specific antibody levels. Interestingly, we also observed a MyD88-dependent increase in the expression of MHC II by surviving plasma cells established during prior immune responses, and increased
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numbers of these cells appearing in the spleen. While these experiments do not conclusively define the mechanism behind the depletion of bone marrow plasma cells during inflammation, or the intriguing possibility that inflammation may impede the entry of plasma cells to the bone marrow, they provide evidence that inflammatory episodes play a role in both. To expand on this data, it would be interesting to firstly pin down the mechanism, or multiple mechanisms behind the depletion of plasma cells. Secondly, further investigations into how boosting with antigens with or without adjuvants can lead to a differing duration of humoral immunity could provide interesting data which could have implications for booster vaccination protocols. Finally, we have demonstrated that secondary immunisation with alum-precipitated FITC-OVA with killed *B. pertussis* leads to the reduced generation of plasma cells that persist in the bone marrow. This may be due to a failure to migrate to the organ, as reduced levels of CXCL12 secreted by bone marrow cells during times of inflammation likely impair the ability of plasma cells to migrate there\textsuperscript{84,334}. Alternatively, the adjuvant used here contains a mixture of TLR-ligands and other innate signals that could influence the outcome of B cell responses and the lifespan of the plasma cells produced. Autoantibody-secreting plasma cells generated during SLE secrete antibody against self-DNA or self-RNA. The B cells that differentiate to these plasma cells are stimulated through different TLRs (TLR7 for RNA and TLR9 for DNA). Interestingly anti-DNA plasma cells are short-lived, while the anti-RNA plasma cells may become long-lived\textsuperscript{262}. Similarly, differing combinations of TLR ligands (e.g. TLR4 + TLR7) can induce greater levels of antibody in the serum, and more detectable long-lived plasma cells than single TLR ligands\textsuperscript{263}. It would be interesting to see if differing innate signals mediate the establishment of greater or fewer long-lived plasma cells, and to determine the mechanism behind these differences.
Chapter 7 - Final Discussion and Further Work

The studies here have investigated a number of areas of plasma cell biology. Initially, we investigated the accumulation of plasma cells in the bone marrow of naïve C57Bl/6 mice over time. These studies arose from the surprising finding that 6-week-old naïve mice had similar turnover of plasma cells in the spleen and bone marrow. While it has been shown that some plasma cells survive for long periods in the spleen\textsuperscript{193}, the majority of long-lived plasma cells reside in the bone marrow, where they persist for months or years without cellular division and maintain serum antibody levels to prior infections and vaccinations\textsuperscript{6,189,193,306,317}.

We saw an accumulation of plasma cells in the bone marrow, but not the spleen over time. These plasma cells did not divide in a 10-day BrdU pulse and corresponded to a similar accumulation in serum antibody levels. This required the expression of MHC II, CD40 and a normal BCR repertoire as mice deficient in MHC II and CD40, as well as MD4 mice (expressing a BCR specific for HEL), did not exhibit the same accumulation of plasma cells in the bone marrow. This data indicates that CD4 T cell activation is likely crucial for the generation of these long-lived bone marrow plasma cells, and this would be in agreement with the literature\textsuperscript{6,246,303,306}. Whether it is the activation of CD4 T cells by professional APC (and the subsequent production of cytokines), or the cognate interactions between T and B cells that occur during the initiation of TD immune responses is unclear. That long-lived plasma cells are thought to arise from germinal centre responses\textsuperscript{248} (which require cognate interactions between activated CD4 T cells and B cells\textsuperscript{100}) implies that germinal centre formation may be necessary to generate long-lived plasma cells in naïve mice. To confirm these hypotheses, chimeric mice in which B cells do not express CD40 or MHC II (allowing for the priming of CD4 T cells by professional APC, but not for cognate interactions with B cells) could be used. Although it is already known that CD40 expression on B cells is essential for germinal centre formation\textsuperscript{341}, it would be interesting to
correlate germinal centre formation in these mice with a lack of long-lived plasma cell formation compared to wild type chimeras.

That these naive mice form germinal centres despite their housing in specific pathogen-free conditions is not surprising; they are not germ free mice and are exposed to environmental antigens, and contain large amounts of microbial flora in the gut. The comparison of these mice to germ-free mice would be interesting; from these experiments we cannot conclude what the accumulated long-lived plasma cells are specific for, and whether they would occur independently of antigen.

As well as bone marrow plasma cells, we performed experiments looking at the requirements for the extrafollicular plasma cell response to SRBC immunisation and infection with *Salmonella*. From this, we observed that plasma cells generated by SRBC were very short-lived, dying within 1-3 days following immunisation. Those generated by *Salmonella* infection persisted for longer periods with a half-life of around 6 days. These were likely to be supported by the local generation of large amounts of the plasma cell survival factor APRIL, and the increased availability of splenic plasma cell niches due to the influx and expansion of innate cell populations such as monocytes, macrophages and dendritic cells. To conclusively show this, blocking antibodies to APRIL, or its receptors TACI and BCMA, could be used; if plasma cell numbers fell, or the turnover of plasma cells increased, it would be likely that APRIL is a key factor in the support of these cells.

We also identified the key signalling required for IgM and IgG plasma cells seen at day 5 of the response to SRBC and at days 4 and 8 of *Salmonella* infection. At day 5 following SRBC immunisation, both IgM and IgG plasma cells were found to be TD, requiring MHC II, CD40 and ICOS expression. Surprisingly, the expression of the TLR adaptor proteins MyD88/TRIF were also required for optimal IgM and IgG responses. It remains unknown why TLR signalling would be important in this immunisation; there are no known TLR ligands in SRBC. As well as TLR signalling, MyD88 plays a role in signalling through TACI\(^{149}\) and the
IL-1 receptor; TACI is known to cause class switching, and IL-1 receptor signalling causes the up-regulation of CD40L on CD4 T cells. These effects may impact both germinal centre and class switched plasma cell formation following SRBC immunisation.

In *Salmonella* infection, two waves of extrafollicular plasma cells were observed. An early IgM wave at day 4 that required MyD88/TRIF but not CD40, ICOS or MHC II and a later IgG wave by day 8 that required CD40, ICOS and MHC II but not MyD88/TRIF. As the mice used for these experiments were total knockouts, we cannot conclude that cognate interactions between T and B cells are required for the generation of these IgG plasma cells. However, CD4 T cell activation is certainly key, and it is known that TD extrafollicular plasma cell responses require cognate interactions between T and B cells. In addition, Cunningham et al have shown that CD40L is required for IgG2c plasma cells in *Salmonella* infection. In addition, we observed that early TD *Salmonella*-specific IgG2c was dependent on TLR signalling, as MyD88/TRIF/- mice switched instead to the Th2 associated isotype IgG1.

In contrast, the day 4 IgM plasma cell response did not require T cell help. While Gil-Cruz et al have shown that B1b cells contribute to the early plasma cell response to *Salmonella*; our data suggest that marginal zone B cells, too, play a role. CD1d/- mice, which lack the marginal zone B cell compartment, failed to generate an IgM plasma cell response at day 4. Indeed, the marginal zone B cell compartment is thought to respond rapidly to systemic infections due to prime location, and lower threshold of activation than follicular B cell types. Whether this is the case in *Salmonella* infection could be definitively tested using chimeras that are reconstituted with both CD1d/- and DS-RED (which express the modified red fluorescent protein ‘DS-RED’ ubiquitously) bone marrow. In these mice, all marginal zone B cells would arise from the DS-RED bone marrow and would therefore be identifiable. If the majority of IgM plasma cells arising at day 4 expressed the DS-RED protein, they would likely arise from the marginal zone compartment.
Interestingly CD1d−/− mice also failed to generate a robust IgG plasma cell response at day 8. This may be due to a deficiency in NKT cells, which aid early plasma cell responses to lipid antigens through both cognate interactions and IL-21 secretion\textsuperscript{82,83,344}. That MD4 mice failed to respond at day 4 could imply that BCR-recognition of antigen is essential for day 4 TI plasma cells, however, these mice responded at day 8, when the response was TD in wild type mice. Cognate interactions with CD4 T cells cannot occur without antigen-specificity, as antigen presented on MHC II molecules of B cells must be recognised by the BCR for uptake, and subsequently by the TCR. Bystander activation, whereby the threshold of activation is reduced by the presence of high levels of inflammatory cytokines, may play a role in the activation of HEL-specific B cells in MD4 mice. In vitro, the stimulation of follicular B cells with LPS alone is enough to induce suboptimal differentiation to plasma cells and antibody secretion\textsuperscript{31,53,59}. The specificity of these B cells is, presumably, not towards LPS. Whether Salmonella infection is sufficient to induce the polyclonal activation of follicular and marginal zone B cells in wild type mice, irrelevant of BCR specificity, remains unknown.

This too could explain why we saw the expansion of NP-specific plasma cells in the spleens of Salmonella infected mice that were previously immunised and boosted with NP-KLH. While known not to occur following the injection of LPS or CpG DNA alone, it is possible that inflammatory cytokines, and other activatory signals (such as BLyS and APRIL) may be enough to stimulate the differentiation of memory B cells to plasma cells.

In these experiments, we did not detect any turnover of NP-specific plasma cells in the spleen at day 6-8 or day 14-16 following infection with Salmonella. This would imply that these cells were pre-existing, although the possibility remains that turnover occurred at other time points (i.e. not during our BrdU pulses). If pre-existing, these plasma cells may have migrated to the inflamed spleen from the bone marrow due to altered chemokine expression in the inflamed spleen.
(increased CXCL9, CXCL10, CXCL11 and CXCL12\textsuperscript{201,345}) and reduced CXCL12 from the bone marrow\textsuperscript{334}.

This would be in accord with our data showing that inflammation depletes bone marrow plasma cells. Although here we have not made estimates concerning the total number of antigen-specific plasma cells depleted from the bone marrow during infection, and the increase in number seen in the spleen, the kinetics of their appearance in the spleen is similar to their depletion from the bone marrow. Experiments where mice were boosted with NP-KLH and provided with BrdU for a period of 10-20 days (to label all NP-specific plasma cells generated by the secondary immunisation), and subsequently infected with \textit{Salmonella}, could be performed. If NP-specific plasma cells appearing in the spleen contained the BrdU label, it is likely that they have migrated there from other organs such as the bone marrow. Similar experiments where NP-specific bone marrow plasma cells are transferred to naïve or \textit{Salmonella} infected hosts would allow the detection of altered migration.

Bone marrow plasma cells are thought to be non-migratory, and this would appear to confound these ideas\textsuperscript{201}. However, that they up regulate certain other markers of immaturity (such as MHC II) during \textit{Salmonella} infection could mean that they revert to a more immature state, and regain the ability to migrate. This idea could be tested by sorting NP-specific plasma cells from the bone marrow of non-infected or \textit{Salmonella} infected mice and performing migration assays towards chemokines such as CXCL12. The precise factor, or combination of factors, that mediates the depletion of bone marrow plasma cells during inflammatory episodes is yet to be identified, but studies looking at the levels of various cytokines in the serum, or from cultured bone marrow cells from naïve or \textit{Salmonella} infected mice could help to identify candidates that are altered in infected mice. By injecting candidate recombinant cytokines to naïve mice and looking at the depletion of plasma cells, or by stopping the action of cytokines with blocking antibodies, we may learn what factors are important to this process.
A similar mechanism may help to determine the observation that the formation of bone marrow plasma cells is impaired during chronic inflammation. This was observed in K/BxN mice undergoing a chronic autoimmune response, mice infected with *Salmonella*, which exhibits high levels of inflammation over a long period, and in mice receiving repeated immunisation with NP-KLH over a sustained period. While there are a number of possible mechanisms that could explain these findings, reduced levels of CXCL12 from the bone marrow caused by inflammation\(^{334}\) is a strong candidate, as CXCL12 has been shown to mediate migration of plasma cells to the bone marrow\(^{84}\). Furthermore, our data in which mice were boosted either with soluble FITC-OVA, or alum-precipitated FITC-OVA with *B. pertussis* showed a reduced accumulation of FITC-OVA-specific plasma cells in the bone marrow, at least in the first 30-days following secondary immunisation. Whether these plasma cells accumulate at later time points, and whether a reduced level of CXCL12 from bone marrow cells can be detected in mice receiving FITC-OVA and adjuvant will be investigated in future experiments.

Other possibilities exist, such as differential responsiveness of plasma cells to chemokines when antigen is still present. Many plasma cells migrate towards inflamed organs, thought to be mediated by CXCL9, CXCL10 and CXCL11 through their expression of CXCR3\(^{201,345}\). It is possible that the persistence of antigen alters the expression of CXCR4 and CXCR3 on plasma cells. In addition, some plasma cells have been reported to be unresponsive towards CXCL12, despite their continued expression of CXCR4\(^{333}\).

Whether the depletion of bone marrow plasma cells in infection is a mechanism by which space is created for the establishment of new bone marrow plasma cells is still unresolved. While we did not observe this during the first 30-days following the injection of soluble or alum-precipitated FITC-OVA (with *B. pertussis*), this may have been due to the reduced migration of plasma cells to the bone marrow during inflammatory episodes. This will require longer time course experiments. Additionally, the infection of mice with *Salmonella* expressing the ovalbumin protein could allow for a greater understanding of
when antigen-specific plasma cells generated by live infection enter the bone marrow.

**Conclusion**

While there is much work to do to dissect the mechanisms behind the observations seen here, this thesis has expanded our knowledge on the factors required for the formation and maintenance of extrafollicular plasma cell responses in *Salmonella* infection as well as the TD response to SRBC. In addition, we have noted the accumulation of bone marrow plasma cells in naïve mice over time, and provided evidence that CD4 T cell activation plays a key role in their generation. Thirdly, we have shown that few bone marrow plasma cells are formed during chronic inflammation compared to acutely immunised mice. This may be due to reduced migration to the organ, or a failure to generate plasma cells with the potential to survive for long periods. Similarly, we have shown that adjuvant (in the form of alum-precipitation and killed *B. pertussis*) delays or reduces the number of bone marrow plasma cells formed in a secondary immune response to protein antigen. Finally, we have shown that inflammation reduces bone marrow plasma cell numbers. While these recover following the resolution of inflammation, humoral immunity to prior immunisation is reduced. Whether this mediates the establishment of increased numbers of newly generated plasma cells or not is still unknown.
Chapter 8 - Bibliography

41. Rodríguez-Pinto, D. B cells as antigen presenting cells. Cellular Immunology 238, 67–75 (2005).


75. Martin, F. & Kearney, J. F. Positive selection from newly formed to


139. Wu, Y. *et al.* Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLYS. *J. Biol. Chem.* **275**, 35478–35485 (2000).


221. Nie, Y. *et al.* The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med* 200, 1145–1156
(2004).


301. Mendiratta, S. K. et al. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6, 469–477 (1997).
302. Müller, U. et al. Functional role of type I and type II interferons in


334. Ueda, Y., Yang, K., Foster, S. J., Konda, M. & Kelsoe, G. Inflammation Controls B Lymphopoiesis by Regulating Chemokine CXCL12


