Regulation of Extrafollicular Immunoglobulin Class Switch Recombination

Laura George

A thesis submitted to

The University of Birmingham

for the degree of

Doctor of Philosophy

School of Immunity and Infection

College of Medicine and Dental Science

University of Birmingham

September 2013

DEDICATION

For my father

ACKNOWLEDGMENTS

Thanks to my excellent supervisor Dr Kai-Michael Toellner for his advice and guidance during this project. I would also like to thank him for allowing me the opportunity to work on this project and for all his support over the last few years.

I would like to acknowledge Dr Yang Zhang for her help and practical tips in the lab. Mahmood Khan for his help with the immunohistochemistry. Dr Saeeda Bobat and Sarah Cook for proof reading the drafts. Dr Jennifer Marshall for the groundwork for the project. Members of the Cunningham group: Jessica Hitchcock, Dr Adrianna Flores-Garcia and Charlie Cook (now part of the Toellner group) who have helped me one way or another over the course of my PhD. All the members of BMSU and members of the IBR 4th floor who have made the last few years an enjoyable experience.

Thanks to Dr Kyoko Nakamura who produced the miR-125b overexpressing mice and helped with much of the experimental work and Professor Peter Lane for his suggestions for this aspect of the project.

Lastly thanks to my family for their support and love, specifically my mum and brothers. Finally thanks to Richard.

ABSTRACT

The humoral immune response is characterised by the production of antibody secreting B cells. Some of these cells have cycled through the germinal centre, diversifying and optimising their antigen receptors to produce affinity-matured, class switched antibody. As this is a relatively slow process, the first class switched antibody is produced by non-mutated B blasts that have differentiated independently of the germinal centre reaction outside B-cell follicles. Extrafollicular foci of plasmablasts provide the first line of defence within the adaptive antibody response.

IRF4 has been shown to be essential for Ig class switching and plasma-cell differentiation. Two expression levels of IRF4 were reported, with intermediate levels proposed to regulate CSR in the GC, while high levels regulate plasma-cell differentiation. We have correlated the two phases of IRF4 induction with specific stages of B-cell differentiation. Following immunisation of quasi-monoclonal mice with NP-Ficoll, intermediate levels of IRF4 protein are expressed by all B blasts as they move to the outer T zone and before the formation of germinal centres or plasma cells. This is followed by expression of AID and CSR. In contrast, plasma-cell differentiation occurs with high level expression of IRF4, expression of Blimp1 and complete suppression of AID and CSR. The NFκB family signalling molecules C-REL and NFκB1 have been shown to be required for the induction of IRF4 protein in B cells following stimulation. We show that these signalling molecules are not necessary to induce the early rapid intermediate level expression of IRF4, and extrafollicular expression of AID and CSR occur normally when they are absent. IRF4-high expression and plasma-cell differentiation, however, are blocked in the absence of these molecules.

Using overexpression of the micro-RNA miR-125b we show that all phases of IRF4 protein expression can be blocked. This leads to inhibition of AID protein induction and CSR , indicating that extrafollicular intermediate level IRF4 expression is related to AID expression and CSR. Further, we show that AID in the GC is expressed normally when IRF4 is repressed, leading to normal affinity maturation This shows that AID expression is regulated differently in extrafollicular B blasts and in the germinal centre.

Common Abbreviations

AID	Activation-induced cytidine deaminase
APC	Allophycocyanin
APRIL	A proliferation inducing ligand
β2m	beta-2 microglobulin
BAFF	B cell activating factor
BAFF-R	BAFF receptor
Bcl6	B cell lymphoma 6
BCMA	B cell maturation antigen
BCR	B cell receptor
Blimp-1	B-lymphocyte induced maturation protein-1
BSA	Bovine serum albumin
Bt	Biotin
C57/BL6	C57BL/6J
CGG	Chicken gamma globulin
CSR	Class switch recombination
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DC	Dendritic cell
Dnky	Donkey
DNA	Deoxyribonucleic acid
EBI2	Epstein-Barr virus-induced gene 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay

F	Follicle
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FC	Flow cytometry
FDC	Follicular dendritic cells
FITC	Fluorescein-isothiocyanate
GC	Germinal centre
Gt	Goat
Hs	Hamster
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Int.	Intermediate
i.p	Intraperitoneal
IRF4	Interferon-regulatory factor 4
i.v	Intravenous
J	Joining
κ	Kappa
λ	Lambda
KO	Knock out
LPS	Lipopolysaccharide
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
MS	Marginal sinus
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells

NK	Natural killer
NP	4-hydroxy-3-nitrophenyl acetyl
NP-CGG	4-hydroxy-3-nitrophenyl acetyl-chicken gamma globulin
PAX5	Paired box gene 5
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PNA	Peanut agglutinin
Prdm1	Positive-regulatory-domain-containing
QM	Quasi-monoclonal
Rb	Rabbit
RNA	Ribonucleic acid
Rt	Rat
RT-PCR	Reverse transcription polymerase chain reaction
Sh	Sheep
SHM	Somatic hypermutation
SRBC	Sheep red blood cells
ST	Switch transcript
STAT	Signal transducers and activators of transcription
Strept AP	Streptavidin-biotin alkaline phosphatase complex
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TCR	T-cell receptor
TD	T cell-dependent
TI-I	T cell-independent type I

TI-II	T cell-independent type II
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
T zone	T cell zone or periarteriolar lymphoid sheath
UNG	Uracil-N-NDA glycoslase
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
V-region	Variable region (of immunoglobulin)
Xbp-1	X-box binding protein 1

Table of Contents

Table of Figures

Table of tables

1.INTRODUCTION

1.1 Overview

The adaptive immune response consists of cellular and humoral immunity. Whilst cellular immunity is provided by T cells, humoral immunity is provided by antibody producing B cells. Antibody elicits its protective function mainly in two ways. The first of these is via opsonisation, antibody coated pathogens are recognised and destroyed by phagocytes such as macrophages and neutrophils. The second method is via neutralisation, antibody binding to specific epitopes on a pathogen blocks the interactions required for bacteria or virus to enter host cells. In addition, antibody is protective via the process of antibody dependent cell-mediated cytotoxicity (ADCC). Infected cells express viral proteins on their surface that are bound by antibody. Natural killer cells recognise antibody bound to infected cells via their Fc receptors and destroy the infected cell. This section will briefly, without references, introduce the project in the context of the immune response.

Antibody affinity increases over the course of an immune response, however this process is relatively slow. Affinity matured antibodies are produced by B cells that have differentiated via the germinal centre (GC) pathway. The GC is a specialised structure within the B-cell follicle where B cells mutate their immunoglobulin genes. Classically, these structures can start to form within four days of antigen exposure. In contrast, during the first days after infection, protection is provided by the rapid extrafollicular response. This occurs independently of the GC reaction and does not produce affinity matured B cells, however some of these antibody-producing cells do undergo class switch recombination (CSR). In the latter process, B cells change their heavy chain gene, but do not alter the affinity of their B-cell receptor (membrane bound Immunoglobulin (Ig)). This produces an antibody with a different heavy chain, but unaltered specificity. The antibody class induced is dependent upon the type of pathogen or antigen, and the route of delivery. This thesis is primarily concerned

with the extrafollicular B-cell response and the role of IRF4 for CSR. IRF4 is a transcription factor that is differentially expressed during B-cell activation and differentiation. By altering the two phases of IRF4 induction, this thesis aimed to test its role in the process of CSR.

1.2 Overview of the murine spleen

The B-cell responses detailed in this thesis are primarily studied in the spleen, this is a highly vascularised organ which lacks afferent lymphatic vessels. The main functions of the spleen are blood filtration, haematopoiesis and defence against blood-borne pathogens. It should be noted that splenic haematopoiesis is a process that occurs in rodents (Cesta, 2006) but not humans.

Together the spleen, lymph nodes and gut associated lymphoid tissue (GALT) comprise the peripheral lymphoid organs. Here, mature naive lymphocytes re---circulate and are able to encounter antigen that has entered the spleen via the blood, or the lymph nodes via the lymphatics. The spleen is comprised of white pulp and red pulp areas, the latter making up the bulk of the organ (Fig 1.1). One function of the spleen is to remove senescent red blood cells; this is performed by macrophages within the red pulp. The white pulp is comprised of the periarteriolar lymphoid sheath (PALS) surrounding the central arterioles. The latter is the site where antigen and lymphocytes enter the white pulp. The PALS is comprised of T---cell areas and B---cell follicles. The B---cell follicle is surrounded by the marginal zone which contains macrophages and non---recirculating marginal zone B cells (Martin and Kearney, 2001; Muppidi et al., 2011).

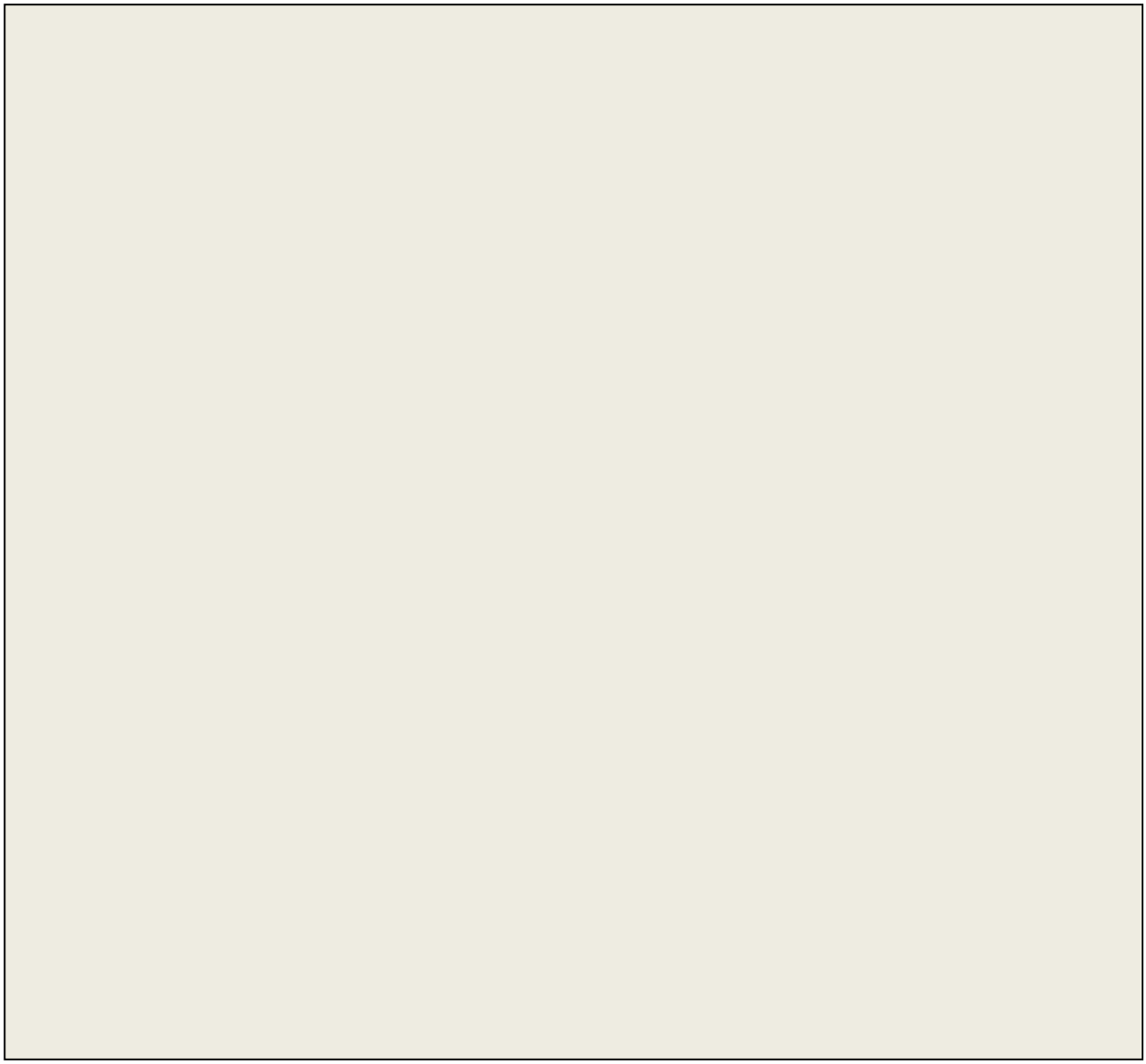


Figure 1 1 The structure of the spleen (A) Blood enters via the afferent splenic artery which branches into a network of central arterioles. These central arterioles end in cords in the red pulp which feed into venous sinuses. These then feed into the efferent splenic vein. (B) The structure of the white pulp in the mouse. The central arteriole is surrounded by the white pulp known as the PALS. This consists of a central T-cell area with an adjacent B-cell follicle. The PALS is then surrounded by the marginal zone containing marginal zone B cells and macrophages (Mebius and Kraal, 2005).

The Red Pulp

The red pulp is perfused by a rich blood supply. Blood enters the spleen via the afferent splenic artery and branches into central arterioles that terminate in cords in the red pulp. The cords are composed of reticular fibres, reticular cells and macrophages (Cesta, 2006; Mebius and Kraal, 2005). The structure of the cords traps ageing erythrocytes between endothelial cells which facilitates easier phagocytosis by red pulp macrophages. This is important for iron recycling, a process that will not be discussed here. Red pulp macrophages also perform a second important function by performing phagocytosis of blood borne particulate matter.

The second role of the spleen is also carried out in the red pulp, as it is here that haematopoiesis occurs. This is the developmental site of erythroid, myeloid and megakaryocytic cells and also will not be discussed further (Cesta, 2006).

Most importantly in the context of this thesis, the red pulp is the site in which plasmablasts and plasma cells produce antibody, following antigen-specific differentiation in the follicles. From this location in the red pulp, antibody can enter the blood stream rapidly. Migration of plasmablasts to the red pulp is dependent upon the network of chemokines expressed and may be mediated by upregulation of CXCR4 and downregulation of CXCR5 and CCR7. CXCR4 binds CXCL12 expressed in the red pulp, whereas CXCR5 and CCR7 bind chemokines in the follicular and T-cell areas of the white pulp respectively (Hargreaves et al., 2001).

The White Pulp

In contrast to the red pulp, the white pulp is the site where B cells are initially activated and T cells are primed on dendritic cells. Activated B cells can then interact with these primed T cells. This area is also the site of B-cell receptor hypermutation that occurs within GC. The white pulp can be further divided into the B-cell follicles− these are surrounded by the marginal zone− and the T-cell area. These separate areas are described in the following sections.

B-cell follicles

Naïve recirculating B cells are attracted to the follicles via the gradient of CXCL13. This chemokine is produced by follicular stromal cells such as follicular dendritic cells (FDC)(Cyster et al., 2000) and marginal reticular cells (MRC) (Katakai et al., 2008). The receptor for CXCL13 is CXCR5, this is expressed on all mature B cells. Activated B cells migrate within the follicles in order to access antigen and T-cell help. In addition, migration to specialised microenvironments is required to support production of antibodies.

B cells can respond in a T-dependent or T-independent manner to antigen. These two types of responses will be discussed in more detail later. The first event which occurs within two hours of B-cell activation in the T-dependent response, is migration to the outer follicle (Gatto and Brink, 2013) (Figure 1.2). This migration is controlled by B-cell expression of EBI2, a G-protein coupled receptor, whose ligand is 7α,25-dihydroxycholesterol (Hannedouche et al., 2011; Liu et al., 2011). Upregulation of EBI2 on B cells occurs within one hour of B-cell activation (Kelly et al., 2011). Concentrations of 7α,25-dihydroxycholesterol are highest in the outer follicle, whilst degradation of this ligand is highest in the T zone, creating a gradient (Gatto and Brink, 2013). The reason for B-cell migration to the outer follicle so early after activation is currently unknown, but may occur in order to allow B cells to capture antigen from the marginal sinus and interact with the resident macrophages present there.

The second migration event after B-cell activation is to the border with the T zone. This occurs within six hours of activation. At this time, B cells downregulate EBI2 (Kelly et al., 2011) and upregulate CCR7 (Reif et al., 2002). The ligands for the CCR7 receptor, CCL21 and CCL19, are highest in the T zone and a decreasing gradient of CCL21 extends into the B-cell follicle (Okada et al., 2005). Although EBI2 transcripts are lower at this stage, the receptor is still required for even distribution of B cells along the T-B boundary (Kelly et al., 2011). Following interactions with T cells, B cells can differentiate via one of two pathways. They can move to the follicles to become GC B cells, or migrate to the red pulp, to form

extrafollicular plasmablast foci. Deficiency of EBI2 leads to a failure of B cells to locate to the outer follicle two days after activation, and leads to decreased GC and plasmablast responses to TD antigens (Pereira et al., 2009) (Gatto et al., 2009).

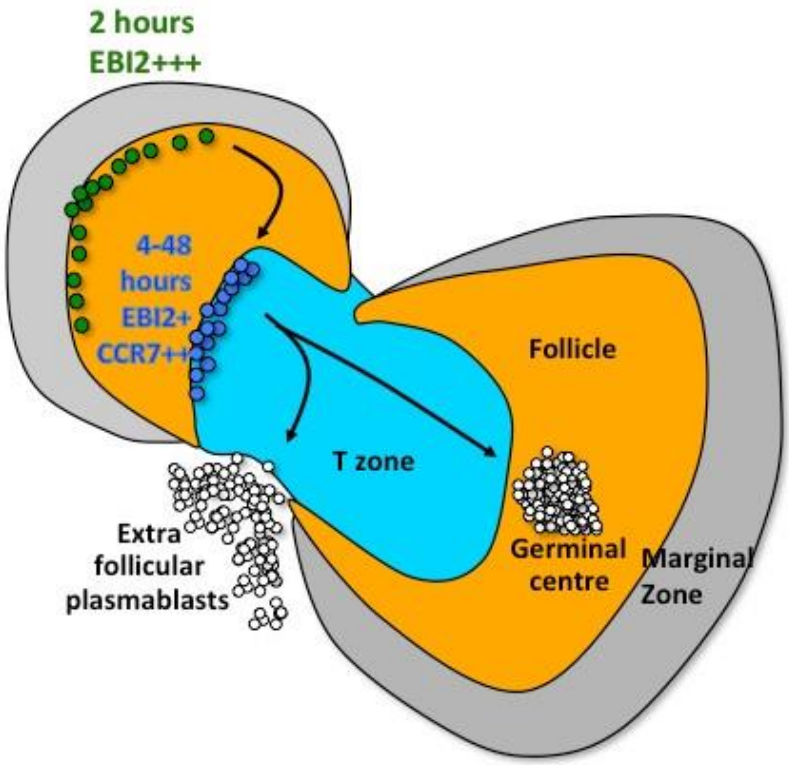


Figure 1. 2 B-cell migration in the first hours post-activation.

Within one hour of activation, B cells upregulate their expression of EBI2 which allows them to migrate to the outer follicle along a gradient of 7α,25-dihydroxycholesterol. Within four hours of activation B cells down-regulate their expression of EBI2, upregulate their expression of CCR7 and migrate to the border with the T zone following the gradient of CCL19 and CCL21. Four days after activation B cells have differentiated into GC B cells or extra-follicular plasmablasts.

B-cell follicles are the location of GC and FDC. The former will be discussed in more detail later. In terms of FDC: these stromal cells capture and display opsonised antigens for long periods of time (Sukumar et al., 2008) and may have a role in secondary responses (Tew et al., 1997). They also act as a reservoir for infectious agents such as HIV (Smith et al., 2001). FDC express complement receptors CD21 and CD35 and the low affinity receptor for IgG

FcγRIIb (Allen and Cyster, 2008). Expression of these receptors allows FDC to retain opsonised antigen for presentation to B cells.

T Zone

The T zone is sandwiched between the follicles and only contacts the red pulp at specific points, known as marginal zone bridging channels. At these points, a break in the marginal sinus and B-cell follicles allows the T zone to directly contact the red pulp.

Antigen and lymphocytes can enter the white pulp of the spleen via the central arterioles, located in the T zone. This allows T cells to become activated by antigen bearing interdigitating DCs that have migrated from the periphery. These T cells are largely $CD4^+$, although some $CD8^+$ cells may also be present. CCL19 and CCL21, produced by T zone fibroblastic reticular cells (FRC) (Siegert and Luther, 2012), attract DCs and T cells expressing CCR7 to the T zone. T cells also enter the spleen via the marginal zone bridging channel (MZBC) (Bajénoff et al., 2008) and are again guided by a gradient of CCL21 on FRC. FRC are a type of stromal cell unique to the TZ and MZBC and these stromal cells drive T-cell migration along their fibres. Expression of CCL19 and CCL21 by FRC requires the expression of LTαβ and TNF on stromal cells. Engagement of these receptors with the ligands, expressed on haematopoietic cells during neonatal development, stimulates signalling via NFκB and production of the chemokines (Mebius and Kraal, 2005; Ngo et al., 2001).

Following antigen activation, T cells can differentiate into T follicular helper cells (T^{FH}). This differentiation requires the transcription factors BCL6, c-Maf and BATF in T cells, with BATF upstream of BCL6 and c-Maf (Betz et al., 2010; Ise et al., 2011; Johnston et al., 2009). These T^{FH} cells downregulate CCR7 and upregulate CXCR5. This drives migration to the B-cell areas for T-B cell interactions.

The marginal Zone

The marginal zone is a transit area. Here, cells leaving the blood enter the spleen in an active G-protein coupled process (Cyster and Goodnow, 1995). Blood enters through the marginal

sinus− a vessel located between the marginal zone and the follicles− and travels in the direction of the red pulp. The marginal zone contains resident B cells and macrophages. MZ B cells will be discussed in more detail later. Macrophage types include marginal zone macrophages (MZM) expressing SIGNR1, and an inner ring of marginal zone metallophilic macrophages (MMM). This latter population express toll-like receptors and the macrophage receptor with collagenous structure (MARCO). B cells and marginal zone dendritic cells (DC) are sandwiched between these two types of macrophage.

1.3 B-cell subtypes

Five types of mature B-cell are found in the mouse spleen, these can first be divided into B-1 and B-2 cells. B-1 cells are sub-divided into B-1a and B-1b, based upon their expression of CD5. B-2 cells include marginal zone and follicular B cells. In addition, regulatory B cells are present in the spleen. It is currently unknown whether these cells constitute a distinct B-cell subset, or are a component of the other subsets.

B---1 Cells

B-1 cells are so called as they are the first B cells to develop during embryonic development (Montecino-Rodriguez and Dorshkind, 2012). They differ from conventional B cells in a number of ways. They have limited diversity in their antigen receptors and are self-renewing outside of the central lymphoid organs, whereas B-2 cells are renewed from the bone marrow (Baumgarth, 2011). B-1 cells are also unique in that they do not form memory; repeated immunisations elicit similar or decreased responses.

B-1 cells are the main B-cell type present in the peritoneal and pleural cavities in mice and are the main producers of natural antibody, however under certain circumstances they have the capacity to migrate to other sites. This natural antibody is cross-reactive for self-antigens and microbial products. In locations such as in the spleen and lymph nodes, B-1 cells are rare and can be difficult to identify. Within the peritoneal and pleural cavities B-1 cells can respond to polysaccharide antigens in a T-independent manner. This antibody response is crucial for

pathogen neutralisation, and inhibition of pathogen replication via the activation of complement. In addition, this natural antibody augments the response of B-2 cells, probably via the deposition of immune complex on FDC. B-1 cells in the peritoneal cavity secrete lower titres of natural IgM than B-1 cells in the spleen and bone marrow; whereas B-1 cells in the gut preferentially secrete IgA. These differences may occur because these cells migrate to the site of infection to become antibody-secreting cells.

Microbial antigens recognised by these cells include LPS and phosphorylcholine of gram-positive bacteria as well as porins and Vi antigens (Marshall et al., 2012). Self-antigens recognised include oxidised lipids, annexin IV and phosphatidylcholine. As these molecules are expressed on apoptotic cells, B-1 cells may function in their clearance, this can occur in two ways. Firstly, secretion of antibodies against apoptotic markers promotes the phagocytosis of those cells by DCs. Secondly, B-1 cells can phagocytose apoptotic cells through BCR recognition or expression of TIM4, a receptor which binds phosphatidylserine on apoptotic cells. Interestingly, there is evidence that, rather than B-1 cells being cross-reactive for self-antigens or failing to be negatively selected during development, they are actually positively selected for self-reactivity. A proportion of B-1 cells in wild-type mice are specific for the Thy1 antigen (CD90) but mice lacking this antigen do not have Thy1 specific B-1 cells or anti-Thy1 antibody. (Hardy and Hayakawa, 2012).

B-1 cells are divided into B-1a and B-1b based on their expression of CD5 which is not expressed by B1b cells. However, both types are slightly larger than B-2 cells and are $IL5R^{+}$,$CD9^{+}$,$CD19^{hi}$, $CD23^{-}$,$CD43^{+}$, IgM^{hi} and IgD^{low}. CD5 expression on B1a cells is, however, lower than the expression observed on T cells, and outside of the peritoneum, a sensitive detection method is required for their identification. In addition, many of the markers used to identify B-1 cells are expressed on other B cells. B-1 cells can therefore be difficult to differentiate from activated, unswitched B-2 cells which can also upregulate CD43 expression. CD5 is also expressed both on anergic B cells and B cells stimulated via their

BCR *in vitro*. IL5R can also be expressed by pre-plasmablasts and CD9 is also expressed on marginal zone B cells.

Maintenance of B-1-cell numbers occurs via a 'self-renewal' process (Krop et al., 1996), whereby cell death is counterbalanced by proliferation. Natural antibody may provide a feedback mechanism for this process.

B---2 B cells

B-2 B cells are constantly renewed from cells produced in the bone marrow. Differentiation into follicular or marginal zone B cells then mainly occurs in the spleen.

Marginal zone and follicular B cells are discussed in more detail below.

Marginal Zone B cells

These B cells respond strongly to T-independent antigens and are specialised to respond to blood borne pathogens, such as bacteria and viruses. Marginal zone B cells are IgM^{hi}, $CD21^{hi}$, IgD^{low}, $CD1d^{hi}$, $CD23^{-}$ and recognise antigen presented by marginal zone macrophages. Loss of these macrophages leads to loss of the marginal zone B-cell compartment (Garraud et al., 2012). Retention of marginal zone B cells is mediated by the integrins LFA-1 and α4β1 (VLA-4), expressed on stromal cells (Lu and Cyster, 2002). These bind to ICAM-1 and VCAM-1 respectively. In addition, S_1P_1 and S_1P_3 are also involved in the localisation of marginal zone B cells (Cinamon et al., 2008).

Marginal zone B cells are important for the early response to blood borne pathogens as upon activation, they rapidly secrete IgM. This class of antibody binds with low affinity but high avidity and is important for the neutralisation of pathogens. Additionally, marginal zone B cells can transport antigens into the follicles, in order to activate a TD response (Cinamon et al., 2008). This shuttling to the follicle is dependent upon the expression of the chemokine receptor CXCR5.

Marginal zone B cells also express high levels of CD1d− a non-classical class I MHC molecule that can present glycolipid antigens to iNKT cells. This molecule has been

implicated in the formation of B-cell memory, with weaker stimulation through the CD1d receptor leading to greater B-cell memory (Lang et al., 2011), this appears to be due to more memory B cells emerging from the GC in this system.

Follicular B cells

Follicular B cells are mature naïve B cells that recirculate between the spleen and lymph nodes. These B cells are CD5-, $CD19^{mid}$, $CD1d^{mid}$, $CD23^{+}$, $CD43^{-}$, IgM^{low}, IgD^{hi} and constitute more than 70% of the total B-cell population in the murine spleen. The decision over whether to become a follicular recirculating B-cell, or a marginal zone B-cell, has been proposed to be decided by the strength of signalling through the BCR. Signalling via BTK, which induces a strong signal, promotes the development of follicular B cells and $BTK^{-/-}$ mice have a greater number of marginal zone B cells. Conversely, mice which lack the transcription factor Aiolos have a stronger BCR signal and produce more follicular cells (Cariappa et al., 2001).

Furthermore, a number of transcription factors are thought to play a part in marginal zone versus follicular B-cell differentiation. NOTCH-2 signalling (Saito et al., 2003) in addition to RBP-J (Tanigaki et al., 2002), promotes the development of marginal zone B cells. MINT competes with RBP-J to bind and block notch signalling, and increased MINT levels lead to more follicular B cells. Moreover, E2A promotes the development of follicular B cells and is antagonised in turn by ID3 (Quong et al., 2004).

Regulatory B cells

Regulatory B cells are defined by their ability to produce IL-10 upon stimulation. IL-10 is known to supress both Th1- and Th2cell polarisation, pro-inflammatory cytokine production and antigen presentation by myeloid cells. IL-10 activated DCs also activate Treg cells (Soyer et al., 2013). B10 regulatory cells are a subset of the $CD1d^{hi}$ $CD5^{+}$ Bcell population in the spleen (Ding et al., 2011). These cells express T-cell domain and mucin domain protein (TIM-1) and ligation of this receptor has been shown to induce B10 cells (Ding et al., 2011).

Regulatory B cells are also increased in number by BAFF signalling and their development in mice is known to require antigen and BCR stimulation (Kalampokis et al., 2013), as well as IL21 (Yoshizaki et al., 2012) and TLR4 or TLR9 stimulation (Kalampokis et al., 2013). IL10 is dispensable for the development of these cells, as they are found in $IL10^{-/-}$ mice (Maseda et al., 2012). Whether B10 cells really constitute a separate B-cell subset, or simply represent a B-cell differentiation state is unknown. There are no distinct markers for B10 cells, and they are defined simply by their ability to produce IL-10 upon stimulation with LPS or CpG, PMA and ionomycin. Whilst IFNα and TGFβ were found to inhibit formation of B10 cells by around 50% (Yoshizaki et al., 2012). Not all B cells become B10 cells after stimulation in B10 generating conditions however, with only around 1-3% of C57 splenic B cells becoming IL-10 producing B cells (Kalampokis et al., 2013).

$CD21^{low}$ B cells

A $CD21^{low/negative}$ CD11c+ population has been reported in mice. These cells have an impaired response to CD40 and BCR stimulation but are responsive to TLR stimulation, particularly TLR7. This population may represent 'exhausted' B cells and are associated with autoimmunity, as they are capable of secreting autoantibodies following activation (Rubtsov et al., 2011). These cells were also found to express low levels of CD138 protein and transcripts for plasma-cell transcription factors *Prdm1, Irf4* and *Xbp1*. Therefore these cells show a plasmablast phenotype (Rubtsov et al., 2011). Perforin, granzyme B and T-bet are also expressed. Similar $CD21^{low}$ IgM^{+}, $CD27^{+}$ cells have been identified in hepatitis C-related autoimmunity in humans (Terrier et al., 2011). These cells do not proliferate following BCR and CD40 stimulation, however they were responsive to TLR9 and were found to be more prone to apoptosis.

1.4 B-cell activation and differentiation

Naive B-cell responses to antigen can be classed into thymus dependent (TD) and thymus independent (TI) immune responses. In the former, B cells require help from an accessory

signal delivered by primed antigen specific T cells. In contrast, the latter requires a signal from antigen alone and strong responses can be induced in athymic mice.

Thymus---independent antigens

These are categorized into two types: TI-I and TI-II antigens. TI-I antigens stimulate B cells in the absence of T-cell help. These B-cell mitogens, such as LPS, do not elicit memory; yet can activate mature and immature B cells independently of co-stimulatory signals from T cells. This is because during BCR ligation they co-ligate co-receptors such as toll-like receptors (Lesinski and Westerink, 2001). In contrast, TI-II antigens extensively cross link B-cell receptors, thereby circumventing the requirement for T-cell help. This cross-linkage is achieved with repeating epitopes, such as those found on the capsulated bacteria such as *pneumococci*, *meningococci* and *Haemophilus influenza b*. These molecules are capable of activating complement, but not T cells, as they cannot be presented to T cells via MHC molecules. B cells which respond to these antigens are mainly marginal zone B cells (Vinuesa et al., 2003) and B-1 cells. As marginal zone B cells develop later in ontogeny: 1-2 weeks in mice and 1-2 years in humans, neonates tend to have poor responses to encapsulated bacteria (Adkins et al., 2004). TI responses normally are not capable of stimulating the formation of GC, however T-independent GC have been described in responses where there are pre-existing high frequencies of antigen-specific B cells. GC formed under these conditions involute when positive selection of centrocytes would normally occur, approximately five days after immunisation (Vinuesa et al., 2000). Although affinity maturation is absent due to GC being non-productive, TI-II responses can produce switched antibody. TI-II responses are important for the opsonisation of encapsulated bacteria. T cells cannot target these bacteria because they do not initially present protein antigen on their surface.

Thymus Dependent antigens

For this type of B-cell response, helper T cells specific for antigen must first be activated via interactions with DCs presenting peptide. When this occurs, T cells upregulate CXCR5 and

downregulate CCR7; this stimulates migration to the edge of the B-cell follicles (Ansel et al., 1999). B cells in turn, bind antigen via their B-cell receptor (BCR), upregulate CCR7 and move to the edge of the B-cell follicle (Reif et al., 2002). BCR bound antigen is internalised, processed and presented on the B-cell surface in the form of peptide bound to MHC class II molecules. If this complex is recognised by a helper T-cell (T_{FH}), activating signals such as CD40L and IL-4 are provided which induce the B-cell to proliferate and differentiate. In the absence of CD40L, GC do not develop after immunisation (Castigli et al., 1994; Chirmule et al., 2000; Kawabe et al., 1994),(Xu et al., 1994). Moreover, memory B-cell formation and affinity maturation to TD antigens are impaired (Renshaw et al., 1994), as is B-cell proliferation and immunoglobulin class switching. In response to CD40 ligation, B cells increase their expression of co-stimulatory molecules. After obtaining signals from T_{FH} cells, differentiation can occur via one of two pathways. The B-cell can become an antibody forming plasma-cell via the extrafollicular response in the red pulp, or can become a GC B-cell. The former pathway rapidly produces low affinity plasma cells. The latter pathway produces antibody producing cells and memory cells. These are affinity matured and often class switched. Recently, antigen presented by DC inhibitory receptor 2 (DCIR2), which is found on marginal zone $CD8\alpha^-$ DCs, has been found to stimulate class-switched antibody responses to TD antigen. This indicates a previously unknown role of marginal zone associated DCs in the TD response. This type of response does not produce GC, or affinity matured antibody however (Chappell et al., 2012).

Antibody structure and function

An antibody molecule is comprised of two heavy and two light chains which form a ‘Y’ shaped protein. In addition, there are two regions known as the constant and variable regions. Antigen specificity is encoded via the variable region; this is the site where point mutations are introduced in order to create antibodies with altered specificities. This is known as SHM.

Light chains can be either lambda (λ) or kappa (κ) and the C region of the light chain gene does not undergo CSR.

There are several different classes of antibody that are defined by their expression of different constant region genes. Initially B cells express Cμ and Cδ, following CSR these genes can be exchanged in mice for Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, or Cα (Chaudhuri and Alt, 2004). These classes of antibody are expressed as either membrane receptors, or secreted antibodies and altering the C_H gene results in antibody molecules with differing effector functions. Antibody class determines whether multimeric units are formed in the serum, IgM forms pentamers resulting in a molecule with a high molecular weight whereas IgA can exist as a monomer or a dimer in sera. Altering the isotype of an antibody results in expression of a different Fc region. These regions interact with different Fc receptors on effector cells leading to altered function. IgM is the most effective class for complement activation followed by IgG subclasses. IgG is also capable of crossing the placenta to provide passive immunity to the foetus. IgA is capable of being secreted in milk, tears and mucous secretions. IgE in contrast binds to mast cells and basophils leading to the release of inflammatory mediators.

1.5 BCR signalling

The BCR is composed of heavy and light immunoglobulin chains that are specific for antigen. Signalling from an IgM or IgD BCR is mediated via two proteins which associate with the BCR to form the B-cell receptor complex. These are the Igβ and Igα subunits. These accessory proteins consist of a single chain with an amino terminal immunoglobulin-like domain, a transmembrane domain and a cytoplasmic tail. Each Igβ and Igα chain contains an ITAM motif. Tyrosine residues within these ITAMs become phosphorylated by associated kinases following ligand binding (Pierce and Liu, 2010).

The first event after BCR ligation is oligomerisation and micro cluster formation. B cells are activated when their BCRs are cross-linked by a multivalent antigen and are capable of being activated by engagement of multiple B-cell receptors (Metzger, 1992). Engagement of

multiple BCR leads to the formation of the immunological synapse. However, monovalent antigen are also capable of triggering BCR signalling (Pierce and Liu, 2010). Additionally, the BCR has a co-receptor which upon engagement strengthens signalling through the BCR. The co-receptor consists of CD19, CD21 and CD81. Engagement of CD21, the receptor for the complement component C3d, allows the cytoplasmic tail of CD19 to be phosphorylated by BCR associated tyrosine kinases. Three protein tyrosine kinases of the Src family are responsible for the phosphorylation of the ITAM sequences on Igβ and Igα. These are Fyn, Blk and Lyn and these protein kinases are activated by receptor cross-linking. The phosphorylated ITAM sequences then recruit Syk via its SH2 domain. Syk molecules transphosphorylate each other due to their close proximity after BCR cross-linking. Syk in turn, phosphorylates BLNK, this scaffold protein then recruits SH-2 containing proteins. Btk activates phospholipase-Cγ which hydrolyses PIP_2 into DAG and IP_3. Cross-linking of the BCR and activation of intracellular signalling pathways also stimulates BCR internalisation. This process is essential for antigen presentation to T cells. Receptor internalisation requires Vav and the GTPase dynamin (Malhotra et al., 2009).

BCR signalling is in turn, negatively regulated by FcγRIIB. This is a low to medium affinity receptor for IgG1, IgG2a and IgG2b. Negative regulation is mediated via an immunoreceptor tyrosine-based inhibitory motif (ITIM) within its cytoplasmic region. Cross-linking of the BCR in conjunction with FcγRIIB leads to phosphorylation of its ITIM by LYN. This recruits the lipid phosphatase SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP). SHIP hydrolyses phosphoinositide intermediates and prevents recruitment of BTK and PLC-γ to the cell membrane, thereby preventing Ca^{2+} signalling. Isolated signalling through this receptor can lead to apoptosis via activation of BTK and JUN N-terminal kinase (JNK) (Nimmerjahn and Ravetch, 2008).

Interestingly FcγRIIB is also expressed at high levels in plasma cells. This could be a mechanism by which plasma-cell numbers are regulated. During a new infection antigen-

specific B cells receive signals through both their BCR and FcγRIIB. In contrast non-specific plasma cells engage circulating IgG only. This occurs via their FcγRIIB and leads to apoptosis of the plasma-cell, thereby creating space in the bone marrow for new antigen-specific plasma cells (Nimmerjahn and Ravetch, 2008).

1.6 Overview of the Germinal Centre

GC develop in secondary lymphoid organs such as the spleen and lymph nodes during a T-cell dependent antibody response, usually within 6 days of primary immunisation. These structures give rise to high affinity antibody secreting plasma cells and memory cells, via the process of affinity maturation (Hauser et al., 2007).

The GC is divided into a dark and light zone and surrounded by the follicular mantle (Figure 1. 3). The follicular mantle is composed of predominantly naive IgD^+ B cells, whereas the dark zone contains rapidly dividing centroblasts. These have down-regulated surface immunoglobulin and are thought to be undergoing somatic hyper mutation (SHM) of the B-cell receptor (BCR) loci at the variable locus of the immunoglobulin gene. This is the process by which point mutations are introduced into the variable region locus of the IgH and IgL genes. Centroblasts have large nuclei and therefore stain darkly with haematoxylin and eosin. These cells are believed to give rise to centrocytes, present in the light zone. B cells which have undergone SHM may express receptors of low affinity, or alternatively receptors which recognise self-molecules and must therefore be deleted from the B-cell repertoire. This selection process is thought to occur in the light zone, as this area contains FDC, follicular helper T cells (T_{FH}) and tingible body macrophages (Hauser et al., 2007). Whilst the latter are known to clear apoptotic cells (Baumann et al., 2002), FDC and T_{FH} cells are thought to provide a survival signal to B cells which would otherwise become apoptotic (Vinuesa et al., 2000). FDC are known to trap antigen in an unprocessed form for presentation to B cells (Sukumar et al., 2008). GCs are also the site of CSR, in which the antibody affinity is not altered but the IgH constant region (C_H) μ exon is replaced with the C region γ, α, ε or δ

exon, resulting in a different isotype antibody. Both CSR and affinity maturation require the enzyme activation-induced cytidine deaminase (AID) in an active form. Both processes occur in activated B cells in response to antigen stimulation and costimulatory signals. *In Vitro* CSR can be induced in response to LPS alone, or in combination with IL-4 or IFN-γ, as well as other cytokines (Chaudhuri and Alt, 2004). CSR involves a recombination event between two switch (S) regions of DNA. These are located upstream of each C_H region gene except $C_H\delta$. AID has been shown to act as a DNA-cytidine deaminase and deaminates cytosines in the donor and acceptor S regions. The targeted bases are excised by uracil DNA glycosylase and the single stranded breaks converted to double stranded breaks (Stavnezer et al., 2008). The S-S regions are recombined, excising the DNA between the two sites (Stavnezer et al., 2008). The process of CSR will be discussed in more detail later. CSR has been shown to occur within 12 hours during a secondary response to antigen (Toellner et al., 1996) but can also occur in a T independent manner outside of GCs. GC classically persist for around three weeks, with involution beginning at 14 days, depending upon the model (Toellner et al., 1996), and can be detected by the ability of the cells within this area to bind the lectin peanut agglutinin (PNA). They can also be detected by their expression of the transcription factor BCL-6.

Figure 1 3 :Overview of the germinal centre.

GC form when antigen specific B cells interact with antigen specific T cells at the B-T zone boundary (Allen et al., 2007). B cells either move into extra follicular foci in the red pulp as short lived plasma cells, or move into follicles and form GC. These structures are the sites of somatic hypermutation which results in altered affinity B cells. The resulting B cells are screened for their ability to recognise antigen, after which they are either eliminated by apoptosis or selected to leave, either as memory or plasma cells (Reproduced from (Klein and Dalla-Favera, 2008).

1.7 Transcription Factors in B-cell differentiation

Cell fate decisions

The cell fate decision between a GC B-cell or plasma-cell phenotype may be regulated in part by the transcription factor PAX5 (Singh and Sciammas, 2006). PAX5 represses the activation of genes associated with plasma-cell differentiation, such as *Prdm1* (BLIMP1) *Igj*, *cd28* and *Ccr2*, whilst positively regulating the transcription of *Aicda* (AID). *Aicda* is also positively regulated by E2A amongst other factors; these will be discussed in more detail later. In GC cells, repression of Blimp1 expression by BCL-6 (Shaffer et al., 2000) maintains the GC phenotype. As cells move towards plasma-cell differentiation, expression of PAX5 is reduced and BLIMP1 is up-regulated. The latter is a zinc finger transcription factor that represses the expression of BCL6 (Figure 1.4) and activates the expression of the chemokine receptor

CXCR4. This receptor is responsible for plasma-cell homing to the red pulp (Hargreaves et al., 2001) which is associated with an increase in the endoplasmic reticulum and protein synthesis. Moreover, de-repression of Xbp1 activates transcription of genes for chaperones and enzymes involved in the secretory apparatus of plasma cells.

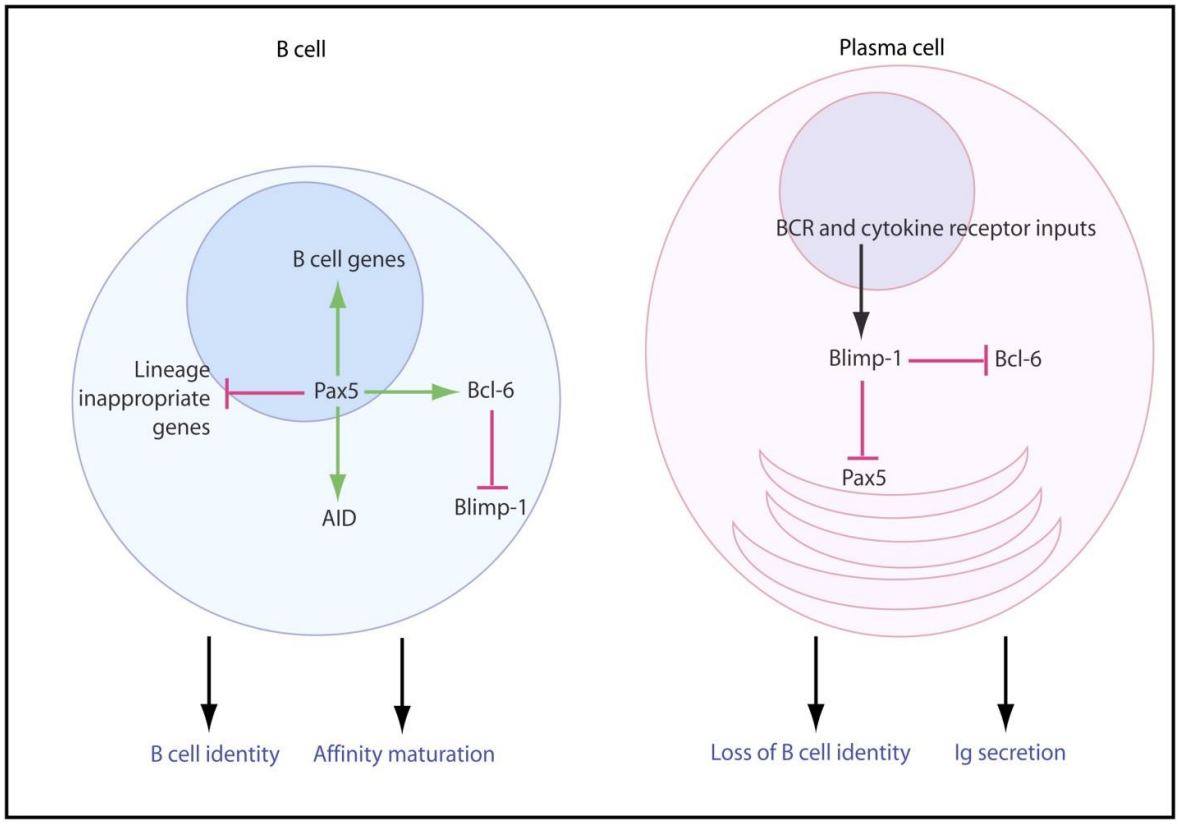


Figure 1 4: Transcription factors involved in cell fate decisions between plasma cells and GC cells.

In the GC B-cell, PAX5 represses genes responsible for alternative lineage decisions. PAX5 maintains expression of genes that maintain the GC-cell phenotype such as BCL6, which in turn represses Blimp-1. In plasma cells Blimp-1 acts to repress BCL6 and Pax5, leading to the loss of B-cell identity and the secretion of immunoglobulin. Reproduced with permission from (Singh and Sciammas, 2006).

The positioning of B cells within the white pulp is also crucial for the differentiation of plasma cells. EBI2 deficient B cells do not migrate to the extrafollicular region and fail to form plasmablasts, although differentiation *in vitro* is not affected (Gatto et al., 2009). Positioning of B cells at the bridging channel and extrafollicular regions may be essential for interactions with other cell types such as DCs (García De Vinuesa et al., 1999) which are known to produce factors such as IL-6 and APRIL which promote plasma-cell differentiation

and survival (Mohr et al., 2009). In addition, strong stimulation via the BCR is required for extrafollicular plasma-cell differentiation. Interestingly the strength of the BCR signal, determined by the affinity of the BCR for the selected antigen, does not affect the size of the germinal centre response (Paus et al., 2006). Previous studies in the quasi-monoclonal mouse (QM) (Cascalho et al., 1996), which has a BCR with high affinity for the model antigen 4-Hydroxy-3-nitrophenylacetyl (NP), showed that smaller antigen doses induced mainly extrafollicular responses whereas high antigen doses induced both germinal centre and extrafollicular responses (Vinuesa et al., 2000).

Extracellular signalling molecules responsible for Plasma---cell differentiation

BAFF and APRIL

Two members of the tumour necrosis factor family (TNF): B-cell activating factor (BAFF) and its homologue APRIL, are membrane associated proteins which have important roles in B-cell and plasmablast differentiation and survival (Mohr et al., 2009; Moisini and Davidson, 2009). Proteolytic cleavage of the membrane proteins converts them to soluble cytokines. These proteins also have a role in FDC development and DC maturation (Moisini and Davidson, 2009). BAFF is capable of binding to three different receptors; these are BAFF-receptor (BAFF-R, BR3), TACI and B-cell maturation antigen (BCMA). APRIL, in contrast, binds only to TACI and BCMA. Plasma cells express TACI and/or BCMA, which are capable of binding BAFF or APRIL. BAFF or APRIL binding to TACI on plasma cells synergises with TLR signalling to activate MYD88 during the T-independent response. This initiates a signalling cascade involving IRAK1, IRAK4 and TRAF6. Downstream NFκB signalling is initiated (p50 and p65 heterodimers) for the induction of AID and CSR. TLR stimulation in turn leads to the upregulation of receptors for BAFF (Rawlings et al., 2012).

Interactions between BAFF and BAFF-R are important for the survival of B2 cells past the T1 stage of their development. The amount of BAFF signal is connected to the BCR signal

strength, as BCR signalling generates p100. This is cleaved to p52, a component of the NFκB signalling pathway that is also required for BAFF signalling. Generation of p100 leads to B-cell survival (Schiemann et al., 2001). Both BAFF and APRIL from trimers which can bind to three independent receptors simultaneously. Signalling is initiated through TRAF proteins. TRAF2 and TRAF6 bind to TACI to activate NFκB1, but only if at least six receptors are in close proximity. BAFFR in contrast recruits TRAF3 and therefore requires less receptor clustering, this leads to degradation of TRAF3 and indirectly leads to activation of the NFκB2 pathway (Mackay and Schneider, 2009).

Cells which are capable of producing BAFF include FDC, monocytes, macrophages, DCs neutrophils and iNKT (Shah et al., 2013) and the receptors for this molecule are upregulated by activated B cells. Expression of BAFF and APRIL can be increased by type I interferons IFN-γ, IL-10 and G-CSF. In addition, both BAFF and APRIL can be produced by plasma cells themselves, both *in vitro* and *in vivo* (Chu et al., 2007). Therefore, plasma cells may sustain their own survival in an autocrine manner. APRIL has also been shown to be required for the survival of class switched B cells in mice and APRIL and BAFF are involved in CD40-independent CSR to IgA. This occurs through TACI and BAFFR (Castigli, 2004). TI-II responses and TD IgM responses also require BAFF binding to TACI.

CD40-CD40L Signalling

Another member of the TNF family involved in plasma-cell differentiation is CD40L. CD40 is a cell surface receptor that was first described on B cells, but is also expressed on monocytes, DCs, endothelial and epithelial cells. The ligand, CD40L, is also expressed on a wide range of cells including monocytes, DC, B cells, mast cells basophils and eosinophils.

Expression of CD40L is tightly regulated. Some pre-formed CD40L is present in T cells before activation and can be rapidly expressed at the cell surface within minutes of activation. A second wave of CD40L is synthesised between 1-8h after activation, after this CD40L levels decrease again. Downregulation occurs via a number of mechanisms; release of soluble

CD40 which binds CD40L, cleavage of CD40L from the cell surface, downregulation of the mRNA for CD40L and finally, receptor mediated endocytosis and lysosomal degradation of CD40L. CD40 also has a second ligand− C4bp, this is a ligand which is expressed by FDC and is critical for TI, B-cell activation and B-cell proliferation (Brodeur et al., 2003).

CD40 has been shown to be important for GC formation and plasma-cell survival and differentiation. CD40 receptor has been shown to associate with various TRAF proteins including TRAF3, TRAF2, TRAF5 and TRAF6 (Jabara et al., 2009; van Kooten and Banchereau, 2000). After engagement by CD40L on T cells, CD40 signals via NFκB1 to induce a high level of IRF4, this is required for plasma-cell differentiation (Klein et al., 2006). $CD40L^{KO}$ mice do not form GC after immunisation (Castigli et al., 1994; Chirmule et al., 2000; Kawabe et al., 1994; Xu et al., 1994), and have impaired memory B-cell formation and affinity maturation to TD antigens, although responses to TI antigens are maintained (Renshaw et al., 1994). B-cell memory can be restored by administration of CD28 AB (Chirmule et al., 2000). In addition, patients with hyper IgM syndrome, caused by a mutation in the CD40L gene, do not produce GC, although aggregates of centroblasts have been observed in lymph nodes (Facchetti et al., 1995). CD40 has been reported to drive B cells towards a memory cell phenotype, rather than a plasma-cell phenotype *in vitro* (Randall et al., 1998).

Interferon regulatory factor 4 in plasma---cell differentiation

IRF4 is a member of the interferon regulatory factor (IRF) family of transcription factors. This family contains nine members: IRF1---9. Unlike other members of this family, IRF4 is not induced by interferon but rather by signalling via antigen---receptor, LPS or CD40 signalling (Paun and Pitha, 2007). Members of the IRF family are characterised by a DNA---binding domain containing five tryptophan repeats and, with the exception of

IRF---1 and IRF---2, all members contain the IRF associated domain (IAD). This domain is required for interactions with the 3' terminal part of the protein (Paun and Pitha, 2007). IRF4 is a crucial transcription factor for plasma-cell differentiation which is also induced in activated T cells (Huber et al., 2008) and macrophages (Satoh et al., 2010). IRF4 binds DNA closely when interacting with members of the Ets or helix-loop-helix family of transcription factors and has a redundant function with IRF8 in pre-B-cell development, but not in GC and plasma-cell development (Lu, 2008). Commitment to plasma or memory cell differentiation is thought to occur when centrocytes in the GC downregulate BCL-6, and activate BLIMP-1 (Shapiro-Shelef et al., 2003; Turner et al., 1994), IRF4 and BMI-1. NFkB1 and STAT3 are also upregulated at this stage. However, BLIMP-1 alone does not appear to be sufficient for the development of plasma cells (Vinuesa et al., 2001) and premature upregulation of BLIMP-1 leads to apoptosis through downregulation of the anti-apoptotic protein Bfl-1/A1, a member of the bcl-2 family. BLIMP-1 acts upstream of XBP-1 and cannot drive plasma-cell differentiation in the absence of XBP-1 (Shapiro-Shelef et al., 2003). When IRF4 is conditionally knocked-out in GC B cells, GC development is unaffected whereas plasma cells lacking IRF4 are completely absent (Klein et al., 2006) (Sciammas et al., 2006). Additionally, XBP-1 expression is abrogated in B cells lacking IRF4 and CSR is deficient (Klein et al., 2006), as is production of antibody (Sciammas et al., 2006). The latter effect is due to an inability to upregulate AID. IRF4 deficient mice also exhibit reduced numbers of memory B cells, indicating a requirement for IRF4 in the maintenance of the memory cell pool. In addition, memory cells developed from IRF4$^{-/-}$ B cells are reported to be unable to differentiate into plasma cells (Klein et al., 2006).

B cells lacking IRF4 are able to express BLIMP-1 mRNA at a similar level to wild-type B cells, however this is not sufficient for plasma-cell differentiation, indicating that BLIMP-1 may act upstream of IRF4 (Klein et al., 2006) However IRF4 has also been reported to have contradictory roles in the induction of BLIMP-1 and AID expression. This appears to be due

to a dose-dependent effect. Transduction of wild type splenic B cells with IRF4 augments the generation of CD138 expressing cells, with high concentrations of IRF4 required for repression of the GC programme via the upregulation of *Prdm1*. Intermediate levels of IRF4, in contrast, induce AID expression and isotype switching (Sciammas et al., 2006). This action of IRF4 for the induction of AID expression has been suggested to be indirect, as AID has not as yet been shown to be a direct target of IRF4 (Sciammas et al., 2011).

1.8 Regulation of AID

AID is required for both SHM and Ig class switching. During SHM, point mutations are introduced to the variable region of antibody genes. During CSR, the antibody heavy chain is altered, whilst the affinity of the BCR remains the same. Both processes require DNA cleavage facilitated by AID. This can potentially have dangerous consequences and must therefore be tightly regulated.

The murine Ig locus contains eight C_H genes. These are Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα. Located upstream of each IgH gene (except Cδ) is a switch (S) region of DNA; these sequences are where breaks are introduced. Switch regions are then joined, and the intervening DNA deleted (Figure 1.5). Characteristically, these regions are highly repetitive with the non-template strands being rich in guanines. Transcription through the specific C_H S region is necessary for CSR to a specific C_H gene, and transcription initiates at an upstream activator/cytokine responsive promoter (Chaudhuri and Alt, 2004).

Figure 1 5: Mechanism of Class Switch Recombination. A promoter, upstream of the VDJ segment drives transcription of the μ heavy chain. This associates with a light chain to form an IgM molecule. Following CSR the constant region of the heavy chain is exchanged for a downstream constant region gene. To enable CSR, double stranded breaks are introduced at the switch regions. These are located in front of the heavy chain genes. Repair of the breaks leads to the rearranged C_H locus with the deletion of the intervening sequence. (Chaudhuri and Alt, 2004).

AID functions in this process by deaminating deoxycytosines, creating U: G mismatches. These are repaired by base excision or mismatch repair, in an error-prone process. This error-prone repair process which leads to CSR requires the DNA polymerase-ζ (Schenten et al., 2009). Class switching can be detected by measuring heavy chain transcripts containing Iμ. During CSR Ig switch regions are exposed by transcription that is initiated from the induce (I) exon which precedes each C_g gene, through the switch region and into the C_g heavy chain genes. Most Ig genes (with the exception of IgG3) are strictly regulated and typically only expressed during induction of CSR. The I μ exon upstream of the IgM switch region is constitutively active, and in non-switched cells produced a Iμ-Cμ transcript. After CSR to IgG3 Iμ-Cγ3 hybrid transcripts are expressed, and these indicate successful Ig class switch recombination to IgG3 (Li et al., 1994).

Commitment to an antibody class is dependent upon both the antigen, and the route of administration. Different cytokines drive commitment to different antibody classes. IL-4, which is the hallmark of a Th2 response, promotes CSR to IgG1 and IgE (Snapper and Paul,

1987), whereas IFNγ, which is characteristic of a Th1 response, activates STAT1 and induces T-bet to promote switching to IgG2a (Mohr et al., 2010; Peng et al., 2002; Snapper and Paul, 1987). TGFβ in turn, signals to RUNX proteins to promote switching to IgA (Cazac and Roes, 2000; Watanabe et al., 2010). LPS in contrast induces switching to IgG2b and IgG3 (Chaudhuri and Alt, 2004).

Furthermore, CSR requires cell division. The frequency of switched B cells increases after two divisions and levels plateau at around six cell divisions (Hodgkin et al., 1996). This correlation with division number appears to be due to a number of factors. Firstly, the IgH constant region only becomes accessible after a single cell division event, this may be because the substrate for AID is ssDNA, AID activity requires active transcription (Chahwan et al., 2012). Secondly, the amount of AID mRNA increases with subsequent divisions and is correlated with CSR (Rush et al., 2005). Moreover, double stranded breaks only occur during G1 phase of cell cycle (Schrader et al., 2007) and homologous repair proteins mainly assemble at AID targets during S-G2/M phase (Yamane et al., 2013). Therefore the events that are required for CSR occur at defined stages of the cell cycle.

AID function and expression has to be tightly regulated by various mechanisms including: transcriptionally, post translational modification, interactions with other proteins and regulation of the subcellular location of AID (Vuong and Chaudhuri, 2012).

Transcriptional regulation of Aicda

AID has been proposed to be regulated by intermediate expression levels of IRF4 protein (Klein et al., 2006; Sciammas et al., 2006; 2011). AID can be induced *in vitro* after stimulation with anti-CD40 in conjunction with IL-4, but only minimally by either factor separately (Dedeoglu et al., 2004). Activation of AID following B-cell stimulation required STAT6 binding to a site in the 5' upstream region of the AID gene (Dedeoglu et al., 2004). IRF4 may regulate AID through this pathway as IRF4 is known to interact and cooperate with

STAT6 (Gupta et al., 1999). Ligation of CD40 stimulates NFκB binding to two sites in the 5' upstream region of the AID gene (Dedeoglu et al., 2004). Other studies support the importance of the 5' region of the *Aicda* locus (Tran et al., 2009). E2A which is expressed in centroblasts (Goldfarb et al., 1996) has been shown to positively regulate AID, this gene encodes two transcription factors: E12 and E47 which are members of the E-protein family: a class of basic helix-loop-helix (bHLH) proteins. These proteins bind to HLH inhibitor of differentiation 1 (Id1) Id2, Id3 and Id4 which block the transcriptional activity of the E-proteins (Sayegh et al., 2003). Transduction of murine B cells with a retrovirus expressing Id3 lead to decreased levels of *Aicda* whilst overexpression of E47 activated *Aicda* expression. Ectopic expression of AID in cells overexpressing Id3 resulted in a partial rescue of Ig CSR, indicating that there may be other roles for E-proteins in CSR besides the induction of AID (Sayegh et al., 2003). Furthermore E47 is a binding partner of IRF4 (Nagulapalli and Atchison, 1998). Binding of E-proteins to the *Aicda* promoter has been reported elsewhere (Tran et al., 2009). Restriction of AID expression to B cells may be mediated by PAX-5 (Tran et al., 2009), recruitment of which to the *Aicda* promoter has been suggested to be mediated by other transcription factors, as the PAX-5 binding site has not been identified (Park et al., 2009). Within the germinal centre Homeobox C4 (HoxC4) is correlated with AID expression and binds to the *Aicda* promoter to induce CSR and SHM (Park et al., 2009). Deletion of forkhead box O1 (FOXO1) in B cells leads to reduced AID and CSR (Dengler et al., 2008). BATF, a member of the activator protein 1 (AP-1) family, regulates both AID expression, with $BATF^{KO}$ B cells expressing 90% less AID protein, and undergoing less CSR. The requirement of BATF in the process of CSR occurs independently of AID. BATF induces I_H-C^H transcripts via interactions with I-region promoters and elements of the 3' *Igh* enhancer (Ise et al., 2011). In addition, within naïve B cells, E2F and c-Myb are bound to the *Aicda* locus and act as transcriptional repressors (Huong et al., 2013).

Post-transcriptional regulation of AID

Negative regulation of AID has been reported by a number of micro-RNAs including miR-155 (Teng et al., 2008; Vigorito et al., 2007), miR-361 (Basso et al., 2012) and miR-181b (de Yébenes et al., 2008).

Interactions with cofactors and post translational modification

The C-terminal region of AID is important for interactions with other proteins as mutating this region affects CSR but not SHM (Ta et al., 2003). Indeed alterations in AID levels have been reported to affect CSR but not SHM indicating that the expression levels of AID required for these two processes may be different (Teng et al., 2008) (Muto et al., 2006). AID may also require post-translational modification for interactions with other proteins (Chaudhuri et al., 2004) and has been proposed to work in conjunction with co-factors and adaptor proteins. For example, the adaptor protein 14-3-3 is recruited with and targets AID to switch regions (Xu et al., 2010). AID protein contains a number of phosphorylation sites and phosphorylation of AID has been shown to have a role in interactions with cofactors in tetrapods but not zebrafish which undego SHM but not CSR (Basu et al., 2008). Phosphorylation at S38 by protein kinase A (PKA) is required for interactions with replication protein A, this interaction is required for AID access to DNA (Basu et al., 2008). Therefore this may indicate a mechanism whereby phosphorylation of AID can control these two different functions.

Subcellular location of AID

AID continually shuttles between the nucleus and the cytoplasm. It is only active within the nucleus but it is also rapidly degraded there (Aoufouchi et al., 2008). In addition to degradation, AID is also exported from the nucleus under the control of CRM1 which binds the nuclear export signal of AID (Geisberger et al., 2009; Ito et al., 2004). In addition there is a cytoplasmic retention mechanism (Patenaude et al., 2009). Some of the proposed regulators of AID are summarised in figure 1.6.

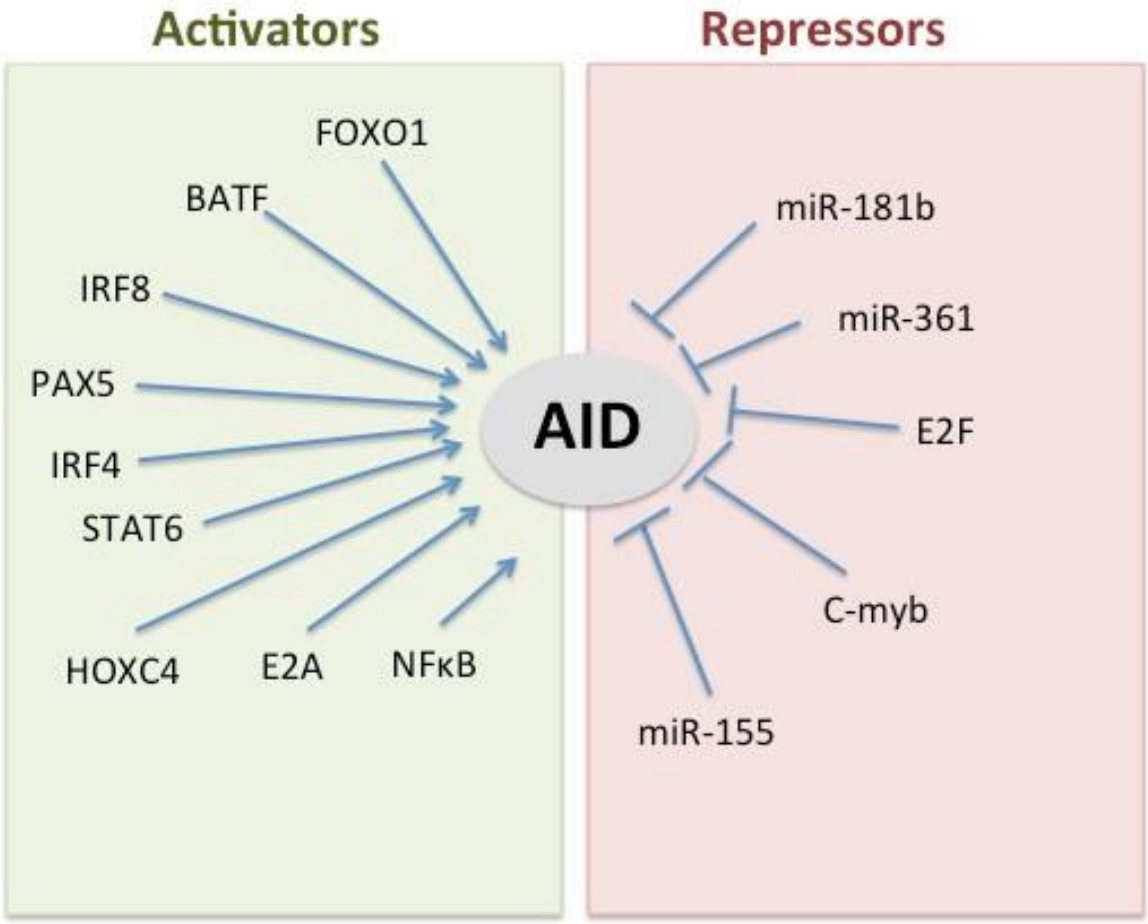


Figure 1 6 The signalling network mediating regulation of AID protein levels includes activators and repressors of transcription/translation

In addition AID is regulated by its sub-cellular location, interactions with cofactors and post-translational modification.

The role of AID and IRF4 in B---cell malignancies

Both AID and IRF4 are associated with a number of malignancies. Aberrant AID expression can lead to mutations in tumour suppressors or other genes resulting in transformation of B cells and giving rise to various lymphomas. Philadelphia chromosome positive ALL, characterised by the expression of BCL-ABL1, is associated with high aberrant AID

expression (Feldhahn et al., 2007). Furthermore Myc-IgH translocations in Burkitt's lymphoma may be attributable to AID (Ramiro et al., 2004).

Multiple myeloma (MM) is a plasma-cell malignancy and IRF4 was first identified as multiple myeloma oncogene 1 (Mum1). This was cloned from an MM cell line with a translocation of t(6;14)(p25;q32). This translocation put IRF4 under the control of the IgH promoter leading to IRF4 overexpression. This was shown to be oncogenic *in vitro* (Iida et al., 1997). RNA-interference of the 3' UTR of IRF4, which leads to a drastic inhibition of IRF4 mRNA and protein, has been shown to lead to death of MM cell lines (Shaffer et al., 2008). This was found to be specific for MM cell lines but had no effect on lymphoma cell lines. IRF4 was found to bind to its own promoter leading to a positive feedback loop and MYC was found to be a direct target of IRF4 in both MM cells and normal activated B blasts. Expression of MYC was required for the survival of myeloma cells (Shaffer et al., 2008). However in MM cells IRF4 regulated a number of signalling pathways which are not active in normal plasma cells. This represents a fusing of normal plasma-cell differentiation pathways and B-cell activation pathways which may also be due to the graded expression of this transcription factor. IRF4 is also highly expressed in activated B-cell-like DLBCL and the IRF4 binding partner, SPIB has been shown to act as an oncogene in this malignancy (Lenz et al., 2007). In addition IRF4 is implicated in CLL with single nucleotide polymorphisms associated with the 3' UTR of IRF4 (Di Bernardo et al., 2008).

Knocking down IRF4 mRNA and protein by 50% was sufficient to kill myeloma cell lines (Shaffer et al., 2008). Therefore it has been proposed that targeting IRF4 therapeutically would kill myeloma cells whilst sparing normal B-cell responses. This is an approach that has been tried with BCL6 in lymphomas with some success (Polo et al., 2004).(Cerchietti et al.,

2010). In order to utilise IRF4 protein in the treatment of malignancies, it is crucial to elucidate its pleiotropic effects within different cell types.

1.9 Memory B-cell development

Memory B cells develop after primary antigen exposure and recirculate in the blood until re-exposure to the same antigen prompts rapid differentiation to antibody secreting cells. In this manner, memory B cells can mount a fast response upon secondary antigen encounter. Memory B cells are derived from the germinal centre but have also been reported to develop independent of the GC reaction (Linterman et al., 2010) (Toyama et al., 2002) (Inamine et al., 2005) (Chan et al., 2009) (Taylor et al., 2012) . Memory cells produced in this way are known as early memory (EM) cells and are detectable within 10 days of immunisation during the T-dependent response. They are characterised as $B220^{high}$, BCR^{high}, GL-7^{low}, FAS^{low} and $CD38^{+}$. These cells are not dependent upon IL21-signaling, unlike GC B cells (Linterman et al., 2010) and whilst they may be class-switched, they tend to be of low affinity (Inamine et al., 2005). Taylor *et al* (2012) defined the memory compartment as CD38+ GL7- cells which had divided in response to antigen stimulation, the peak production of these cells was found to be between days five and seven after immunisation.

There is also a second phase of memory B-cell formation however, that of affinity-matured memory B cells derived from the GC reaction (Inamine et al., 2005). One week after a primary immunisation with NP-KLH, switched antigen-specific B cells with a memory phenotype can be detected in the blood. These contain mutations in their V_H gene (Blink et al., 2005). As the immune response continues, the numbers of mutations also increase, indicating that these cells are GC derived.

Memory B cells have been shown to require anti-apoptotic signalling for their survival (Vikstrom et al., 2010) and BCR signalling may also be important for their persistence, as depletion of phospholipase C leads to a decreased memory B-cell compartment (Hikida et al., 2009). Upon recall, memory B cells expand rapidly and differentiate into high affinity plasma

cells. Their migration is dependent upon inflammatory signals, such as LPS (Shenoy et al., 2012). The ability to differentiate rapidly may be due to the enhanced affinity of the memory BCR, as well as altered surface receptor class. For example, there is evidence that signalling via the IgM BCR is functionally different to signalling via the IgG1 BCR. IgG1 BCRs have enhanced signal initiation due to micro clustering, a process which requires the cytoplasmic tail of IgG1(Liu et al., 2010). In contrast, the short cytoplasmic tails of IgM and IgD have not been shown to directly signal into the cell. The signalling pathway through IgG1 is further augmented as the IgG receptor is not sensitive to down-regulation of signalling through CD22 (Wakabayashi et al., 2002).

1.10 Conclusion

The production of class switched antibody is crucial for the generation of an effective humoral immune response. Class switch recombination is facilitated by the protein AID which in turn is tightly controlled to prevent the introduction of potentially damaging point mutations into the genome. IRF4 protein has been reported to modulate contradictory gene regulatory networks including the induction of AID protein (Klein et al., 2006; Sciammas et al., 2006; 2011) and plasma-cell differentiation (Klein et al., 2006). These gene regulatory networks are contradictory as plasma cells do not express AID (Marshall et al., 2011). These contradictory effects are associated with different expression levels of IRF4 protein within B cells *in vitro* (Sciammas et al., 2011) with intermediate-level IRF4 associated with AID induction whilst high level IRF4 is associated with plasma-cell differentiation. Intermediate-level IRF4 has been proposed to be expressed by GC B cells (Ochiai et al., 2013), however data from our group has shown that B blasts upregulate IRF4 to an intermediate level within minutes of activation before the formation of GC (Yang Z, UOB thesis 2010) and that extrafollicular B blasts temporarily acquire a capacity for Ig CSR in the first days post-immunisation (Marshall et al., 2011). Therefore, this thesis aims to test the role of differential

levels of IRF4 for the regulation of AID protein and Ig class switching within extrafollicular B blasts. We tested the hypothesis that —

(i) Intermediate levels of IRF4 protein regulate AID and CSR within extrafollicular B blasts.

(ii) High levels of IRF4 protein are dispensable for AID and extrafollicular CSR but are required for plasma-cell terminal differentiation.

Chapter 3 explores the timing of IRF4 intermediate and high protein expression within activated B cells and correlates these two phases with:

(a) Stages of B blast differentiation and

(b) Expression of AID protein.

To test the role of intermediate-level IRF4 for extrafollicular AID induction and CSR we used two approaches. Firstly we blocked high-level but not intermediate-level IRF4 protein expression using mice lacking the NFκB family signalling molecules NFκB1 (chapter 4) and C-REL (chapter 5) and tested extrafollicular AID induction and class switch recombination when only intermediate level IRF4 is induced normally.

Secondly, to test extrafollicular AID induction and CSR when intermediate-level IRF4 is blocked we repressed IRF4 with the micro-RNA miR-125b. We then tested AID induction and CSR within extrafollicular B blasts (chapter 6).

2.MATERIALS AND METHODS

2.1 Animals

In all cases of animal studies mice were age and sex matched and used between the ages of 6-12 weeks. Wild type (WT) C57Bl6/j, NFκB1-/-x $QM^{NP/NP}$ K-/- xC57Bl6, $QM^{NP/NP}$ K-/- x C57Bl6 $F_{1,}$ NFκB1-/-x $QM^{NP/WT}$ K-/- xC57Bl6, and $QM^{NP/WT}$ K-/- x C57Bl6 F_1mice were bred and housed in specific pathogen free conditions in the animal facility at the University of Birmingham Biomedical Services Unit. All procedures were approved by the University Ethics Committee and covered by Home Office licenses'.

2.2 Mouse Models

Quasi-monoclonal $^{NP/NP}$ κ^{KO}

Quasi-monoclonal (QM) mice were utilised to test early B-cell activation, due to their high frequency of B cells specific for the hapten 4-Hydroxy-3-nitrophenylacetyl (NP). QM mice contain a targeted insertion of the rearranged V_HDJ_H 17.2.25 segment derived from an NP-specific BALB/c hybridoma (Cascalho et al., 1996). This heavy chain, combined with a λ light chain, confers specificity for NP with high affinity. However, due to secondary heavy chain rearrangements only around 60% of peripheral B cells are specific for NP (Cascalho et al., 1996). These mice produce a strong extra follicular response to NP-Ficoll. They also develop GCs in response to NP-Ficoll that show little evidence of hypermutation. Consequently B cells do not undergo affinity maturation during these responses (Toellner et al., 2002).

QMxC57BL/6 F1 hybrid mice were also used in experiments. These mice have one copy of the rearranged V_HDJ_H genes and one copy of the κ light chain gene. In these mice around 5% of B cells are NP-specific. Immunisation with NP-Ficoll induces germinal centres and plasma-cell foci with similar kinetics to the secondary T-dependent response when T-cell help is available.

NFκB1^{KO}

As an approach to test Ig CSR and AID induction in a system lacking plasma-cell differentiation, NFκB1^{KO} mice were used. These mice were first described by Sha *et al* (1995). Part of the gene encoding the p50 subunit is ablated via a targeted insertion. A PGK-neo-cassette is inserted into exon 6 of the NFκB1 gene. Exon 6 encodes residues 134-187 within the Rel homology domain, this is designed so as not to affect the synthesis of IκB-γ. Disruption produces a truncated polypeptide that is unable to bind other NFkB members, dimerise, or bind DNA. These mice are more prone to death from infection but otherwise have no difference in lifespan up to a year (Sha et al., 1995).

NFκB1^{KO} mice were also bred onto the QM background. This results in mice that possess B cells specific for NP but lacking NFκB1 protein.

C-REL^{KO}

This mouse was utilised to test class switching when IRF4^{high} expression is deficient. C-REL ^{KO} mice were as described (Liou et al., 1999). Briefly, the genomic DNA fragment containing exon 5, which encodes amino acids 102-130 of the Rel homologous domain, was deleted and replaced with an insertion of the neomycin resistance gene. These mice do not express C-REL protein and contain equivalent amounts of other NFκB family members such as p50, p65 and IκBα. B and T-cell compartments are normal, although T-cell proliferation is defective.

eYFP reporter strain

This strain was first described in 2001 (Srinivas et al., 2001). In brief: a targeted insertion of eYFP into the ROSA26 locus preceded by a *loxP*-flanked stop sequence leads to ubiquitous expression of this autofluorescent protein when crossed with transgenic strains expressing Cre in a ubiquitous pattern (*β-actin-Cre*). This allows the tracking of eYFP^{+} cells following transfer into non-EYFP^{+} hosts. These mice were crossed with QM mice to produce QM x eYFP^{+}. These were then crossed with NFκB1^{KO} or CREL^{KO} to produce QM x NFκB1^{KO} eYFP^{+} or QM x CREL^{KO} eYFP^{+} mice.

2.3 Antigens and Immunisations

Immunisations were performed intravenously (i.v) or intraperitoneally (i.p). Mice were primed with 50 µg of alum precipitated chicken γ globulin (CGG, Sigma) per mouse i.p. This was prepared by mixing one part CGG to one part 9 % aluminium hydroxide (alum). The pH was adjusted to 6.5 with NaOH, and left in the dark at room temperature for one hour to precipitate. This was followed by two washes in sterile pH 7.4 1x phosphate buffered saline

(PBS) in a centrifuge. After which the precipitated CGG was re-suspended to a final volume of 200 µl/mouse in PBS with 10^7 *Bordetella Pertussis* (Dako) per mouse for i.p injection.

Secondary responses were induced via immunization with 50 µg of 4-Hydroxy-3-nitrophenylacetyl (NP) conjugated to CGG in a volume of 200 µl of PBS per mouse. NP-CGG was prepared by Ms Chandra Raykundalia. Briefly NP-OSu (Cambridge Research Biochemicals, UK) was dissolved in dimethylformamide (DMF) (Sigma, Poole, UK) at 200 mg/ml. CGG (Sigma) was dissolved in PBS at a concentration of 5 mg/ml. 200 µl of NP in DMF was added to 1 ml of CGG solution followed by incubation for 2 hours at room temperature on a rotating mixer. Protein was dialysed against PBS pH 7.4. The resulting conjugation ratio is NP_{18}-CGG.

For the study the of TI-II responses, sterile NP-Ficoll (Biosearch Technologies, Novato, CA) was diluted in sterile PBS to a concentration of 30µg in 200µl of PBS per mouse. This was administered i.p.

Sheep red blood cells (SRBC, TCS Biosciences) were injected i.v at $2x10^8$/200µl. SRBC were washed three times and resuspended in sterile PBS.

Mice were sacrificed at the indicated time points and spleen and whole blood harvested.

2.4 Tissue Sections

Spleens were harvested from sacrificed mice at time points indicated and immediately frozen in liquid nitrogen (N_2) before storage at -80°C until required. When ready for use, tissues

were mounted on a chuck through a bed of pre-cooled OCT compound (Bright Instruments, Huntingdon, UK) and 5 µm sections obtained on a cryostat (Bright Instruments, Huntingdon, UK) and mounted onto multispot glass slides (Hendley, Essex). Sections were air dried under a fan at room temperature for one hour and then directly frozen or fixed in cold acetone (4°C) for 20 minutes before further air drying at room temperature for 10 minutes. Slides were stored in polythene bags at -20°C until ready for use.

2.5 Immunohistochemistry

Frozen sections were allowed to reach room temperature and rehydrated in Tris Buffer (pH 7.6: Table 2.3) for 10 minutes. For AID and IRF4 staining sections were fixed in 4% paraformaldehyde for 20 minutes before washing in Tris buffer supplentented with 0.1% saponin (Sigma). Appropriately diluted primary antibodies (Table 2.1) were added, and slides were placed in an incubation chamber for 1 hour at room temperature. Sections were washed again in two changes of Tris buffer for 10 minutes before addition of secondary antibody for 45 minutes at room temperature. Secondary antibodies (Table 2.2) were pre-adsorbed for 30 minutes with normal mouse serum to remove any non-specific binding. Tertiary stains were used when necessary and incubated as per secondary antibodies. Sections were again washed for 10 minutes before the addition of Streptavidin complex (Vector Laboratories Inc, Burlingame) pre-mixed 30 minutes before use. Stains were developed with 3,3 diaminobenzidine tetrahydrochloride (DAB) for horseradish peroxidise conjugated antibodies. After washing for 5 minutes in Tris buffer (pH 7.6) slides were developed with fast blue for biotinylated antibodies. Both substrates are made up freshly before use. After washing in distilled water, slides were mounted with immunomount (Thermo, Basingstoke, UK).

Table 2.1 *Primary antibodies used in immunohistochemical staining*

Target	Isotype	Dilution	Clone	Supplier
AID	Rat anti-Human/ms	1/50	mAID-2	eBioscience
BCL6	Rb anti-ms	1/30	N-3	Santa-Cruz Biotechnology
CD3	Rat anti-ms	1/1000	17A2	Gift of Professor Peter Lane (University of Birmingham)
FDC-M1	Rat anti-ms	1/300	FDC-M1	BD PharMingen
Idiotype 17.2.25	Rat anti-ms	1/100	R2.438.8	T. Imanishi-Karni
IgD	Shp anti-ms	1/1500	Polyclonal	Abcam
IgD	Rat anti-ms	1/1000	11-26C.2a	BD PharMingen
IRF4	Goat anti-ms	1/200	M-17	Santa Cruz
Ki67	Rb anti Ms	1/400	N/A	J. Gerdes, Germany
NP	NP conjugated Shp	1/2500	N/A	Gift of Professor Peter Lane (University of Birmingham)

Table 2.2 *Secondary and tertiary antibodies.*

Antibody specificity	Dilution	Supplier
Donkey anti-sheep biotin	1/100	The Binding Site
Donkey anti-sheep peroxidase	1/100	The Binding Site
Rabbit anti-rat peroxidase	1/50	Dako
Rabbit anti-rat biotin	1/600	Dako
Sheep anti-rat biotin	1/400	The Binding Site
Swine anti rabbit biotin	1/400	The Binding Site
Swine anti-rabbit Igs	1/100	Dako
Rabbit PAP	1/100	Dako

Table 2.3 *Buffers and substrates required for immunohistochemical staining*

Buffer	
Tris buffer pH 7.6	1.5L Physiological NaCl 1.5L 0.1M Hcl 1.0L 0.2M Tris
Tris Buffer pH 9.2	As for Tris buffer pH 7.6 but made to pH 9.2
Peroxidase Substrate	One 15mg tablet of DAB (Sigma) is dissolved in 15mls of TBS pH 7.6. Filter 10mls into a clean universal and add one drop of 30% hydrogen peroxide solution (sigma) to activate the enzyme.
Alkaline Phosphate Substrate	8mg of Levamisole (-)-tetramisole hydrochloride (Sigma) is dissolved in 10mls of Tris buffer pH 9.2. 4mg of naphthol AS-MX phosphate (Sigma) is then dissolved in 370µl of dimethyl formamide (Sigma) in a glass bijoux. This was then added to the centre of the Levamisole solution. To this 10mg of fast blue BB salt (Sigma) was added and the solution filtered.

2.6 Assessment of spleen and germinal centre size. Quantification of antigen specific B cells and plasma cells (NP^+).

Slides were stained for NP, active Caspase3 and BCL6, and counterstained for IgD.

Germinal centres were identified as IgD^- areas surrounded by an IgD^+ follicular mantle. The GC area was quantified by counting the number of intercepts on a $100mm^2$ eyepiece graticule at a magnification of x10. Numbers of NP specific plasma cells were counted in the red pulp over a $100mm^2$ area at x25 magnification and expressed as the average number of cells counted per $100mm^2$ section at a magnification of x 25. The size of the total spleen section was measured by counting intercepts on a $100mm^2$ eyepeice graticule at x4 magnification.

2.7 Immunofluorescent staining

Frozen tissue sections were cut at 5 µm. Sections were firstly washed in PBS for 5 minutes before adsorption with 10% goat serum in PBS for 10 minutes. Antibodies were diluted in PBS supplemented with 10% BSA and incubated with sections in a dark shaking chamber at room temperature. Primary antibodies were added for one hour with the exception of anti-AID which was added overnight at 4°C. Sections were washed for 5 minutes in PBS and subsequent antibody steps were added for 45 minutes. Sections were mounted with Prolong® Gold antifade reagent (Life technologies). Slides were cured at room temperature in the dark for 24 hours then stored at −20°C. Visualisation of staining was performed on a Leica DM6000 microscope. Images were processed with ImageJ software.

Table 2.4: *Primary antibodies not listed for IHC staining*

Antibody specificity	Dilution	Clone	Supplier
B220 Rat anti-ms	1/300	RA36B2	BD bioscience
CD11c Hamster anti-ms	1/500	MCA1369	ABD serotec
FDC-M2 Rat anti-ms bt	1/200	FDC-M2	
IgG Rat anti-ms	1/200	Polyclonal	Serotec
IgM Gt anti-ms FITC	1/100	1021-02	Southernbiotech
MOMA-1 Rat anti-ms	1/30	Moma-1	Abcam

Table 2.5: *Secondary antibodies for immunofluorescent staining*

Antibody specificity	Dilution	Supplier
Dky anti rat cy3	1/300	Jackson Immunoresearch
Rb anti-bt 1/200	1/200	Dako
Gt anti-hmstr Cy5	1/150	Jackson Immunoresearch
Dnky anti-sheep Cy5	1/200	Jackson Immunoresearch

2.8 Quantification of AID protein levels by immunofluorescence

Following staining of tissue sections with anti-AID, images were captured using a Leica DM6000 fluorescent microscope. Images were captured using the same exposure settings for all pictures. QM BCR expressing B cells within follicles were measured for their expression of AID protein by drawing gates around the area of interest within an individual cell and quantifying the median fluorescent pixels. This is proportional to the quantity of protein within the cell.

2.9 Measurement of serum antibody titres

(a) Serum preparation

For analysis of antibody, blood samples were collected at times of sacrifice of each animal and allowed to clot for 1 hour in a 37°C incubator. Serum was separated from clotted blood by centrifugation at 265 x g for 5 minutes.

(b) ELISA

96 well flat-bottomed Nunc Maxisorp plates (Fisher) were coated with 100 µl of NP_{15}-BSA (to detect NP specific antibodies of all affinities) or NP_2-BSA (to detect high affinity NP specific antibodies) at a concentration of 5 µg/ml in coating buffer (Table 2.4). Conjugated proteins were prepared by Mrs C Rayundalia.

Plates were washed x3 in wash buffer, then 200 µl of blocking buffer added for 1 hour in a 37°C incubator. Plates were washed again in wash buffer x3 before the addition of serum (1:10 or 1:50 starting dilution) in tripling dilutions made up in dilution buffer (Table 2.6). Plates were incubated for a further hour at 37°C and after washing x3 in wash buffer, 100 µl of the diluted AP conjugated antibody was added (Table 2.7). After one hour at 37°C the wells were washed x3 and 100 µl of substrate solution added to each well (Table 2.6). After developing in a 37°C incubator the absorbance of the plates was measured at 405nm. Standard calibration curves were plotted from the negative and positive control sera, and the

test serum titres calculated as a relative value of these standard controls. Development was between 20 minutes and 2 hours.

Table 2.6 *Substrates and Buffer solutions required for ELISA*

Buffer	
Coating Buffer pH 9.6	Dissolve 1.95g (0.015M) Na_2Co_3 and 2.93g (0.035M) $NaHCO_3$ in 1L of distilled H_2O.
Wash buffer	0.1M PBS (pH 6.8) with 15.6g of NaH2PO4x2H2O in 1L of distilled water
Blocking buffer	0.1M PBS + 1% BSA (Sigma)
Dilution Buffer	0.1M PBS + 1% BSA + 0.05% Tween (Sigma)
Substrate Solution	Dissolve NpNN (N2770 Sigma) in 20ml distilled H_2O

Table 2.7 *Antibodies utilised in ELISA*

Antibody Specificity	Dilution	Supplier
Goat anti-mouse IgG	1:1000	SouthernBiotech
IgG1	1:1000	SouthernBiotech
IgG1a	1:500	BD bioscience
IgG2a	1:1000	SouthernBiotech
IgG3	1:1000	SouthernBiotech
IgM	1:2000	SouthernBiotech
IgMa	1:500	BD bioscience

2.10 Flow cytometry staining

Spleens were collected into cold RPMI-1640 (Sigma) supplemented with 10% heat inactivated fetal calf serum (HIFCS) with 1% penicillin/streptomycin solution (sigma). Splenocytes were dissociated from the splenic capsule through a 70µm nylon mesh cell

strainer (Falcon) in a sterile 6 well culture plate (Falcon). Cells were re-filtered back through the 70µm cell strainer and washed twice in a centrifuge with cold RPMI. All procedures were carried out at 4°C or on ice to halt the cell cycle reduce cell death. Numbers of cells were calculated using a haemocytometer and approximately $1x10^6$ cells were used for staining in 100µl of FACS buffer (Sterile PBS pH 7.4 supplemented with 0.5% FCS and 0.2mg EDTA). Antibodies used were fluorochrome conjugated or biotinylated for use with a fluorochrome conjugated secondary antibody. Splenocytes were incubated with purified anti-CD16/32 to block non-specific antibody binding by Fc receptors. After incubation with primary antibody (see table 2.8) for 20-30 minutes, cells were washed twice in cold FACS buffer (1% fetal calf serum with 2mM EDTA in sterile PBS) and centrifuged to form a pellet. After removal of the supernatant, cells were resuspended in FACS buffer and stored in the dark at 4°C until analysis, or if required, a secondary antibody was added as described for the primary. In addition single colour controls were included for compensation and a negative control including secondary antibodies if used. Flow cytometric analysis was performed using a Dako Cyan analyser and FlowJo software (FlowJo, Ashland, OR). The number of events collected was dependent upon the frequency of the cell population of interest but was usually around $3x10^5$

Table 2.8 Antibodies used for flow cytometric analysis

Antibody specificity	Dilution	Clone	Supplier
AID	1/50	m-AID-2	eBioscience + Lightning-Link PerCP/Cy.5 (Innova Biosciences)
B220-Pacific Blue	1/50	RA3-6B2	BD Pharmingen
B220-PE-Cy5.5	1/300	RA3-6B2	BD pharmingen
CD16/32	1/200	24g2	BD Pharmingen
CD138-APC	1/100	281-2	BD Pharmingen
CD138-bt	1/100	281-2	BD Pharmingen
CD138-PE	1/400	281-2	BD Pharmingen
CD138-Brilliant violet	1/100	281-2	Biolegend
CD38-Pacific Blue	1/400	90	BD Pharmingen
CD38-Alexa647	1/1000	90	Biolegend
CD69-APC	1/200	H1.2F3	eBioscience
CD62L-PeCy7	1/400	MEL-14	eBioscience
CD86-PerCP-Cy5.5	1/400	GL1	eBioscience
GL7-FITC	1/100	MP1-22E9	BD Pharmingen
Fas-PE-Cy7	1/100	Jo2	BD Pharmingen
IgG1-APC	1/100	X56	BD Pharmingen
IgG3-PeCy7	1/400	Polyclonal	Southern Biotech
NP-PE	1/30,000	N/A	Gift of Professor P Lane

In addition, annexin V-Cy5 protein and 7AAD for FC were obtained from BD biosciences and used according to the manufacturers protocol.

2.11 Intracellular staining for flow cytometric analysis

For intracellular staining of IRF4 and AID, cells were washed and stained as in section 2.7. After two washes with FACS buffer cells were fixed and permeabilised in one step with Fixation/permeabilisation buffer (paraformaldehyde and saponin) (BD Pharmingen) followed by two washes with permeabilisation wash (Fetal bovine serum plus saponin) (BD Pharmingen) and incubation for 30 minutes with goat anti-mouse IRF4 (1/200) followed by anti-goat Cy5 (1/500), or rat anti-mouse AID-PerCP-Cy5.5 (1/50). Rat anti-AID was conjugated to PerCP-Cy5.5 using Lightning-Link PerCP/Cy5.5 (Innova Biosciences) according the manufacturers instructions.

2.12 Cell transfer

Cells were isolated from the spleen as described in section 2.10. Red cells were lysed with ACK lysing buffer (life technologies) for 2 minutes at room temperature then washed in supplemented media as before. Splenocytes were counted and resuspended to a density of 10^7cells/90 µl in sterile de-gassed MACS buffer (0.5% BSA, 2mM EDTA in PBS without Ca^{2+} Mg^{2+}) and labeled with 10µl of anti-CD43 beads per 10^7 splenocytes (Miltenyi Biotech). B-cell enrichment was performed as per manufacturers instructions by incubation at 4°C for 15 minutes followed by one wash in MACS buffer and centrifugation at 300g for 10 minutes. Supernatant was removed completely and splenocytes were resuspended to 500µl /10^8 cells. Splenocytes were passed through a magnetic column (Miltenyi Biotech) to collect unlabeled $CD43^-$ resting B cells. Purified B cells were stained with NP-PE and anti-B220-PE-Cy5.5 (Table 2.6) and analysed by flow cytometry to obtain the percentage of antigen specific B cells. B cells were resuspended in sterile PBS to give a minimum concentration of $1x10^5$ antigen specific B cells per 200µl for carrier primed mice and $5x10^5$ antigen specific B cells per 200µl for TI-II responses. 200µl of cell suspension was injected i.v. into the tail vein per mouse. Where stated, cells were labeled following MACS with CellTrace® (life technologies) according to the manufacturers protocol.

2.13 mRNA detection

Total mRNA extraction

One 8 µm section of spleen was cut on a cryostat and stored at -80°C until use. RNA was extracted with the RNeasy kit (Qiagen, Crawley, UK) after homogenization of tissue using a QIAshredder column (Qiagen) according to the manufacturer's instructions. The resulting RNA was eluted into 30µl of RNAse free water and stored at -80°C until required. For mRNA extraction from FACS sorted cell populations, cells were washed once with ice cold PBS and pelleted at 6000rpm for 15 minutes. The supernatant was discarded and the dry cell pellet was snap frozen in liquid N_2 and stored at -80 °C until processing for RNA extraction. Cells were homogenized with a QIAshredder column (Qiagen) according to the manufacturer's instructions RNA was extracted with an RNeasy micro kit (Qiagen, Crawley, UK).

Production of cDNA

30µl of RNA solution was mixed with 3µl of random oligo-dN6 (1µg/µl) (Promega) and denatured at 70°C for 10 minutes. Samples were then cooled by placing on ice. After this 27µl of reverse transcription mix is added as follows:

12µl 5x First strand buffer (Invitrogen)

6µl 0.1M DTT

3µl dNTP (10mM) (Invitrogen)

3µl Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen)

1.5µl RNAse inhibitor (Promega)

1.5µl RNAse free H_20

This is mixed and heated at 41°C for one hour followed by 90°C for 10 minutes to inactivate the reverse transcriptase. cDNA was then stored at -20°C until required.

Semiquantitative real time PCR

1.5µl of cDNA template was added to each well of a 384 well plate (Applied Biosystems) followed by primers and probes for the appropriate sequence to be amplified (table 2.9 and TaqMan universal PCR mastermix (Applied Biosystems) before covering the plate with clear adhesive film (Applied Biosystems) and centrifuged for 5 seconds at 1200rpm to remove any air bubbles. In addition to amplifying the target sequence, an internal housekeeping gene (β-actin or β_2-Microglobulin) were also amplified to normalize the result for the amount of starting template. These were labelled with Yakima Yellow so as not to interfere with the FAM-labelled probes. Real-time PCR was performed in an ABI 7900 Real-time PCR analyser (Applied Biosystems) with the following cycling programme:

- 2 minutes at 50°C
- 10 minutes at 95° C

Followed by 40 cycles of:

- 15 seconds at 95°C
- 1 minute at 60°C

Data analysis was performed with SDS 2.2 software (Applied Biosystems). Thresholds (i.e cycle number (C_t) at which the signal for the housekeeping and target genes were recorded) were set within the logarithmic phase of the cycle. The relative quantity of target gene mRNA was calculated by subtracting the C_t for the housekeeping gene from the C_t of the target gene (ΔCt) and then calculating $2^{-\Delta Ct}$.

Table 2.9 *Primers and probes required for real time PCR*

Name of target sequence		Nucleotide sequence
IgG1 switch transcripts	Forward Primer	CGAGAAGCCTGAGGAATGTGT
	Reverse Primer	GGAGTTAGTTTGGGCAGCAGAT
	TaqMan probe	TGGTTCTCTCAACCTGTAGTCCATGCCA
IgG3 switch transcripts	Forward Primer	GACCAAATTCGCTGAGTCATCA
	Reverse Primer	ACCGAGGATCCAGATGTGTCA
	TaqMan probe	CTGTCTATCCCTTGGTCCCTGGCTGC
Recombined gamma3 heavy chain transcript	Forward Primer	TCTGGACCTCTCCGAAACCA
	Reverse Primer	ACCGAGGATCCAGATGTGTCA
	TaqMan probe	CTGTCTATCCCTTGGTCCCTGGCTGC
Prdm1	Forward Primer	TTTGGAGGATCTGACCCGAAT
	Reverse Primer	CTCCACCATGGAGGTCACATC
	TaqMan probe	CGCTGATGTCGAACCTCTCAATTTCTTCA
Aicda	Forward Primer	GTCCGGCTAACCAGACAACTTC

	Reverse Primer	GCTTTCAAAATCCCAACATACGA
	TaqMan probe	TGCATCTCGCAAGTCATCGACTTCGT
Irf4	Forward Primer	GGAGGACGCTGCCCTCTT
	Reverse Primer	TCTGGCTTGTCGATCCCTTCT
	TaqMan probe	AGGCTTGGGCATTGTTTAAAGGCAAGTTC

2.14 *In Vitro* B-cell stimulation

$CD43^-$ resting B cells were enriched from total splenocytes as described in section 2.12. B cells were added to a 96-well sterile u-bottomed plate (Corning) at a density of $1x10^5$ cells/well in 200 µl of pre-warmed media containing 10% heat inactivated fetal calf serum (HIFCS) with 1% penicillin/streptomyacin solution (sigma) and stimulated with 2 µg per well of NP-Ficoll. At selected timepoints after incubation with antigen, cells were harvested by centrifugation. This was followed by either fixation with Fixation/permeabilisation buffer (BD Pharmingen) as described in section 2.11 for intracellular IRF4 staining, or by washing with ice cold PBS and snap freezing in liquid N_2 for quantification of IRF4 mRNA levels by real-time RT-PCR.

2.15 Preparation of miR-125b over-expressing QM B cells

Lethally irradiated C57 mice were reconstituted with QM bone marrow over-expressing the micro-RNA miR-125b, these mice were directly immunised or used as donors for cell transfer experiments. Bone marrow transfections and chimera mice were prepared by Kyoko Nakamura (University of Birmingham). In brief: PlatE cells were infected (Morita et al.,

2000) with an MSCV-based retrovirus plasmid vector using Lipofectamine 2000 (Invitrogen) in accordance with manufacturer's instructions. This was either MIG control vector or contained the 500-bp gene segment for the miR-125b hairpin. The 500bp miR-125b containing gene segment was amplified with the primers:

5'-agatatgtgtgttgcatatacagggctg-3'

5'-gaattcatctaacaatggaagcctcaaggg-3' by AccuPrime Taq DNA polymerase, High Fidelity (Invitrogen). The DNA fragment was cloned into *Bgl*II and *Eco*RI site of the MIGR1 vector. Chimeras were prepared as follows; QM mice were injected with 5-fluorouracil (5-FU) (Sigma) at a concentration of 150 mg/kg body weight. 5-FU has a cytotoxic effect on dividing bone marrow progenitors; therefore after treatment with 5-FU, bone marrow is enriched for 5-FU resistant stem cells (Wieder et al., 1991). Femurs were harvested four days after 5-FU treatment and bone marrow was flushed out of the bone using a syringe containing supplemented media (RF10 supplemented with 10% FCS, 1% penicillin/streptomycin solution and 2 mM L-glutamine). Following red cell lysis, bone marrow cells were washed and resuspended in supplemented media before filtration through a 70 µm cell strainer. Sca1 was used as a marker to isolate hematopoietic stem/progenitor cells (Spain and Mulligan, 1992). Bone marrow cells were incubated with anti-Sca1-PE antibody for 30 minutes on ice at a concentration of 1 µl anti-Sca-1-PE per 10^7 cells in 200 µl of MACS buffer. Cells were washed with 10 ml of MACS buffer followed by resuspension in 40 µl MACS buffer and 10 µl of anti-PE magnetic beads per 10^7 cells for 20 minutes at 4°C. Cells were washed before passing through a magnetic MS column as per manufacturer's instructions. Purified Sca-1^+ cells were plated into 24 well plates with SCF (50 ng/ml), IL6 (50 ng/ml), IL3 (10 ng/ml). Cells were incubated at 37°C at 5% CO_2 density. Plasmid-containing supernatant from PlatE cells was then used to infect QM bone marrow before transfer into lethally irradiated C57 or RAG^{KO} host mice.

2.16 Statistical analysis

Statistical significance was calculated using a one-tailed Mann-Whitney U test where * p=<0.05, ** p=<0.01, *** p=<0.0001. A one-tailed test was deemed appropriate in order to test whether CSR and AID induction were reduced when intermediate or high-level IRF4 are blocked.

3: INDUCTION OF IRF4 AND AID EXPRESSION FOLLOWING B-CELL ACTIVATION

3.1 Introduction

Ig class switching is a process that occurs with high frequency within the germinal centre (Liu et al., 1996). However, this process can also occur early in the B-cell response before the formation of germinal centres (Marshall et al., 2011; Toellner et al., 1996). The initiation of CSR by extrafollicular B blasts is preceded by the upregulation of *Irf4* mRNA (Sciammas et al., 2006) and *Aicda* mRNA (Honjo et al., 2002).

Activation-induced cytidine deaminase (AID) protein, encoded by the *Aicda* gene, was first identified by the subtraction of cDNAs derived from switch-induced and uninduced murine B lymphoma CH12F3-2 cells, the majority of which switch to IgA following stimulation. AID expression was subsequently found to be restricted mainly to the germinal centre (Muramatsu et al., 1999). This protein is required for both class switch recombination (CSR) and somatic hypermutation (SHM) (Muramatsu et al., 2000). AID facilitates CSR by deaminating deoxycytosines in S regions to create uracils, this creates U:G mismatches. Repair of these mismatches leads to the creation of double stranded breaks into the DNA template (Stavnezer et al., 2008).

IRF4 is a transcription factor that is essential for plasma-cell differentiation (Klein et al., 2006). *Irf4*-Cγ1-Cre mice have a conditional knockout of IRF4 in cells that have switched to IgG1. Following immunisation, these mice have no defect in germinal centre formation, yet completely lack plasma cells (Klein et al., 2006). Although $IRF4^{KO}$ B cells express the transcription factor BLIMP-1, which is crucial for plasma-cell differentiation (Turner et al.,

1994) (Shapiro-Shelef et al., 2003), this is not sufficient for further differentiation into plasma cells (Klein et al., 2006).

It has previously been shown that IRF4 is differentially expressed *in vitro* (Sciammas et al., 2006; 2011). High levels of IRF4 facilitate plasma-cell differentiation, whilst low levels are required for Ig class switching (Sciammas et al., 2006). It has recently been shown in our group (Zhang Y UOB thesis 2010, Marshall J UOB thesis 2009) that these two phases of IRF4 induction during the B-cell response correlate with certain stages of B-cell differentiation.

Within the first hours of activation, during B blast migration to the border with the T zone, all B blasts upregulate intermediate levels of IRF4 protein. Plasmablasts in contrast, express high concentrations of IRF4 protein.

Our aim was firstly to characterise the expression patterns of both IRF4 and AID during extrafollicular B blast and plasmablast differentiation (reviewed in (Maclennan et al., 2003)). Secondly, due to the published role of IRF4 for *Aicda* induction and Ig class switching, to test whether there is a correlation between the expressions of these two proteins during the early B-cell response.

We aimed to address

1. The timing of IRF4 protein and mRNA expression.
2. Whether this correlates with the induction of AID mRNA and protein and the onset of Ig class switching.

3.2 Results

3.2.1 IRF4 protein and mRNA are rapidly induced *in vivo* upon antigen-stimulation

To determine the timing of IRF4 protein and mRNA induction, quasi-monoclonal x C57BL/6 F1 mice (QMxB6 mice) were immunised with the TI-II antigen NP-Ficoll. QM mice (Cascalho et al., 1996) have one copy of a VDJ recombined heavy chain gene that codes for a heavy chain specific for NP (Grosschedl et al., 1984), a Jh deletion on the other allele (Chen et al., 1993a) and kappa light chain deficiency (Chen et al., 1993b). Due to the high frequency of antigen-specific B cells in these mice (60-80%) and the massive response induced post-immunisation, tracking of individual activated cells is difficult. Therefore, QM mice were crossed with B6 mice to produce offspring with one copy of the rearranged NP-specific Ig heavy chain and one copy of the kappa allele ($QM^{NP/WT}$ $\kappa^{-/WT}$) which results in a frequency of antigen-specific B cells of around 5-10% (Vinuesa et al., 2001). Due to more numerous antigen-specific B cells than normally physiologically present prior to immunisation (Less than 0.5% of lymphocytes are NP-specific B cells in C57 mice: Chapter 6 Fig. 6.5), sufficient numbers of activated cells can be tracked in the first hours and days after immunisation, before cell division has expanded the numbers.

Following immunisation of QMxC57BL/6 mice with NP-Ficoll, IRF4 mRNA was tested in whole spleen sections 1, 2, 4 and 8 hours after immunisation by real-time RT-PCR (Figure 3.1A). IRF4 mRNA is increased by 8x within 1 h, and remains high 2 h post-immunisation. IRF4 mRNA levels are slightly decreased at 4 h post-immunisation, however mRNA levels remain higher than in non-immunised spleens by nearly 3x. IRF4 is also expressed by activated macrophages (Satoh et al., 2010) and T cells (Li et al., 2012) (Bollig et al., 2012). To determine the expression of IRF4 within the activated B-cell population, NP-binding B cells were sorted before immunisation and 1, 2, 4, 24 and 48 h after activation (Figure 3.1B).

In addition, three days after activation, plasma cells (NP-binding, $CD138^+$) and germinal centre cells (NP-binding, $CD138^-$ $B220^+$) were tested for IRF4 expression. Similar to whole spleen sections, 1 h post-immunisation, IRF4 mRNA is rapidly and strongly induced in sorted antigen-specific B cells. IRF4 mRNA levels remain high 2 h post-immunisation before decreasing again at 4 h post-immunisation. IRF4 mRNA levels remain largely unchanged until B cells differentiate further into germinal centre cells (Figure 3.1B red) and plasma cells (Figure 3.1B blue). Germinal centre cells express low amounts of IRF4 mRNA, whereas plasma cells express IRF4 mRNA levels similar to that seen at 1 h post-immunisation.

In the QM spleen, NP-Ficoll immunisation activates follicular and MZ B cells which migrate to the border of the T zone after activation (Vinuesa et al., 2001). Before immunisation, NP-binding cells are visible in the marginal zone and the follicle and these cells express low levels of IRF4 protein (Figure 3.1C, top panel). Some background plasma cells are located at the bridging channel (interphase between T zone and red pulp) and show high IRF4 protein levels (Figure 3.1C, top panel, arrow). Four hours post-immunisation, antigen-specific B cells have migrated to the border with the T-zone and are expressing IRF4 (Figure 3.1C, bottom panel, arrow). Antigen specific B cells remain in the outer T zone until they form germinal centres within the follicles or plasma-cell foci in the red pulp. IRF4 protein expression within the marginal zone and follicles, outer T zone and both plasma and germinal centre B-cell populations were quantified by measuring immunostaining from tissue sections. Within 1 h of immunisation, B cells in the marginal zone and follicles upregulate IRF4 protein (Figure 3.1D). IRF4 protein levels continue to increase until 8h post-immunisation within activated B blasts which have migrated to the outer T zone (Maclennan et al., 2003), thereafter IRF4 protein remains at an intermediate level until differentiation to germinal centre or plasma cells occurs four days after immunisation. Germinal centre cells do not express IRF4 protein, whilst IRF4 protein levels are high in plasma cells (Figure 3.1D). Therefore, whilst IRF4

mRNA peaks between 1 h and 2 h post-immunisation in both spleen sections and sorted B cells, IRF4 protein levels continue to increase to an intermediate level, until expression of both IRF4 protein and mRNA stops in germinal centre B cells. Alternatively, upon plasma cell differentiation, IRF4 mRNA is upregulated to levels seen in B blasts 1 h post-immunisation, whereas IRF4 protein is expressed at a much higher level than seen in B blasts during the first hours and days of activation.

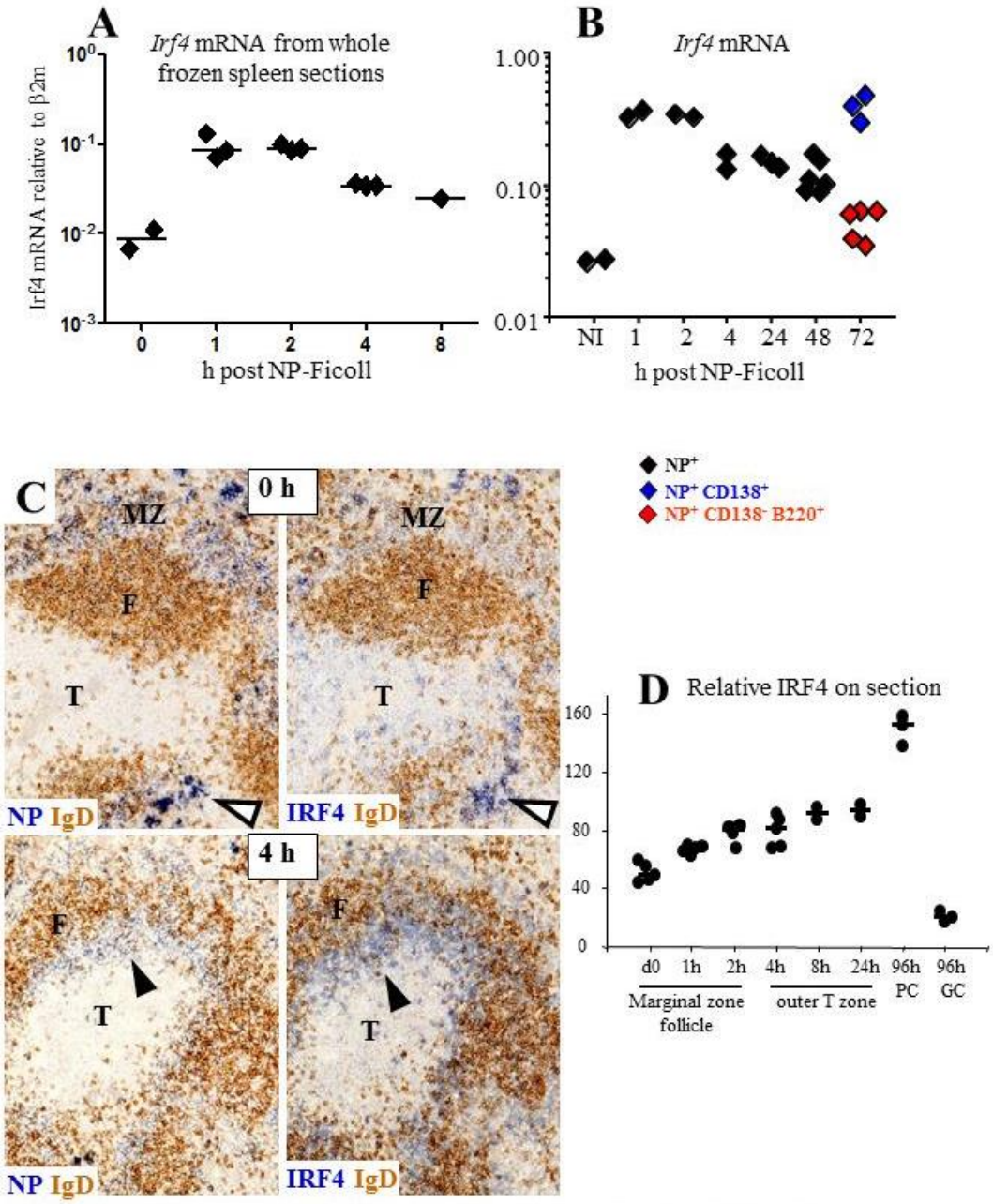


Figure 3.1: *Rapid IRF4 induction in differentiating B cells*. QMxC57BL/6 mice were immunised with NP-Ficoll and spleens collected pre-immunisation and 1, 2, 4, 8, 24 and 96 hours post-immunisation. **A:** IRF4 mRNA levels were measured from 8 µm spleen sections by real time RT-PCR. **B:** Antigen-specific B cells were sorted at stated timepoints post-activation and tested for IRF4 mRNA by real-time RT-PCR. Each data point represents one mouse. **C:** Splenic tissue sections were stained with antibodies against NP and IgD (left) or IRF4 and IgD (right). Before immunisation (top panel) NP-binding cells are located in the follicles and marginal zone and express low amounts of IRF4 protein. Some background plasma cells are visible near the bridging channel (arrow) and these cells express high levels of IRF4 protein. Four hours after immunisation, antigen specific B cells have migrated to the follicular/T-zone border and express intermediate amounts of IRF4 protein. **D**: IRF4 protein was quantified from tissue sections using ImageJ software. Experimental data courtesy of Zhang,Y, unpublished.

B cells internalise their BCR immediately after activation (Malhotra et al., 2009), this makes identification of antigen-specific B cells by staining of their antigen-specific B-cell receptors difficult. To address this problem, QMxB6 mice were immunised with Fluorescein labelled NP-Ficoll (NP-Fluorescein-AECM-Ficoll). This enabled the identification by flow cytometry (FC) of $FITC^+$ cells that had bound antigen *in vivo.* In addition, splenocytes were incubated with PE conjugated with the hapten NP (NP-PE) and stained with antibodies against B220 and IRF4 0, 1, 2 and 4 hours after immunisation. $FITC^+$ and $B220^+$ B cells (Figure 3.2A left plots) were tested for binding to NP-PE. The intensity of NP-PE staining was found to decrease in the hours following immunisation probably due to receptor internalisation (Figure 3.2A right plots). NP-PE binding $B220^+$ cells (pre-immunisation) or $FITC^+$ $B220^+$ B cells (post-immunisation) were tested for expression of intracellular IRF4 protein by flow cytometry (FC). The median fluorescence intensity of IRF4 staining within this population was significantly increased ($p<0.05$) by approximately 2x within 1h of immunisation (Figure 3.1B and C). The median fluorescence intensity continues to increase by another 30% between one and two hours post-immunisation and by 70% between two and four hours post-immunisation ($p<0.05$). The median fluorescence intensity of IRF4 staining increases by a median of 3.4x between B cells pre-immunisation and four hours post-immunisation (Figure 3.2B and C). To address whether IRF4 protein levels increase post-immunisation, or whether the increased median fluorescence intensity of IRF4 reflects an increase in the proportion of $IRF4^+$ cells, $B220^+$, NP-binding B cells were gated which expressed >95% the IRF4 protein expressed by NP-binding $B220^+$ cells pre-immunisation (Figure 3.2D). The percentage of NP-binding $IRF4^+$ cells increased within 1 h of immunisation by >2x. The percentage of NP-binding $IRF4^+$ cells further increased at 2 h and 4 h post immunisation. Therefore increased median fluorescent intensity of IRF4 protein following immunisation was found to be due to

an increase in the percentage of IRF4 expressing B cells. $FITC^{+}B220^{+}$ B cells (antigen-activated) or $B220^{+}$ B cells from non-immunised mice were FACS sorted using the gating strategy in Figure 3.2A and tested for expression of IRF4 mRNA by real time RT-PCR (Figure 3.2E). IRF4 mRNA is strongly increased within one hour of immunisation ($p<0.05$) with median mRNA levels increasing by 6.6x. In contrast to protein levels, which continue to increase after one hour, mRNA levels significantly decrease from the peak levels at one hour. Between one and two hours post-immunisation, IRF4 mRNA levels decrease by 16% and decrease by a further 20% between two and four hours post-immunisation ($p<0.05$). However within four hours of immunisation, IRF4 mRNA remains around 4x higher than that expressed in non-activated cells. This indicates that IRF4 protein levels are transcriptionally regulated. Slightly lower transcription levels follow the initial strong burst of IRF4 transcription that occurs within the first hour. This facilitates intermediate expression levels of IRF4 protein in B blasts.

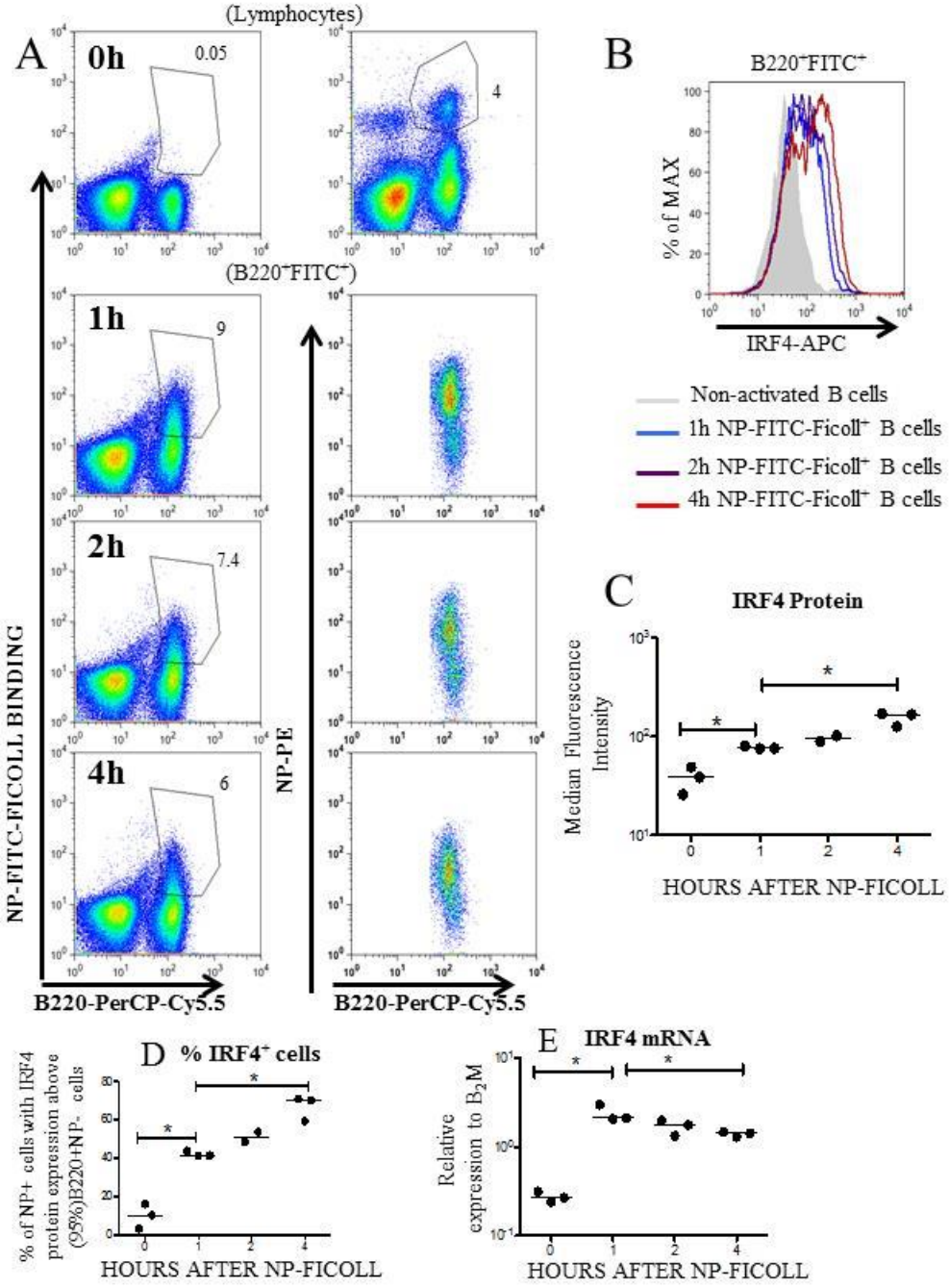


Figure 3.2: *Kinetics of IRF4 induction in B cells*. QM mice were immunised with NP-FITC-Ficoll i.p. **A:** 0, 1, 2 and 4 hours after immunisation, splenocytes were stained with antibodies against B220 and IRF4 and incubated with NP-PE. Left plots: $B220^+$ B cells which were $FITC^+$ were gated and tested for NP-PE binding (right plots) **B:** Representative histogram of IRF4 protein from FC at stated timepoints after immunisation. **C:** Median fluorescence intensity of IRF4 protein in B cells gated as $FITC^+B220^+$ as in (A). **D:** The % of NP-binding B cells which expressed >95% the levels of IRF4 protein seen in $B220^+$ non-NP-binding cells **E:** $B220^+FITC$-binding cells were sorted according to the gating strategy in (A), 0, 1, 2 and 4 hours after immunisation. Sorted cells were tested for expression of IRF4 mRNA by real-time RT-PCR. Each data point represents one mouse. Data are representative of two experiments.

3.2.2 BCR stimulation is sufficient to rapidly induce IRF4 mRNA and protein

To test whether the microenvironment is important for B-cell activation and IRF4 induction, or whether IRF4 can be induced solely via BCR stimulation *in vitro*, B cells were incubated for 0.5, 1, 2, 4 and 48 h with NP-Fluorescein-AECM-Ficoll. To exclude contamination by other cell types capable of capturing FITC-conjugated polysaccharides (Sallusto et al., 1995) such as dendritic cells, $CD43^-$ resting B cells were purified by MACS for stimulation. Expression of the B-cell marker B220 was analysed on total $CD43^-$ purified splenocytes to confirm B-cell purity. More than 90% of $CD43^-$ purified splenocytes expressed B220 (Figure 3.3A). Post-stimulation, cells were fixed and incubated with NP-PE and stained with antibodies specific for B220 and IRF4. $FITC^+$, NP-binding $B220^+$ cells were analysed by FC (Figure 3.3B). Antigen specific B cells express little IRF4 before stimulation (Figure 3.3C and D). Within 30 minutes of stimulation however, IRF4 protein is significantly increased by a median of 90%. Similar to that seen *in vivo* (Fig. 3.2C) there is a significant increase in IRF4 protein expression by one hour post-activation ($p<0.05$) (Figure 3.3C and D). There is a further significant increase in IRF4 protein by 1.6x between four and 48 hours after activation of B cells *in vitro* ($p<0.05$). IRF4 protein is upregulated to a similar degree *in vitro* 30 minutes after antigen stimulation as that observed *in vivo* one hour post-immunisation. This more rapid IRF4 upregulation *in vitro* may be due to an acceleration of B-cell activation, as similar IRF4 protein levels are observed after 4 h whether B cells were stimulated *in vitro* or *in vivo* (140% *in vitro* vs 180% *in vivo*). After i.p immunisation, antigen must travel to the spleen in order to activate B cells (Kool et al., 2008) and this delay *in vivo* may explain the relatively faster IRF4 upregulation *in vitro.*

IRF4 mRNA peaks within one hour *in vivo* (Figure 3.2E). To test the kinetics of IRF4 mRNA expression *in vitro,* real-time RT-PCR was performed on B cells stimulated with NP-

Fluorescein-AECM-Ficoll. IRF4 mRNA was significantly increased ($p<0.05$) by a median of 5.9x within 30 minutes of activation *in vitro* (Figure 3.3E). IRF4 mRNA further increases significantly by 3x one hour post-activation and 1.60x two hours post-activation ($p<0.05$). This represents a median increase above baseline of 50x within two hours of activation. This is followed by a small (40%) but significant decrease in IRF4 mRNA from the peak levels observed at two hours post-activation. Therefore *in vitro* IRF4 protein levels increase within 30 minutes of antigen-activation and continue to increase until 1 h post-activation after which levels remain steady before further increasing 48 hours after antigen activation. Whereas *in vivo,* protein levels increase within 1 h of immunisation but continue to increase at 2 and 4 h after immunisation. mRNA levels *in vitro* increase within 30 minutes and are highest 2 h after incubation with antigen before decreasing at 4 hours after activation. This is similar to IRF4 mRNA expression *in vivo* where levels peak 1-2 h after immunisation but are decreased by 4 h post-immunisation.

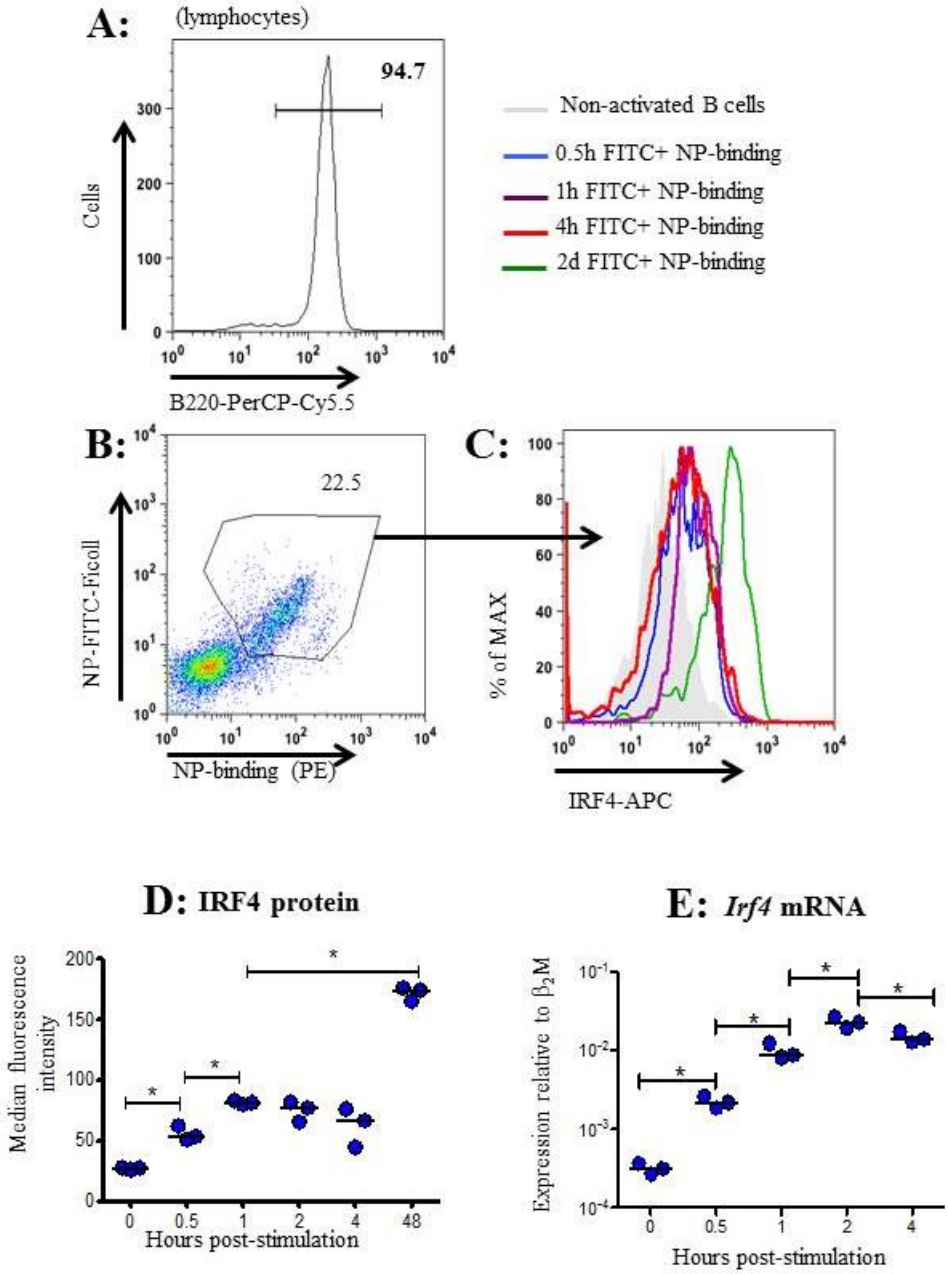


Figure 3.3: *Kinetics of IRF4 induction in B cells in vitro*. Resting B cells from QM mice were isolated via negative selection on the basis of CD43 expression with magnetic beads. These were plated into a 96-well round-bottomed plate and stimulated with 2μg of NP-FITC-Ficoll at a concentration of 10μg/ml. **A:** $CD43^-$ splenocytes are >90% $B220^+$ B cells **B:** B cells were stained with antibodies against B220 and IRF4 and incubated with PE labelled antigen. Activated cells were gated as $FITC^+$ and NP-PE binding.. **C:** Representative expression of IRF4 protein at stated timepoints after incubation with antigen as measured by FC. Cells were gated as shown in (**A**). **D:** Median fluorescence intensity of IRF4 in B cells gated as $FITC^+B220^+$ as in (**A**). **E:** *Irf4* mRNA from B cells at 0, 0.5, 1, 2 and 4 hours after incubation with antigen as measured by real-time RT-PCR. Data are representative of two experiments. Each data point represents one well.

3.2.3 In later stage B-cell differentiation IRF4 protein increases within the plasmablast population whereas germinal centre B cells are IRF4 negative

To test IRF4 expression in emerging germinal centre B-cell and plasmablast populations, QM mice were immunised with NP-Ficoll as described before. Spleens were processed by FC 2, 3, and 4 d after immunisation. B cells were stained with antibodies against B220, IRF4, FAS and CD138, followed by incubation with NP-PE. Activated B cells were gated as NP-binding $B220^{high/int}$ (Fig. 3.4.A). Within two days of immunisation, a small proportion of antigen-specific B cells express CD138 protein (Figure 3.4A top panel) and these express higher levels of IRF4 protein. This population appears at a similar percentage three days post-immunisation (Figure 3.4A middle panel), but is increased four days after immunisation (Figure 3.4A bottom panel). In addition, a $B220^{high}$, NP-binding FAS^{+} germinal centre population has developed (Figure 3.4A, bottom panel). Whilst non-activated B cells express low amounts of IRF4 protein, IRF4 protein is up-regulated two days after immunisation within the NP-binding population (Figure 3.4B, purple). By three days post-immunisation there is a slight loss of IRF4 expression in the majority of B cells (Figure 3.4B, green), while a small population of cells have increased IRF4 protein levels further. These IRF4 low and high populations correspond to emerging germinal centre B cells and plasmablasts respectively. The differential IRF4 expression is more apparent four days after immunisation, following further differentiation. Four days after immunisation, germinal centre B cells express similar levels of IRF4 protein to non-activated B cells (Figure 3.4B, blue) whereas plasma cells express the highest levels of IRF4 (Figure 3.4B, red).

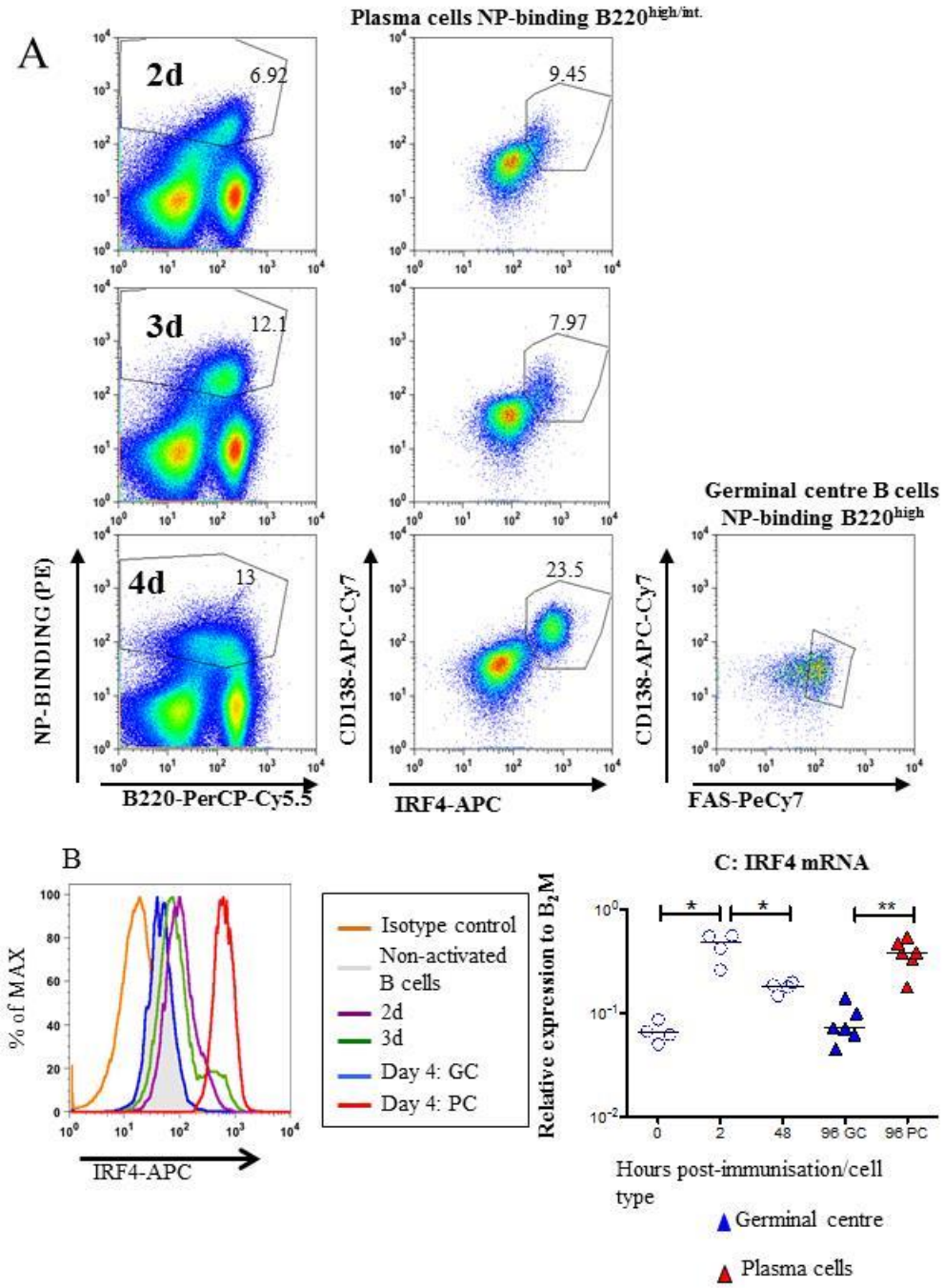


Figure 3.4: *IRF4 induction in differentiating B cells*. QM mice were immunised with NP-Ficoll. **A:** Splenocytes were incubated with NP-PE and stained with antibodies against B220, IRF4 and CD138. Activated cells were first gated as NP-binding. Plasma cells were gated as IRF4^{+} and CD138^{+} whereas germinal centre cells are B220^{high}, NP-binding FAS^{+} CD138^{-}. **B:** Histogram showing representative IRF4 protein induction by FC in all NP-binding B cells at D2 and D3 after immunisation and in GC cells and PC at D4 after immunisation (gated as in A). **C:** Induction of IRF4 mRNA in sorted antigen-specific cells two and 48 hours after immunisation with NP-Ficoll, or in sorted GC (FAS^{+}NP-low) and PC (CD138^{+}), four days after immunisation. Each data point represent one mouse. Data are representative of two experiments, with four mice for each timepoint.

To test whether IRF4 mRNA shows a similar expression pattern, cell sorting experiments were performed. Resting $CD43^{-}$ $eYFP^{+}$ QM B cells were transferred into wild-type hosts and immunised with NP-Ficoll i.p. Two hours and two days after immunisation, $eYFP^{+}$ and NP-binding B cells were sorted. Four days post-immunisation, germinal centre B cells ($NP\text{-}PE^{low}$, $B220^{high}$, FAS^{+}) or plasmablasts ($NP\text{-}PE^{high}$, $B220^{low}$, $CD138^{+}$) were sorted. Antigen-specific B cells were found to significantly upregulate IRF4 mRNA by 6x ($p<0.05$) within two hours of immunisation (Figure 3.4C). IRF4 mRNA then significantly decreases two days post-immunisation to 1.8x higher than background ($p<0.05$). Four days after immunisation, germinal centre B cells express similar levels of IRF4 mRNA as non-activated B cells, whereas plasma cells express significantly higher amounts of mRNA (Figure 3.4C) ($p<0.01$).

3.2.4 Timing of AID mRNA expression

To follow the timing of *Aicda* mRNA expression during the earliest stages of the B-cell response, QM mice were immunised with NP-Ficoll and RNA extracted from spleen sections at selected timepoints. *Aicda* mRNA levels decrease within 4 h of immunisation (Figure 3.5A). However QMxB6 mice contain background GC before immunisation, these GC rapidly shut down upon the initiation of a specific immune response. Therefore it is likely that in frozen tissue sections the *Aicda* signal is so low in activated B blasts that it is masked by background GC, and a specific increase in *Aicda* can only be detected at 72 hours post-immunisation when GC founder cells appear, at this time *Aicda* mRNA levels are increased by around 1.40x compared to two days post-immunisation (Figure 3.5A). To test which B-cell populations were expressing *Aicda* and to remove contamination from non-specific background GC, we performed cell sorting. Resting $CD43^{-}$ $eYFP^{+}$ QM B cells were transferred into wild-type hosts as described above and immunised with NP-Ficoll one day later. Antigen-specific B cells (two days post-immunisation), germinal centre B cells and plasma cells (four days post-immunisation) were sorted by FACS from the spleen as

previously described. *Aicda* mRNA was found to be strongly increased two days post-immunisation in activated antigen-specific B cells (Fig. 3.5B). Levels remained high in germinal centre B cells four days post-immunisation, whereas plasma cells lost *Aicda* mRNA (Figure 3.5B). Figure 3.5C shows the gating strategy and representative samples of the purity of sorted populations.

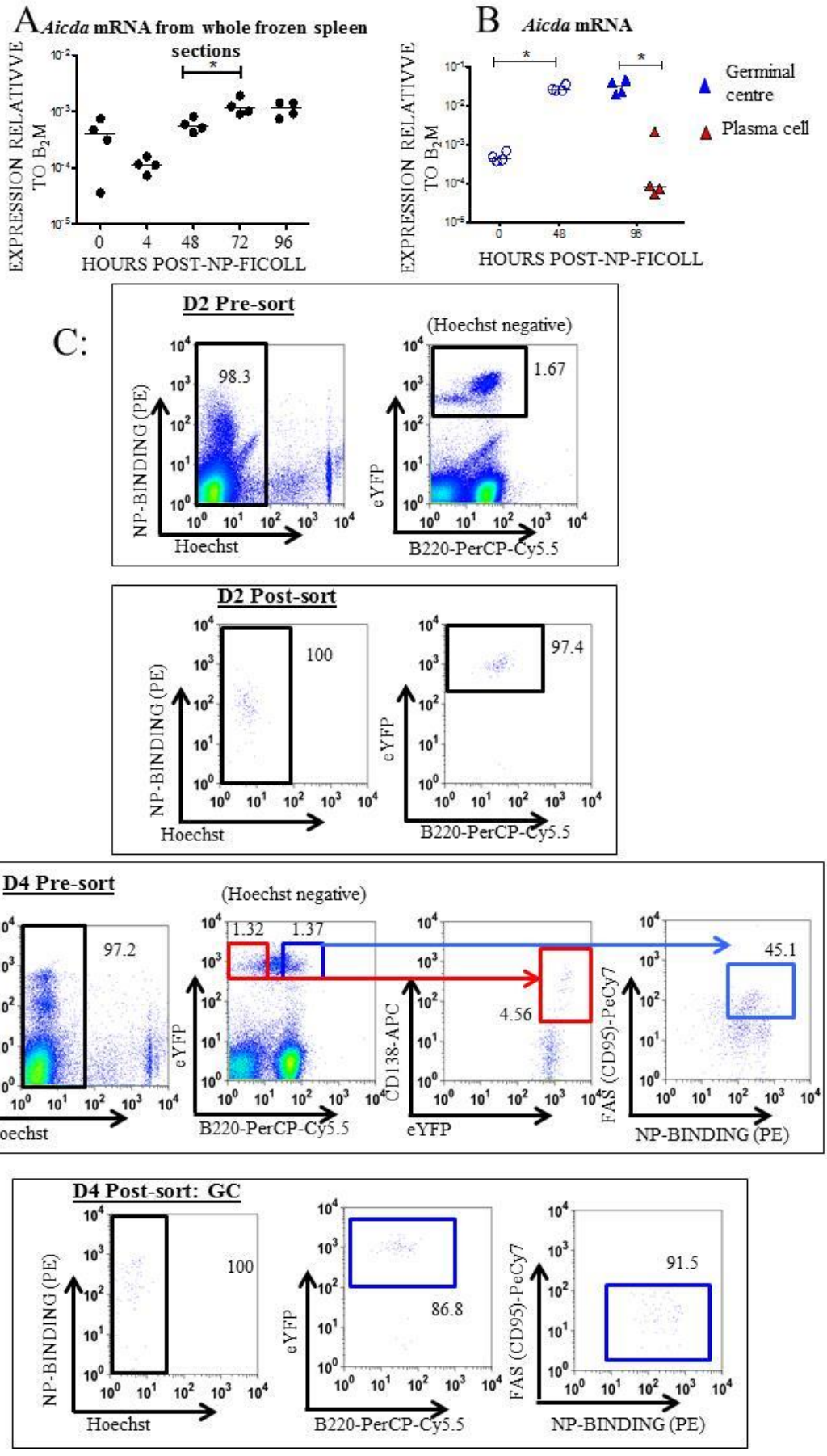

A
Aicda mRNA from whole frozen spleen sections
EXPRESSION RELATIVVE TO B2M
HOURS POST-NP-FICOLL
B
Aicda mRNA
Germinal centre
Plasma cell
C:
D2 Pre-sort
(Hoechst negative)
98.3
1.67
NP-BINDING (PE)
Hoechst
eYFP
B220-PerCP-Cy5.5
D2 Post-sort
100
97.4
D4 Pre-sort
97.2
1.32
1.37
4.56
45.1
CD138-APC
FAS (CD95)-PeCy7
NP-BINDING (PE)
D4 Post-sort: GC
100
86.8
91.5

Figure 3.5: *Kinetics of AID mRNA expression during the TI-II response*. **A:** QMxB6 mice were immunised with NP-Ficoll. RNA was isolated from spleen sections and analysed for *Aicda* mRNA expression by real-time RT-PCR pre-immunisation and 4, 48, 72 and 96 hours after immunisation. **B:** $eYFP^{+}$ QM B cells were transferred into C57 hosts and immunised with NP-Ficoll**.** At selected timepoints post-immunisation, QM B cells were sorted by FACS, based on the gating in (C). Sorted cells were tested for AID mRNA by real-time RT-PCR, relative to B_2-microglobulin. **C**: Top panel, example of purity of antigen-specific B blasts two days post-immunisation (left) and purity post-FACS (right). B cells were sorted as Hoechst^{-}, $B220^{+/int}$ $eYFP^{+}$. Middle panel: representative plot of pre-sort sample four days after immunisation. Cells were gated as Hoechst^{-}, plasma cells were defined as $B220^{low}$, $CD138^{+}$, whereas germinal centre B cells were defined as $B220^{high}$ NP-binding^{low}, FAS^{+}. Bottom panel: example of purity of germinal centre B cells four days after immunisation.

Immunohistochemistry was used to confirm AID protein expression within the spleen. QM mice were immunised with NP-Ficoll and spleens collected two or four days later. Two days after immunisation, sections were stained with antibodies against IgD and NP or IgD and AID. NP-specific B cells are located within the follicles and at the border of the follicles with the T zone (Figure 3.6A blue) and AID^+ B cells are visible in similar locations (Figure 3.6B blue). Four days post-immunisation, large antigen-specific germinal centres have developed, these bind low levels of NP compared to plasma cells located in the red pulp (Figure 3.6C, blue), and contain some $CD3^+$ T cells (Figure 3.6D blue). Germinal centres express high levels of AID protein whereas plasma cells are AID^- (Figure 3.6E).

This shows that AID mRNA and protein are induced within two days of immunisation, while B cells reside in the follicles and T zone. It is highly expressed in germinal centre B cells, while plasma cells do not express AID mRNA any longer, and therefore do not undergo further CSR.

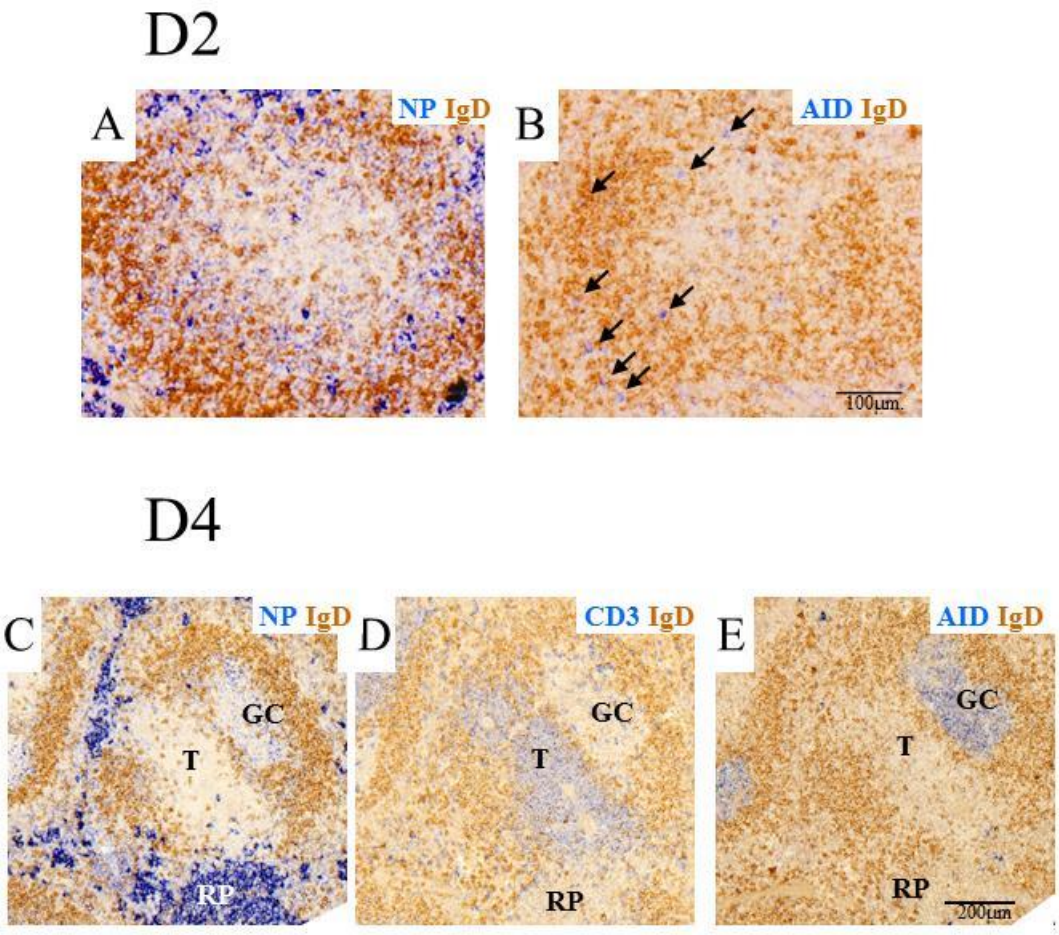


Figure 3.6: *AID induction in differentiating B cells*. QMxB6 mice were immunised with NP-Ficoll i.p. Two and four days post-immunisation spleens were sectioned and analysed by immunohistochemistry. Two days after immunisation spleens were stained with antibodies against IgD (brown) in conjunction with: NP (**A**) and AID (**B**) (blue). Four days after immunisation spleens were stained with antibodies against IgD in conjunction with NP (**C**), CD3 (**D**), and AID (**E**) (blue). Four days post-immunisation, antigen-specific germinal centres and plasma cell foci have formed. Germinal centres express high levels of AID. Plasma cells in contrast do not express AID.

3.2.5 IRF4 and AID are co-expressed in B blasts two days post-immunisation

As IRF4 and AID are both expressed within two days of immunisation, we tested whether IRF4 expression correlates with AID expression within single cells. AID and IRF4 were stained and analysed by FC and immunohistology.

Resting QM eYFP$^+$ B cells were transferred into wild-type hosts and immunised with NP-Ficoll i.p. Two days later splenocytes were stained with antibodies against B220, AID and IRF4 and incubated with PE labelled NP. Activated B cells were defined as NP-binding and B220$^+$ and these cells were collected for FC and found to co-express AID and IRF4 protein at higher levels than non-antigen specific B cells (Figure 3.7A red vs blue). Similar observations were done on splenic B blasts differentiating in situ. Spleen sections were stained with labelled antibodies against IRF4 and AID (Figure 3.7B). As described above, two days post immunisation; activated B cells are located within B220$^+$ inner follicles near the T-zone, or in the outer follicles. Approximately half of activated B blasts express nuclear AID protein compared to cytoplasmic AID protein, with slightly more nuclear AID expression when B cells are located at the border with the T zone compared to the outer follicle. The cellular location of AID protein is one of the mechanisms in which AID function is regulated. AID continually shuttles between the nucleus and cytoplasm and whilst it is only active when located in the nucleus, it is also more sensitive to degradation (Aoufouchi et al., 2008). The export of AID from the nucleus is mediated by CRM1 which binds to a nuclear export signal on AID (Geisberger et al., 2009; Ito et al., 2004). AID is also maintained in the cytoplasm by a cytoplasmic retention mechanism(Patenaude et al., 2009). Interestingly all AID$^+$ B cells co-expressed nuclear IRF4. After quantification of AID and IRF4 staining intensity within

individual cells there was found to be a positive correlation between the quantity of AID and IRF4 protein (Figure 3.7C).

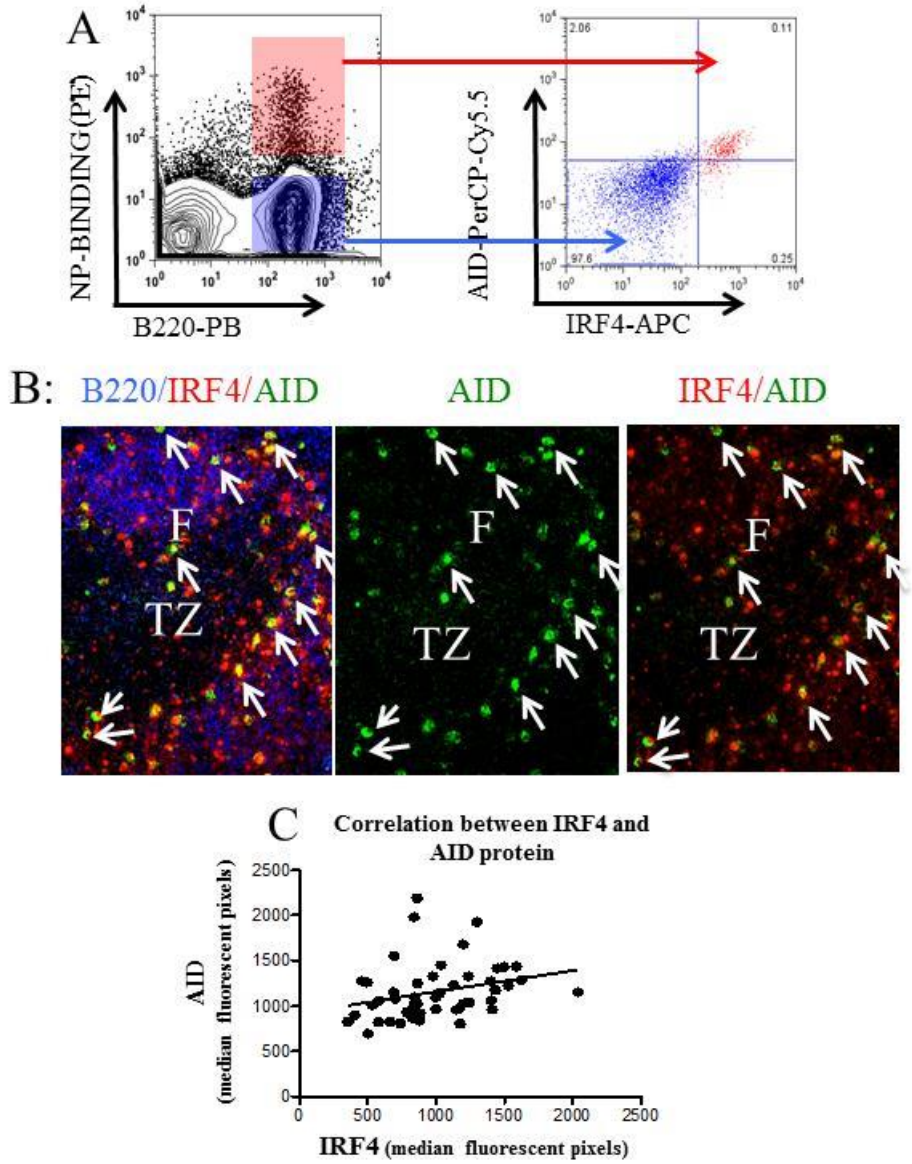


Figure 3.7 : *IRF4 and AID protein are co-expressed in responding B cells*. QM B cells were transferred into WT hosts and immunised with NP-Ficoll.

A: Two days after immunisation, splenocytes were stained with antibodies against B220, AID and IRF4 and incubated with PE labelled NP. Antigen-specific $B220^+$ B cells (red) were tested for co-expression of IRF4 and AID. This was compared to non-antigen specific B cells (blue). Data are representative of four different mice.

B: Immunofluorescence of spleen sections two days post NP-Ficoll immunisation. Spleen sections were stained with antibodies against B220, IRF4 and AID. Activated cells are located in the inner and outer follicle (left, arrows). AID protein is detectable in the nucleus and cytoplasm of activated cells (centre). Most AID^+ cells are also expressing nuclear IRF4 (Right), the positive correlation from IF staining and quantification of median fluorescent pixels with ImageJ is shown in **C**. Each data point represents one cell.

TZ: T zone; F: follicle.

3.3 Summary

IRF4 and AID are expressed within antagonistic gene regulatory networks during terminal B-cell differentiation to germinal centre B cells or plasma cells. Plasma-cell differentiation is regulated by the transcription factors BLIMP1 encoded by the gene *Prdm1* (Turner et al., 1994) and IRF4(Klein et al., 2006). BLIMP1 (Klein and Dalla-Favera, 2008) and IRF4 (Saito et al., 2007) supresses BCL6 which is the master regulator of the germinal centre phenotype (Klein and Dalla-Favera, 2008). BCL6 in turn represses *Prdm1* (Calame, 2008). Whereas IRF4 is required for plasma-cell generation and is not expressed by most germinal centre cells with the exception of some centrocytes, AID is highly expressed in the germinal centre with higher levels expressed in the GC dark zone (Leuenberger et al., 2010). Here it is required for somatic hypermutation and Ig class switching (Muramatsu et al., 2000). Conversely, it is not expressed in plasma cells. We have shown however, that both IRF4 and AID are also expressed at an earlier stage of B-cell activation. IRF4 is expressed within minutes of activation, whereas AID is upregulated within two days of immunisation. This occurs whilst B blasts are located in the follicles.

4: INTERMEDIATE LEVEL IRF4 IS SUFFICIENT FOR NORMAL EXTRAFOLLICULAR IMMUNOGLOBULIN CSR

4.1 Introduction

In order to test the role of the two expression levels of IRF4 protein for extrafollicular Ig CSR, we used mice lacking the transcription factor NFκB1. NFκB1 has been reported to be required for the upregulation of IRF4 (Berberich et al., 1994; Saito et al., 2007). Therefore we aimed to test:

1. Whether expression of intermediate or high level IRF4 is altered in mice lacking NFκB1
2. Whether blocking intermediate or high levels of IRF4 led to reduced extrafollicular Ig CSR.

4.1.1 NFκB family members

The *NFκB* family are pleiotropic transcription factors first described in 1986 (Sen and Baltimore, 1986). These factors have been implicated in inflammation (Karban et al., 2004) and are required for B-cell activation and differentiation (Saito et al., 2007).

The NFκB family consists of 5 members: RelA (p65), RelB, C-REL, NFκB1 (p50/p105) and NFκB2 (p55/p100) (Caamano and Hunter, 2002). NFkB1 (p50) is synthesised as a large precursor of 105 kDa (p105). This precursor is processed to the p50 subunit, whereas NFκB2 (p52) is processed from a 100 kDa precursor (p100), the active subunits p50 and p52 are encoded within the N terminal region of both precursor proteins. Processing of both p100 and p105 to p52 and p50 requires cleavage of the C terminus ankyrin repeats which are then degraded (Fan and Maniatis, 1991). These ankyrin repeats function as IκB-like inhibitors of NFκB. However, a cotranslational mechanism of p50 production has also been described

which does not require proteolytic cleavage from p105 (Lin et al., 1998). Proteins of this family have the ability to induce or repress gene transcription by binding to DNA regions known as κB elements in gene promoters or enhancers. The family is characterised by an N-terminal Rel homology domain (RHD) of 300 amino acids which is important for DNA binding, nuclear localisation and subunit dimerisation (Figure 4.1).

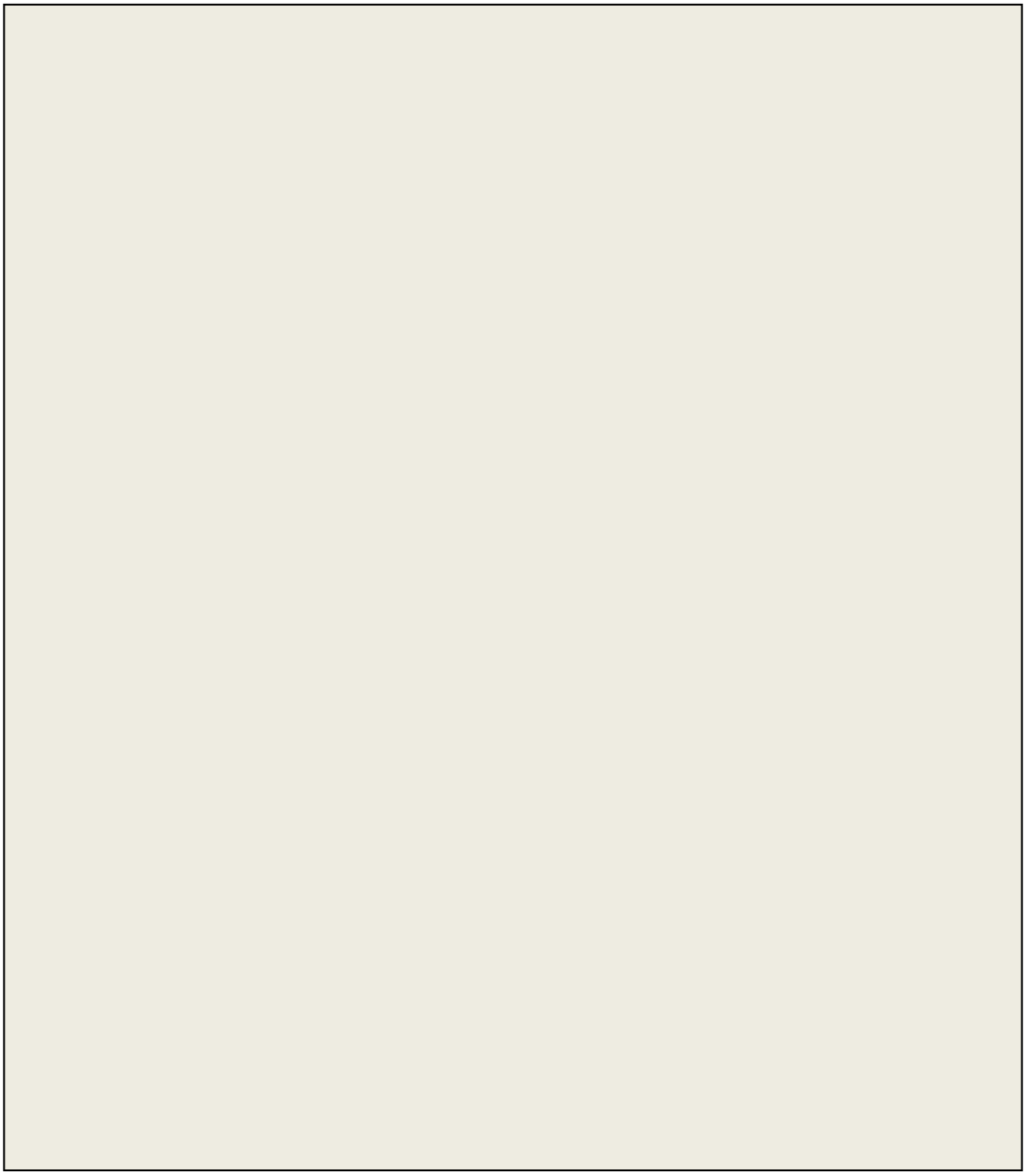


Figure 4 1 The Structure of the NFκB, IκB and IKK families of proteins

A: The NFκB family includes RelA (p65), RelB, C-REL, p105 (NFκB1) and p100 (NFκB2). p50 and p52 are created from the proteolytic cleavage of p105 and p100 respectively. The family are characterised by the presence of a REL-homology domain (RHD). The RHD contains the nuclear localisation domain essential for protein function. C-terminal transactivation domains (TAD) are only present in the REL subfamily of NFκB. TA1 and TA2 are subdomains of the RelA transactivation domain. **B:** The inhibitors of NFκB family (IκB). This family is comprised of IκBα, IκBβ, IκBε and BCL-3. Ankyrin-repeat motifs (ANK) are located at their c-termini; these are also present in p100 and p105. **C**: The IκB Kinase (IKK) complex is comprised of three subunits: NEMO (IKKγ), IKKα and IKKβ. IKKα and IKKβ are catalytic subunits whereas NEMO is a regulatory subunit. CC, coiled coil; DD, region with homology to a death domain; HLH, helix-loop-helix; LZ, RElB-transactivation-domain containing a putative leucine-zipper-like motif; NBD, NEMO-binding domain; PEST, domain rich in proline (P), glutamate (E), serine (S) and threonine (T); ZF, zinc finger domain (Reproduced from Perkins, 2007).

In resting cells, NFκB homo- or heterodimers are maintained in the cytoplasm by the inhibitors of NFkB (IkBs) of which there are three mammalian forms: IkBα, IkBβ, and IkBε (Figure 4.1). These can prevent movement to the nucleus by masking the nuclear localisation sequence contained in the RHD. However the nuclear localisation sequence of p50 remains accessible when bound to IkBα. Therefore IkBα also contains a nuclear export sequence (Hayden, 2004). Uncleaved p105 and p100 can also act in an inhibitory manner, retaining their subunit partners in the cytoplasm, as these proteins have ankyrin-repeat motifs like those in IκB proteins ((Baldassarre et al., 1995).

When cells are stimulated, the IkB kinase complex (IKK) is activated. IKK is comprised of at least three components: IKKα, IKKβ and NFκB essential modifier (NEMO). This complex phosphorylates and targets IkB protein for degradation, releasing NFκB protein and exposing its nuclear localisation sequence. This leads to NFkB translocation to the nucleus where it can bind κB motifs in gene promoters. NFκB dimers also induce the transcription of IκB genes, providing a feedback loop for their own regulation (Caamano and Hunter, 2002). Activation is mainly via four pathways. These are the canonical, atypical and non-canonical pathways which are all IKK dependent and the atypical, IKK independent pathway (Figure 4.2). The canonical pathway is induced by pro-inflammatory cytokines such as TNFα and interleukin 1 (Hayden, 2004). In this pathway the IKK complex is activated by the ubiquitinylation of a regulatory subunit, NEMO. This does not lead to protein degradation, but rather interactions with proteins that contain ubiquitin-binding domains. Various kinases are recruited to the IKK complex such as TGFβ activated kinase 1 (TAK1) which phosphorylates IKKβ in its activation loop (Perkins, 2007). Yet the role of IKKβ in the canonical pathway remains unclear. The phosphorylation of IκBα at Ser32 and Ser36 by the IKK complex, leads to the degradation of IκBα. This occurs within the 26S proteasome in an ubiquitin dependent manner due to polyubiquitination on Lys21 and Lys22 by the E3 $SCF^{\beta\text{-}TrCP}$ and E2UbcH5.

In the atypical, IKK dependent pathway, NEMO activates the canonical pathway in response to genotoxic stimuli. NEMO moves into the nucleus and is sumoylated and phosphorylated by ataxia telangiectasia mutated ATM (Figure 4.2). It then moves back to the cytoplasm and activates IKKβ. IKK independent atypical pathways of NFκB activation can also be induced in response to hypoxia or UV damage. In these pathways IκBα is phosphorylated via casein kinase-II (CK2) or tyrosine kinase.

The non-canonical pathway is activated by stimuli such as BAFF, LPS, and CD40 (Bonizzi and Karin, 2004) which leads to the activation of NFκB inducing kinase (NIK). This activates IKKα and causes the formation of p52 from p100. p52 then associates with RelB for the induction of genes, including chemokines and cytokines.

Members of the NFκB family can form various homodimers or heterodimers after activation. Homodimers of p50 or p52 act as transcriptional repressors unless they are bound to BCL-3 as they contain a DNA binding domain but not a transactivation domain. Homodimers of p50 or p52 also evade regulation by the IκB proteins, as when bound to IκB proteins, the nuclear localisation sequence is not masked (Hayden, 2004). p50 and p52 can however be regulated by the IκB proteins if they form heterodimers with a Rel subunit. RelB in contrast, does not form homodimers but forms heterodimers with p100, p52 and p50. Heterodimers of the latter have a role in reducing inflammation (Hayden, 2004). C-REL may form homodimers and heterodimers with p50 or p65.

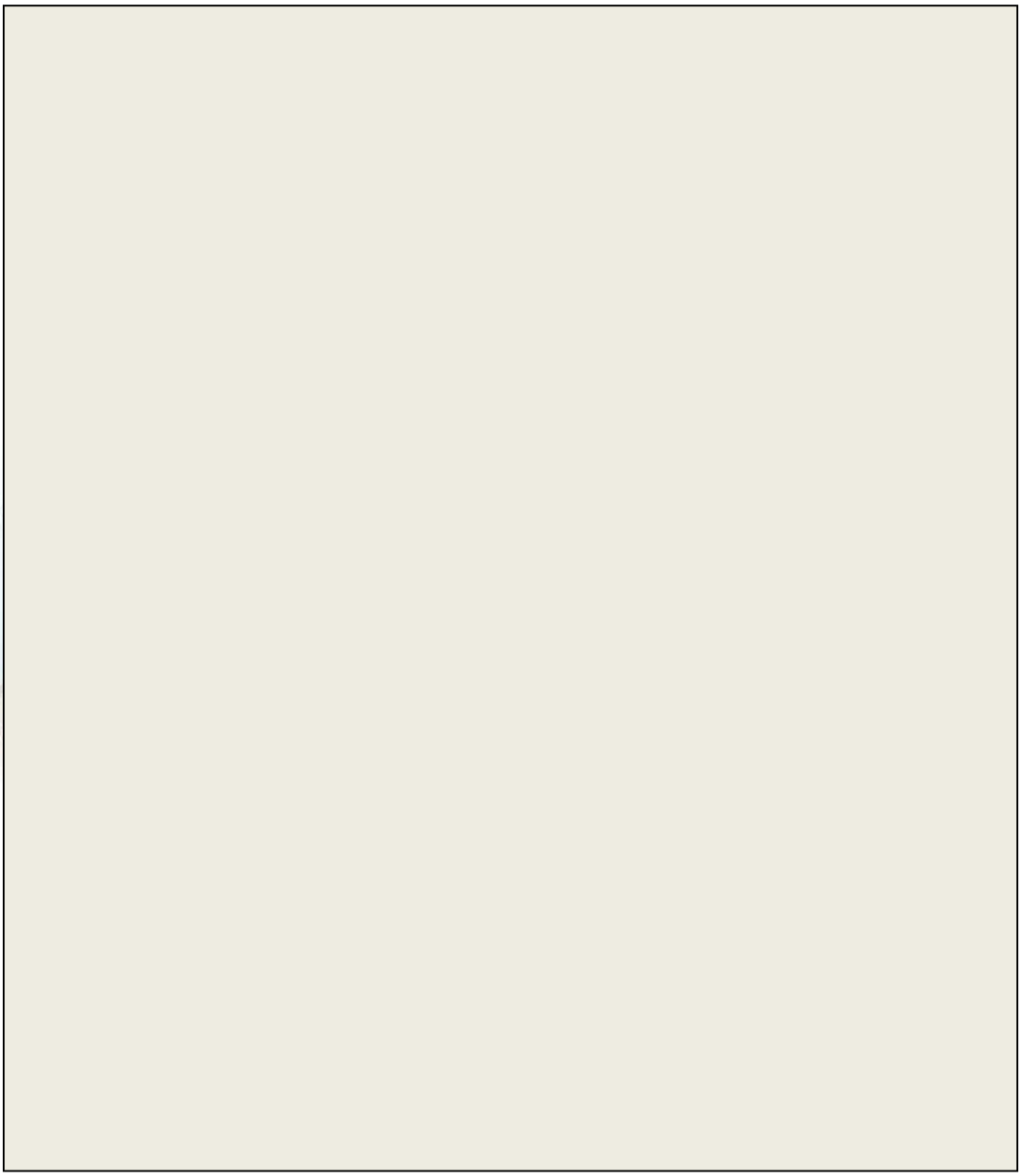


Figure 4 2 The atypical, canonical and non-canonical pathways lead to the activation of NFκB

Upon initiation of the canonical pathway, IKKβ is activated to phosphorylate IκBα at serines 32 and 36. This leads to degradation of the latter and the release of the RelA/p50 complex which moves to the nucleus. In the atypical pathway, genotoxic stress leads to the relocation of NFκB essential modifier (NEMO) to the nucleus. Here it is sumoylated and ubiquitinylated in a process requiring ATM (ataxia telangiectasia mutated) checkpoint kinase. NEMO and ATM move back to the cytoplasm and IKKβ is activated as before. There are also IKK independent atypical pathways which require casein kinase-II (CK2) and tyrosine kinase pathways. In the non-canonical pathway NFκB inducing kinase (NIK) activates IKKα which leads to the phosphorylation of the p100 subunit and cleavage to p52, enabling p52-RelB heterodimers to form (Perkins, 2007).

An added level of regulation is present in this family of transcription factors besides the various hetero- and homodimer combinations available. This is facilitated via the cell type and tissue specific expression patterns of the proteins. NFκB1/p50 and RelA are expressed in all tissues, whereas NFκB2/p52, Rel-B and C-REL are expressed only in lymphoid cells and tissues. During B-cell development the p50/C-REL heterodimer becomes the dominant NFkB complex in mature B cells (Liou et al., 1994).

4.1.2 Regulation of IRF4 by NFκB family members

IRF4 has been reported to be regulated by members of the NFκB family (Berberich et al., 1994; Grumont and Gerondakis, 2000; Saito et al., 2007).

Saito *et al* (2007) studied the regulation of BCL6 by CD40-NFκB-IRF4. When cells were stimulated with CD40, two potential NFκB regions of the *Irf4* promoter could be immunoprecipitated by antibodies to p50 and p65. This indicates that CD40 signals via the canonical NFkB pathway to activate IRF4 as has been previously reported (Berberich et al., 1994). Interestingly, although no C-REL binding site was identified, IRF4 upregulation was blocked in the absence of functioning C-REL (Saito et al., 2007). This is supported by several other studies which found absent IRF4 protein in C-REL^{KO} lymphocytes (Grumont and Gerondakis, 2000) and a role for p50/C-REL in signalling from CD40 (Berberich et al., 1994). In addition, the BCL6 promoter was found to contain IRF4 binding sites. Cotransfection experiments demonstrated that NFkB activation was associated with IRF4 upregulation and BCL6 reduction. This may indicate the pathway by which T-cell derived signals (CD40L) induce germinal centre B cells to differentiate into plasma cells and exit the germinal centre. NFκB can also be activated directly via the BCR, a process which requires BCL-10 expression. BCL-10 in turn regulates the IKK complex. BCL-10^{KO} B cells have normal Ca^{2+} influx following BCR stimulation but fail to upregulate NFκB (Ruland et al., 2001). Activation of NFκB following BCR stimulation with anti-IgM requires PKCβ (Su et

al., 2002), phosphorylation of IKKα (Saijo et al., 2002) and degradation of IKBα (Bajpai et al., 2000; Petro et al., 2000).

4.1.3 Aims

The aim of this project is to test whether NFκB1/p50 has a role for Ig class switching via induction of IRF4 protein expression, and whether changes in IRF4 expression impact on AID expression and CSR. The detailed aims are as follows:

1. Does NFκB1 regulate the early intermediate phase of IRF4 protein expression?
2. Does NFκB1 regulate the late high phase of IRF4 protein expression?
3. Does blocking either of these phases of IRF4 block AID induction and Ig class switching?

To address these questions we used a variety of techniques. AID and IRF4 protein induction were measured by flow cytometry (FC) and immunofluorescence (IF). IRF4 and Ig switch transcripts were detected by real-time RT-PCR following fluorescence activated cell sorting (FACS). B-cell differentiation was measured via FC and immunohistochemistry. In addition, ELISA and ELIspot were used to measure antibody production and frequency of antibody producing cells respectively.

4.2 Results

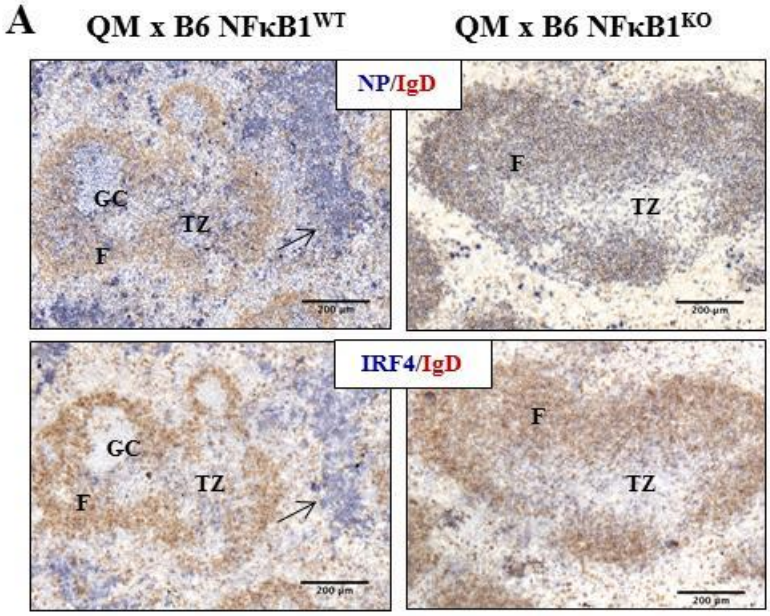
4.2.1 NFκB1 deficient B cells have reduced plasma-cell differentiation and germinal centre formation

In order to test the role of NFκB1 for B-cell differentiation, we immunised QM x B6 NFκB1^{WT} or QM$^{NP/WT}$ κ$^{-/WT}$ NFκB1^{KO} (henceforth referred to as QMxB6 NFκB1^{WT} or QMxB6 NFκB1^{KO}) with the TI-II antigen NP-Ficoll i.p. This induces the synchronous onset of differentiation of large numbers of B cells (5% of all B cells), resulting in large plasma-cell foci and the development of large germinal centres that are sustained without T-cell help until day 6 post immunisation (Vinuesa et al., 2000). This was followed by histological staining of spleen sections. Four days after immunisation, QMxB6 NFκB1^{WT} B cells have formed plasma-cell foci in the red pulp (Figure 4.3A, arrows) and germinal centres within the IgD^{+} follicles. Plasma-cell foci and germinal centres are NP-binding (Figure 4.3A, top left panel). WT germinal centre B cells do not express IRF4, whereas plasma cells in the red pulp express high levels of IRF4. There are also IRF4 intermediate NP-binding blasts in the outer T zone, and IRF4 intermediate T cells in the T zone (Figure 4.3A, bottom left panel). Mice deficient in NFκB1 fail to form germinal centres or plasma cells (Figure 4.3A right panel). Instead a large number of NP-binding B cells are located in the IgD^{+} follicles and express intermediate levels of IRF4 protein (Figure 4.3A bottom panel).

To better quantify altered IRF4 protein levels in the absence of NFκB1, flow cytometry (FC) analysis of splenocytes was performed four days after immunisation. In QMxB6 NFκB1^{WT} spleen, NP-specific B cells differentiate into IRF4^{high} CD138^{+} plasma cells that express lower levels of B220 (Figure 4.3B, top panel). In addition, B cells that express intermediate levels of IRF4 are also found (Fig. 4.3B). Further, many B cells lose IRF4 expression and become

IRF4^{neg} similar to non-activated B cells (Figure 4.3 C). In contrast, in QMxB6 NFκB1^{KO} spleens NP-binding B cells constitute a homogenous, undifferentiated population. This population expresses higher levels of B220 compared to the antigen-activated B cells from QMxB6 NFκB1^{KO} mice; whilst levels of IRF4 protein after activation remain at a level which is slightly higher than intermediate levels observed in WT cells with no differentiation of IRF4^{high} plasma cells or IRF4^{neg} GC B cells (figure 4.3B, lower panel and C). CD138 expression is absent.

The reduced number of plasma cells due to deficiency of NFκB1 leads to profound reduction of antibody titres in blood. Sera were collected 4, 48, 72 and 96 hours after immunisation. Due to their high frequency of B cells specific for NP, QM mice already possess low titres of circulating anti-NP specific antibodies before immunisation. Four hours after immunisation, titres of anti-NP IgM decrease by >95% in QMxB6 NFκB1^{WT} mice and >85% in QMxB6 NFκB1^{KO} mice (Figure 4.4A). This is probably due to immune complex formation following the injection of free antigen. Anti-NP IgM antibody is detectable again in sera from QMxB6 NFκB1^{WT} and QMxB6 NFκB1^{KO} mice two days after immunisation, however B cells lacking NFκB1 produce significantly lower titres compared to QMxB6 NFκB1 wild-type mice (Figure 4.4A). Whereas titres of anti-NP IgM increase by >140x in sera from QMxB6 NFκB1 wild-type mice, titres of anti-NP IgM from QMxB6 NFκB1^{KO} mice increase by only around 14x. Three and four days after immunisation, titres of NP-specific IgM continue to increase in sera from QMxB6 NFκB1^{WT} mice and to a lesser degree in sera from QMxB6 NFκB1^{KO} mice, with QMxB6 NFκB1^{KO} sera containing around 1x times less antibody at both time points.


A
QM x B6 NFκB1WT
QM x B6 NFκB1KO
NP/IgD
IRF4/IgD
GC
TZ
F
200 µm

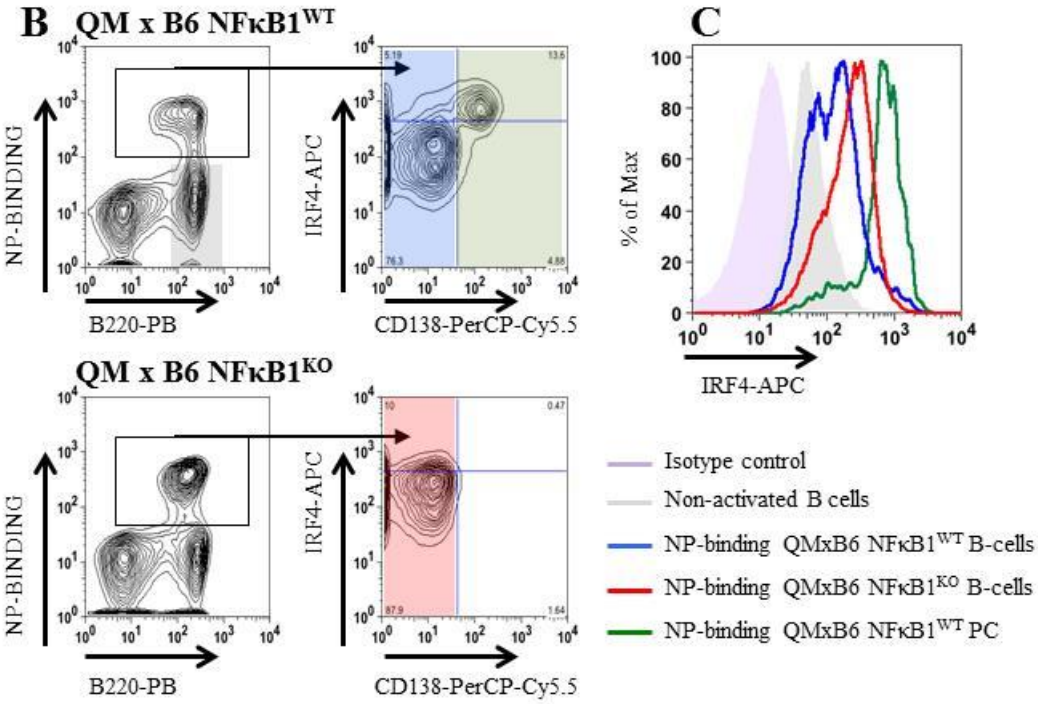

B
QM x B6 NFκB1WT
QM x B6 NFκB1KO
NP-BINDING
B220-PB
IRF4-APC
CD138-PerCP-Cy5.5
C
% of Max
IRF4-APC
Isotype control
Non-activated B cells
NP-binding QMxB6 NFκB1WT B-cells
NP-binding QMxB6 NFκB1KO B-cells
NP-binding QMxB6 NFκB1WT PC

Figure 4.3: *NFκB1 deficient B cells do not form germinal centres or plasma cells*. **A:** Four days after immunisation with NP-Ficoll i.p, spleen tissue from QMxB6 NFκB1^{WT} (left) or QMxB6 NFκB1^{KO} (right) were stained for NP (Blue) and IgD (Brown) (top panel) or IRF4 (Blue) and IgD (Brown) (bottom panel). Plasma cells have developed in QMxB6 NFκB1^{WT} spleens (arrow). GC: germinal centre, TZ: T cell zone, F: follicle
B: Representative FC plots of QMxB6 NFκB1^{WT} (top) or QMxB6 NFκB1^{KO} (bottom) splenocytes four days after NP-Ficoll immunisation. NP-binding, B220^{+} B cells were tested for expression of IRF4 and CD138. Antigen-specific QMxB6 NFκB1^{WT} B cells contained a CD138^{+} IRF4^{+} population (green) and an IRF4 intermediate population (blue). QMxB6 NFκB1^{KO} B cells lack a CD138^{+} population and express intermediate/high levels of IRF4 (red). **C**: Representative histogram of IRF4 protein expression within the cell populations shown in (B). Purple: goat isotype control, Grey: NP^{neg}, B220^{+} B cells, Blue: NP-binding QMxB6 NFκB1^{WT} B cells; Green: NP-binding, CD138^{+} QMxB6 NFκB1^{WT} B cells; Red: NP-binding CD138^{-}, QMxB6 NFκB1^{KO} B cells.

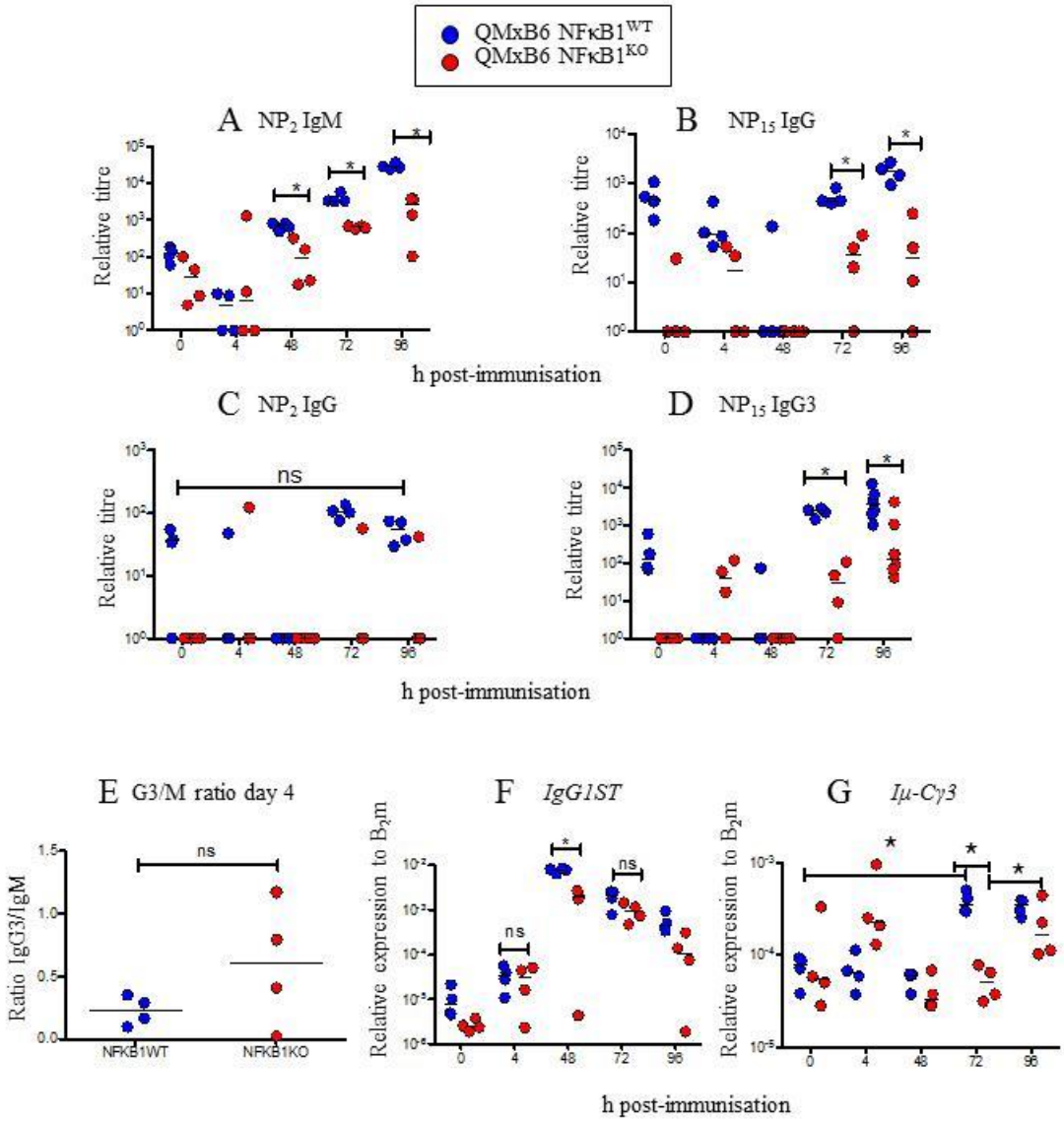


Figure 4.4: *Antibody responses are decreased in NFκB1 deficient mice.* QMxB6 NFκB1^{WT} (blue) or QMxB6 NFκB1^{KO} (red) mice were immunised with NP-Ficoll and sera collected at stated timepoints after immunisation. ELISAs were performed to test binding of antigen specific IgM to NP_2-BSA (**A**), binding of IgG to NP_{15}-BSA (**B**), binding of IgG to NP_2-BSA (**C**) and binding of IgG3 to NP_{15}-BSA (**D**). **E**: The ratio of Ig class switching to IgG3, quantified from ELISA. Antibody titres of class switched IgG3 four days after immunisation were divided by the titre of non-switched IgM. **F**: RNA was extracted from whole spleen sections at stated timepoints post-immunisation and tested for the expression of IgG1 switch transcripts by real time RT-PCR. Transcripts levels are normalised to β_2-microglobulin levels. **G**: RNA was extracted as before and tested for expression of Iμ-Cγ3 transcripts by real-time RT-PCR. Each data point represents one mouse. Data representative of two experiments.

Absence of NfkB1 leads to severe deficiency of IgG. The reduction in total NP-specific IgG (tested by binding to NP_{15}-BSA coated ELISA plates) in QMxB6 $NF\kappa B1^{KO}$ deficient sera was even more than the defect observed in IgM three and four days after immunisation (Figure 4.4B). Higher affinity NP-specific IgG (tested by binding to NP_2-BSA) was not detectable in QMxB6 $NF\kappa B1^{KO}$ deficient sera. However, during the TI-II response germinal centres are not productive due to lack of help from Tfh cells. Therefore affinity matured B cells are not produced. This is reflected by the fact that titres of high affinity IgG do not significantly increase beyond those present in non-immunised mice (Figure 4.4C). The main class of antibody induced in the TI-II response is IgG3 (Vinuesa et al., 2000). This was also significantly reduced in NFκB1 deficient mice by >98% compared to wild-type controls three days after immunisation. Four days after immunisation, sera from QMxB6 $NF\kappa B1^{KO}$ mice contain on average >89% less IgG3 (Figure 4.4D). Despite reduced titres of IgG and IgG3, there is no significant decrease in the ratio of IgG3/IgM in the absence of NfkB1 (Figure 4.4E). This indicates that there is no defect in IgG3 class switching when NFκB1 is absent.

To further test whether there was a defect in Ig class switching in the absence of NFkB1, we measured the expression of Ig switch transcripts (heavy chain germline). While IgG3 is the most abundant isotype induced during the QM B-cell response to NP-Ficoll, IgG3 switch transcripts are not good indicators of induction of IgG class switching, as there is high background activity of the Ig3 exon in non-activated B cells(Rothman et al., 2004). IgG1 is the second most common isotype produced in responses to NP-Ficoll (Vinuesa et al., 2000), therefore, we tested induction of IgG1 switch transcripts. IgG1 switch transcripts are significantly increased within four hours of immunisation ($p=0.029$) in both QMxB6 $NF\kappa B1^{WT}$ and QMxB6 $NF\kappa B1^{KO}$ spleens (Figure 4.4F). Two days after immunisation, when

IgG1 switch transcript production peaks, QMxB6 NFκB1^{KO} spleens contain significantly less IgG1 switch transcripts compared to QMxB6 NFκB1^{WT} spleens, however this difference is small and not significant during the following days. Heavy chain transcripts for Cγ3 containing Iμ were also quantified. The Iμ exon upstream of the IgM switch region is constitutively active, and non-switched cells produce an Iμ-Cμ transcript. After CSR to IgG3 Iμ-Cγ3 hybrid transcripts are expressed, and these indicate successful Ig class switch recombination to IgG3 (Li et al., 1994). Recombined Iμ-Cγ3 transcripts have been previously found to be induced within 48 hours of immunisation in the TI-II response in FACS-sorted activated B blasts compared to FACS-sorted non-activated B cells (Marshall et al., 2011). When testing RNA extracted from spleen sections we found that Iμ-Cγ3 transcripts were not significantly increased above levels found in non-immunised mice until three days after immunisation (Figure 4.4G). This may be because the Iγ3 exon is not as strictly regulated as the other Iγ exons, there are also some background IgG3 secreting plasma cells pre-immunisation. Interestingly, whilst Iμ-Cγ3 transcripts do not further increase between three and four days after immunisation in QMxB6 NFκB1^{WT} mice, transcripts significantly increase in QMxB6 NFκB1^{KO} spleens. This may be due to some ongoing CSR in non-differentiated B blasts in follicles of QMxB6 NFκkB1^{KO} mice, while CSR ceases after plasma-cell differentiation in QMxB6 NFκkB1^{WT} mice.

The absence of splenic germinal centres and plasma cells in mice lacking NFκB1 may be attributed to a stromal deficiency causing an inability to support B-cell differentiation, or due to B-cell intrinsic effects. Follicular dendritic cells (FDCs) are important for germinal centre formation and B-cell selection, due to the ability of FDC to bind and present antigen to B cells. It has been proposed that FDC defects are responsible for the defective GC development in NfkB1 deficient mice (Sukumar et al., 2008; Tew et al., 2001). Indeed the canonical

pathway of NFκB1 activation is known to be required for FDC differentiation − deletion of IKKβ in FDC prevents the upregulation of the adhesion molecules ICAM-1 and VCAM-1 which characterise light zone FDC in secondary follicles (Victoratos et al., 2006). In addition, immune complex trapping by FDC is less efficient in the absence of NFκB1 (Ferguson and Corley, 2005). Therefore to test the status of FDC in mice lacking NFκB1 we performed IF and IHC staining on spleen sections. Anti-FDC-M2 detects the complement component C4 (Taylor et al., 2002) and can be utilised to specifically detect FDC via the immune complex that are deposited on FDC. Two days after immunisation, spleens were analysed by IF for FDC-M2 expression and CD11c, the latter is expressed on dendritic cells. We found a reduction in FDC-M2, and therefore immune complex deposition on FDC, in mice lacking NFκB1 compared to QMxB6 NFκB1^{WT} spleens (Figure 4.5A).

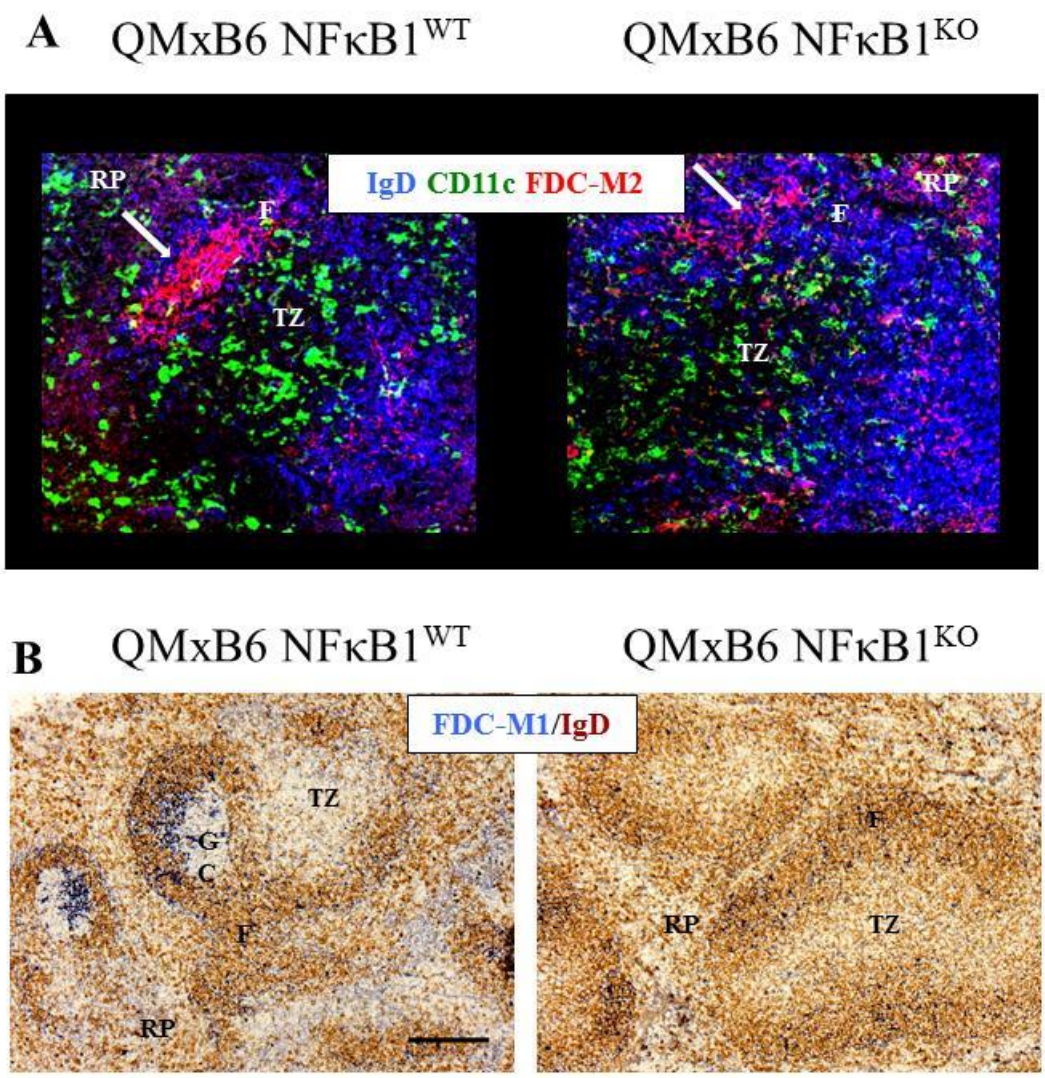


Figure 4.5: *Splenic FDC are abnormal in NFκB1 mice.* QMxB6 NFκB1^{WT} or QMxB6 NFκB1^{KO} mice were immunised with NP-Ficoll i.p. **A:** Two days after immunisation, spleens from QMxB6 NFκB1^{WT} (left) and QMxB6 NFκB1^{KO} mice (right) were stained by IF with antibodies against IgD, CD11c and FDC-M2. Spleens from QMxB6 NFκB1^{WT} demonstrate immune complex trapping on follicular dendritic cells, shown by FDC-M2 staining (red), this complex is located within the B cell follicles (IgD^{+}, blue). In contrast, spleens from QMxB6 NFκB1^{KO} mice demonstrate reduced complement binding.

B: Three days after immunisation, spleens from QMxB6 NFκB1^{WT} (left) and QMxB6 NFκB1^{KO} mice (right) were stained by IHC with antibodies against FDC-M1 and IgD. Only QMxB6 NFκB1^{WT} B cells have formed IgD^{-} GC with extensive FDC networks demonstrated by FDC-M1 staining (blue).

Scale bar indicates 200μm. GC: germinal centre, TZ: T cell zone, F: follicle, RP: red pulp

Anti-FDC-M1 recognises *mfge8* on macrophages and FDC and their processes in the light zone of the germinal centre (Kranich et al., 2008). This network is already apparent at day 3 after NP-Ficoll immunisation in NFκB1 sufficient mice which have started to form IgD^- germinal centres within the follicles. In contrast, there are no FDC dendrites visible in QMxB6 NFκB1 deficient spleens although there is FDC-M1 staining, some of which may be due to the presence of macrophages (Figure 4.5B). As this defect is already published, no further analysis of FDC function was carried out.

4.2.2 NFκB1 deficient B cells upregulate IRF4 in the first hours after activation

As shown in chapter 3, in the response to the TI-II antigen NP-Ficoll IRF4 mRNA production peaks in activated B blasts within 1h of immunisation. This is followed by a gradual decrease in IRF4 mRNA expression over the following hours. Simultaneously, IRF4 protein gradually increases in quantity between one and 24 hours following immunisation. In order to test the effect of NFκB1 deficiency on IRF4 mRNA upregulation, we immunised QMxB6 $NF\kappa B1^{WT}$ and QMxB6 $NF\kappa B1^{KO}$ mice with NP-Fluorescein-AECM-Ficoll. $FITC^+$ $B220^+$ B cells were sorted 1h post-immunisation (Figure 4.6A). Both QMxB6 $NF\kappa B1^{WT}$ and QMxB6 $NF\kappa B1^{KO}$ B cells upregulate IRF4 mRNA at similar levels within 1h post-immunisation. This indicates that initial IRF4 upregulation does not require NFκB1 in either the stromal or lymphocyte compartments. To confirm that intermediate IRF4 protein upregulation is normal, we measured expression of IRF4 by FC six hours later. Splenocytes were stained with fluorescent antibodies against B220 and IRF4 and incubated with fluorescently labelled NP. We found similar upregulation of IRF4 protein in QMxB6 NFκB1 sufficient or deficient splenic antigen-specific B cells (Figure 4.6B and C) and this was significantly higher than that found in non-activated B cells.

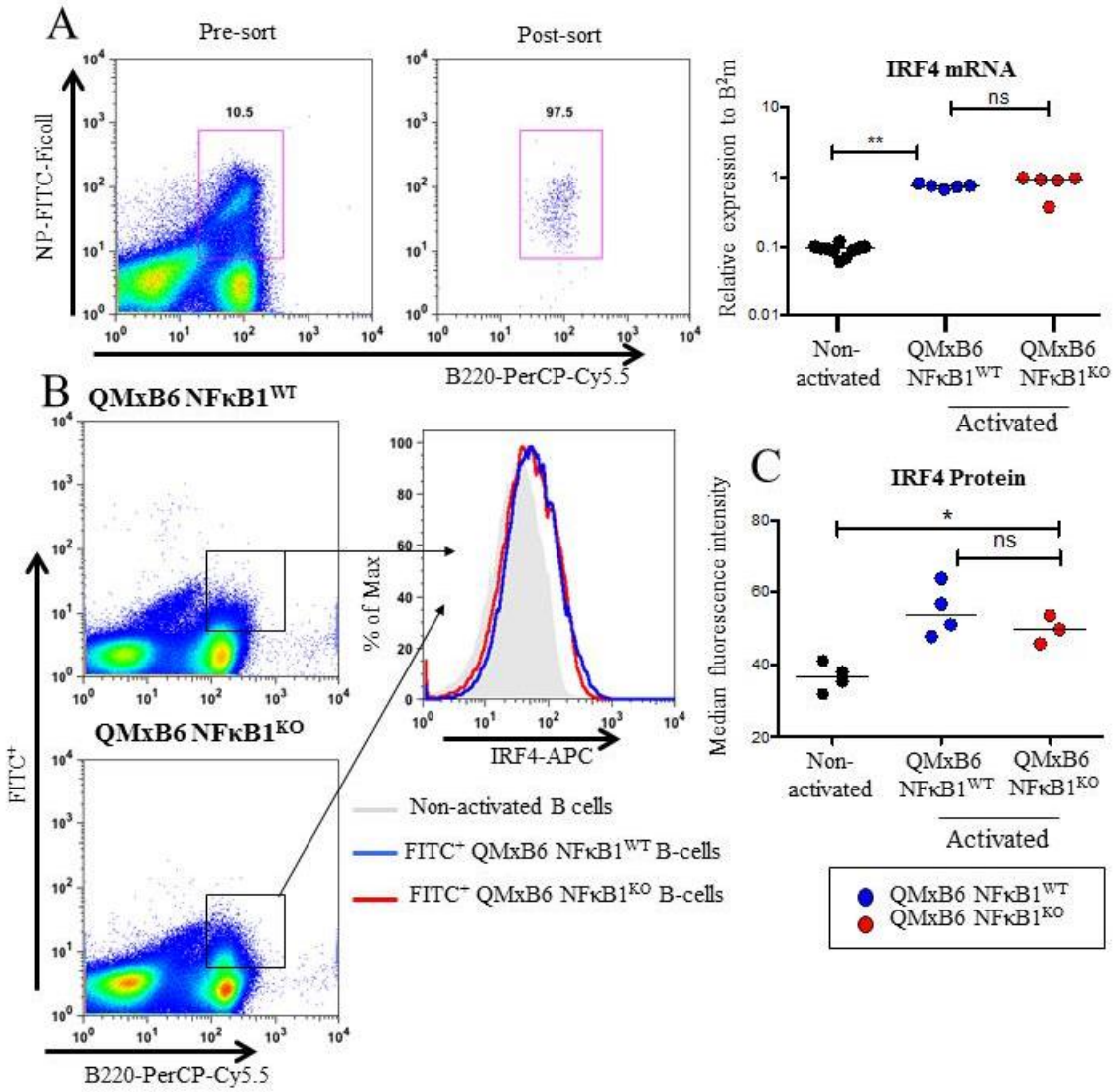


Figure 4.6: *Initial IRF4 upregulation is normal in NFκB1^{KO} B cells*. **A**: QMxB6 NFκB1^{WT} or QMxB6 NFκB1^{KO} mice were immunised with NP-FITC-Ficoll and 1h later FITC^{+} B220^{+} cells were sorted by FACS. Left plot: representative FACS plot of QMxB6 NFκB1^{WT} splenocytes showing the pre-sort gating strategy. Centre: representative plot of sort purity achieved. Right: sorted cells from 1h post-activation and non-activated B cells from QMxB6 NFκB1^{WT} (blue) or QMxB6 NFκB1^{KO} (red) mice were tested for levels of *Irf4* mRNA by real-time RT-PCR. **B**: QMxB6 NFκB1^{WT} (top) or QMxB6 NFκB1^{KO} mice (bottom) were immunised with NP-Ficoll i.p. Six hours later splenocytes were stained with antibodies against B220, IRF4 and incubated with NP-PE then analysed by FC. NP-binding B220^{+} B cells (left) were tested for expression of IRF4 protein (right) **C:** QM^{WT} (blue) and QM^{KO} B cells (red) express similar levels of IRF4 protein 6h post-immunisation as assessed by Median fluorescence intensity from FC. Each data point represents one mouse. Data are representative of two experiments.

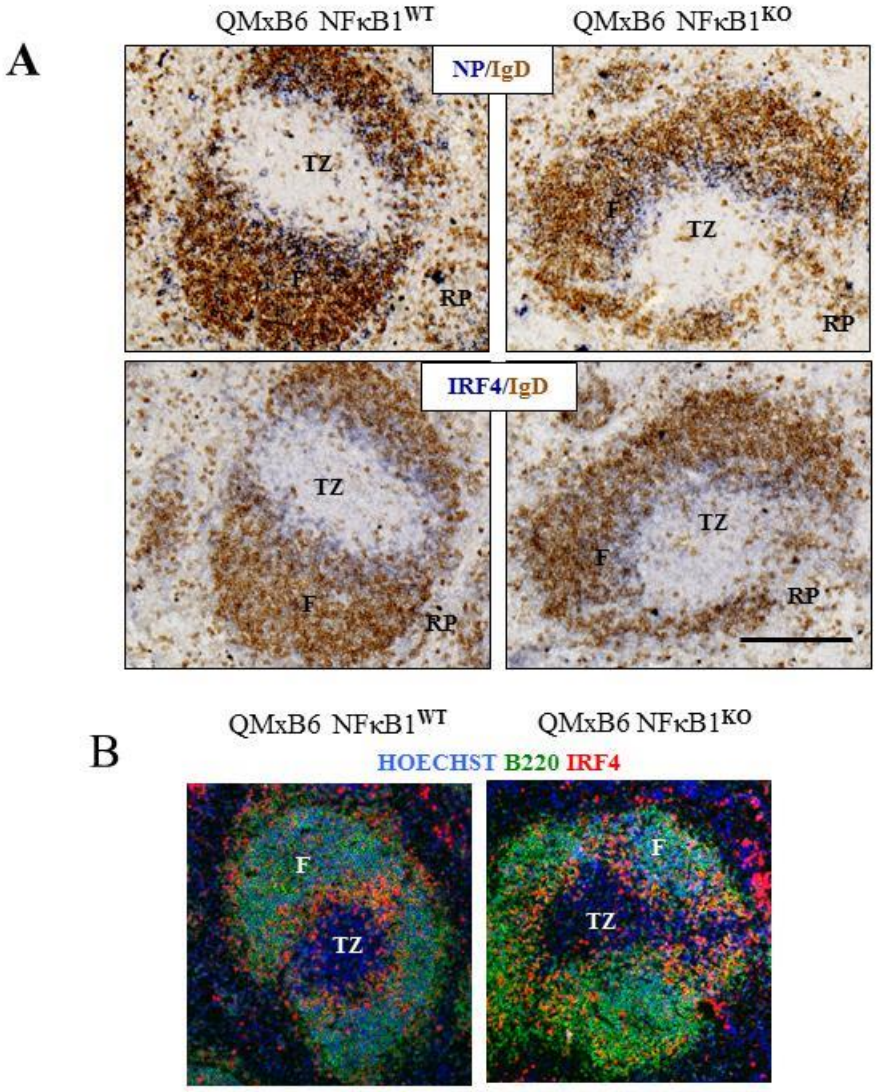


Figure 4.7: *QMxB6 NFκB1^{KO} B cells migrate to the T zone-follicle border normally 6h after immunisation.* QMxB6 NFκB1^{WT} or QMxB6 NFκB1^{KO} mice were immunised with NP-Ficoll and spleens were collected six hours later. **A**: Spleen sections were stained with antibodies against IgD and NP (Top panel) or IRF4 and IgD (bottom panel). Migration and IRF4 upregulation in antigen specific cells occurs in the absence of NFκB1. **B**: Spleen sections were stained with fluorescently labelled antibodies against B220 (green) and IRF4 (red) and counterstained with Hoechst (blue). Six hours post NP-Ficoll both QMxB6 NFκB1^{WT} and QMxB6 NFκB1^{KO} B220^{+}IRF4^{+} cells have migrated to the B-T zone border. Data are representative of four mice per group. Scale bar indicates 200μm. F: follicle, TZ: T zone, RP: red pulp.

To assess whether early migration to the T zone-follicular border was normal, and to confirm IRF4 upregulation in the population of responding B cells, we performed immunohistochemical staining. Six hours after immunisation, NP-specific cells (Figure 4.7A, top panel) are located at the follicular T zone border and express intermediate levels of IRF4 protein (Figure 4.7A, bottom panel). This occurs similarly in QMxB6 NFκB1 sufficient or deficient spleens. As other cell types including macrophages (Satoh et al., 2010) and T cells (Bollig et al., 2012) can also express IRF4, we confirmed with fluorescent staining that these migrating cells are B lymphocytes. $IRF4^{+}$ cells at the follicle-T-zone border were found to express the B-cell marker B220 (Figure 4.7B). This confirmed that early IRF4 upregulation and migration of B cells to the B-T border was normal in the absence of NFkB1.

Induction of other markers of early B-cell activation also occurred normally in the absence of NFκB1: CD69 is upregulated on B cells within 4 hours of stimulation (Arvå and Andersson, 1999). This receptor appears to have a negative regulatory role, at least in T cells, with a lack of CD69 causing an exacerbated phenotype in models of autoimmune disease (la Fuente et al., 2012). As measured by FC, QMxB6 NFκB1 deficient B cells had no defect in the upregulation of this molecule after 6h and actually expressed significantly more CD69 receptor in the responding population of B cells (Figure 4.8A).

In contrast to CD69, CD62L is lost from B cells within 2h of TLR stimulation (Morrison et al., 2010) and is required for lymphocyte entry into lymph nodes across high endothelial venules. Loss of this receptor following activation occurs via receptor shedding and is important for the targeting B cells to the spleen after BCR/TLR signalling (Morrison et al.,

2010; Tang et al., 1998). QMxB6 NFκB1 deficient B cells had no defect in CD62L shedding after activation (Figure 4.8B).

The co-stimulatory molecule CD86 is expressed on B cells and binds to CD28. This molecule has been shown to be upregulated within the first hours of B-cell activation after simulation via the BCR(Lenschow et al., 1994). QMxB6 NFκB1 deficient B cells expressed significantly higher protein levels of CD86 post-activation (Figure 4.8C).

Taken together, these results indicate that there is no defect in the initial induction of IRF4 and transmigration into the T zone in QMxB6 NFκB1 deficient B cells, and if anything there is stronger B-cell activation, as assessed by expression of CD69, CD62L or CD86. However, late B-cell differentiation into germinal centre and plasma cells is defective when mice are deficient in NFκB1. This may be due in part to stromal defects such as absence of functional FDC, or due to a role for NFκB1 expression in B cells for differentiation of GC phenotype, and for the induction of high level expression of IRF4 in plasmablasts.

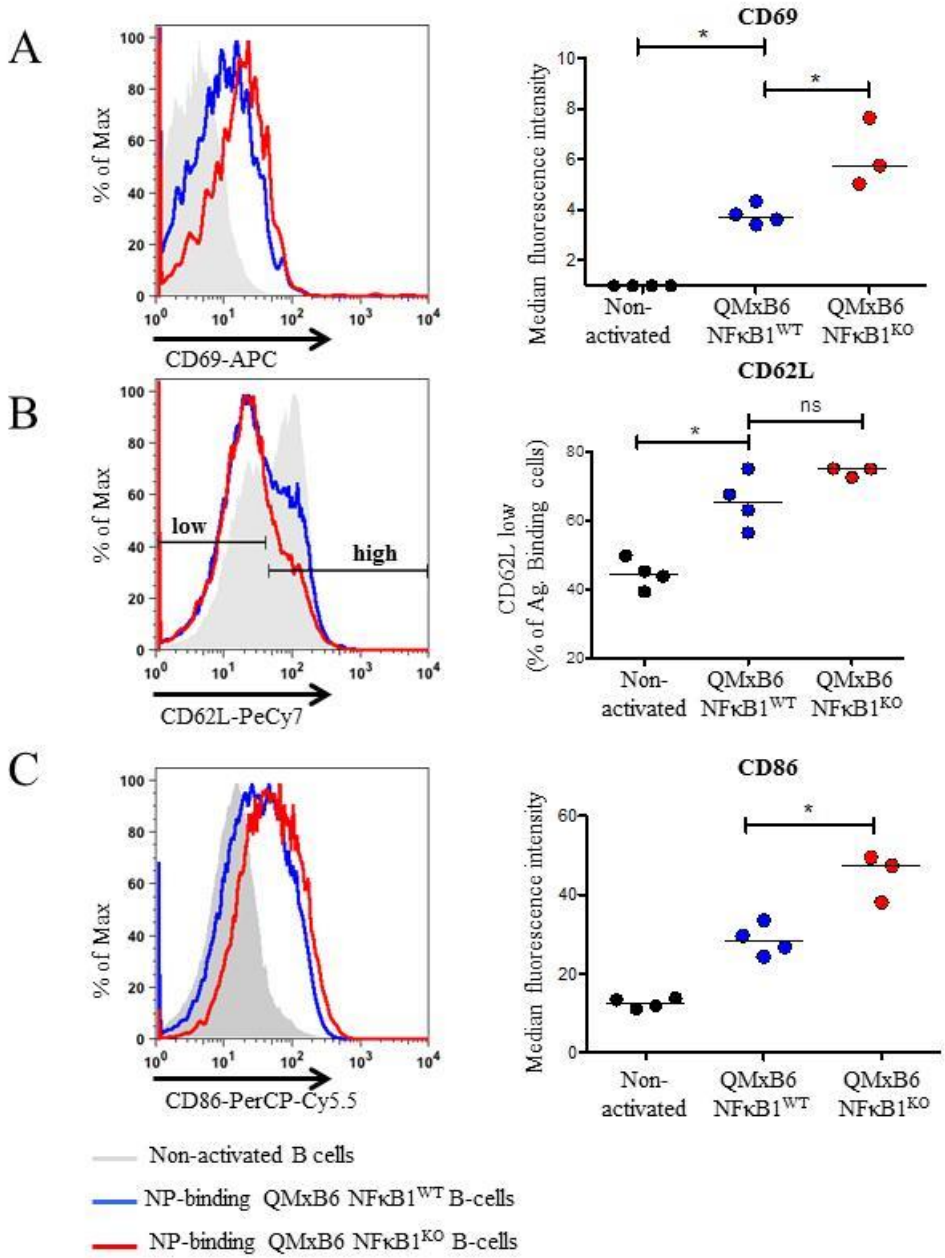


Figure 4.8: *NFκB1 deficient B cells are activated normally in the first hours post-immunisation*. QMxB6 NFκB1^{WT} or QMxB6 NFκB1^{KO} mice mice were immunised i.p with NP-Ficoll as before. Six hours later splenocytes were labelled with fluorescent antibodies against B220, CD69, CD62L and CD86 and incubated with NP-PE, followed by analysis by FC. B cells were gated as in Figure 4.6 for FITC^{+} B220^{+} cells and representative histograms are shown of expression of CD69 (**A**) CD62L (**B**) and CD86 (**C**) for QMxB6 NFκB1^{WT} (blue) or QMxB6 NFκB1^{KO} (red) B cells. Right hand plots show protein levels expressed as the median fluorescence intensity (**A** and **C**) or the percentage of antigen-specific cells which have down-regulated CD62L (**B**). Each data point represents one mouse and the experiment was performed once.

4.2.3 Defects in plasma-cell differentiation are B-cell intrinsic

NFκB1 is involved in signalling in many different cell types. To test whether B-cell intrinsic or B-cell environmental effects are responsible for the defects seen in QM x B6 NFκB1^{KO} mice, antigen specific QM$^{NP/NP}$ κ$^{-/-}$ NFκB1^{WT} or QM$^{NP/NP}$ κ$^{-/-}$ NFκB1^{KO} (hereafter referred to as QM^{WT} and QM^{KO}) B cells were transferred into C57BL/6 or NFκB1^{KO} host mice. This was followed by antigen challenge 24 hours later with NP-Ficoll (Figure 4.9A). Before cell transfer resting B cells were enriched from total splenocytes using negative selection with anti-CD43 magnetic beads. The percentages of NP-binding B cells were measured by FC to ensure similar numbers of antigen-specific cells were transferred (Figure 4.9B).

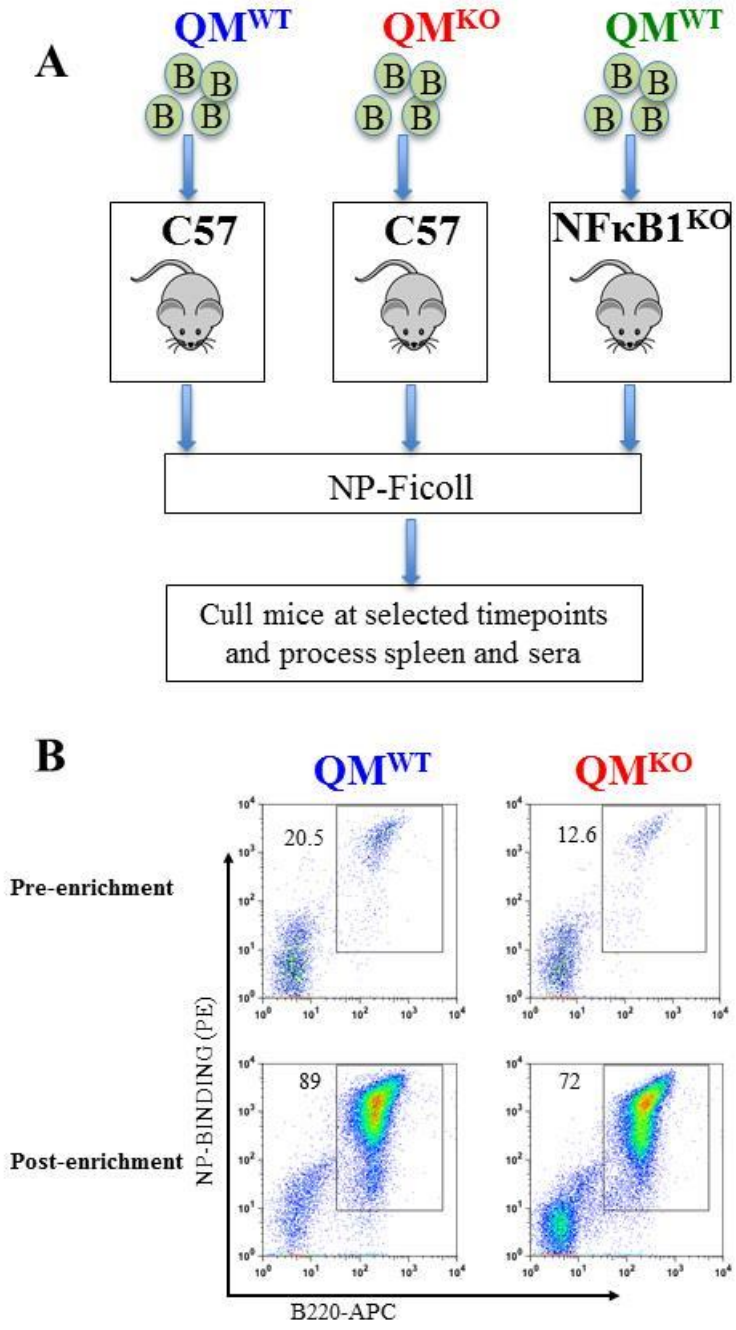


Figure 4.9: *Experimental protocol to study the effect on B cell differentiation when NFκB1 is absent in only B cells or stroma*. **A**: Schematic of experimental design. eYFP expressing QM^{WT} or QM^{KO} B cells were transferred into C57 or $NF\kappa B1^{KO}$ hosts. 24h later mice were immunised i.p with NP-Ficoll. Spleens and sera were harvested at selected timepoints. **B**: Donor B cells before transfer: QM^{WT} or QM^{KO} spleens were harvested and $CD43^-$ B cells were enriched from donor splenocytes using MACS®. An aliquot from the enriched B cells were stained for NP-binding and B220 expression. $5x10^5$ NP-binding B cells were transferred into each host.

Four days following immunisation, QM^{WT} B cells transferred into WT hosts have formed germinal centres and plasma-cell foci (Figure 4.10A left panel). Germinal centres and plasma-cell foci are both NP-binding (blue). In contrast, $NF\kappa B1^{KO}$ B cells transferred into a WT environment have formed smaller germinal centres and lack plasma-cell foci (Figure 4.10a, centre panel). QM^{WT} B cells transferred into an $NF\kappa B1^{KO}$ environment form large plasma-cell foci yet produce small germinal centres (Figure 4.10A right panel). Quantification from tissue sections confirms that lack of NFκB1 in QM B cells results in significantly fewer plasma cells, whereas NFκB1-deficiency in the B cells' environment produces similar numbers of plasma cells to QM^{WT} B cells in a WT environment (Figure 4.10B left). In contrast, germinal centre formation is partly dependent upon NFκB1 expression in both B cells as well as stromal cells (Figure 4.10B, right).

Flow cytometry was performed to quantify the number of exogenous cells and their specificity for NP. Transferred cells can be tracked by flow cytometry by their expression of eYFP. Four days after immunisation antigen-specific QM cells have expanded. In addition, QM^{WT} cells contain two populations: $B220^{int./low}$ $CD138^{+}$ plasma cells and $B220^{high}$ germinal centre B cells (Figure 4.10C, D).

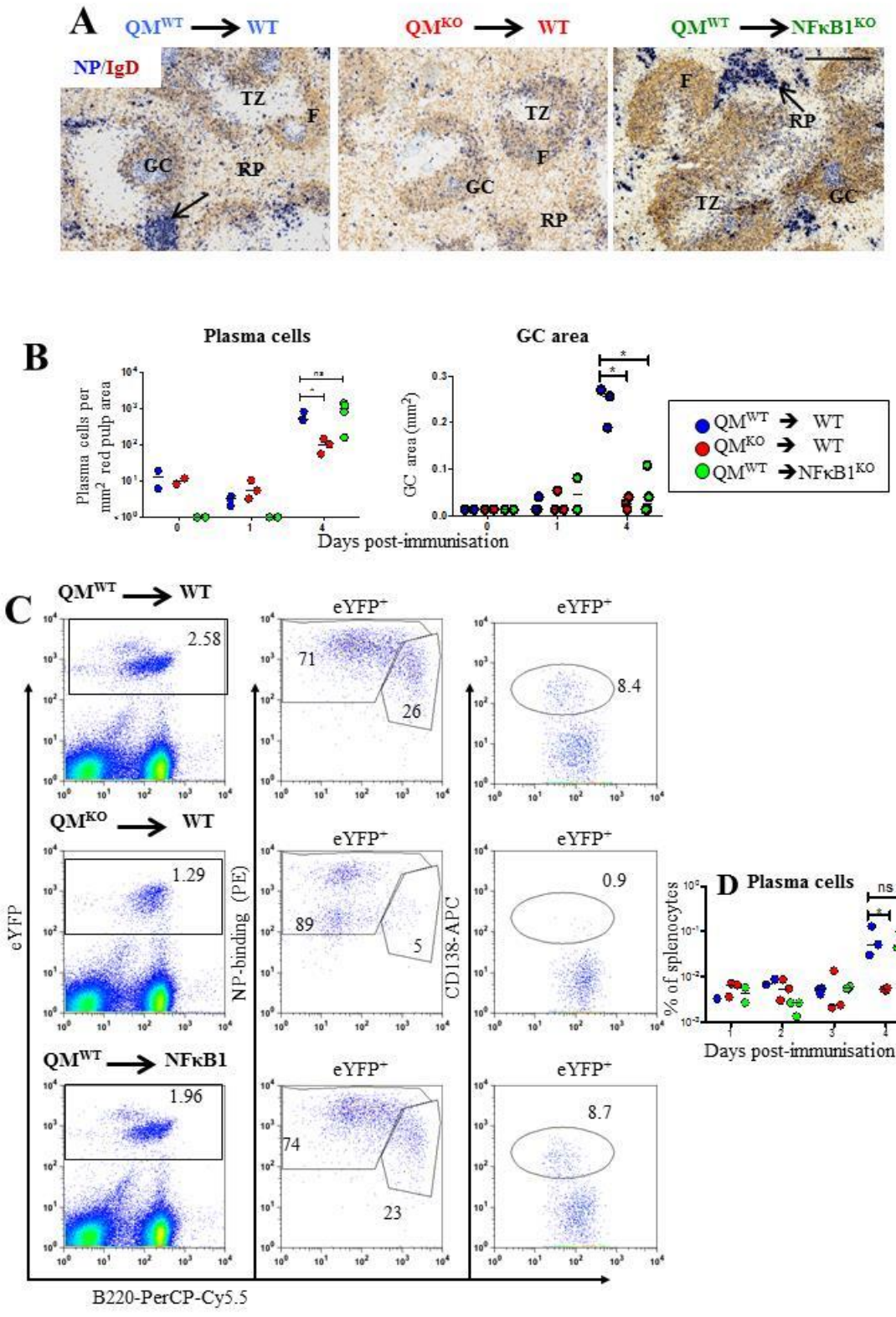

A
QM^WT → WT
QM^KO → WT
QM^WT → NFκB1^KO
NP/IgD
TZ
F
GC
RP
B
Plasma cells
GC area
Plasma cells per mm² red pulp area
GC area (mm²)
Days post-immunisation
QM^WT → WT
QM^KO → WT
QM^WT → NFκB1^KO
C
QM^WT → WT
QM^KO → WT
QM^WT → NFκB1
2.58
1.29
1.96
eYFP⁺
71
26
89
5
74
23
8.4
0.9
8.7
eYFP
NP-binding (PE)
CD138-APC
B220-PerCP-Cy5.5
D
Plasma cells
% of splenocytes
Days post-immunisation
ns

Figure 4.10*: NFκB1 deficient B cells have impaired germinal centre and plasma cell differentiation four days after NP-Ficoll immunisation*. B cells were transferred as described in Figure 4.9. **A**: Four days after NP-Ficoll, spleen sections were stained from WT mice which received QM^{WT} B cells (left), WT mice that had received QM^{KO} B cells (centre) and $NF\kappa B1^{KO}$ mice which had received QM^{WT} B cells (right). Spleen sections were stained for antigen-specific cells (NP: blue), and counterstained with IgD (brown). **B**: Quantification of plasma cell numbers and germinal centre area from histology sections, stained as in (**A**). **C**: Representative FC plots of spleens from mice receiving QM^{WT} (top) and QM^{KO} B cells (centre) and $NF\kappa B1^{KO}$ mice receiving QM^{WT} B cells (bottom). FC was performed on splenocytes labelled with antibodies against B220 and CD138 and incubated with NP-PE. Transferred cells were gated as $eYFP^{+}$ (left plots). The majority of $eYFP^{+}$ cells were B220 low and NP-binding in both QM^{WT} and QM^{KO} populations (centre plots). Plasma cells were quantified as $eYFP^{+}$ $CD138^{+}$ (right plot) **D**: Plasma cells were quantified from FACS as shown in (**C**) for WT mice receiving QM^{WT} (blue) or QM^{KO} (red) B cells and $NF\kappa B1^{KO}$ mice receiving QM^{WT} B cells (green). Scale bar indicates 200μm. GC: germinal centre, TZ: T cell zone, F: follicle, RP: red pulp.

Testing of antigen-specific IgM revealed that titres are similar in all groups until three days after immunisation, yet four days post-immunisation there is no further increase in IgM titres in mice receiving QM^{KO} B cells, while mice receiving QM^{WT} B cells produce 25x more IgM (Figure 4.11A). There in no difference in antibody production between QM^{WT} B cells differentiating in a $NF\kappa B1^{KO}$ environment compared to QM^{WT} B cells transferred to a WT host. QM B cells produce allotype IgM^a and specific detection of IgM^a excludes host-derived antibodies. As expected, anti-NP IgM^a titres were significantly reduced in the sera from mice receiving $NF\kappa B1^{KO}$ B cells (Figure 4.11B). The production of similar levels of IgM between QM^{WT} and QM^{KO} B cells early in the response indicates that the early differentiation of B blasts occurs normally in the absence of NFκB1.

The main antibody class stimulated during a TI-II response is IgG3 (De Vinuesa *et al*, 2000). IgG3 production has been reported to be deficient in $NF\kappa B1^{KO}$ animals (Kenter, 2004; Sha et al., 1995; Snapper et al., 1996). IgG3 production was found to be deficient when NfkB1 was absent from B cells (Figure 4.11C). $NF\kappa B1^{KO}$ mice which received QM^{wt} splenocytes did not have any defect in IgG3 production, showing that class switching to IgG3 is not dependent on NFκB1-expression in stromal cells. Switching to other Ig classes in the response to NP-Ficoll occurs less frequently. Low titres of IgG1 (Figure 4.11D) and IgG2a (Figure 4.11E), and although not as easily detectable showed similar results.

Although deficiency of NfkB1 in B cells leads to significantly reduced production of IgG, this seems not due to a reduction of CSR, but solely due to less efficient plasma-cell differentiation: the ratio of IgG3 to IgM, which indicates of the efficiency of class switching independently of plasma-cell numbers, was not decreased in the sera of mice which received $NF\kappa B1^{KO}$ B cells (Figure 4.11F).

ELISA produces good quantitative data on the amount of antibody secreted by plasma cells. However, it provides no information on numbers of antibody secreting plasma cells or efficiency of antibody production by individual plasma cells. ELIspot quantifies both the number of antibody producing cells and the quantities antibody produced by individual cells. NFκB1^{KO} B cells formed 90% fewer IgM^{a} secreting and 65% fewer IgG secreting cells than both QM^{WT} cells in a WT environment or NFκB1^{KO} environment (Figure 4.11G-H). If anything, the ratio of Ig class switched to non-switched cells was increased when B cells lack NFκB1, again indicating that there is no defect in CSR in the absence of NFκB1 in B cells (Figure 4.11H, right panel).

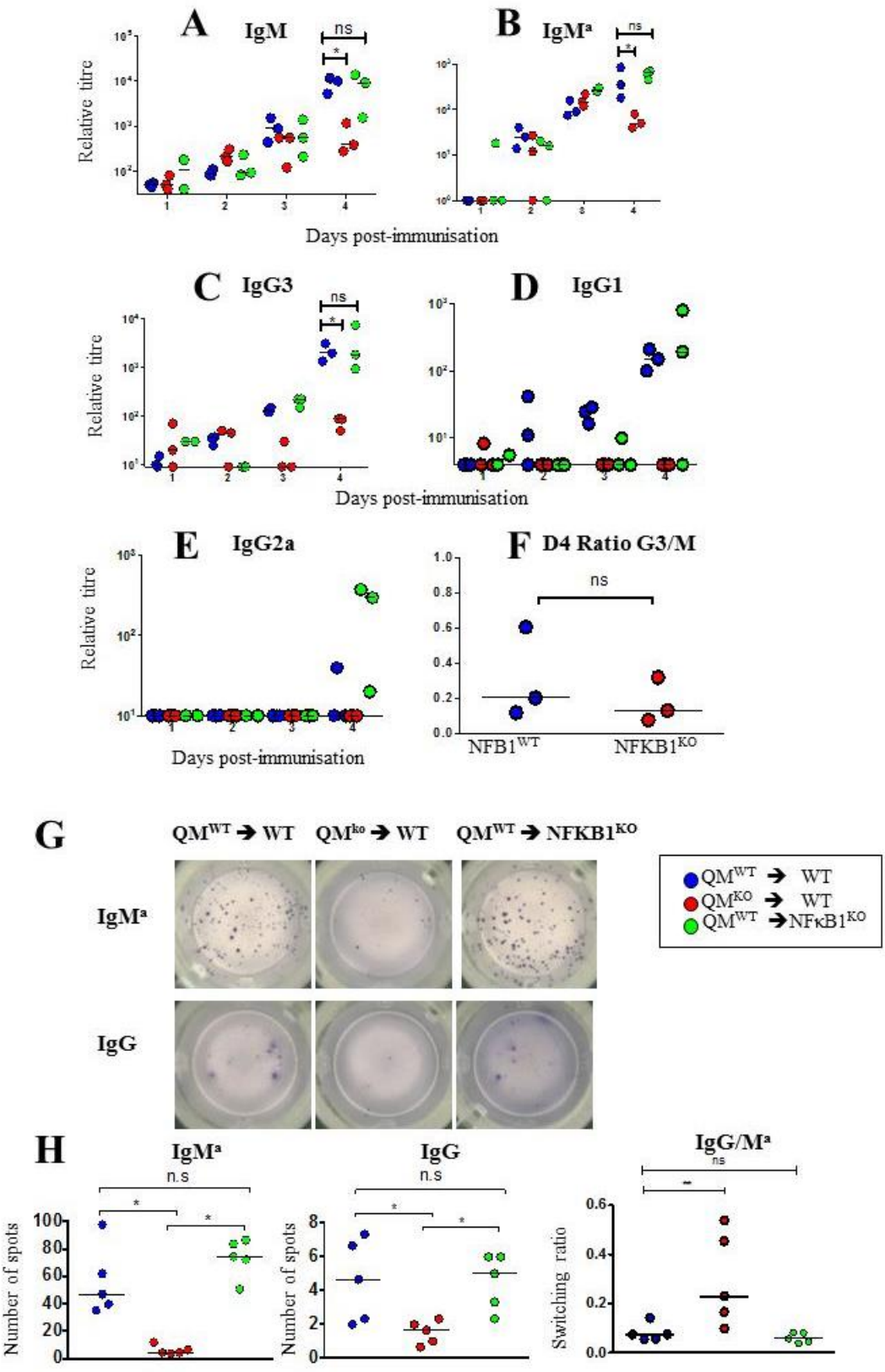

A IgM
B IgMa
C IgG3
D IgG1
E IgG2a
F D4 Ratio G3/M
Relative titre
Days post-immunisation
ns
NFB1WT
NFKB1KO
G
QMWT → WT
QMko → WT
QMWT → NFKB1KO
IgMa
IgG
QMWT → WT
QMKO → WT
QMWT → NFκB1KO
H
IgMa
IgG
IgG/Ma
n.s
Number of spots
Switching ratio

Figure 4.11*: NFκB1 deficient B cells have impaired plasma cell differentiation and antibody responses*. B cell transfers were carried out as shown in Figure 4.9 and mice were immunised with NP-Ficoll. ELISAs were performed for the detection of anti-NP antibodies using sera from stated timepoints: IgM binding to NP_2-BSA (**A**), IgM^a binding to NP_{15}-BSA (**B**), IgG3 binding to NP_{15}-BSA (**C**), IgG1 binding to NP_{15}-BSA (**D**), IgG2a binding to NP_{15}-BSA (**E**). **F**: Ratio of class switching to IgG3 calculated from the relative antibody titres obtained from ELISA four days after immunisation. **G**: Representative ELIspot photograph of IgM^a (top) and IgG (bottom) plasma cells four days after immunisation from spleens from WT mice which received QM^{WT} (left) or QM^{KO} B cells (centre) or $NF\kappa B1^{KO}$ mice which received QM^{WT} B cells (right). **H**: Quantification of plasma cell numbers from ELIspot for IgM^a (left), IgG (centre) and the ratio of Ig class switching to IgG (right). Each data point represents one mouse. Data are representative of two experiments.

4.2.4 Early IRF4 induction is independent of NFκB1, whereas late IRF4 induction is dependent on B-cell intrinsic NFκB1 .

As shown in chapter 3, two days after cell transfer and immunisation, IRF4 protein is present at intermediate levels and at this stage may be essential for induction of *Aicda* expression. To test whether NFκB1 has a role during this phase QM^{WT} and QM^{KO} B cells were tested for IRF4 expression at the mRNA and protein level. Similar to what was seen in complete NFκB1 deficient animals (Fig. 4.7), QM^{WT} and QM^{KO} B cells upregulate IRF4 protein to similar levels two days after cell transfer and immunisation (Figure 4.12A). However, four days after immunisation QM^{KO} B cells have formed a homogenous IRF4 intermediate population (Figure 4.12B) similar to that found in $NF\kappa B1^{KO}$ mice (Figure 4.3C). $B220^{+}$ NP-binding, $CD138^{-}$, $eYFP^{+}$ B cells were sorted by FACS and tested for their expression of IRF4 mRNA. This was expressed at a similar level in all groups tested (Figure 4.12C).

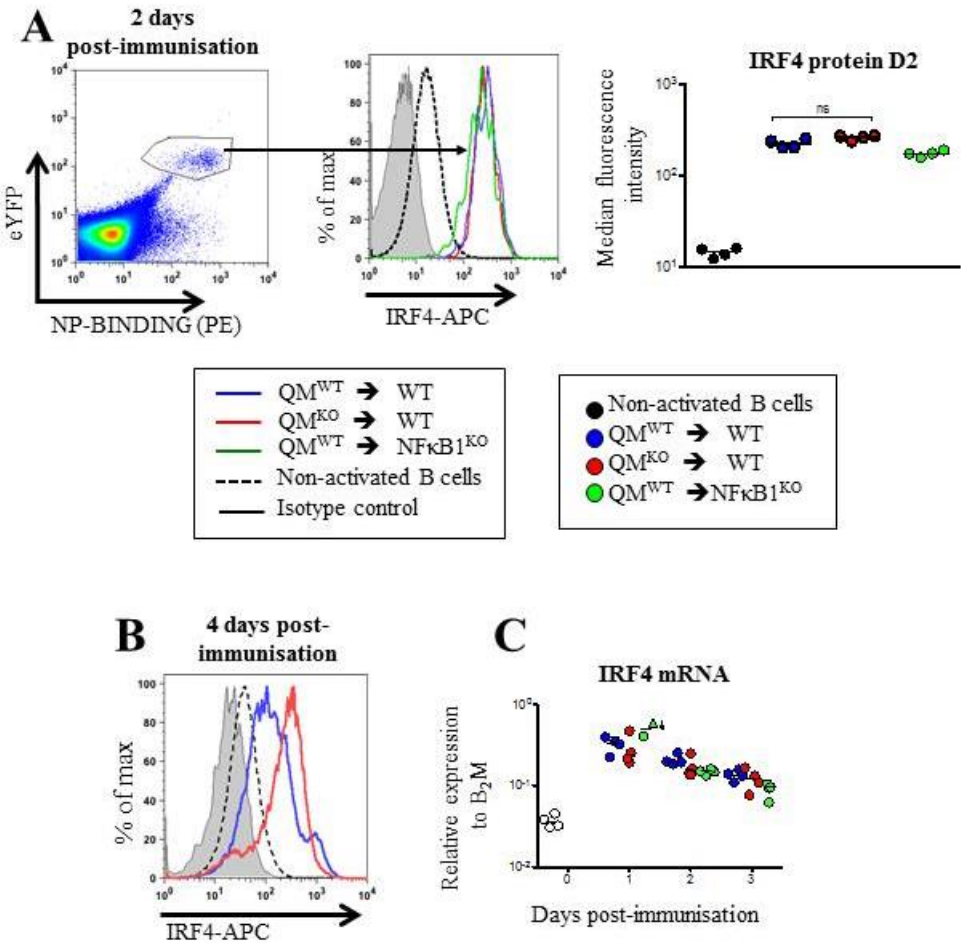


Figure 4.12: *The intermediate phase of IRF4 upregulation is normal after transfer of NFκB1^{KO} B cells into a WT environment, but upregulation of high level IRF4 is abnormal.* B cells were transferred into host mice as in Fig 4.9 followed by immunisation with NP-Ficoll i.p. **A:** Representative FACS plot, left: eYFP$^{+,}$ NP-binding cells from WT mice receiving QM^{WT} or QM^{KO} B cells or NFκB1^{KO} mice receiving QM^{WT} B cells, were tested for expression of IRF4 protein by FC two days after immunisation. Right: levels of IRF4 protein, expressed as the median fluorescence intensity, two days after immunisation in the responding eYFP^{+} NP-binding population. **B**: Representative histogram of IRF4 expression four days after immunisation within the eYFP^{+} B220^{+} NP-binding population of QM^{WT} or QM^{KO} B cells. **C**: NP-binding, CD138^{-}, eYFP^{+} B cells from WT mice receiving QM^{WT} (blue) or QM^{KO} B cells (red) or NFκB1^{KO} mice receiving QM^{WT} B cells (green) were sorted by FACS at stated timepoints after immunisation and RNA isolated. QM^{KO} B cells express similar levels of IRF4 mRNA at all timepoints. Arrow indicates undetectable value, true value may be as stated or lower.

As plasma cells do not undergo Ig class switching (Marshall et al., 2011), it is likely that late high-level IRF4 induction − associated with plasma-cell differentiation – is dispensable for extrafollicular Ig class switching. As intermediate level IRF4 was expressed at normal levels in QM^{KO} B cells and class switching by ELIspot and ELISA appear normal (Fig. 4.11), we tested whether AID was induced at normal levels in NFκB1 deficient B cells two days after activation. AID protein levels were quantified from spleen sections by IF. Spleens were stained with labelled antibodies against AID, IRF4, IgD and the QM BCR (Figure 4.13A). AID protein was quantified from IF sections by gating on individual cells with ImageJ software and measuring the median fluorescence. Two days after immunisation, transferred B cells are located within the IgD^{+} follicle and express IRF4 and AID protein (Figure 4.13A). Normal upregulation of AID (Figure 4.13B) and IRF4 (Figure 4.13C) occur in the absence of NFκB1. This was confirmed by FC staining: $NF\kappa B1^{KO}$ $eYFP^{+}B220^{+}$ expressed similar amounts of AID protein as $NF\kappa B1^{WT}$ B cells (Figure 4.13C and D).

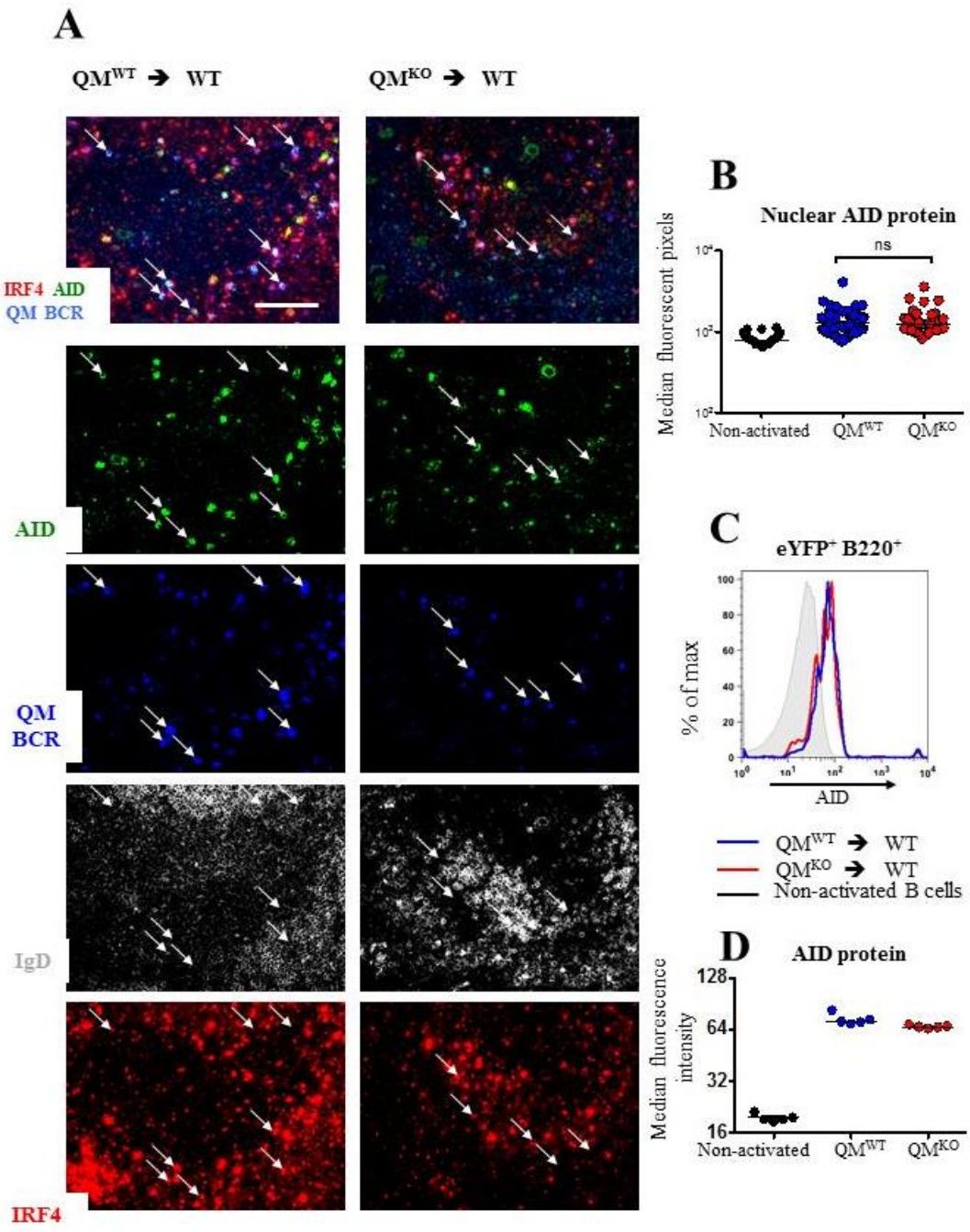

A
QM^WT → WT
QM^KO → WT
IRF4 AID
QM BCR
AID
QM
BCR
IgD
IRF4
B
Nuclear AID protein
ns
Median fluorescent pixels
Non-activated
QM^WT
QM^KO
C
eYFP+ B220+
% of max
AID
QM^WT → WT
QM^KO → WT
Non-activated B cells
D
AID protein
Median fluorescence intensity
128
64
32
16
Non-activated
QM^WT
QM^KO

Figure 4.13*: AID protein induction is normal in B cells lacking NFκB1.* **A**: Spleen sections from mice receiving either QM^{WT} or QM^{KO} B cells were stained with antibodies against the QM BCR (transferred cells), AID, IRF4 and IgD. Scale bar indicates 50μm. **B:** AID induction was measured in individual cells from images using ImageJ software and expressed as the median number of fluorescence pixels. Each data point represents one cell and data are taken from a minimum of two different mice for each group. **C**: Representative histogram of AID protein expression within $eYFP^{+}$ $B220^{+}$ QM^{WT} or QM^{KO} cells transferred into a WT environment 1.5 days after immunisation. **D**: The median fluorescent intensity of AID protein by FC within $eYFP^{+}$ $B220^{+}$ B cells gated as before (**C**). Each data point represents one mouse.

As intermediate level IRF4 and AID protein were induced normally when B cells lack NFκB1, we wished to confirm that early, GC independent Ig class switching also occurred within the spleen, despite low numbers of plasma cells. As discussed previously, Ig switch transcripts begin to be expressed two days after B-cell activation. B cells were transferred into wild-type hosts and immunised with NP-Ficoll before being sorted 1.5 days after immunisation. Figure 4.14A shows that IgG3ST are already upregulated 1.5 days after immunisation and this occurs in both QM^{WT} and QM^{KO} B cells equally. To confirm that this translates to switching in the few plasma cells that develop, we stained tissues sections with antibodies against the QM BCR, IgG and IgM (Figure 4.14B). $NF\kappa B1^{KO}$ B cells switch to IgG with the same frequency as $NF\kappa B1^{WT}$ B cells.

As NFκB1 has a role in apoptosis and proliferation (Sha et al., 1995; Snapper et al., 1996) we tested $NF\kappa B1^{KO}$ B cells for changes in cell division and death. Apoptosis was measured by FC utilising the marker annexin V. This is a phospholipid binding protein with a high affinity for phosphatidylserine, the latter becomes exposed at the cell surface in the early stages of apoptosis. In conjunction with a dead cell marker 7AAD, this can be used to differentiate live cells from both dead cells and those in the early stages of apoptosis (van Engeland et al., 1996). QM^{WT} or QM^{KO} B cells were transferred into wild-type hosts and immunised with NP-Ficoll. Two days after activation, splenocytes were incubated with annexin V, 7AAD and PE-labelled NP. B cells were gated as $eYFP^{+}$ and NP-binding and tested for the percentage of annexin V^{+} $7AAD^{-}$ cells. Two days after activation $NF\kappa B1^{KO}$ B cells are undergoing apoptosis at higher frequency (Figure 4.15A). However by three days after immunisation frequencies of apoptotic cells are similar (Figure 4.15B).

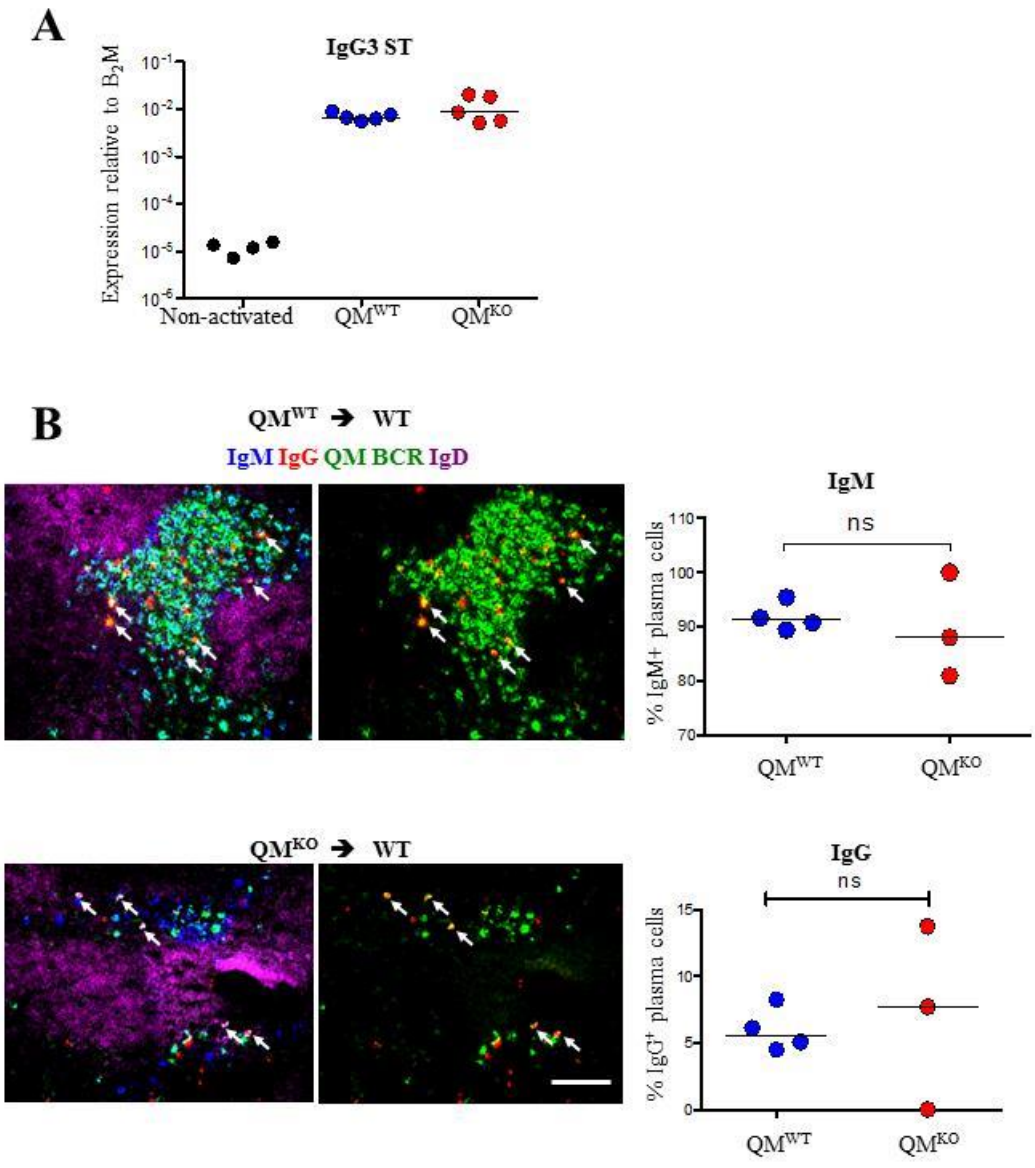


Figure 4.14: *Normal early intermediate level IRF4 is sufficient for normal Ig class switching.* B cells were transferred into host mice as in Fig 4.9 followed by immunisation with NP-Ficoll i.p. **A**: QM B cells were sorted at 1.5 hours after NP-Ficoll immunisation following cell transfer of QM^{WT} or QM^{KO} B cells as previously described. RNA was isolated from sorted cells for real-time RT-PCR analysis of IgG3ST. **B**: Four days after immunisation spleen sections from mice which received QM^{WT} (top) or QM^{KO} B cells (bottom) were stained with antibodies against IgG, IgM, IgD and the QM BCR. Scale bar represents 50μm. The percentage of QM cells which had switched to IgG were quantified from these sections (right plots). Each data point represents one mouse.

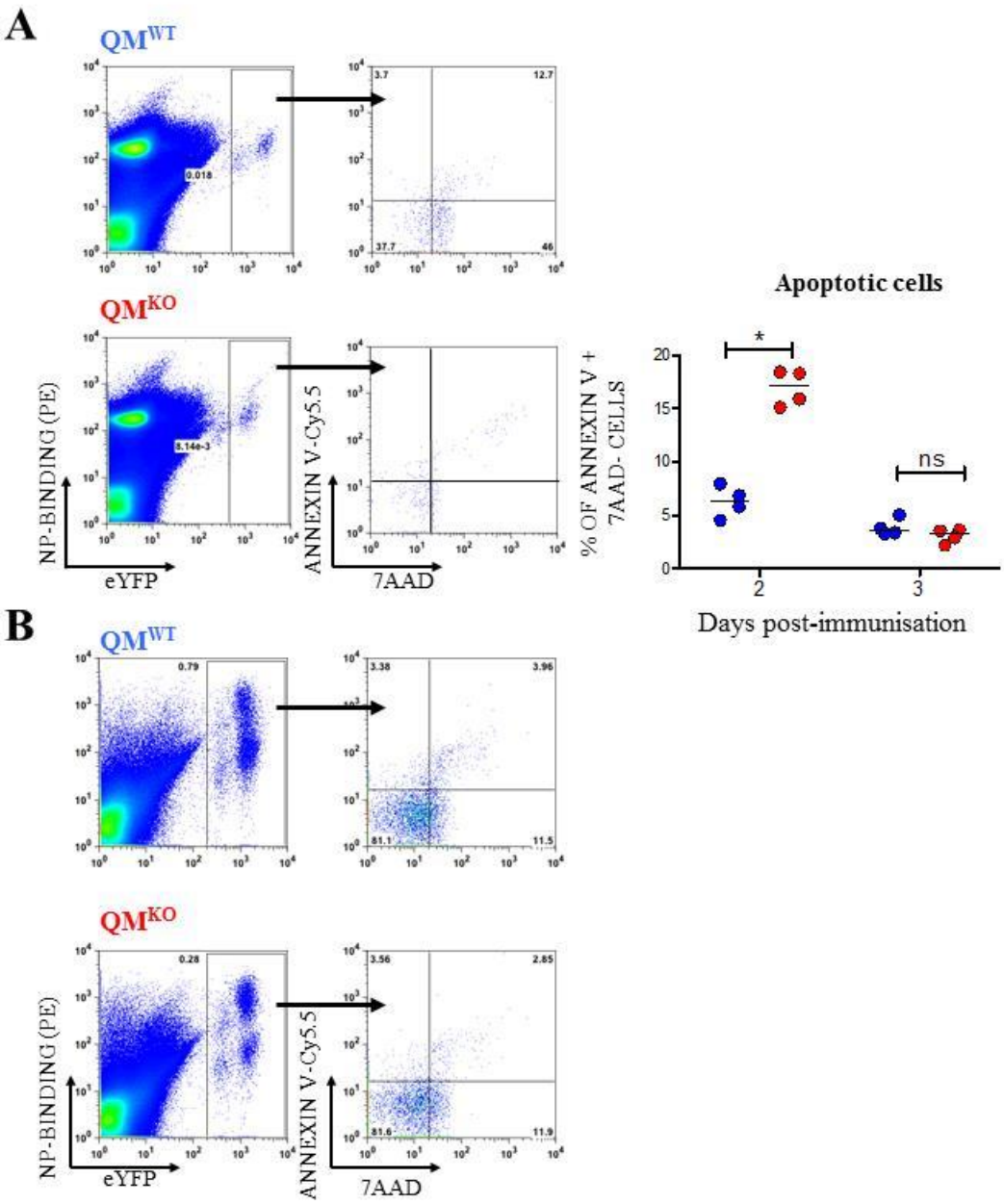


Figure 4.15: *More frequent apoptosis of NFκB1^{KO} B cells two days post-immunisation.* QM^{WT} or QM^{KO} B cells were enriched from total splenocytes before transfer into WT hosts and immunisation with NP-Ficoll. **A and B:** Apoptosis of transferred B cells, recovered from the spleen, was measured by FC two (**A**) and three days (**B**) after immunisation. Representative flow cytometry plot of eYFP^{+} NP-binding cells (left) from mice receiving QM^{WT} (top) or QM^{KO} B cells (bottom). These were tested for 7AAD and Annexin V binding (centre plot). (**C**) The percentage of eYFP^{+} cells binding annexin V^{+}, but not 7AAD were quantified. Each data point represents one mouse.

In order to test induction of cell division, enriched B cells from the spleens of QM^{WT} or QM^{KO} mice were labelled with the division tracker dry CellTrace^{TM}. Before transfer into WT hosts and immunisation with NP-Ficoll, QM^{WT} or QM^{KO} B cells express similar levels of CellTrace^{TM} dye (Figure 4.16A). $B220^{+}eYFP^{+}$ cells were tested for cell division. Two days post-immunisation NFκB1 deficient B cells had undergone the same number of divisions whether they expressed NFκB1 or not (Figure 4.16B). However after three days post-immunisation when B cells are beginning to differentiate into plasma cells and germinal centre cells, cell division slows down in $NF\kappa B1^{KO}$ B cells (Figure 4.16C). Four days after immunisation the cell tracker dye has diluted too much to analyse cell division, however significantly more QM^{KO} B cells are still labelled stronger with the dye (Figure 4.16D). To test whether $NF\kappa B1^{KO}$ B cells have reduced proliferation four days after immunisation, Ki-67 staining was performed on tissue sections. Ki-67 is a marker that is expressed in dividing cells only. Four days after immunisation there is no defect in the proliferation of $NF\kappa B1^{KO}$ B cells located in the splenic red pulp (Figure 4.16E). This indicates that extrafollicular plasmablasts are capable of cell division. Ki67 labels cells in active phases of cell cycle such as: G_1, S, G_2 and mitosis but is not expressed by resting G_0 cells (Scholzen and Gerdes, 2000). Therefore it is still possible that $NF\kappa B1^{KO}$ B cells may be arrested in one of these phases of cell division despite expressing this division marker.

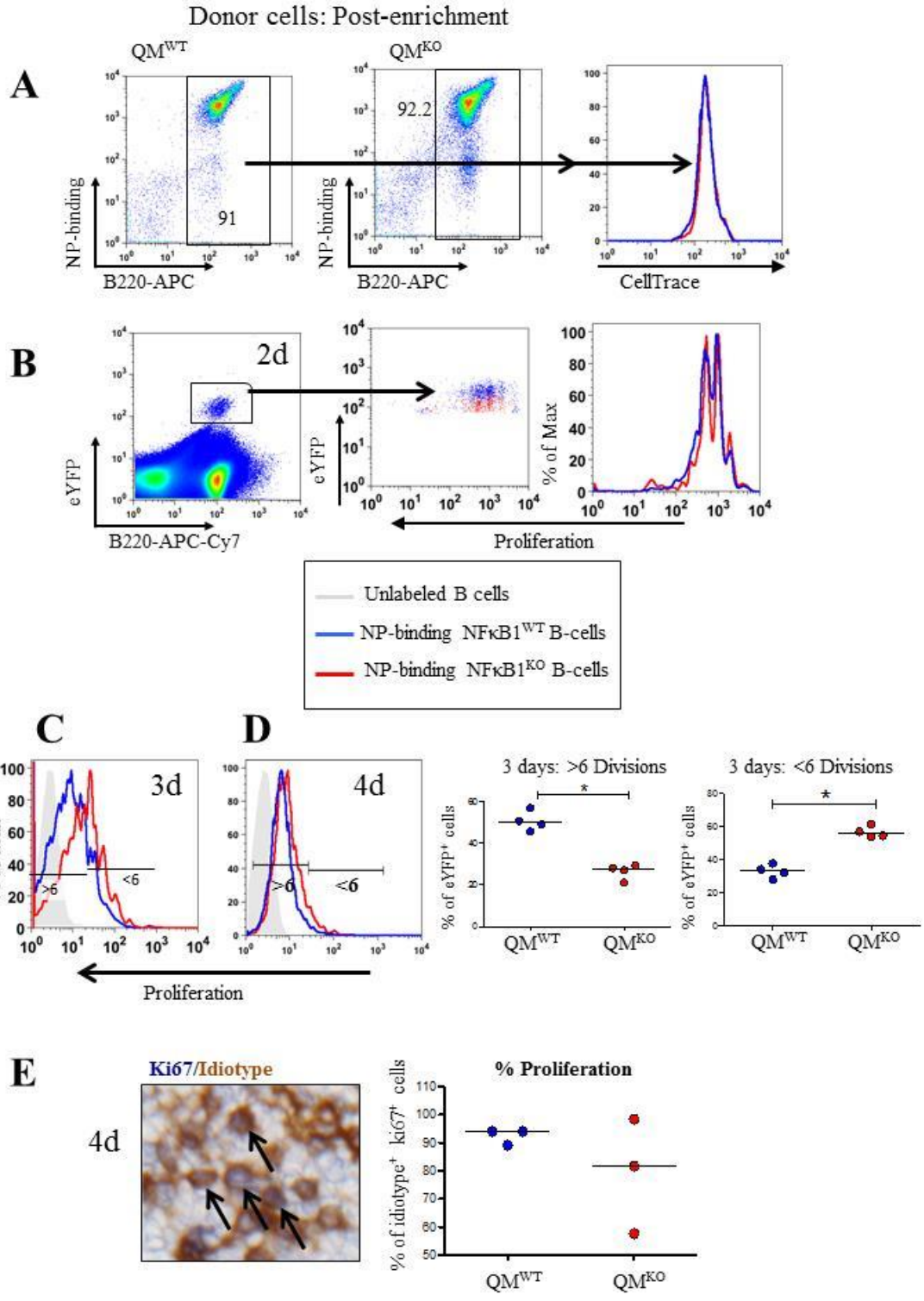

Donor cells: Post-enrichment
A
QM^WT
QM^KO
92.2
91
NP-binding
B220-APC
CellTrace
B
2d
eYFP
B220-APC-Cy7
% of Max
Proliferation
Unlabeled B cells
NP-binding NFκB1^WT B-cells
NP-binding NFκB1^KO B-cells
C
D
3d
4d
>6
<6
3 days: >6 Divisions
3 days: <6 Divisions
% of eYFP+ cells
E
Ki67/Idiotype
4d
% Proliferation
% of idiotype+ ki67+ cells

Figure 4.16*: NFκB1*^{KO} *B cells have an intrinsic defect in proliferation from three days post-immunisation.* **A**: QM^{WT} (left) or QM^{KO} B cells (centre) were enriched from total splenocytes and labelled with the cell division tracker CellTrace^{TM} before transfer into WT hosts. The percentage of NP-binding B220^{+} B cells were calculated by staining a small number of donor cells. 1x10^{6} NP-binding B220^{+} cells were transferred into each host mouse followed by immunisation with NP-Ficoll. **B-D**: Transferred B cells were analysed by FC two (**B**), three (**C**) and four days (**D**) after immunisation. Proliferation was assessed within the eYFP^{+} donor derived population and measured by dilution of CellTrace^{TM} dye. **D**: Right hand plots: the percentage of eYFP^{+} QM^{WT} (blue) or QM^{KO} (red) B cells which had undergone more than six divisions (Centre) or less than six divisions (right hand plot) were quantified using the gates shown in (**C)**. **E**: Spleens from mice receiving NFκB1^{KO} B cells were stained for transferred B cells (idiotype: brown) and expression of Ki67 (blue). The percentage of transferred cells undergoing proliferation within the red pulp was quantified from tissue sections. Each data point represents one mouse.

4.3 Summary

B cells lacking the signalling protein NFκB1 exhibit impaired survival and proliferation. In addition, the proportion of B cells which differentiate into germinal centre B cells and plasma cells are reduced. Defects in germinal centre formation were found to be due partly to stromal defects and partly due to B-cell intrinsic defects. Stromal defects were due to the failure of FDC to differentiate and trap immune complex. Plasma-cell defects in contrast, were due to a failure to fully upregulate IRF4 protein levels, although IRF4 mRNA levels were not altered when B cells lacked NFκB1.

In contrast, early migration and activation of NFκB1^{KO} B cells was normal and this leads to normal IRF4 intermediate level induction. We have shown that even in the absence of normal plasma-cell differentiation, expression of intermediate level IRF4 is sufficient for normal AID protein induction and this leads to a normal frequency of CSR.

5: C-REL^{KO} MICE LACK HIGH BUT NOT INTERMEDIATE LEVEL IRF4 AND SHOW NORMAL CSR

5.1 Introduction

The results described in the previous chapter used mice lacking NFκB1 to indicate that intermediate level IRF4 protein was sufficient for extrafollicular CSR. To confirm these results, we utilised a different mouse model whereby the signalling molecule C-REL is absent.

C-REL is also a member of the NFκB family, the structure and signalling of which are described in detail in chapter 4. In unstimulated, or mitogenically stimulated B cells, the main NFκB complexes are formed of p50 and C-REL heterodimers (Grumont and Gerondakis, 1994; Liou et al., 1994). C-REL^{KO} splenocytes fail to upregulate IRF4 two hours after stimulation with LPS or anti-IgM *in vitro* and induction of IRF4 by REL proteins was found to require two NFκB elements within the IRF4 promoter at −1733 and −686 (Grumont and Gerondakis, 2000). Additionally C-REL may act in synchrony with IRF4 to induce IRF4 target genes: in T cells, C-REL protein has been shown to form a complex with IRF4 in order to induce the expression of IL-4 and IL-2 (Shindo et al., 2011). This interaction occurs between the N-terminus of C-REL and the C-terminal IRF association domain of IRF4. Therefore, as for NFκB1, we predicted that high level IRF4 induction and plasma-cell differentiation would be deficient in mice lacking C-REL protein.

Within B cells, C-REL is also expressed in the nucleus of BCL6^{+} IRF4^{-} centrocytes in the light zone of the human tonsil, but also in some IRF4^{+} PRDM1^{+} cells (Cattoretti et al., 2006). Therefore C-REL may have a role within the GC, inducing genes that determine cell fate decisions. In addition, C-REL protein is required for signalling via BAFF-R (Rickert et al.,

2011), which is important for PC differentiation and survival (Mohr et al., 2009; Moisini and Davidson, 2009).

Mice lacking C-REL protein maintain normal weight and have normal percentages of lymphoid cells under pathogen free conditions. In contrast, C-REL deficient mice exhibit reduced survival in a non-pathogen-free environment and show defective clearance of *L.monocytogenes* (Carrasco et al., 1998), yet LMCV clearance, which requires cytotoxic T lymphocytes, (CTL) is normal. Decreased immunity to *L.monocytogenes* may be due to reduced nitric oxide (NO) production by C-REL^{KO} macrophages (Carrasco et al., 1998). B cell responses and germinal centre formation are impaired and interestingly, naïve C-REL^{KO} mice have increased titres of IgG2a, IgM and IgA, whilst titres of IgG1, IgG2b and IgG3 are reduced (Carrasco et al., 1998).

Similar to the experiments described in the last chapter for NFkB1, in this series of experiments I tested whether C-REL deficient mice (Hsia et al., 2002) express normal levels of intermediate and/or high level IRF4 protein following immunisation. This was then correlated to extrafollicular AID protein induction and CSR.

5.2 Results

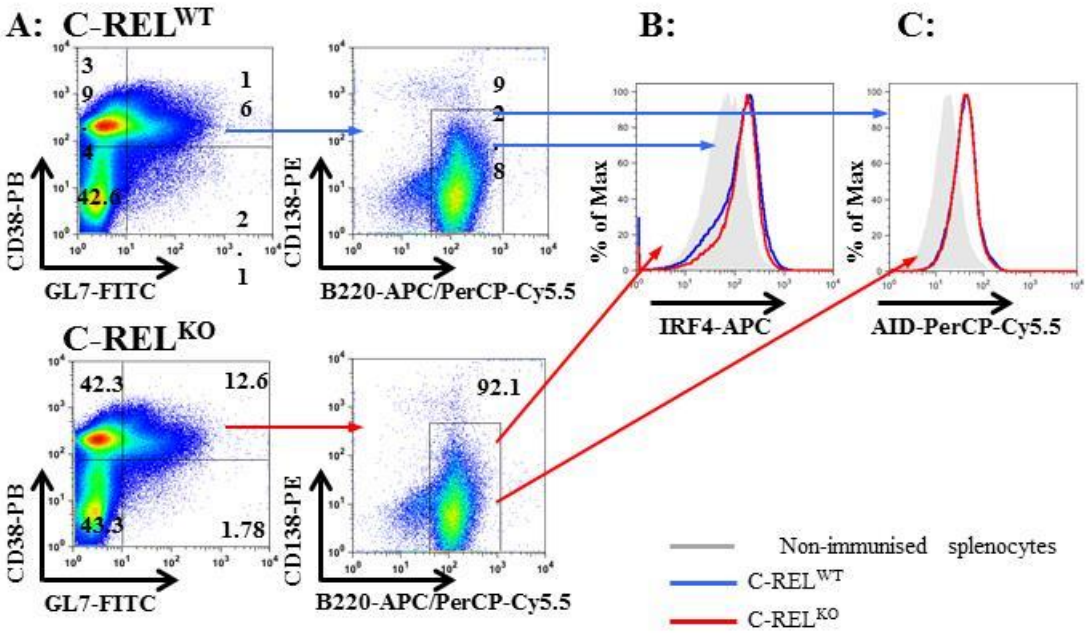
5.2.1 C-REL protein deficiency leads to impaired TD responses

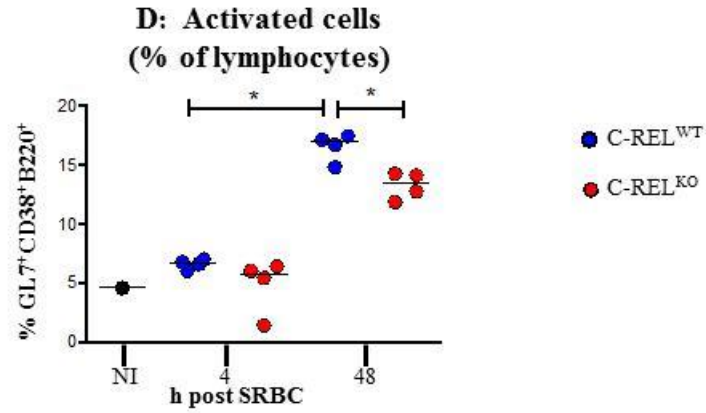
To test whether C-REL is required for B-cell differentiation, we immunised C-REL^{WT} or C-REL^{KO} mice with $2x10^8$ sheep red blood cells i.v and tracked subsequent B-cell differentiation. Immunisation with SRBC induces a large polyclonal TD response (Stamm et al., 2013), the kinetics of which are similar to that induced within lymph nodes by alum precipitated NP-CGG following footpad immunisation (Toellner et al., 1998). By three days

after immunisation, B cells have started to expand and migrate into the follicles. This is followed by further proliferation and differentiation into germinal centre B cells and plasma cells five days post immunisation.

Activated B blasts upregulate expression of GL7 whilst still expressing CD38 (Taylor et al., 2012), in contrast to germinal centre B cells which express GL7 but lose CD38 expression. Two days after immunisation, $GL7^{+}CD38^{+}B220^{+}$ activated B cells become detectable by FC in the spleens of $CREL^{WT}$ as well as $CREL^{KO}$ mice (Figure 5.1A). The percentage of activated B cells two days after immunisation was significantly lower when mice lacked C-REL expression (Figure 5.1D). Despite their lower numbers, activated antigen-specific B cells show similar upregulation of IRF4 protein between 4 h and 2 d post-immunisation in both C-REL^{WT} and C-REL^{KO} B cells (Figure 5.1E and F).

B cells during extrafollicular differentiation upregulate AID mRNA and protein within two days of activation (Chapter 3). We found significant upregulation of AID protein two days after immunisation and this occurred similarly in both C-REL^{WT} and C-REL^{KO} B cells (Figure 5.1E and F).


A: C-REL^WT
3
9
4
42.6
1
6
2
1
CD38-PB
GL7-FITC
CD138-PE
B220-APC/PerCP-Cy5.5
9
2
8
B:
C:
% of Max
IRF4-APC
AID-PerCP-Cy5.5
C-REL^KO
42.3
12.6
43.3
1.78
92.1
Non-immunised splenocytes
C-REL^WT
C-REL^KO


D: Activated cells
(% of lymphocytes)
% GL7+CD38+B220+
NI
4
48
h post SRBC
C-REL^WT
C-REL^KO

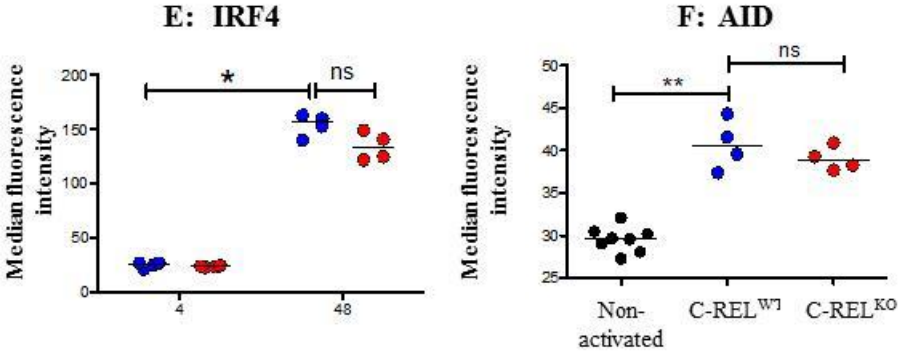

E: IRF4
Median fluorescence intensity
ns
F: AID
Non-activated
C-REL^WT
C-REL^KO

Figure 5.1. *Mice lacking C-REL have fewer activated cells, but these have similar IRF4 and AID induction compared to WT***.** C57 or C-REL^{KO} mice were immunised with SRBC i.v. **A**: Representative flow cytometry analysis of the number of activated B cells (GL7^{+} CD38^{+} B220^{+}) in the spleens of C-REL^{WT} (top panel) or C-REL^{KO} mice (bottom panel), two days after immunisation. **B and C:** Representative histogram of IRF4 protein expression (**B**) or AID protein expression (**C**) within the activated population shown in (**A**) for C-REL^{WT} (blue) and C-REL^{KO} cells (red). **D:** The percentage of activated splenic (GL7^{+} CD38^{+} B220^{+}) B cells gated according to (**A**) are fewer two days after immunisation in C-REL^{KO} mice (red) compared to C-REL^{WT} mice (blue). **E** and **F**: Median fluorescence intensity of IRF4 (**E**) and AID protein (**F**) within activated B cells gated according to (**A**), two days after immunisation
Each data point represents one mouse.

To test whether downstream B-cell differentiation into plasma-cell and germinal centre cells was affected, we examined C-REL^{WT} and C-REL^{KO} spleens five days after immunisation by FC. Plasma-cell numbers were significantly reduced in C-REL^{KO} spleens compared to C-REL^{WT} spleens (Figure 5.2 A and B). In addition, germinal centre B cells (CD38-GL7+) were strongly reduced in number in mice lacking C-REL protein (Figure 5.2C and D). Tissue sections from C-Rel deficient mice five days after immunisation show no detectable germinal centres and no IRF4^{high} plasma-cell foci in the T zone – red pulp junction (Figure 5.2E).

To be able to follow IRF4 protein expression in large cohorts of antigen-specific B cells during the early extrafollicular differentiation *in vivo*, QM$^{NP/NP}$ $\kappa^{-/-}$ C-REL^{WT} were crossed with C-REL^{KO} mice to create QM$^{NP/WT}$ $\kappa^{-/WT}$ C-REL^{KO} (henceforth referred to as QM^{WT} or QM^{KO}). QM^{WT} or QM^{KO} mice were immunised with the TI-II antigen NP-Fluorescein-AECM-FICOLL. Six hours after immunisation, fluorescein^{+} B cells were tested by FC for their expression of IRF4 protein. Similar proportions of QM^{KO} as QM^{WT} B cells had taken up the antigen 6 h after immunisation (Figure 5.3A). There was no significant difference in IRF4-protein induction between QM^{WT} and C-Rel deficient QM B cells within six hours of immunisation (Figure 5.3B and C).

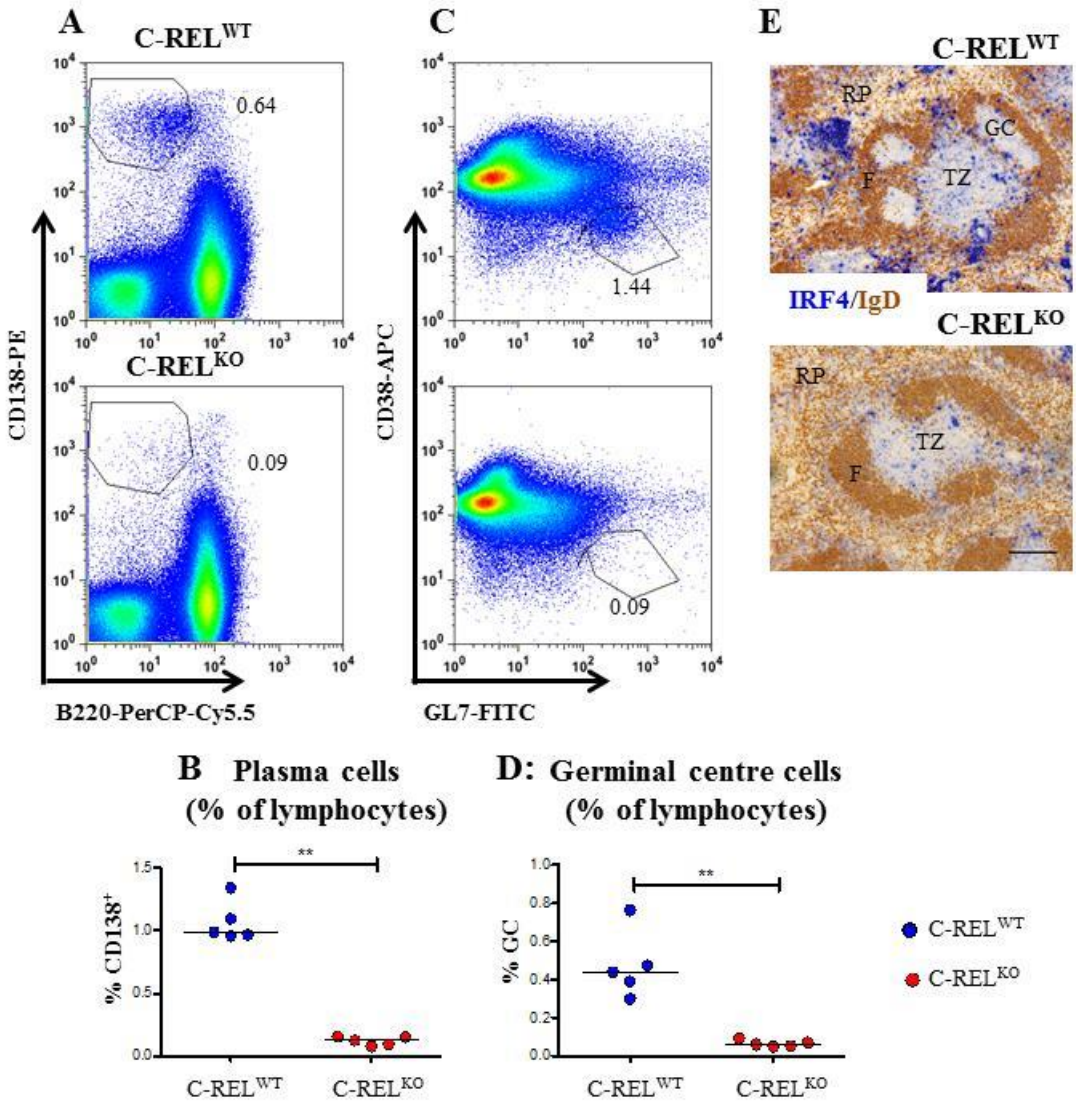


Figure 5.2. *Mice lacking C-REL have impaired late primary B cell responses to TD antigen.* C57 or C-REL^{KO} mice were immunised with SRBC i.v. **A**: Representative FC plot: five days after immunisation, numbers of plasma cells from the spleens of C-REL^{WT} (top), or C-REL^{KO} mice (bottom) were quantified by flow cytometry as B220^{int} CD138^{+}. **B**: Quantity of plasma cells, expressed as a percentage of the total population of lymphocytes. Each data point represents one mouse **C:** Representative FC plot: five days after immunisation, numbers of germinal centre cells from the spleens of C-REL^{WT} (top), or C-REL^{KO} mice (bottom), were quantified by flow cytometry. Germinal centre cells were defined as CD38^{low} GL7^{+}. **D**: Quantity of germinal centre B cells from C-REL^{WT} (blue) or C-REL^{KO} (red) expressed as a percentage of the total population of lymphocytes. Each data point represents one mouse. **E:** Spleen sections from C-REL^{WT} (top) or C-REL^{KO} mice (bottom) five days after SRBC. Spleen sections were stained with antibodies against IgD and IRF4. GC: germinal centre, TZ: T zone, RP: red pulp, F: follicle. Scale bar indicates 200µm.

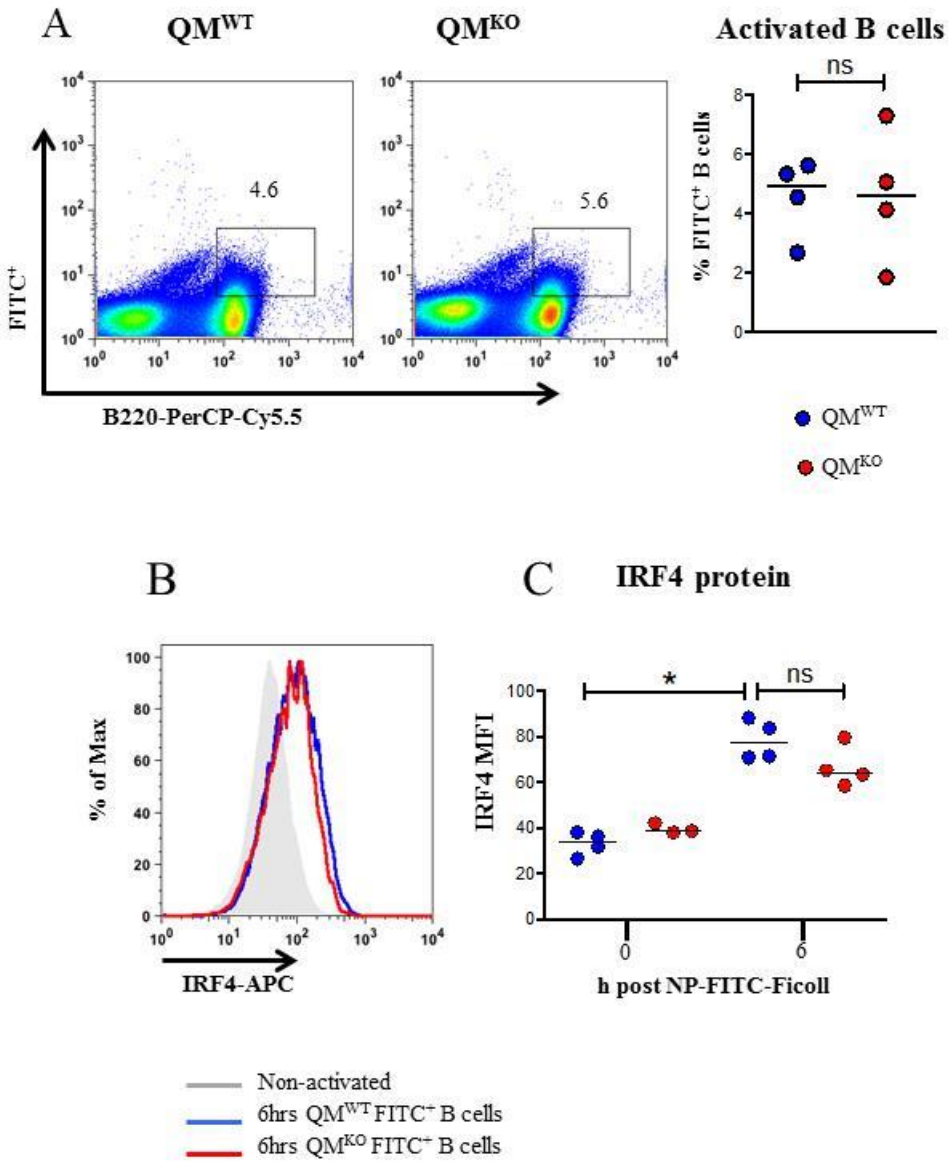


Figure 5.3. *C-REL signalling is dispensable for up-regulation of IRF4 in the first hours after B cell activation.* QM^{WT} or QM^{KO} mice were immunised i.p with NP-Fluorescein-AECM-Ficoll. **A**: Representative FC plot of splenocytes pre-immunisation and six hours after immunisation. Activated cells are $B220^{+}FITC^{+}$. **B**: Representative histogram of IRF4 protein expression at six hours after immunisation, measured in activated B cells (gated as in **A**.). **C:** Median fluorescence intensity of IRF4 protein, derived from FC. NP-binding B cells were gated pre-immunisation whereas FITC-binding B cells were gated six hours after immunisation as shown in (**A**). Each data point represents one mouse. MFI: median fluorescence intensity.

To further test whether early B-cell activation was normal 6 h post immunisation, expression of B-cell activation markers were analysed by FC. After gating on cells which had bound the antigen *in vivo* ($FITC^+$) significant loss of CD62L is seen. This occurred at similar levels in QM^{WT} as well as QM^{KO} B cells (Figure 5.4A). CD86 protein is significantly upregulated (Figure 5.4B) and this occurred to the same degree in QM^{WT} or QM^{KO} B cells. However there were significantly fewer cells that were $CD69^+$, leading to significantly lower CD69 mean fluorescence intensity in the QM^{KO} population on activated B cells (Figure 5.4C).

Although the experiments in BCR non-transgenic mice (Fig. 5.1) showed a significant reduction of antigen-specific B cells 2 days post immunisation, the experiments with QM B cells show that C-Rel-deficient B cells bind antigen as efficiently as QM^{WT} B cells. Further, following antigen binding, IRF4 protein induction is normal. B cells show grossly similar expression of activation markers (CD62L, which is required for their retention at the site of activation and the expression of the co-stimulatory molecule CD86), although expression of CD69 is slightly reduced. CD69 is reported to have a negative regulatory role on T cells (la Fuente et al., 2012) and has been shown to be regulated by NFκB family members. The discrepancy between initially similar numbers of $C\text{-}REL^{WT}$ and C-REL deficient B cells being activated and significantly lower numbers 2 days post immunisation probably means that proliferation in C-REL deficient B cells in the extrafollicular response in inhibited.

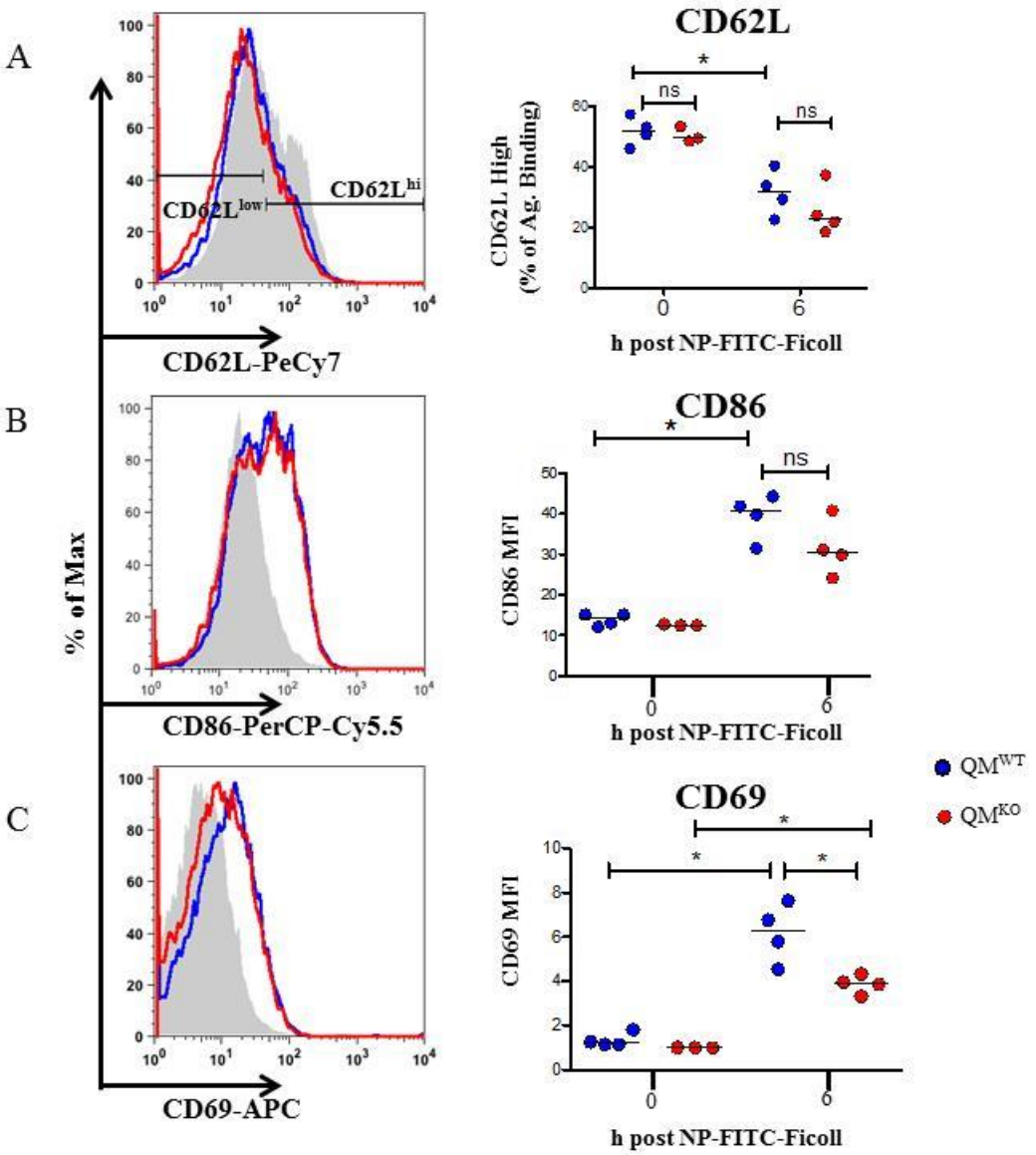


Figure 5.4. *Initial activation of B cells in the absence of C-REL protein.*
QM^{WT} or QM^{KO} mice were immunised i.p with NP-FITC-Ficoll. Six hours later spleens were collected and flow cytometry performed to test expression of activation markers. Activated B cells were gated as $B220^+$, FITC-binding, as in figure 5.3. Representative histograms of the percentage of antigen-binding $CD62L^{low}$ cells (**A**), and protein expression of CD86 (**B**) and CD69 (**C**) shown for QM^{WT} (blue) and QM^{KO} (red) B cells. The median fluorescence intensity for each protein is shown in the plots on the right. Each data point represents one mouse.

5.2.2 Defects in plasma-cell and germinal centre formation are B-cell intrinsic

C-REL is implicated in signalling pathways in many cell types, and the slight reduction in numbers of activated B cells discussed in the last paragraph may be due to an impact of C-REL deficiency on accessory cells. Therefore, adoptive transfer experiments were done to study the responses of C-REL^{KO} B cells in a wild-type environment. 5x10^{5} antigen-specific QM$^{NP/NP}$ $\kappa^{-/-}$ C-REL^{WT} or QM$^{NP/NP}$ $\kappa^{-/-}$ C-REL^{KO} (hereafter referred to as QM^{WT} and QM^{KO}) B cells were transferred into C57BL/6 mice. This was followed 24 hours later by challenge with NP-Ficoll. Resting B cells were enriched from total splenocytes using negative selection with anti-CD43 magnetic beads. The percentages of NP-binding B cells were measured by FC to ensure similar numbers of antigen-specific cells were transferred.

Two hours post-immunisation, eYFP^{+} NP-binding donor cells were tested for their expression of IRF4 protein. We found that QM^{KO} B cells did not have any defect in their early upregulation of IRF4 protein. QM^{KO} B cells expressed similar amounts of IRF4 protein compared to QM^{WT} B cells two hours (Figure 5.5A) and four hours post immunisation (Figure 5.5B).

To exclude that differences in the numbers of transferred, C-REL deficient antigen-specific B cells were causing the lower amounts of cells developing from transferred QM^{KO} cells, the percentage of eYFP^{+} transfer cells were measured over the first 5 days in a single experiment. Two hours post-immunisation, B cells have not yet started to divide, and similar numbers of QM^{WT} and QM^{KO} B cells were detected within spleens (Figure 5.6A). Likewise two days post-immunisation the percentage of transferred cells were also similar, whether B cells were QM^{WT} or QM^{KO}. Still, five days after immunisation there are significantly fewer eYFP^{+} cells (>80% reduction) when C-REL is absent in B cells ($p<0.05$).

To test whether defects in plasma-cell differentiation observed in C-REL^{KO} mice were B-cell intrinsic, numbers of plasma cells were quantified by FC. The percentage of QM^{KO} transfer cells which differentiated into plasma cells (Figure 5.6B and C) were reduced by around 70% ($p<0.001$) and plasma-cell numbers are reduced by >95% (Figure 5.6D, $p<0.001$). Spleen sections of hosts of QM^{KO} B cells were analysed by immunohistology five days after immunisation. Spleen sections were stained with antibodies against the QM idiotype BCR (blue) and IgD (brown) to visualise donor-derived germinal centres and plasma cells. Whilst QM^{WT} B cells form large germinal centres and plasma-cell foci, QM^{KO} B cells produce small germinal centres and plasma cells are mostly absent (Figure 5.6E).

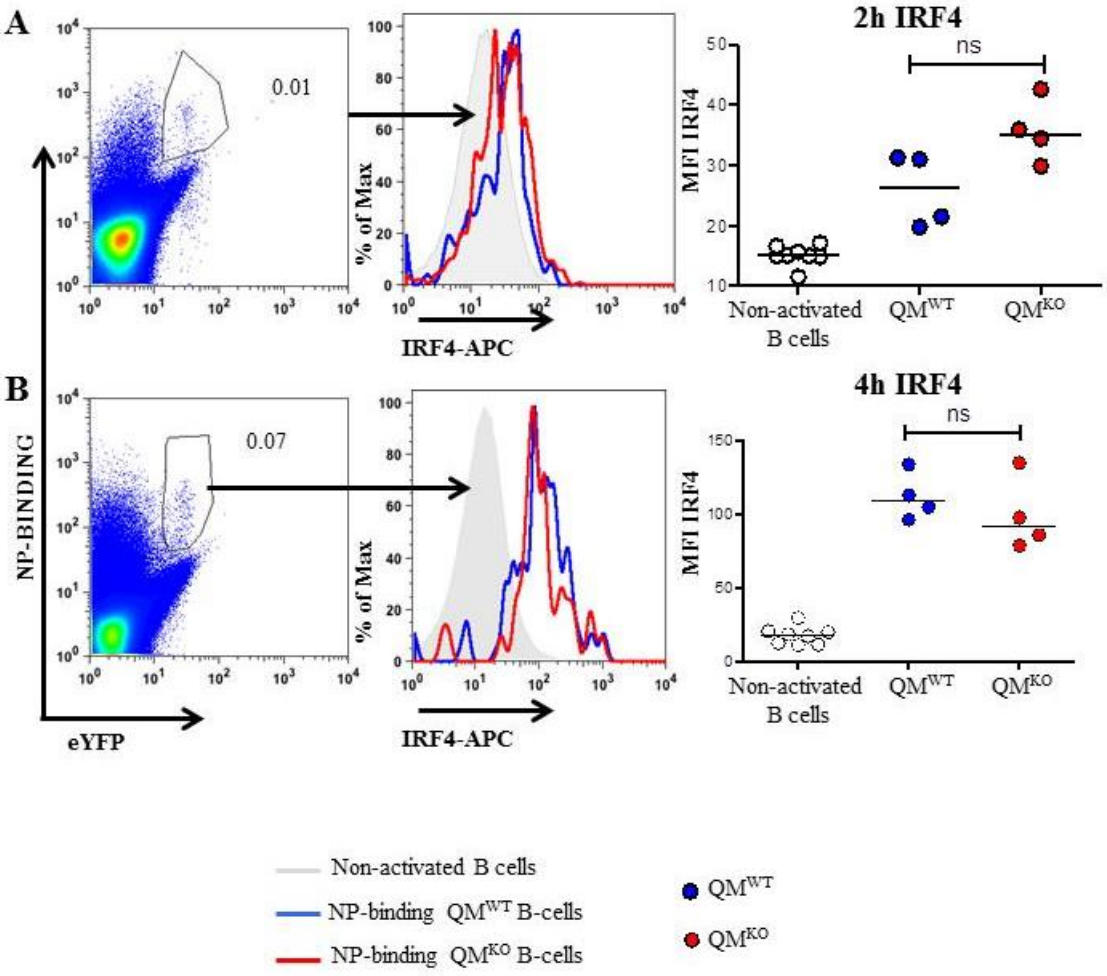


Figure 5.5. *Normal initial activation of B cells when C-REL signalling is absent in B cells only*. QM^{WT} or QM^{KO} B cells were transferred into C57 hosts and immunised 24 hours later with NP-Ficoll i.p.

A: Representative flow cytometry plots from splenocytes harvested two hours post-immunsiation. Activated transfer cells were defined as $eYFP^+$ and NP-binding (left). Centre: activated QM^{WT} (blue) or QM^{KO} (red) splenic B cells were tested for expression of IRF4 protein. Right: IRF4 protein levels were quantified from FC and expressed as median fluorescence intensity. Each data point represents one mouse. **B**: IRF4 protein is further up-regulated four hours after immunisation, gated as before. Each data point represents one mouse.

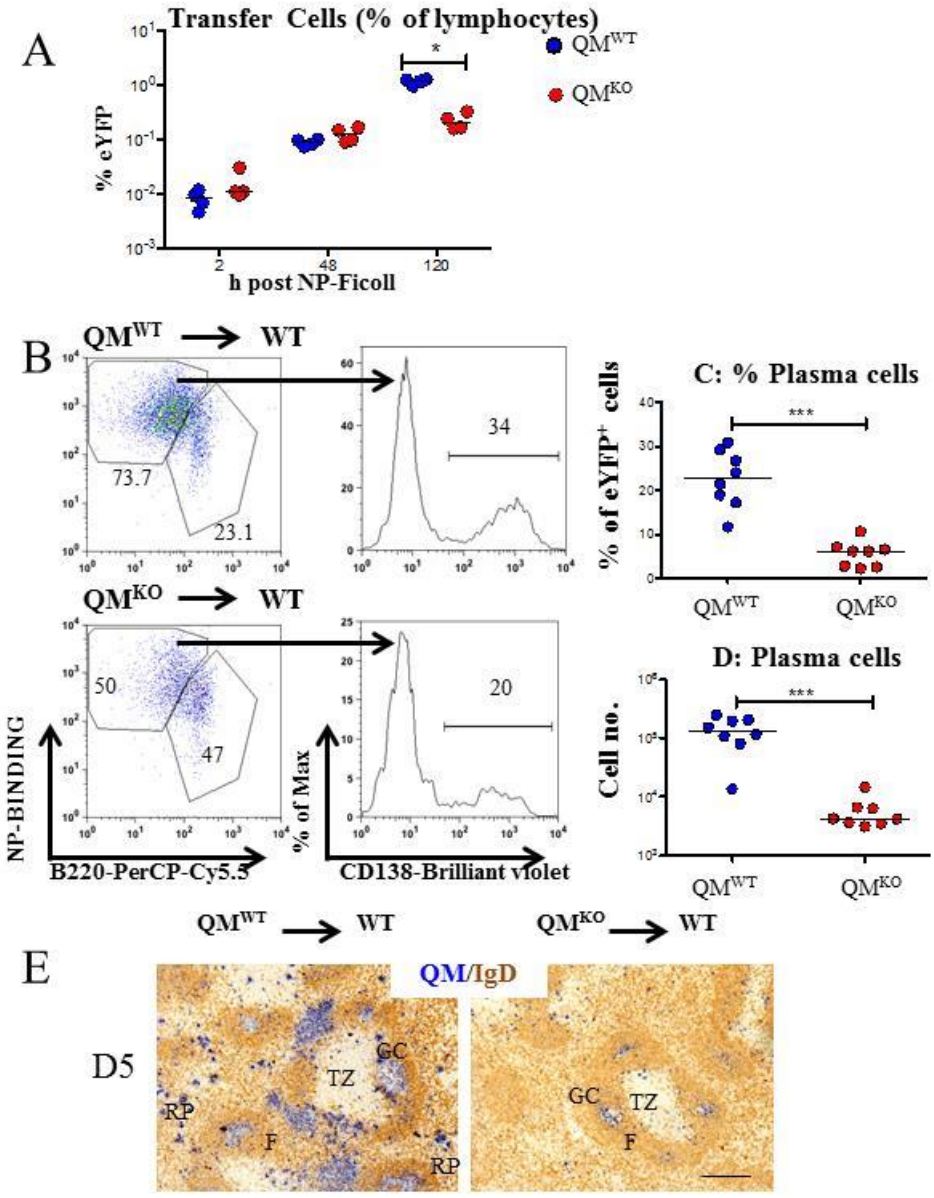


Figure 5.6. *Plasma cell production is deficient when B cells lack C-REL protein.* QM^{WT} or QM^{KO} B cells were transferred into WT hosts and immunised with NP-Ficoll. **A**: % of $eYFP^+$ transfer cells quantified by FC. **B:** Representative flow cytometry plots of QM^{WT} (top) and QM^{KO} (right) splenocytes. $eYFP^+$, $B220^{low}$, NP-binding, $CD138^+$ plasma cells were tested for expression of IRF4. **C**: Plasma cell numbers obtained by FC as shown in (B) expressed as a percentage of the $eYFP^+$ transfer cell population. Data are merged from two experiments with n=4 **D**: Absolute numbers of plasma cells calculated from flow cytometry. Data are merged from two experiments with n=4 Each data point represents one mouse, data are representative of two experiments. **E:**. Five days after immunisation, spleen sections from mice receiving QM^{WT} (left) or QM^{KO} cells (right) were stained with antibodies against the QM BCR and IgD. GC: germinal centre, TZ: T zone, RP: red pulp, F: follicle. Scale bar indicates 200µm.

To test whether levels of IRF4 protein were sustained two days post immunisation, IRF4 protein levels were tested by FC. QM^{KO} B cells were found to express similar levels of IRF4 protein two days after immunisation (Figure 5.7A). We found that similar to early intermediate level IRF4 expression, upregulation of AID protein is not impaired in QM^{KO} B cells either (Figure 5.7A). Two days after immunisation, antigen specific B cells are still located at the border between the T-zone and the follicles. There is no significant difference in B-cell location between QM^{KO} and QM^{WT} B cells (Figure 5.7B).

To test whether effects of early or late stage IRF4 expression cause defective plasma-cell differentiation in C-REL deficient B cells, QM^{WT} or QM^{KO} B cells were FACS sorted at selected time points after immunisation. There is no difference in IRF4 mRNA expression within the first 2 days post immunisation, with a normal rapid peak in *Irf4* expression 2 h after immunisation (Figure 5.8A) and normal loss of IRF4 mRNA levels two days post-immunisation. Five days post immunisation IRF4 is down-regulated equally within the germinal centre population. When the few plasma cells that develop from QM^{KO} B cells 5 d post immunisation were sorted, there was not significant difference in *Irf4* mRNA levels compared to QM^{WT} plasma cells. *Prdm1* mRNA, encoding the transcription factor BLIMP1, is essential for the plasma-cell differentiation programme (Nutt et al., 2011). Lack of plasma cells may be due to failure to upregulate *Prdm1*. To test if changes in *Prdm1* expression cause the reduction in the number of plasma cells developing, *Prdm1* mRNA was measured in sorted QM^{WT} and QM^{KO} B cells and plasma cells. *Prdm1* mRNA becomes detectable within two days of immunisation, due to the first differentiation of small numbers of plasma cells (Marshall et al., 2011). Two days after immunisation both QM^{WT} and QM^{KO} B cells have similar upregulation of *Prdm1* mRNA ($p<0.05$) (Figure 5.8B). Five days after immunisation, *Prdm1* mRNA is further strongly induced in both QM^{WT} and QM^{KO} plasma cells ($p<0.05$).

Therefore reduced plasma-cell numbers do not seem to be due to a defect in *Prdm1* induction, at least not in the small numbers of plasma cells that do develop from QM^{KO} B cells.

IRF4 protein expression was also tested by FC within QM^{KO} derived plasma cells and germinal centre B cells. Germinal centre B cells showed similar absence of IRF4 protein whether they expressed C-REL protein or were C-REL deficient (Figure 5.8C), while plasma cells (gated as shown in figure 5.6B) express similar high levels of IRF4 protein whether derived from B cells that are QM^{KO} or QM^{WT} (Figure 5.8C and D) ($p<0.05$).

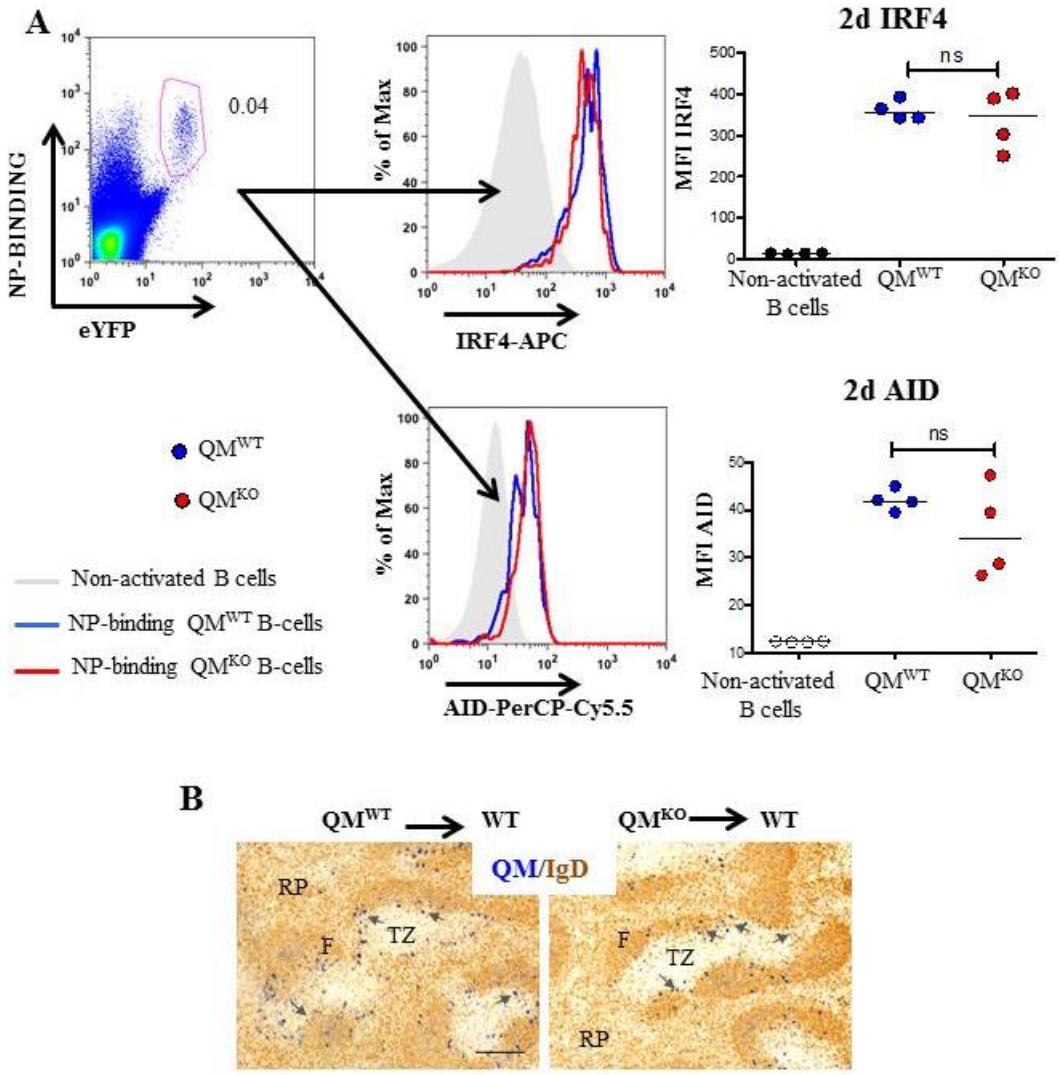


Figure 5.7. *Normal IRF4 and AID induction when C-REL signalling is absent in B cells only*. QM^{WT} or QM^{KO} B cells were transferred into C57 hosts and immunised 24 hours later with NP-Ficoll i.p. **A:** Representative flow cytometry plots from splenocytes harvested two days post-immunsiation. Activated transferred cells were defined as $eYFP^+$ and NP-binding (left). Centre top: activated QM^{WT} (blue) or QM^{KO} (red) splenic B cells were tested for expression of IRF4 protein. Top right: IRF4 protein levels were quantified from FC and expressed as median fluorescence intensity. Each data point represents one mouse. Activated $eYFP^+$ B cells were also tested for expression of AID protein (bottom centre). Protein levels were expressed as median fluorescence intensity (bottom right). Each data point represents one mouse. Data are representative of two experiments. **B**: Spleen sections harvested two days post-immunisation from QM^{WT} (left) or QM^{KO} (right) were stained for the QM BCR and IgD. TZ: T zone, RP: red pulp, F: follicle. Scale bar indicates 200μm.

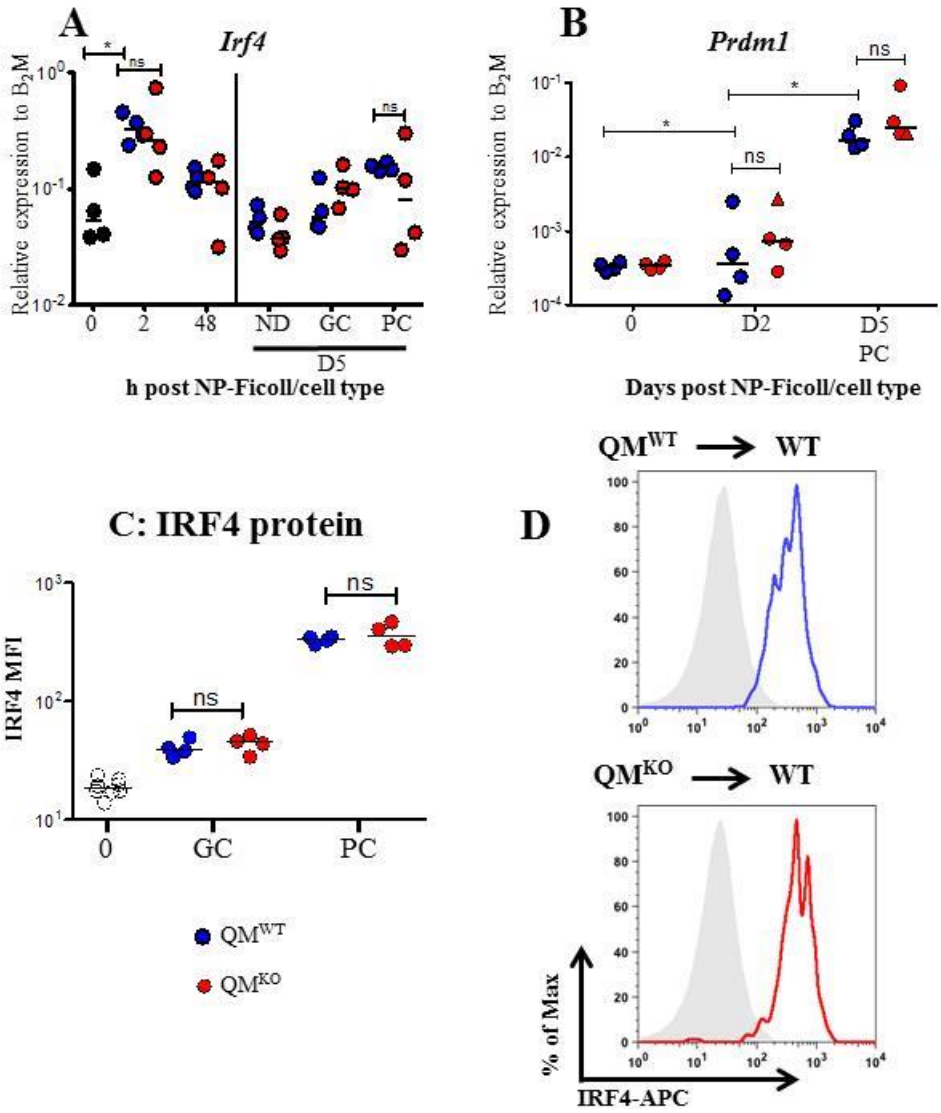


Figure 5.8. *Normal induction of IRF4 and BLIMP1 in B cells which lack C-REL protein*. QM^{WT} (blue) or QM^{KO} (red) B cells were transferred into WT hosts and immunised with NP-Ficoll. Two hours, two days and five days after immunisation, transferred cells were sorted from QM^{WT} and QM^{KO} spleens. Two hours and two days after immunisation, $eYFP^+$ NP-binding cells were sorted by FACS. Five days after immunisation, B cells which did not express either germinal centre or plasma cell markers (ND), germinal centre B cells (GC) and plasma cells (PC) were sorted by FACS. Sorted cells were tested by real-time RT-PCR for the expression of *Irf4* (**A**) and *Prdm1* which encodes BLIMP1 (**B**). **C**: Median fluorescence intensity of IRF4 protein quantified by FC within $eYFP^+$ NP-binding cells (4 and 48 hours post-immunisation), or five days after immunisation: within the GC population ($eYFP^+$, NP-binding, $B220^+$ FAS^+, $CD138^-$) or the PC population ($eYFP^+$, NP-binding, $B220^+$, $CD138^+$). **D:** Representative histogram of IRF4 protein expression quantified by FC within $CD138^+$ plasma cells as gated in figure 5.6B.

5.2.3 Normal AID protein and mRNA induction when B cells are C-REL deficient

The initial upregulation of IRF4 protein to intermediate levels is normal when B cells lack C-REL protein (Figure 5.1D, Figure 5.3C). However, plasma-cell differentiation is impaired (Figure 5.7A-D). Two days after immunisation, AID protein becomes detectable within differentiating antigen-specific B blasts within the follicles (Figure 3.7). To test effects of C-REL deficiency on AID protein induction the adoptive transfer system was used. Spleen sections were stained with antibodies against the QM BCR idiotype in conjunction with AID and IgD in order to locate AID expressing cells in the outer T zone and follicles (Figure 5.9A). Similar proportion and similar levels of AID protein expression were detected within nuclei of individual QM^{WT} and QM^{KO} B cells (Fig. 5.9B).

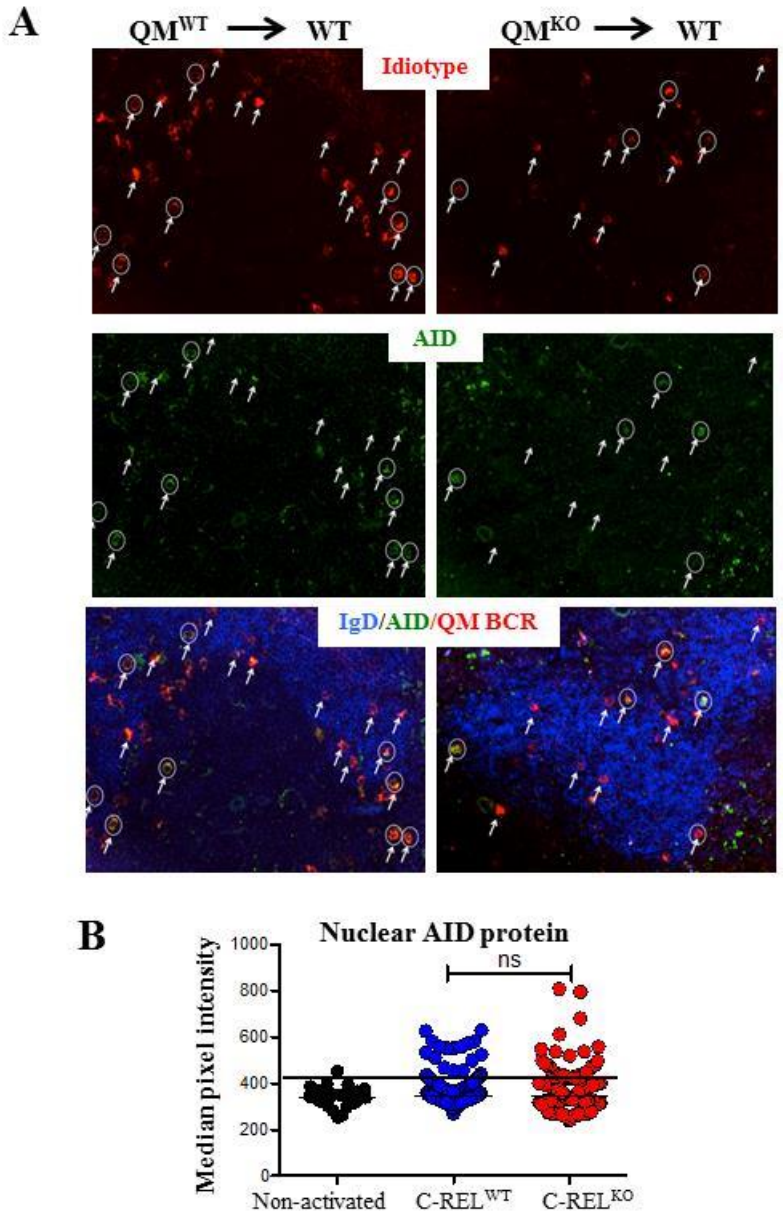


Figure 5.9. *AID protein induction is normal two days after activation when B cells lack C-REL protein.* QM^{WT} or QM^{KO} B cells were transferred into WT hosts and immunised with NP-Ficoll. **A:** Two days after immunisation, spleens from mice which received QM^{WT} B cells (left) or QM^{KO} B cells (right) were stained by IF with antibodies against the QM BCR (idiotype: red, top panel), AID (green, centre panel) and IgD (blue, bottom panel). Transferred cells are located within the IgD^+ follicles (arrows) and many are expressing AID protein (circles). **B:** AID protein was quantified within the nucleus of individual QM BCR^+ transfer cells located within the follicles using ImageJ software. Each data point represents one cell, three mice were quantified for each group.

5.2.4 Normal induction of Ig Class Switching in C-REL deficient B cells

NFκB1 deficiency in B cells does not affect IRF4 intermediate level protein or AID induction, and early GC independent Ig class switching is not diminished. Following transfer of QM^{WT} or QM^{KO} B cells into WT hosts, Ig class switching was quantified by measuring the titres of unswitched and class switched antibodies in sera. NP-specific IgM titres increase within five days of immunisation. Although there is a trend to lower IgM levels from QM^{KO} B cells, the difference was not significant (Figure 5.10A). When testing NP-specific IgM^a only, which is derived solely from the transferred QM B cells, lower production derived from QMKO cells becomes clearly detectable by two days after immunisation (Figure 5.10B).

NP-specific IgG and $IgG1^a$ produced by QM^{KO} cells show a similar reduction, however, due to the low number of mice analyzed differences between WT and C-REL deficiency are never significant (Figure 5.10C, D,). There is no significant difference the ratio of $IgG1^a$ to IgM^a indicating that there is no change in the frequency of Ig class switching events of QM^{KO} B cells before they differentiate into plasma cells (Figure 5.10E). As $IgG1^a$ titres were undetectable in the sera of several mice, Ig class switching frequency was confirmed by FC and IF staining. For FC, $eYFP^+$, NP-binding, $CD138^+$ plasma cells were gated (Figure 5.11A) and analysed for expression of IgG3 and IgG1. The main isotype produced in TI-II responses is IgG3 (Vinuesa et al., 2000). Significantly fewer IgG switched plasma cells were seen in spleens from mice receiving QM^{KO} B cells. $IgG3^+$ and IgG1 switched plasma-cell frequencies are reduced by >97% when B cells lack C-REL protein ($p<0.05$, Figure 5.11B, C). Unswitched plasma cells are also reduced by >95% in QM^{KO} B cells compared to QM^{WT} B cells ($p<0.05$, Figure 5.11D). While there was no obvious reduction of switched cells when QM^{WT} B cells were transferred into a $C\text{-}REL^{KO}$ environment, QM^{WT} B cells did produce significantly fewer $IgG1^-/IgG3^-$ negative plasma cells in a $C\text{-}REL^{KO}$ environment ($p<0.05$, Figure 5.11D). This may represent increased Ig class switching to other isotypes such as IgG2a when C-REL is absent in stromal cells.

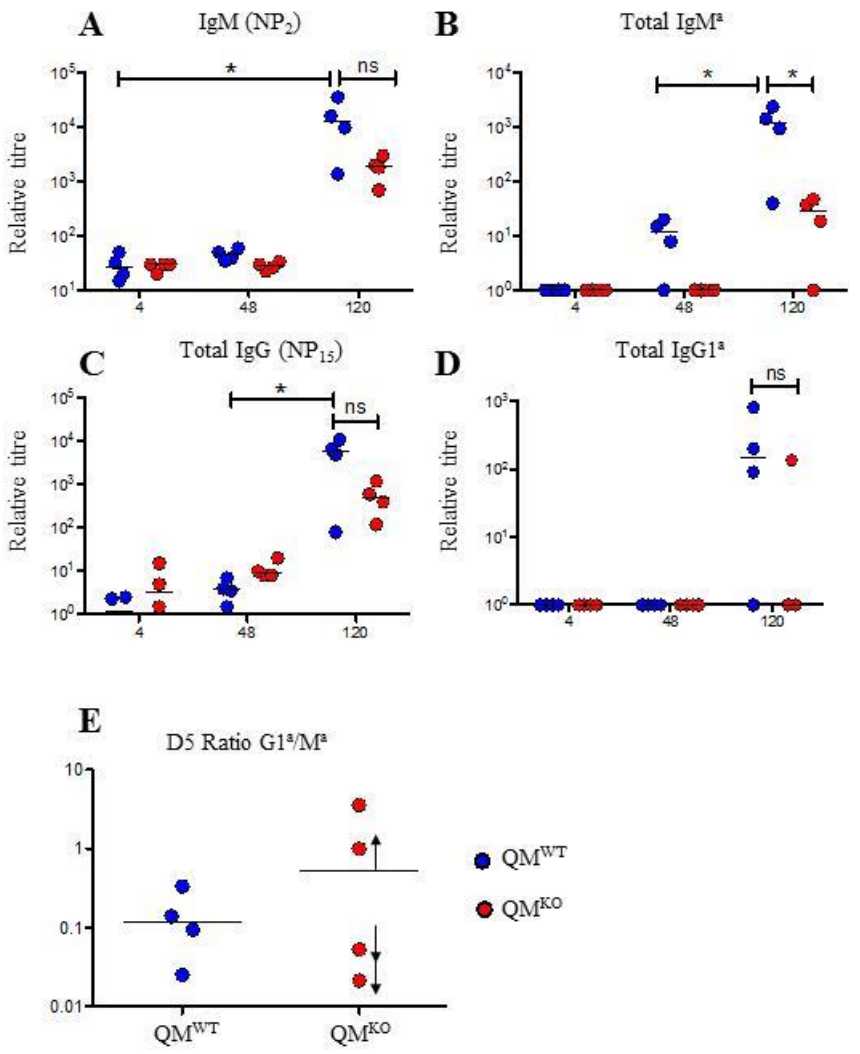


Figure 5.10. *A deficiency of C-REL in B cells leads to reduced antibody titres but switching is unaffected.* QM^{WT} (blue) or QM^{KO} B cells (red) were transferred into WT hosts and immunised with NP-Ficoll. Sera were collected 4, 48 and 120 hours after NP-Ficoll. Sera were tested for titres of antigen-specific antibody by ELISA. **A:** IgM binding to NP_2-BSA, **B:** IgM^a binding to NP_{15}-BSA, **C:** IgG binding to NP_{15}-BSA **D:** $IgG1^a$ binding to NP_{15}-BSA, **E:** Switching efficiency calculated as $IgG1^a$ titres divided by IgM^a titres. Arrows demonstrate that the result is of stated ratio or more/less. Each data point represents one mouse.

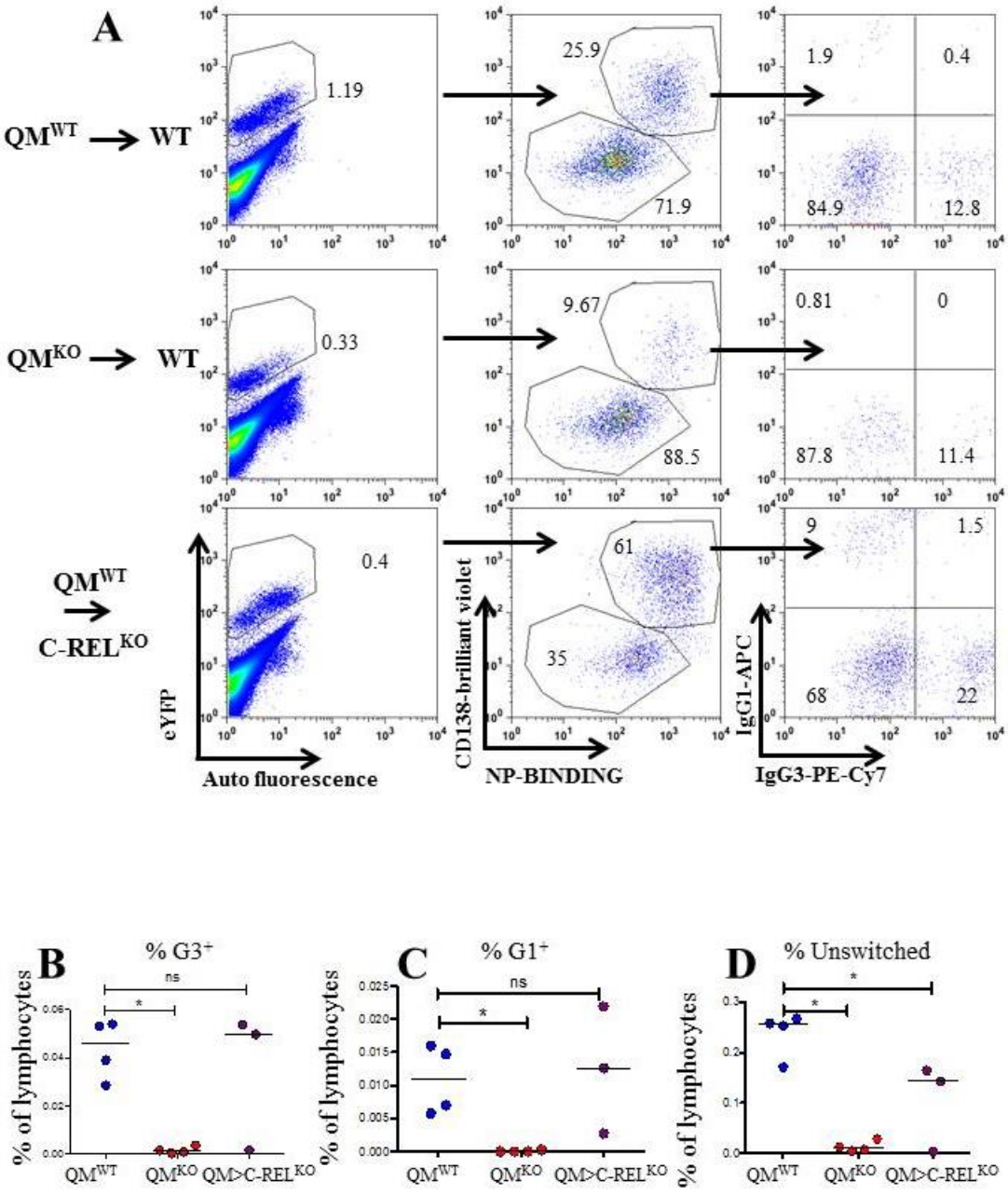


Figure 5.11. *C-REL^{KO} B cells produce fewer switched and unswitched plasma cells.* QM^{WT} (blue) or QM^{KO} B cells (red) were transferred into WT hosts or C-REL^{KO} hosts and immunised with NP-Ficoll. Five days after immunisation switched B cells were quantified from FC staining. **A:** QM^{WT} (top), QM^{KO} (centre) or QM^{WT} in a C-REL^{KO} environment (bottom), plasma cells were defined as eYFP^{+} (left), NP-binding and CD138^{+} (centre). These were tested for expression of IgG1 and IgG3 (right). Switched cells were expressed as a percentage of the lymphocytes in the spleen for IgG3 (**B**), IgG1 (**C**), and unswitched plasma cells (**D**). Each data point represents one mouse.

To confirm results from FC and to quantify class switching specifically in extrafollicular plasma cells, IF staining was performed on spleen sections from mice that received QM^{WT} or QM^{KO} B cells followed by immunisation with NP-Ficoll. Transferred B cells were identified by their expression of the QM BCR idiotype. Counterstaining with antibodies against IgM and IgG was used to identify non-switched or switched plasma cells (Figure 5.12A). Five days after immunisation, QM^{WT} B cells have formed large plasma-cell foci in the red pulp. In contrast, few QM^{KO} extrafollicular plasma cells are visible (Figure 5.12A). Transfer of QM^{KO} B cells result in a slightly lower frequency of IgM expressing extrafollicular plasma cells than transfer of QM^{WT} B cells ($p<0.05$, Figure 5.12B). Further, if anything, higher percentages of IgG switched plasma cells form (Figure 5.12C), resulting in a slightly increased ratio of IgG to IgM expressing cells. (Figure 5.12D), confirming that C-REL-deficient B cells have no defect in CSR.

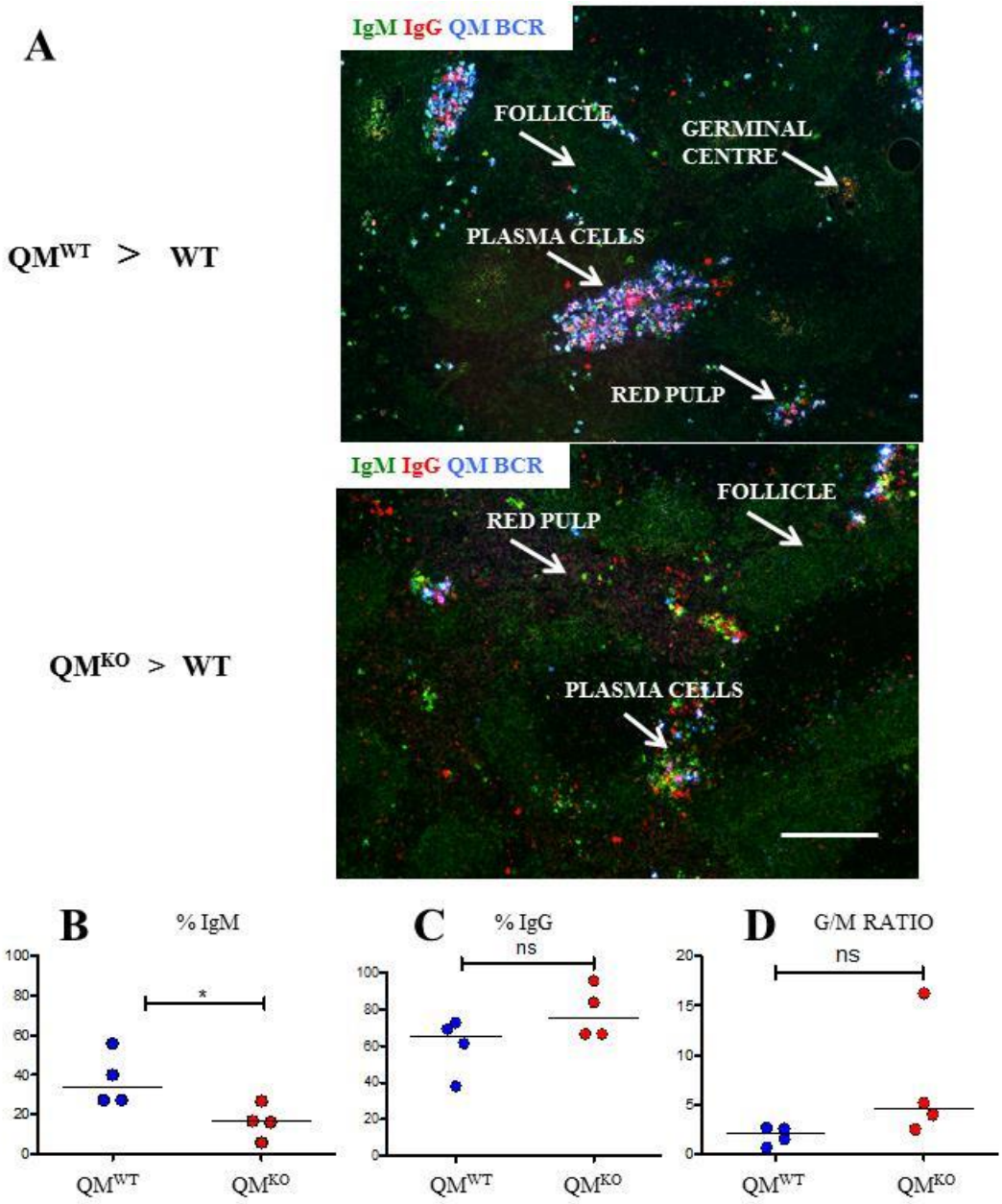


Figure 5.12. *Class switching to IgG is normal when B cells lack C-REL protein.* QM^{WT} or QM^{KO} B cells were transferred into WT hosts and immunised with NP-Ficoll. Spleens were taken five days after immunisation. **A:** Immunofluorescent staining of Spleen sections. Sections from QM^{WT} (top) or QM^{KO} recipients (bottom) were stained with labelled antibodies against IgM (green), IgG (red) and the QM BCR (blue). Scale bar indicates 200µm. **B-D** Switching was quantified on sections and the percentage of IgM (**B**) and IgG (**C**) expressing cells counted in mice which received QM^{WT} (blue) or QM^{KO} (red) B cells. The ratio of IgG to IgM (**D**) was calculated by dividing the percentage of IgG^+ cells by the percentage of IgM^+ cells. Each data point represents one mouse.

To further confirm this we tested the induction of IgG3 germline heavy chain transcripts in sorted QM^{WT} and QM^{KO} B cells after in WT environments after immunization. $eYFP^{+}$ NP-binding B cells were FACS sorted before and 2 or 48 hours after immunisation with NP-Ficoll. Five days after immunisation, plasma and germinal centre B cells were sorted. As expected, IgG3ST are significantly increased within two hours of immunisation (Figure 5.14A). Increase in IgG3ST expression is similar in both QM^{WT} and QM^{KO} B cells. IgG3ST are further upregulated two days after immunisation in both QM^{KO} and QM^{WT} B cells. Five days after immunisation both QM^{KO} germinal centre and QM^{KO} plasma cells express significantly higher quantities of IgG3ST than QM^{WT} cells ($p<0.05$). To test the frequency of cells which have completed switching to IgG3 five days after immunisation, expression of rearranged Iμ-Cγ3 hybrid transcripts were tested (Figure 5.14B). Both QM^{WT} and QM^{KO} B cells produce increased levels of rearranged Iμ-Cγ3 transcripts, in all populations tested, and transfer of QM^{KO} B cells resulted in at least the same or more CSR.

5.3 Summary

Similar to the phenotype observed in B cells lacking NFκB1, B cells which lack functional C-REL protein upregulate intermediate level IRF4 mRNA and protein normally. Far fewer plasma are produced, but in the few plasma cells that do develop IRF4 is induced normally. The presence of normal intermediate level IRF4 protein results in induction of normal induction of AID. This leads to normal, or possibly slightly enhanced, Ig class switching to IgG3 and IgG1.

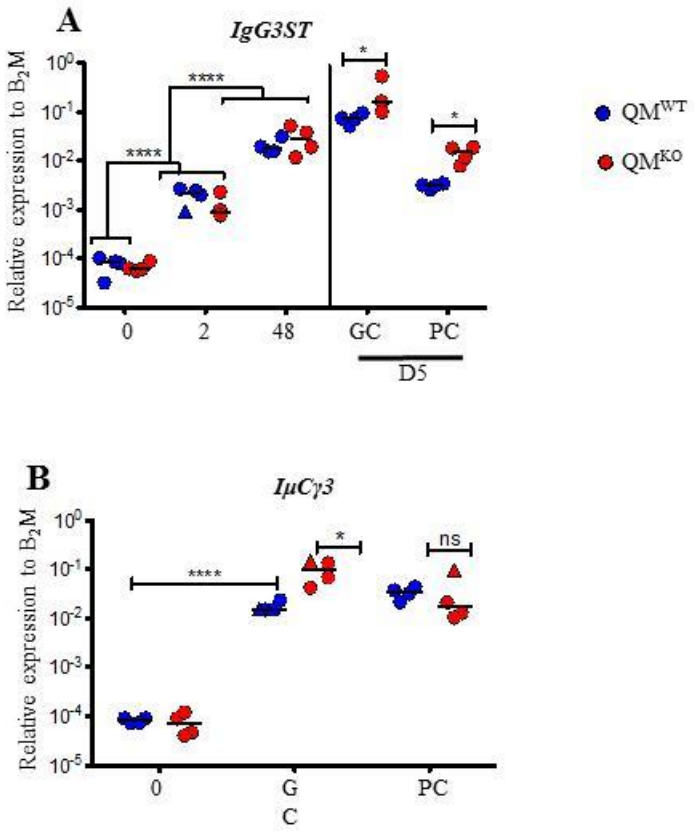


Figure 5.13. *Normal induction switch transcripts in B cells which lack C-REL protein.* QM^{WT} (blue) or QM^{KO} (red) B cells were transferred into WT hosts and immunised with NP-Ficoll. Two hours, two days and five days after immunisation, transferred cells were sorted from QM^{WT} and QM^{KO} spleens. Two hours and two days after immunisation $eYFP^+$ NP-binding cells were sorted. Five days after immunisation, plasma cells and germinal centre B cells were sorted. Sorted cells were tested by real-time RT-PCR for expression of IgG3ST (**A**) and I**μ**Cγ3 (**B**). Triangle indicates that transcript level is as stated or lower.

6: IRF4 INTERMEDIATE EXPRESSION IS REQUIRED FOR AID INDUCTION AND CLASS SWITCHING

6.1 Introduction

The previous two chapters indicate, using mice lacking NFκB family members, that intermediate IRF4 protein levels are sufficient for normal AID upregulation and extrafollicular CSR. In order to test whether extrafollicular CSR is decreased when intermediate level IRF4 protein is blocked, we repressed IRF4 protein with a micro---RNA. We chose the micro---RNA miR---125b for our study as it is predicted to bind the 3' UTR of IRF4. Furthermore gururajan *et al* (2010) showed direct binding of miR---125b to a target sequence (GGGACUC) in the 3' UTR of IRF4 that is conserved between humans and mice. Moreover, overexpression of miR---125b led to inhibition of terminal plasma cell differentiation in vitro (Gururajan et al., 2010).

6.1.1 Protein regulation by miRNAs

Small, single-stranded, non-coding RNAs can modulate the expression of genes (Ambros, 2004; Zhang et al., 2009). These highly conserved micro-RNAs (miRNA) are typically 18-25 nucleotides long and can negatively regulate target protein levels post-transcriptionally through two mechanisms. The first mechanism is by facilitating the degradation of target mRNA, the second mechanism is via inhibition of translation (Mishima et al., 2012). Both of these processes are mostly mediated by binding of miRNA to the 3’ untranslated regions (UTR) of the target gene. miRNAs are encoded within genomic DNA and their transcription occurs via RNA polymerase II. The main pathway of mature miRNA development is shown in figure 6.1. The unprocessed, primary miRNA transcript (pri-miRNA), must be converted to the pre-miRNA by the enzyme drosha and DiGeorge syndrome critical region gene 8 (DGCR8). The pre-miRNA is then exported from the nucleus to the cytoplasm by exportin 5.

Within the cytoplasm, Dicer processes the pre-miRNA into a duplex. Only one strand (the guide strand) forms part of the RNA-induced silencing complex (RISC). The RISC then binds to the target 3' UTR to inhibit translation and direct the mRNA for degradation (O'Connell et al., 2010b).

miRNAs are regulated not only at the transcriptional level, but also via their subcellular localisation and at the processing stage. For instance, interferons can inhibit Dicer expression (O'Connell et al., 2010b). miRNAs can be up-regulated or down-regulated by TLR signalling and other stress response elements (O'Neill et al., 2011). IL-10 has been shown to downregulate miR-155 (McCoy et al., 2010), this miRNA has an important role in B-cell differentiation (Turner and Vigorito, 2008). miR-155 has been shown to be necessary for the production of IgG1 switched cells and for the formation of GC. Furthermore miR-155 was found to directly repress Pu.1 (Vigorito et al., 2007). In addition miR-155 represses AID expression (Teng et al., 2008; Vigorito et al., 2007), therefore miR-155 may be important for the terminal differentiation and the shutting down of AID expression within plasma cells.

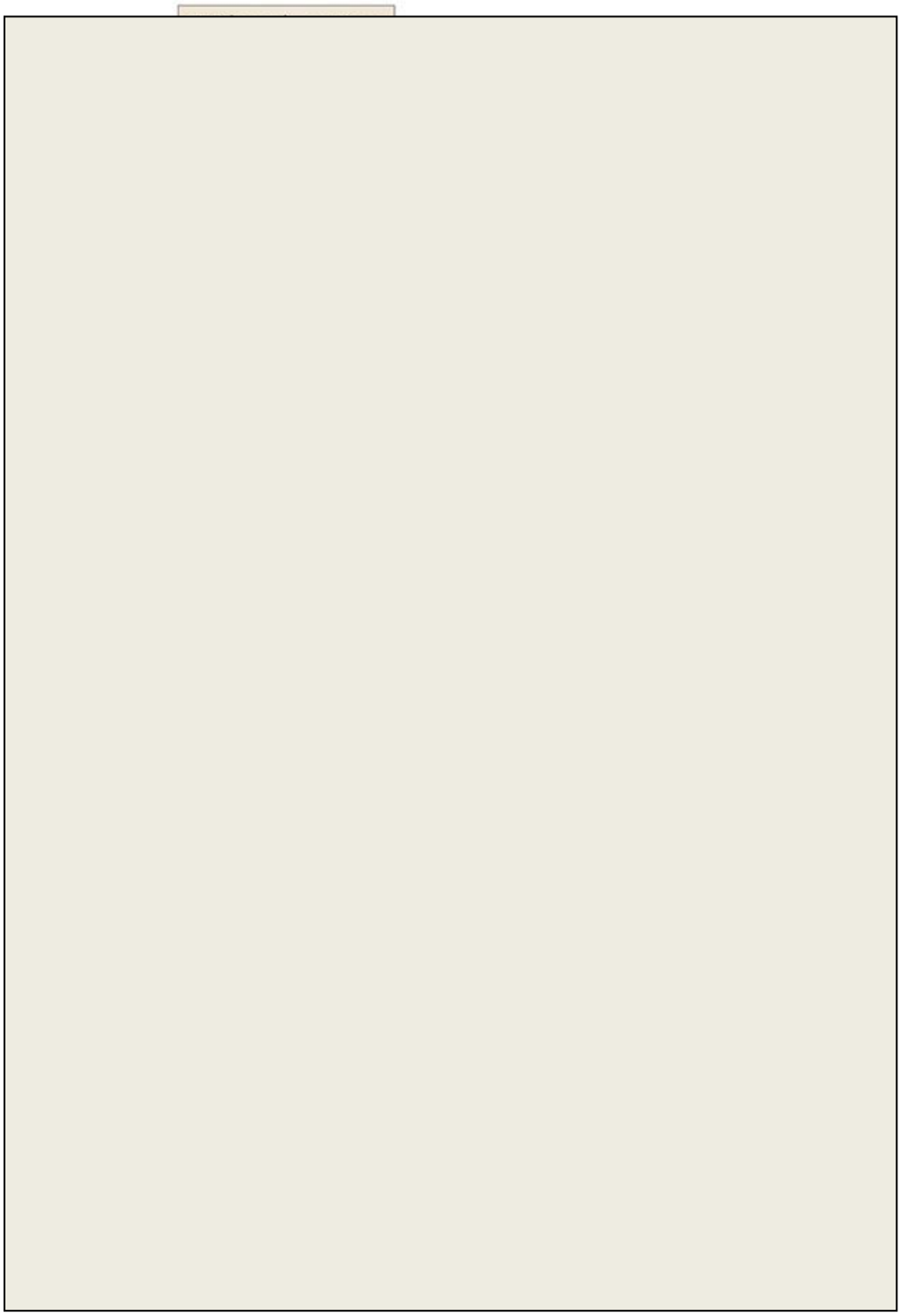


Figure 6 1 *Processing and regulation of miRNAs*

Pri-miRNA is transcribed in the nucleus following signalling via TLR ligands, antigen or cytokines. Pri-miRNAs are then processed within the nucleus to Pre-miRNA. This is regulated by a number of factors such as Drosha, which are themselves subject to regulation during inflammation. Pre-miRNA is exported to the cytoplasm by exportin 5. Within the cytoplasm pre-miRNA is processed by Dicer. This creates a miRNA duplex which must be unwound. The guide strand is incorporated into the RNA-induced silencing complex (RISC). Importin 8 transports the complex to the target 3' UTR. Image taken from {(O'Connell et al., 2010b).

6.1.2 miRNA functions in B cells

Regulation of several events in B-cell differentiation is thought to be under the control of miRNAs. This includes B-cell development in the bone marrow where miRNAs are required at the transition of pro-B cell to pre-B cell stages. In addition, in mature B cells micro-RNAs are involved in cell fate decisions and maintenance of the germinal centre phenotype, this will be discussed in more detail later. As well as these roles, miRNAs have been shown to have an important role in inflammation and response to TLR signalling.

In haematopoiesis, miR-181 is expressed in normal B-lymphoid cells in mouse bone marrow. Over-expression of miR-181 causes an increased number of B cells, but no increase in other cell types such as T cells or myeloid cells (Chen et al., 2004). In support of this role of miR-181 for B-cell differentiation, when Dicer is ablated in early B-cell progenitors there is a complete block in B-cell differentiation at the pro- to pre-B cell stage. This may be due to increased levels of the pre-apoptotic protein Bim (Koralov et al., 2008). In contrast when miR-150 is constitutively expressed there is a differentiation block at the pro-B to pre-B transition. Deficiency of miR-150 leads to an accumulation of B-1 B cells in the spleen and peritoneal cavity and a decrease in B-2 cells (O'Connell et al., 2010b). In mature B cells, miR-150 is expressed at a 10 fold lower level in GC B cells than in naïve and memory B cells (Tan et al., 2009) and it is responsible for reducing levels of c-myb, survivin and Foxp1 in B cells (Tan et al., 2009).

Several studies have aimed to define the miRNAs expressed in different mature B-cell populations (Basso et al., 2009; Tan et al., 2009) and many miRNAs have specific expression profiles. Germinal centre B cells express high levels of miR-17-5p, miR-181b (Tan et al., 2009; Zhang et al., 2009), miR-106a (Tan et al., 2009), miR-20b, miR-28 and miR-93 (Zhang et al., 2009). Moreover, miR-17-5p, miR-106a and miR-181b are expressed at higher levels in

dark zone centroblasts, than in light zone centrocytes. In addition, miR-125b is highly expressed in centroblasts located in the dark zone of the germinal centre (Gururajan et al., 2010) (Malumbres et al., 2009) (Tan et al., 2009). Memory cells in contrast, express high levels of miR-223 and this micro-RNA down-regulates the expression of the transcription factor LMO2. LMO2 is expressed in germinal centre B cells (Malumbres et al., 2009) (Zhang et al., 2009). During antigen-specific responses, miR-9 and members of the miR-30 family (miR-30b, miR-30d) block plasma-cell differentiation via repression of BLIMP1 (Zhang et al., 2009). miRNAs have also been reported to regulate AID protein, these include miR-155 (Teng et al., 2008; Vigorito et al., 2007), miR-361 (Basso et al., 2012) and miR-181b (de Yébenes et al., 2008). miR-155 also regulates PU.1 (Vigorito et al., 2007).

Dysregulation of micro-RNAs have been implicated in a number of B-cell cancers such as Burkitt lymphoma, diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL) (Zhang et al., 2009). For example, high expression of miR-222, miR-146b-5p, miR-146a, miR-21, miR-155, miR-500, miR-363, miR-574-3p and miR-574-5p have been detected in activated B-cell (ABC)-like DLBCL cell lines. In addition, high expression of miR-222 is correlated with inferior patient survival (Malumbres et al., 2009). miR-17-92 is over-expressed in acute lymphoblastic leukaemia (ALL) DLBCL and chronic myeloid leukaemia (CML) (O'Connell et al., 2010b) whilst miR-155 is overexpressed in CLL, DLBCL and acute myeloid leukaemia (AML)(O'Connell et al., 2010b).

6.1.3 miR-125b in B-cell differentiation and IRF4 repression

MiR-125b is a 22 nucleotide long, highly conserved micro-RNA found amongst mammals, vertebrates and nematodes. The seed region is unchanged throughout these species. There are three homologues of miR125: hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2. miR-125a is located on chromosome 17 in mice, whereas miR-125b-1 is located on chromosome 9

and miR-125b-2 is located on chromosome 16. miR-125b-1 and miR-125b-2 have differing pre-miRNA sequences but identical sequences post-DICER processing.

miR-125 is over-expressed in B-ALL with a translocation of t(11;14)(q24;q32) and has been shown to enhance proliferation by repressing B-cell regulator of immunoglobulin heavy-chain transcription (Bright)/ARID3a. This protein activates immunoglobulin heavy chain transcription. Repression of this gene blocks differentiation and provides a survival advantage. Caspase activation is blocked and therefore apoptosis is reduced when miR-125 is over-expressed (Puissegur et al., 2012). Another target of miR-125 in B-ALL that is involved in apoptosis, is *Trp53inp1,* a pro-apoptotic gene induced by cell stress (Enomoto et al., 2011).

Studies addressing the role of miR-125 in haematopoietic stem cells have been contradictory. Over-expression of miR-125 can skew cells towards the lymphoid lineage. This is characterised by an increase in the number of early B-progenitor cells in the spleen and a reduction in the mRNA expression of two pro-apoptotic genes: Bmf and KLF13 (Ooi et al., 2010). Other reports have found increased numbers of $CD11b^{+}$ $Gr\text{-}1^{+}$ myeloid cells after overexpression of miR-125 in bone marrow progenitors. (Enomoto et al., 2012). Additionally, studies have shown that in hepatocellular carcinoma (HCC) miR-125b actually suppresses proliferation and promotes apoptosis by inhibiting gene expression of Bcl-2 which is anti-apoptotic (Zhao et al., 2012). In addition the negative NFκB regulator *TNFAIP3* is a direct target of miR-125a and miR-125b in diffuse large B-cell lymphoma (DLBCL) and expression of these micro-RNAs increased levels of NFκB (Kim et al., 2012). All these studies use over-expression of miR-125 to define its role in the immune system, however this may render some of these results misleading, as the micro-RNA is not necessarily expressed at physiological levels, and the temporal expression as well as cell types which normally express it are different.

In mature human B cells, miR-125 is highly expressed in centroblasts within the germinal centre (Gururajan et al., 2010). This micro-RNA has been shown to bind to and down-regulate the expression of both IRF4 and BLIMP-1, which are both required for plasma-cell differentiation. Therefore miR-125 may be required for the maintenance of the germinal centre phenotype and preventing premature differentiation into plasma cells (Gururajan et al., 2010; Malumbres et al., 2009). The experiments described here aim not to study the effects of expression of miR-125 in a physiological situation, but rather to use the micro-RNA as a tool to repress IRF4 and study the effects of IRF4 absence.

Our aims during this project were:

1. To repress both early intermediate and late level IRF4 induction
2. To test whether AID protein and mRNA can be upregulated in a system where IRF4 is deficient
3. To test downstream Ig class switching after repression of IRF4 protein levels

To address these questions we utilised a system whereby miR-125 is over-expressed by a retroviral vector. We then performed cell transfer experiments and tested IRF4 and AID expression, Ig class switching and B-cell differentiation. These were tested by flow cytometry, immunohistochemistry, immunofluorescence, real-time RT-PCR and ELISA.

6.2 Results

6.2.1 Forced expression of miR-125b leads to splenic expansion

Due to the published role of miR-125b in the repression of IRF4 gene expression, bone marrow was infected *in vitro* with an MSCV-based retrovirus vector which was either empty (control) or contained a 500-bp gene segment for the miR-125b hairpin. Both vectors also co-express GFP to enable the sorting of successfully infected cells. Lethally irradiated C57 host mice were reconstituted with GFP^+ infected bone marrow and between five and eight weeks later, mice were culled and spleen and bone marrow analysed. As this micro-RNA is highly expressed in haematopoietic stem cells (O'Connell et al., 2010a; Ooi et al., 2010), we

questioned whether B-cell development in the bone marrow was altered when miR-125b is over-expressed. To test this, bone marrow was analysed by FC. Late pro-B cells express the markers B220, CD43, HSA and LY51 (Dautigny et al., 1996). B cells expressing these markers were found to be reduced in mice with forced expression of miR-125b compared to control mice (Figure 6.2A). In addition, percentages of immature B cells (IgM^+B220^+) were also reduced.

We next sought to test whether the reduced proportion of pre-B and immature B cells leads to fewer B cells in the spleen and whether the numbers of other myeloid and lymphoid cells in the spleen are normal, as there have been conflicting reports on the effect of miR-125b over-expression in haematopoietic cells within the bone marrow. Whilst miR-125b has been reported to cause a myeloid cell expansion (O'Connell et al., 2010a) with no effect on the lymphoid compartment, another study showed expansion in the lymphoid compartment, specifically of $CD8^+$ T cells (Ooi et al., 2010). Spleens were expanded when miR-125b was over-expressed (Figure 6.2B). Spleens from mice over-expressing miR-125b contain significantly more splenocytes than control spleens ($p=0.0039$), by a median value of 1.65x (Figure 6.2C). To address whether this could be due to increased numbers of lymphocytes, FC was performed. B-cell numbers were found to be unaltered in the spleens of miR-125b over-expressing mice compared to control spleens (Figure 6.2D). Likewise T-cell numbers were also similar between the two groups (Figure 6.2E). In contrast, $CD11c^+$ cell numbers are significantly increased ($p=0.0465$) by a median of 3x when miR-125b is over-expressed. CD11c is an integrin which binds to the complement fragment iC3b, fibrinogen, ICAM-1, ICAM-2, VCAM-1 (Sadhu et al., 2007), ICAM-4 (Ihanus et al., 2007), LPS (Ingalls and Golenbock, 1995) and collagen (Garnotel et al., 2000). This integrin is mainly expressed by dendritic cells, although it is capable of being expressed by inflammatory spleen monocytes (Drutman et al., 2012) and neutrophils under certain circumstances ((Loike et al., 1991)). In

addition, we found that over-expression of miR-125b led to significantly increased numbers of natural killer (NK) cells ($p=0.0043$) by a median of 75%. Therefore splenic expansion is partly due to increased cell numbers in both the lymphoid and myeloid compartments. However this does not account for the total increase in splenocytes, therefore other cell types may also be increased in number besides $CD11c^+$ and NK cells

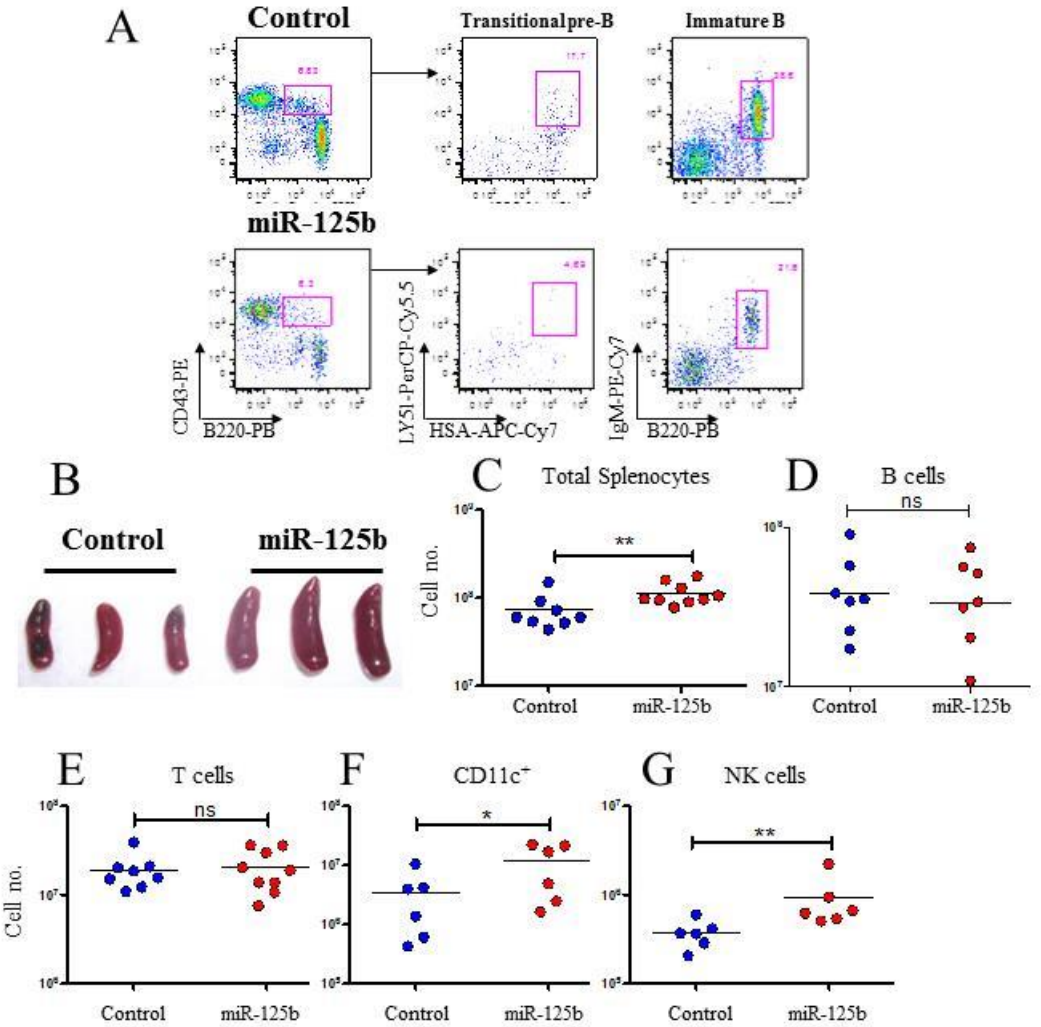


Figure 6.2: *Forced expression of miR-125b causes splenic expansion, but not greater numbers of lymphocytes.* Lethally irradiated C57 mice were reconstituted with empty vector or miR-125 retrovirus infected bone marrow. Between 5-8 weeks later spleens were analysed for myeloid and lymphoid cell development. **A:** B cell development within the bone marrow. Bone marrow cells were stained with labelled antibodies against B220, CD43, LY51, HSA and IgM. Representative FC plots are shown for bone marrow infected with empty vector (control, top) or miR-125b (bottom). Transitional B cells (left and centre) were gated as $B220^{+}CD43^{+}HSA^{+}LY51^{+}$. Immature B cells (right) were gated as $B220^{+}$ IgM^{+}. **B:** Spleens from empty vector infected mice (control, left) or miR-125b infected mice (right) were harvested. Picture shows one representative experiment. **C:** Total splenocyte numbers from control or miR-125b infected mice. **D:** Splenic B cells were quantified by flow cytometry with labelled antibodies against CD19. **E:** T cells were quantified by flow cytometry with labelled antibodies against CD3. **F:** $CD11c^{+}$ cells were quantified by flow cytometry. **G:** Natural killer cells (NK) were quantified by flow cytometry as $CD3^{-}NKp46^{+}NK1.1^{+}IL7Ra^{-}$.Each data point represents one mouse.
Data courtesy of Kyoko Nakamura (Unpublished).

6.2.2 Over-expression of miR-125b leads to enlarged red pulp volume following immunisation

We next aimed to test whether increased numbers of dendritic and NK cells led to an altered splenic architecture. C57 mice were lethally irradiated and reconstituted with GFP^+ infected bone marrow. Mice were immunised three times with NP coupled to chicken γ–globulin (NP-CGG) and spleens were processed four days after the final immunisation. IHC staining was performed with antibodies against antigen specific B cells and IgD^+ follicles (Figure 6.3A). Whilst mice which received control vector infected B cells have well defined IgD^+ follicles, mice infected with miR-125b have disorganised follicles and larger red pulp areas. Quantification from histology reveals miR-125b over-expression leads to significant expansion of the red pulp ($p=0.05$) by a median of 57% compared to control spleens (Figure 6.3B). B-cell follicles are significantly reduced as a proportion of the spleen area ($p=0.05$) by a median value of 73% when miR-125b is over-expressed (Figure 6.3C). The proportion of the spleen comprised of GC are not significantly reduced however (Figure 6.3D). However because miR-125b over-expression leads to larger spleens composed mainly of red pulp, this leads to a reduction in the relative GC area as a percentage of the spleen. Accordingly the absolute GC area is very similar (Figure 6.3E). Additionally, plasma cells per mm^2 of red pulp area are significantly reduced ($p=0.05$) by a median of 89% when miR-125b is over-expressed (Figure 6.3F). However this is most likely due to the larger red pulp area in miR-125b over-expressing mice.

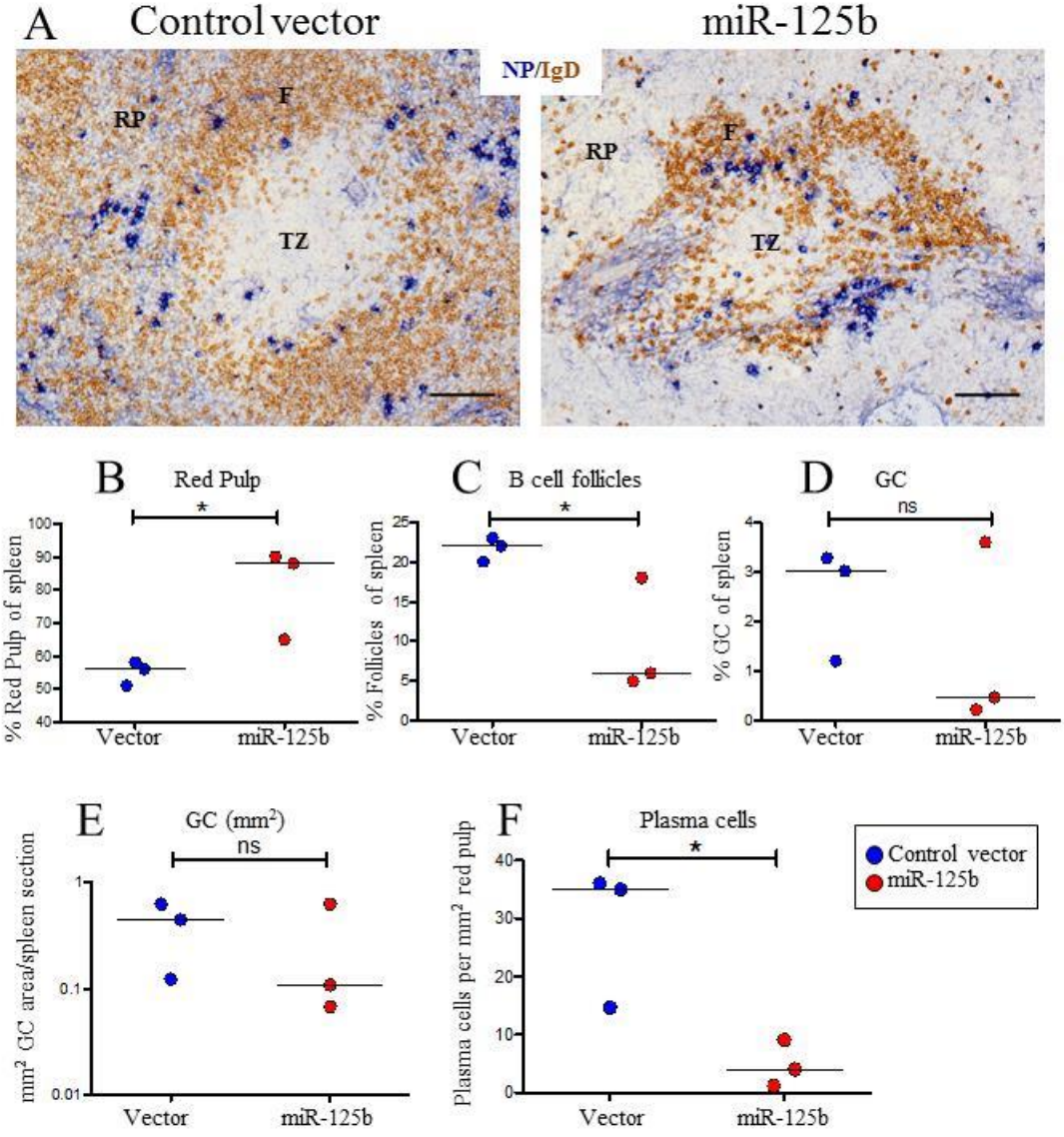


Figure 6.3: *Smaller B cell follicles, expanded red pulp and reduced numbers of plasma cells in mice over-expressing miR-125b.* C57 mice were irradiated and reconstituted with empty vector or miR-125 retrovirus transduced bone marrow. Five weeks later mice were immunised three times with 50µg of NP-CGG. Four days after the third immunisation, spleens were analysed for the generation of germinal centres and plasma cells. **A**: Spleen sections from mice that had received control bone marrow (left) or miR-125b over-expressing bone marrow (right). Sections were stained by immunohistochemistry for NP (blue) and IgD (brown). Scale bar indicates 100µm. TZ: t-zone F: follicles RP: red pulp. Four days after the final immunisation, histology sections were used to quantify the percentage of the spleen consisting of red pulp (**B**), IgD^+ follicles (**C**), NP^+ germinal centres (**D and E**) and the number of plasma cells (**F**). Each data point represents one mouse. The experiment was performed once.

6.2.3 Over-expression of miR-125b leads to a block in plasma-cell differentiation and larger germinal centres

To test the role of miR-125b on B cells undergoing an immune response, C57 wild-type mice were primed with CGG in alum. Five weeks later, mice received control or miR-125b infected QM B cells, followed by immunisation with NP-CGG twenty-four hours later. Mice were culled at selected timepoints after immunisation (Figure 6.4A). Control or miR-125b over-expressing donor cells were isolated from splenocytes using CD43 beads to enrich for resting B cells. These were FACS sorted for GFP expression before transfer and equal numbers of NP-binding B cells were transferred into host mice (Figure 6.4B). Priming hosts with CGG five weeks before cell transfer allows the generation of T-cell memory. Memory T cells are rapidly activated following immunisation to provide survival and differentiation signals to naïve NP-specific B cells. In this system, the B-cell response occurs with similar dynamics to the T-independent responses described earlier, with GC and extrafollicular plasma cells generated four days after immunisation. Extrafollicular plasma cell numbers peak at between four and five days after immunisation whereas GC continue to increase in size and reach a peak at 14 days after immunisation.

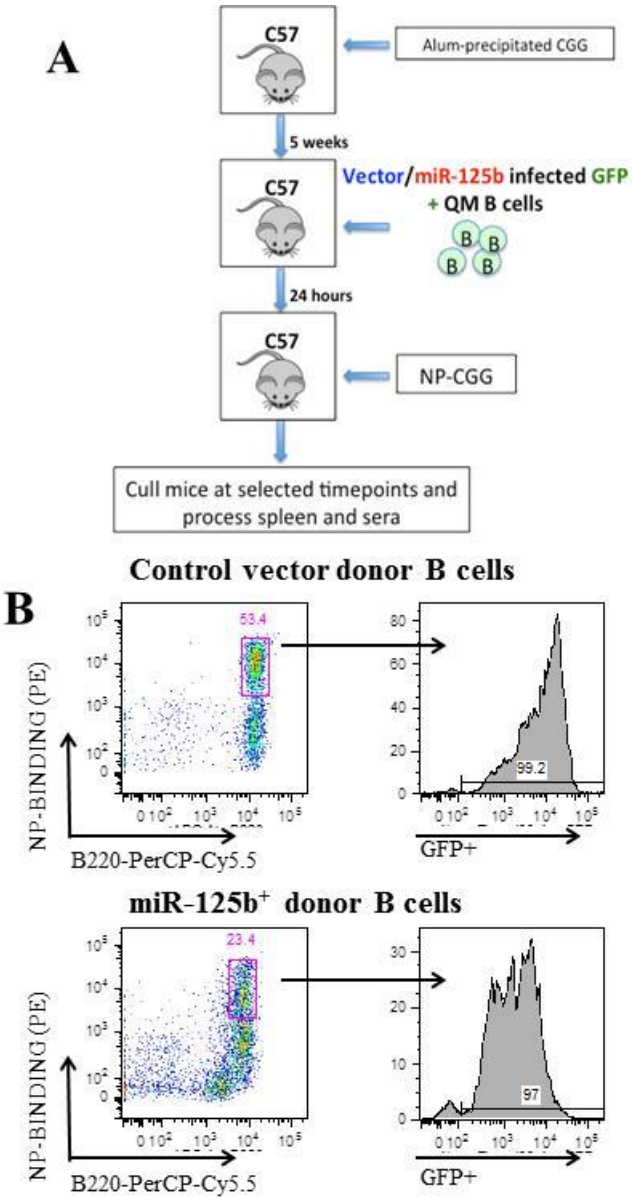


Figure 6.4: *Schematic of experimental design for T-dependent cell transfer response.* **A**: C57 mice were immunised with 50μg CGG in alum i.p. Five weeks later, the same mice received control or miR-125b over-expressing B cells from the spleen i.v. Spleens and sera were collected at selected timepoints. **B**: $CD43^-$ B cells were enriched from the spleens of control (top) or miR-125b infected donor mice (bottom) as in (A) using magnetic beads. Empty vector or miR-125b infected cells express GFP and enriched splenic B cells were sorted by FACS for GFP expression. A small number of $CD43^-$ GFP^+ cells were stained with antibodies against B220 and incubated with NP-PE to test the % of GFP^+ NP-binding B cells. Identical numbers of control or miR-125b over-expressing antigen-specific B cells were transferred into WT hosts.

To test whether B-cell activation and differentiation occur normally in this model, FC staining was performed on splenic B cells five and eight days after immunisation. To test the development of germinal centres, splenocytes were stained with antibodies against B220, FAS and GL7 and cells were incubated with NP-PE. GC B cells were gated as NP-binding, $B220^{high}$ cells expressing both FAS and GL7 (Figure 6.5A). Five days after immunisation, numbers of GC cells are similar whether mice received control cells or miR-125b over-expressing cells (Figure 6.5B). However, numbers of GC B cells are 1.5x higher ($P<0.05$) eight days after immunisation in mice receiving miR-125b over-expressing cells. While this increase seems very low, further experiments (described below) confirm an effect on the size of the germinal centre population due to overexpression of miR-125b.

GFP^{+} antigen-specific plasma cells were identified by their down-regulation of B220 and expression of CD138 (Figure 6.5C). In contrast to germinal centres, plasma cells are significantly reduced ($P<0.05$) by 80% five days after immunisation when B cells over-express miR-125b (Figure 6.5D). Extrafollicular plasma-cell numbers reach a peak during the TD response at between four and five days after immunisation before decreasing again due to onset of apoptosis in non-sustainable plasma cells. This is followed by a plateau phase with constant number of plasma cells sustainable by the splenic environment (Sze et al., 2000). Between five and eight days after immunisation, plasma-cell numbers in recipients of control infected QM B cells have decreased by a median of 52%. However plasma-cell numbers increase further between five and eight days after immunisation in mice which received miR-125b over-expressing B cells, reaching similar levels to control infected plasma cells by day 8. The further expansion of plasma cells after day 5 is probably due to plasma-cell numbers not having filled niches available in the spleen in recipients of miR-125b infected B cells by day 5 (Sze et al., 2000).

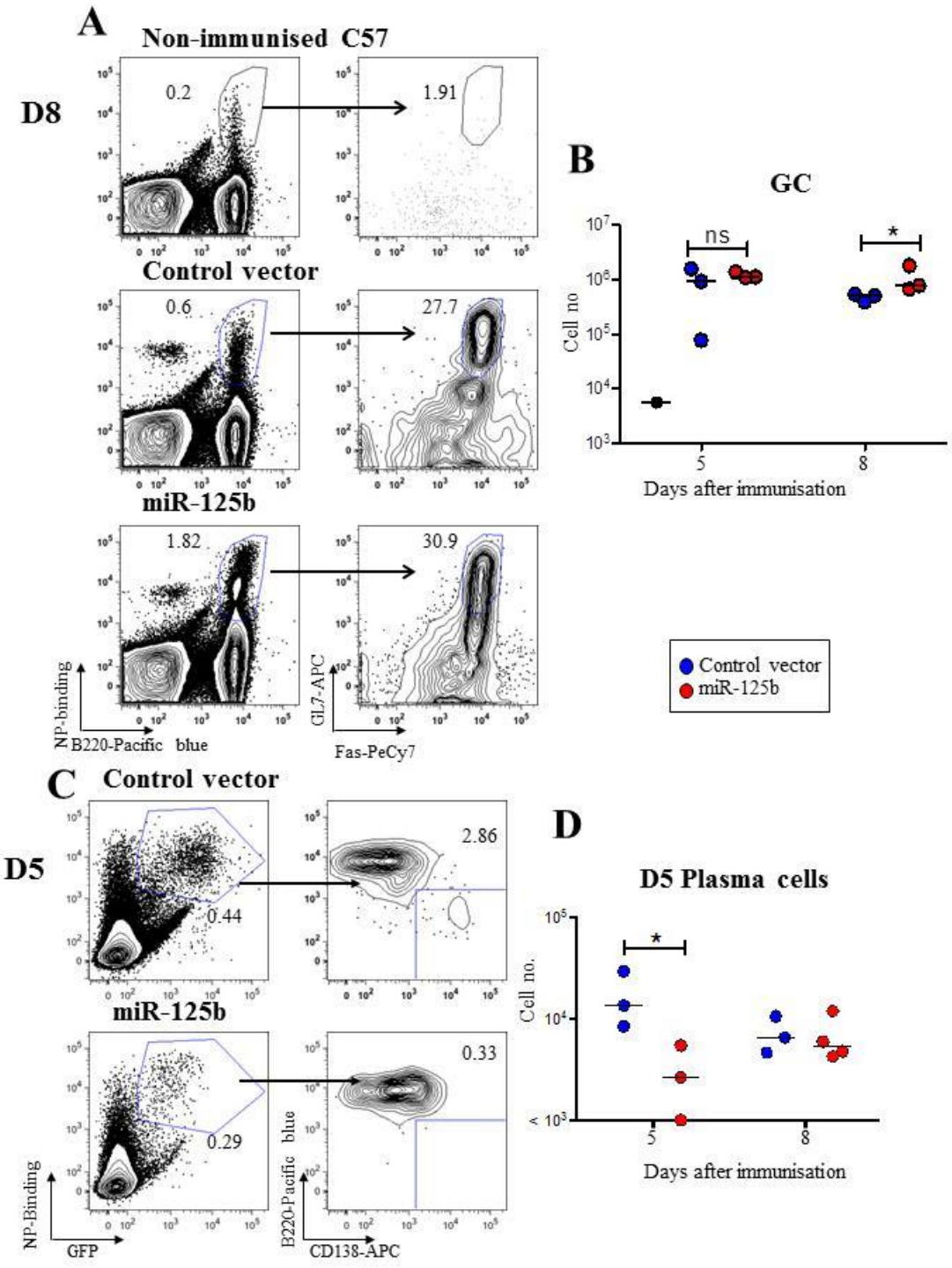

A
Non-immunised C57
D8
0.2
1.91
Control vector
0.6
27.7
miR-125b
1.82
30.9
NP-binding
B220-Pacific blue
GL7-APC
Fas-PeCy7
B
GC
ns
*
Cell no
Days after immunisation
Control vector
miR-125b
C
Control vector
D5
0.44
2.86
miR-125b
0.29
0.33
NP-Binding
GFP
B220-Pacific blue
CD138-APC
D
D5 Plasma cells
*
Cell no.
Days after immunisation

Figure 6.5: *miR-125b over-expressing B cells form larger germinal centres and produce fewer plasma cells than control B cells.* $CD43^-$ GFP^+ infected B cells were isolated by MACS column and transferred into wild-type hosts. 24 hours later, mice were immunised with NP-CGG i.p. **A**: Representative FC plots from either non-immunised C57 mouse (top), or eight days after immunisation with NP-CGG in mice which had received control QM B cells (centre) or miR-125b over-expressing QM B cells (bottom). Germinal centre B cells were identified by binding of NP-PE and by staining with labelled antibodies against B220, FAS and GL7. **B:** Absolute numbers of control (blue) or miR-125b over-expressing germinal centre cells five and eight days after immunisation gated as shown in (**A**). **C**: Representative FC plots of control B cells (top) or miR-125b over-expressing plasma cells (bottom) within the spleen, five days after immunisation. Plasma cells were identified as NP-binding, GFP^+ (left plot) and $B220^{low}$, $CD138^+$ **D**: Number of $CD138^+$ plasma cells within the spleen five and eight days after immunisation gated as in (**C**).

To confirm the results obtained by FC and to identify the location of transferred B cells within the splenic microarchitecture, spleens were analysed by IHC, four, five and eight days after immunisation. Antibodies against IgD and the QM BCR (idiotype) were used to detect B-cell follicles and transferred B cells. Five days after immunisation, small germinal centres are detectable in mice that received control B cells and in those receiving miR-125b over-expressing B cells (Figure 6.6A top panel). Mice receiving control B cells have also formed plasma-cell foci five days after immunisation (Figure 6.6A, arrow), whereas plasma cells are infrequent in mice receiving miR-125b over-expressing B cells. Eight days after immunisation, numbers of extrafollicular plasma cells have decreased, whereas germinal centres remain visible (Figure 6.6A bottom panel). Following quantification of tissue sections stained as in Figure 6.6A, germinal centre sizes four and five days after immunisation were found to be similar between mice which received miR-125b over-expressing B cells and mice which had received control B cells (Figure 6.6B and C). However, eight days after immunisation GCs from mice receiving miR-125b infected B cells are significantly larger ($p=0.0383$), by a median of 100% (Figure 6.6C).

In contrast to germinal centre sizes, miR-125b infected plasma-cell numbers are significantly decreased throughout the response ($p=0.0383$) compared to control B cells. While control plasma-cell numbers decrease after day 5, due to onset of apoptosois of non-sustainable B cells (Sze et al., 2000), there is a slight increase in plasma-cell numbers from miR-125b overexpressing B cells between day 5 and day 8. This is probably due to plasma-cell niche space (Sze et al., 2000) still available for expansion of the few plasma cells that differentiate despite over-expressing miR-125 (Figure 6.6E).

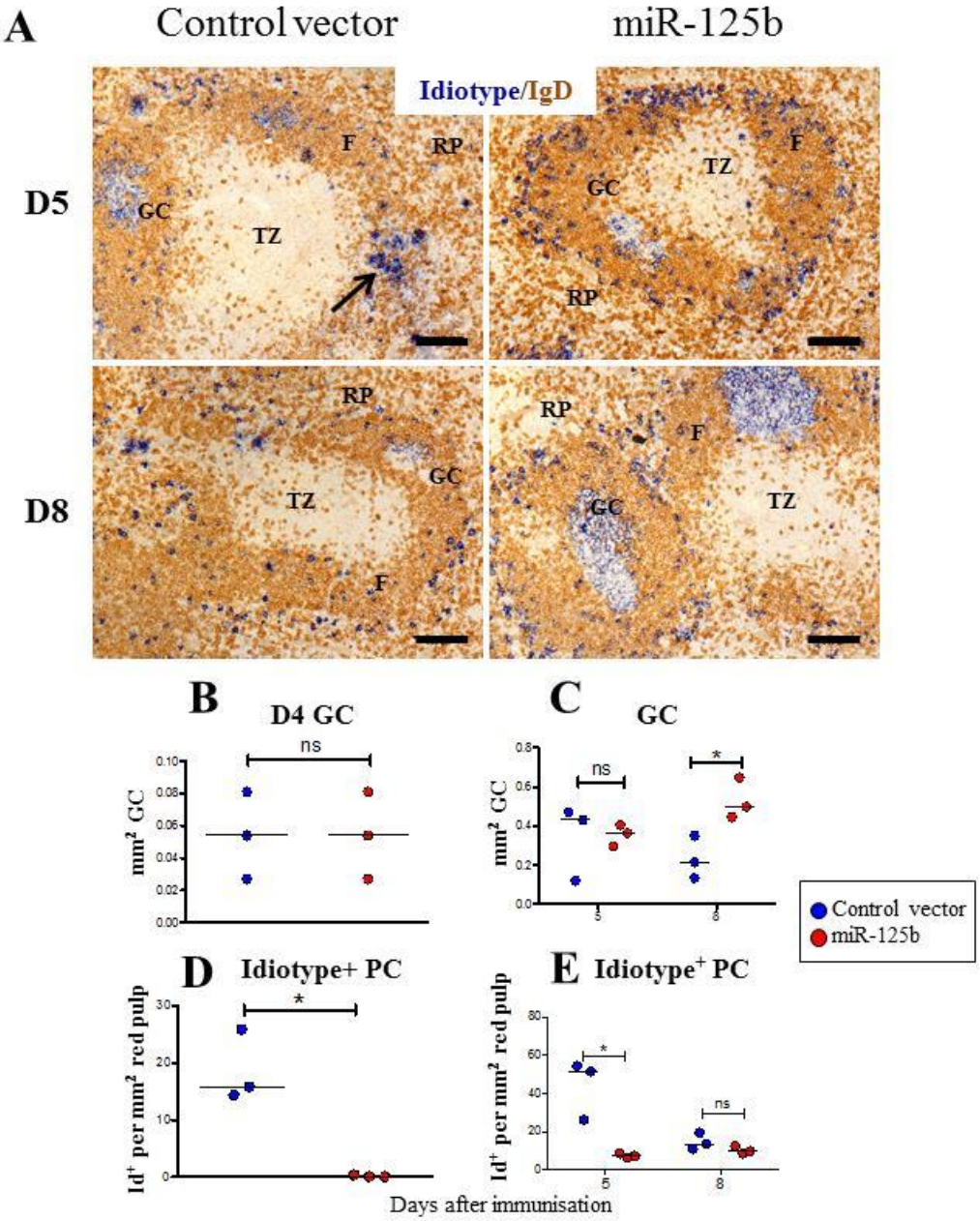

A
Control vector
miR-125b
Idiotype/IgD
D5
D8
F
RP
GC
TZ
B
D4 GC
ns
mm² GC
C
GC
ns
*
mm² GC
Control vector
miR-125b
D
Idiotype+ PC
*
Id+ per mm² red pulp
E
Idiotype+ PC
*
ns
Id+ per mm² red pulp
Days after immunisation

Figure 6.6: *miR-125b over-expressing B cells form larger germinal centres and produce fewer plasma cells than control B cells.* $CD43^-$ GFP^+ infected B cells were isolated by MACS and transferred into wild-type hosts. 24 hours later, mice were immunised with NP-CGG i.p. **A**: Spleen sections from mice which had received control B cells (left) or miR-125b over-expressing cells (right) were cut and stained for the QM BCR (idiotype) and IgD at five days after immunisation (top) and eight days after immunisation (bottom). Scale bar indicates 100μm. **B**: Four days after immunisation ID^+ germinal centres sizes were quantified from histology. **C**: $Idiotype^+$ germinal centre sizes 5 and 8 days after immunisation were measured from tissue sections. **D:** $Idiotype^+$ plasma cells were quantified within the red pulp. from IHC staining four days after immunisation. **E**: $Idiotype^+$ plasma cells were counted on tissue sections 5 and 8 days after immunisation and expressed as the number of cells per mm^2 of red pulp area. Each data point represents one mouse. Data are representative of at least two experiments. Data from four days after immunisation and data from 5 and 8 days after immunisation are from two separate experiments.

6.2.4 B cells over-expressing miR-125b locate to the marginal zone of the spleen

Within four days of immunisation, B cells have differentiated into germinal centre cells or plasma cells. However some idiotype$^+$ cells are located in the outer follicles (Figure 6.7A). At four and five days after immunisation, numbers of these cells are similar (Figure 6.7B and C), however, eight days after immunisation there are significantly more of these cells in the spleens of mice which received miR-125b over-expressing B cells (Figure 6.7C, increase=89% P=0.05). Memory B cells locate to the marginal zone and follicles during T-dependent responses (Anderson et al., 2007) and are visible as early as four days after immunisation when T-cell help is readily available (Liu et al., 1988). As activated B cells also initially migrate to the outer follicles (Gatto and Brink, 2013), IF staining was used to test whether the idiotype$^+$ cells visible eight days after immunisation are located within the marginal zone or the outer follicles. The marginal zone is defined as a ring of IgD$^-$ IgM$^+$ B cells separated from the follicles by a ring of MOMA-1 expressing macrophages which are localised at the marginal sinus (Kraal and Janse, 1986). NP-binding, QM BCR expressing transferred cells were found to be located preferentially outside of the ring of MOMA-1 macrophages (Figure 6.7D) within the IgD$^-$ IgM$^+$ marginal zone (Figure 6.7E). Upon closer investigation, transferred cells within the marginal zone were found to be largely IgM$^+$ but not IgD$^+$ whether they were infected with control vector or miR-125b (Figure 6.8). Antigen-specific memory B cells have been shown to be IgD$^-$ IgM$^+$ and express marginal zone markers (LIU et al., 1988), whereas recirculating cells which transit through the marginal zone are IgD$^+$.

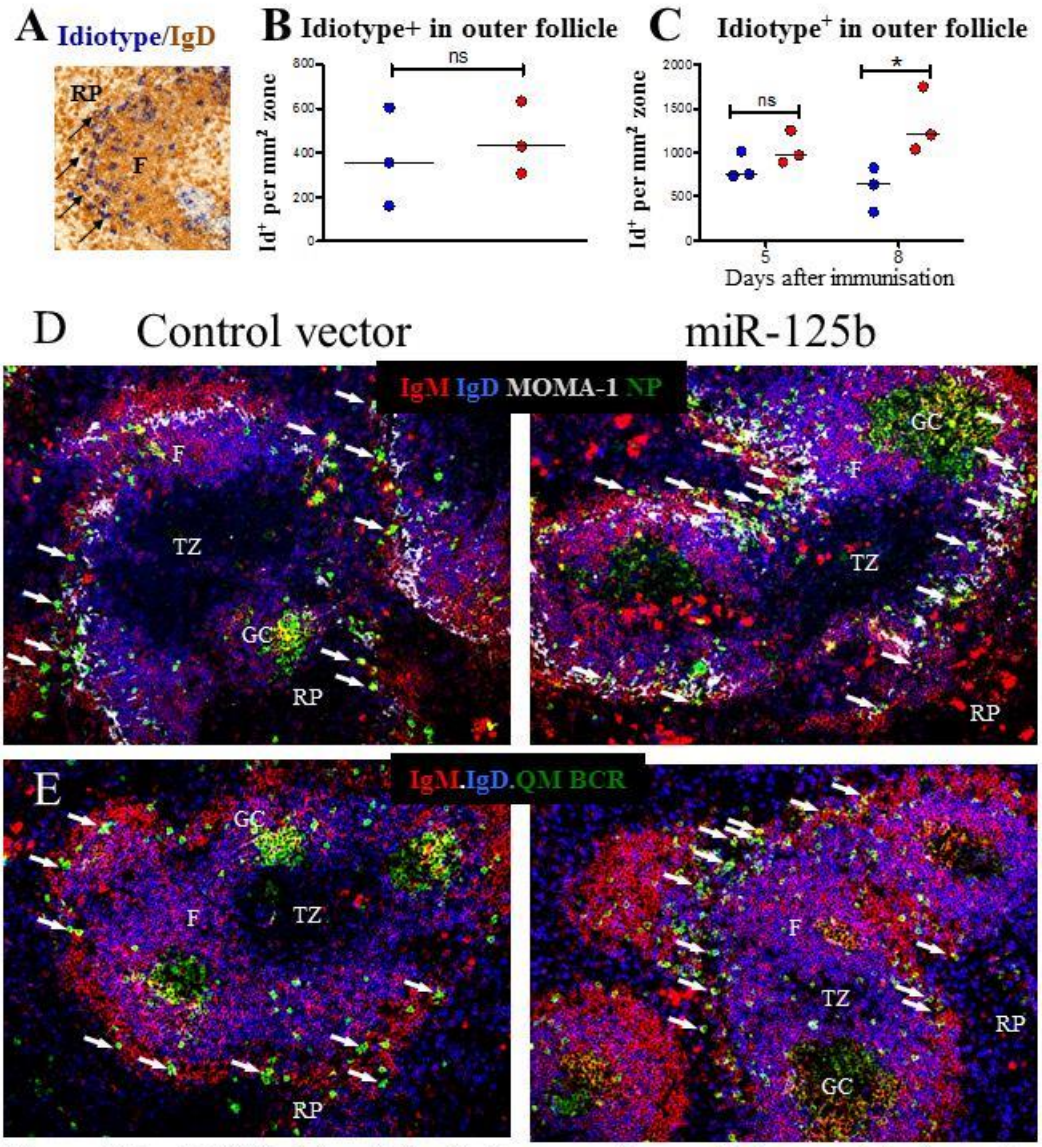


Figure 6.7: *miR-125b infected B cells locate to the marginal zone eight days after immunisation*. Control or miR-125b infected B cells were transferred into CGG primed C57 mice. 24 hours later mice were immunised with NP-CGG i.p. **A:** ID$^+$ cells in the outer follicle were quantified four days after immunisation following IHC staining of spleens with antibodies against the QM BCR and IgD. **B**: Idiotype$^+$ cells located in the outer follicle were measured as before on spleen sections four days after immunisation and (**C**): 5 and 8 days after immunisation. Each data point represents one mouse. Data are representative of at least two experiments.

D and **E**: Eight days later, spleens from mice receiving control B cells (left) or miR-125b over-expressing cells (right) were collected and stained with labelled antibodies against IgM (red), IgD (blue), MOMA-1 (grey) and NP (green) (**D**) or IgM (red), IgD (blue) and the QM BCR (idiotype, green)(**E**). RP: red pulp, TZ: T zone, F: follicle, GC: germinal centre.

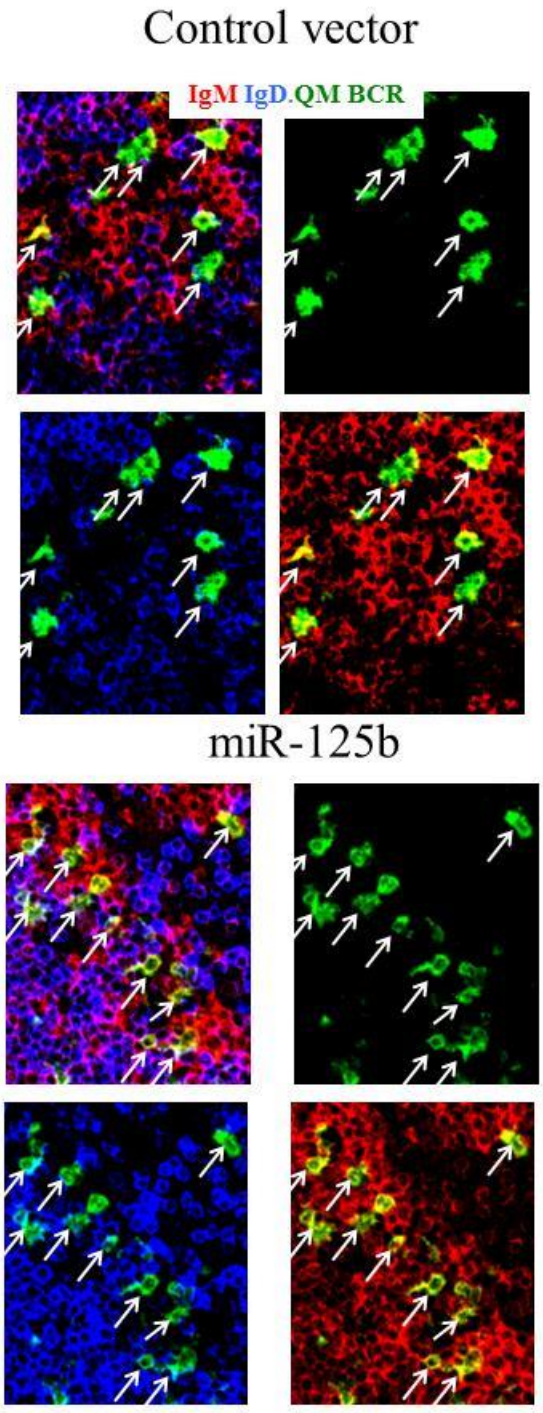


Figure 6.8: *miR-125b infected B cells locate to the marginal zone eight days after immunisation and are IgM$^+$ IgD$^-$*. Control (top) or miR-125b infected B cells (bottom) were transferred into CGG primed C57 mice. 24 hours later mice were immunised with NP-CGG i.p. Eight days later, spleens were collected and stained with labelled antibodies against IgM (red), IgD (blue), and the QM BCR (idiotype, green).

To test whether over-expression of miR-125b increases the number of memory cells we tested the expression of memory cell markers in the blood. Memory cells can be detected within the blood as early as one week after primary immunisation (Blink et al., 2005). There are few markers available for memory B-cell identification, however a larger proportion of memory B cells than naïve antigen-specific cells express the cell surface proteins FAS (CD95), CD80 and CD62L. In addition, memory B cells express a higher quantity of these proteins on their surface (Anderson et al., 2007). GFP^+ cells in the blood (derived from control cells or miR-125b over-expressing cells) were more numerous in mice which received miR-125b over-expressing B cells (Figure 6.9A) These cells were tested by FC for expression of memory markers five days after cell transfer and immunisation with NP-CGG (Figure 6.9A). The majority of antigen-specific B cells in the blood were found to express high levels of CD80 (Figure 6.9B), CD62L (Figure 6.9C) and FAS (Figure 6.9D) compared to endogenous non-activated B cells. miR-125b over-expressing B cells within the blood expressed similar levels of CD80 and CD62L protein on their surfaces compared to control infected B cells (Figure 6.9B and C). However miR-125b over-expressing B cells did express significantly more FAS protein ($p=0.05$, increase=64%) (Figure 6.9D). This indicates that miR-125b may affect the generation of memory cells. In contrast, eight days after immunisation the proportion of 'memory-type' cells in the blood were found to be similar (data not shown).

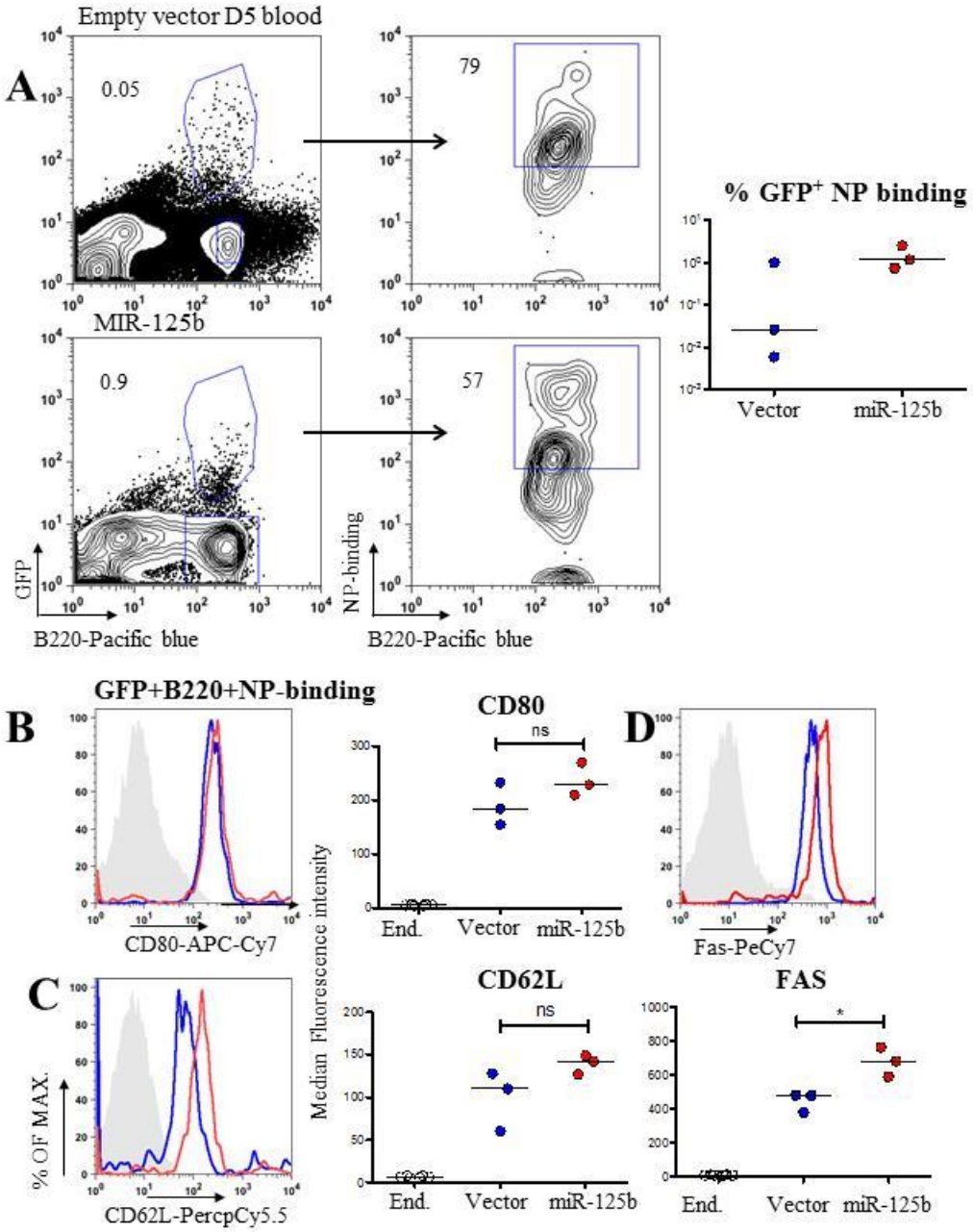


Figure 6.9: *miR-125b over-expressing B cells in the blood adopt a memory phenotype.* CD43^{-} GFP^{+} infected B cells were isolated by MACS column and transferred into wild-type hosts. 24 hours later, mice were immunised with NP-CGG i.p. **A**: Representative FC plots from the blood of mice which received control infected (top) or miR-125b over-expressing B cells (bottom) five days after immunisation with NP-CGG. Transfer cells were identified by their expression of GFP and B220 (left) and binding of antigen (centre). Right hand plot shows the % of antigen-specific GFP^{+} B cells in the blood. Antigen-specific transfer cells were then tested for their expression of CD80 (**B**), CD62L (**C**) and FAS (**D**) and protein levels are expressed by the median fluorescence intensity. Endogenous cells were gated as GFP^{-}, B220^{+}, NP^{neg}. Each data point represents one mouse.

6.2.5 miR-125b directly regulates IRF4 mRNA and protein levels

In human GC B cells, miR-125b is highly expressed in centroblasts and can inhibit IRF4 and BLIMP-1 expression in human cell lines (Gururajan et al., 2010; Malumbres et al., 2009). Malumbres et al (2009) show repression of IRF4 at the protein level, whilst mRNA levels of IRF4 were minimally affected and mRNA transcripts for BLIMP1 were actually increased. To confirm that miR-125b reduces protein levels of IRF4, a luciferase reporter assay was performed using HEK293 cells. Both miR-125b-1 and miR-125b-2 were shown to reduce levels of IRF4 (Figure 6.10A) by approximately 23%. Interestingly miR-125a shows a strong reduction of IRF4. Similarly to miR-125b, this micro-RNA is also expressed in human centroblasts, although at much lower quantities, but has never before been shown to bind BLIMP-1 or IRF4 (Gururajan et al., 2010).

Repression of IRF4 by miR-125b has never been shown in murine B cells. To investigate whether miR-125b over-expression is capable to repress IRF4 expression *in vivo*, we FACS sorted antigen-specific B cells two days after immunisation from the spleens of mice that received control or miR-125b over-expressing B cells. These were tested for their expression of *Irf4* transcripts (Figure 6.10B). Two days after immunisation, *Irf4* transcripts are significantly upregulated in control infected B cells compared to non-activated B cells ($p=0.0076$). At the same time miR-125b over-expressing B cells contain significantly less *Irf4* mRNA transcripts compared to control B cells ($p=0.0325$).

Transcript levels are not always directly proportional to protein concentration; therefore to test whether decreased mRNA transcripts lead to reduced translation to IRF4 protein, FC staining was performed. Transferred cells were identified as GFP^+ NP^-binding and $B220^+$ (Figure 6.10C). Two days after immunisation protein levels of IRF4 are 61% lower in miR-

125b infected B cells compared to control infected B cells (p=0.05) (Figure 6.10D). Four days after immunisation, IRF4 protein levels are still decreased by 84% (p=0.0143) in miR-125b over-expressing B cells. Taken together this shows that miR-125b decreases mRNA transcripts of *Irf4* and this leads to reduced translation of IRF4 protein.

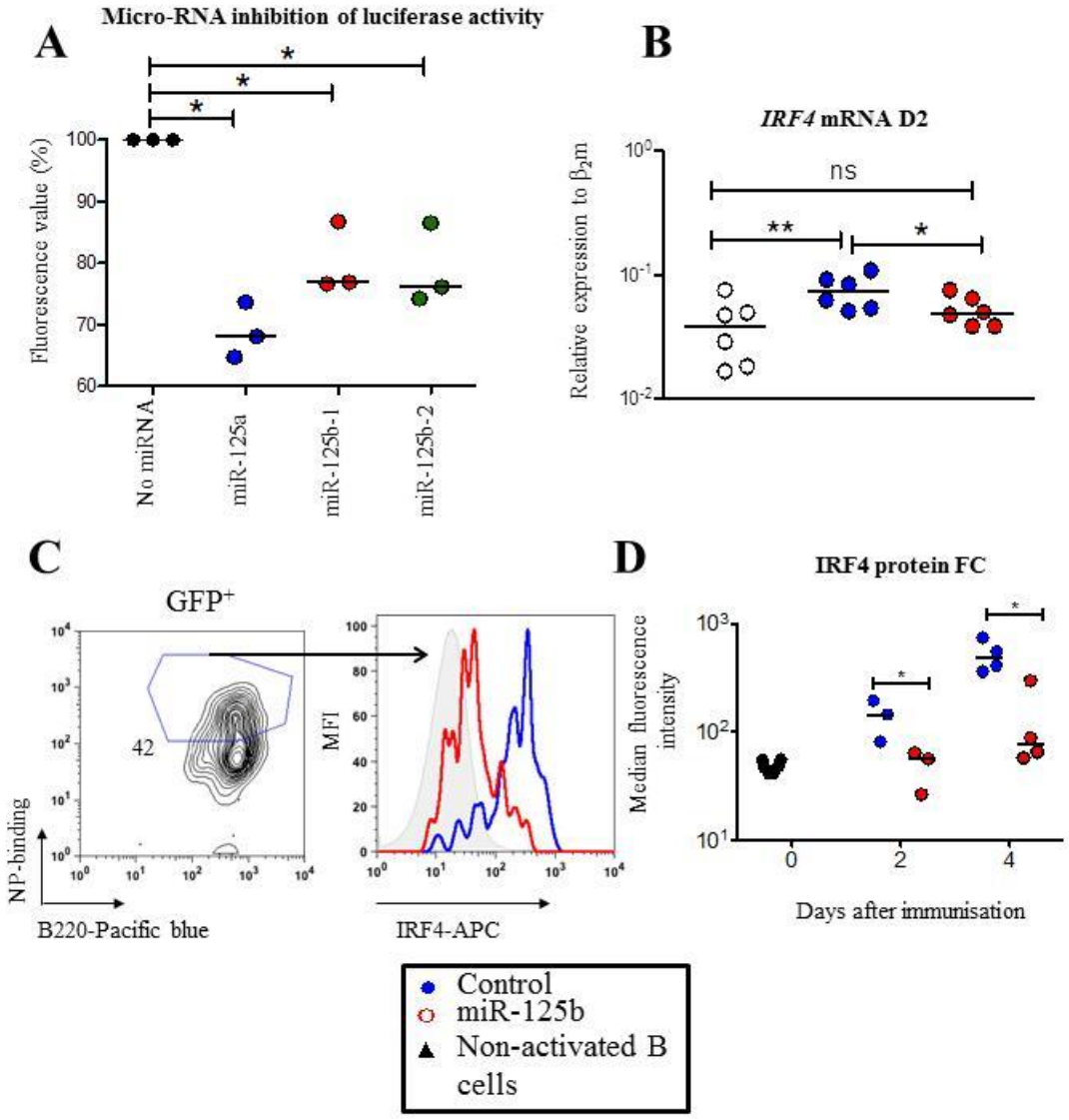


Figure 6.10: *miR-125b blocks both phases of IRF4 induction.* **A**: Renilla/firefly dual luciferase reporter assay for micro-RNA inhibition of IRF4 performed by Juergen Wittmann. Each point represents one independent experiment. **B**: Control (blue) or miR-125b infected (red) B cells were FACS sorted two days after transfer into CGG primed hosts and immunisation with NP-CGG. Non-activated B cells were sorted as GFP^- $B220^+$ and compared to GFP^+ $B220^+$ NP-binding B cells. IRF4 expression is related to β_2M. **C**: Representative histogram of IRF4 expression in $B220^+$ NP-binding control cells (blue) or miR-125b over-expressing cells (red) two days after transfer into CGG primed hosts and immunisation with NP-CGG . **D**: Median fluorescence intensity of IRF4 protein in control cells (blue) or miR-125b over-expressing cells gated as in (C). Each data point represents one mouse. Data are representative of two experiments.

6.2.6 Repression of IRF4 by miR-125b leads to decreased *Aicda* mRNA and AID protein levels

Our hypothesis was that the early intermediate phase of IRF4 protein expression is required for the upregulation of AID protein and Ig class switching. As shown in chapter 3, AID mRNA and protein become detectable as early as 48 hours after B-cell activation. As we found that miR-125b repressed the expression of IRF4 protein at all stages of its bimodal expression, we questioned whether this led to decreased *Aicda* transcripts and AID protein levels. MiR-125b over-expressing and control B cells were FACS sorted two, five and eight days after immunisation with NP-CGG. Plasma cells were excluded. We found that two days after immunisation *Aicda* mRNA levels were significantly reduced by a median of 86% ($p=0.05$) when B cells over-express miR-125b, compared to mock-infected control cells. *Aicda* transcripts remain lower throughout the response five and eight days after immunisation. To test whether this leads to reduced expression of AID protein, tissue sections were fluorescently labelled with antibodies against IgD, AID and the QM BCR to identify transferred cells (Figure 6.11B). Whilst AID protein is detectable within mock-infected B cells, very little AID protein is detectable when miR-125b is over-expressed in B cells (Figure 6.11C). This is a significant decrease in AID protein (Figure 6.11D) ($p=<0.0001$) by a median value of 10%.

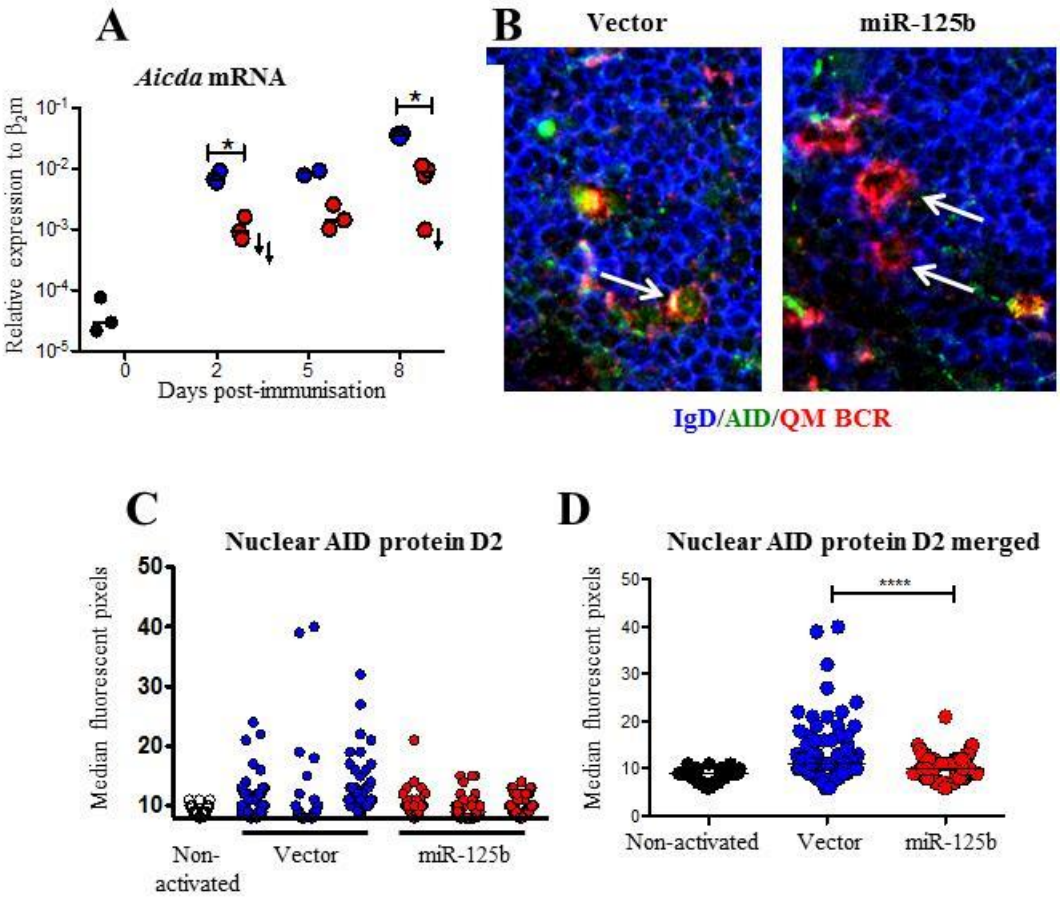


Figure 6.11: *Aicda expression in activated B blasts*. Control (blue) or miR-125b infected (red) B cells were transferred into CGG primed hosts and immunised with NP-CGG. **A**: Control infected (blue) or miR-125b infected B cells were sorted after transfer into CGG primed hosts. B cells were stained for FC with antibodies against B220, FAS, CD138 and incubated with NP-PE. Two days after immunisation, GFP^+ B cells which expressed B220 and bound NP-PE were sorted from spleens. Five and eight days after immunisation GFP^+ cells with a GC phenotype ($B220^+FAS^+$, $CD138^-$ and NP-binding) were sorted from the spleen. Each data point represents one mouse. Arrow indicates that true value is as stated or lower.
B: Two days after immunisation, spleens were stained with labelled antibodies against IgD (blue), AID (green) and the QM BCR (red). **C**: AID protein levels were quantified in individual cells from immunofluorescence staining as in (B). Each data point represents one cell from n=3 for each group. **C**: AID protein levels quantified from IF staining as in (B) each data point represents one cell from three mice per group.

6.2.7 Inhibition of early stage IRF4 and AID leads to reduced extrafollicular class switching

Over-expression of miR-125b leads to diminished plasma-cell responses. To test whether CSR is reduced, serum titres of switched antibodies and NP-specific IgM[a] were tested. A difference of the ratio of these would indicate a block in Ig class switching. Two days after immunisation NP-specific IgM is largely undetectable, whereas four days after immunisation, titres have increased in the sera of all mice (Figure 6.12A). Interestingly, sera from mice which received miR-125b over-expressing B cells actually contain significantly higher levels of anti-NP IgM antibodies despite containing fewer plasma cells ($p=0.0454$, increase= 67%). Titres of anti-NP IgM are similar however eight days after immunisation with NP-CGG whether mice received control or miR-125b over-expressing B cells (Figure 6.12A). Titres of total NP-specific IgG become detectable four days after immunisation and are further increased eight days after immunisation (Figure 6.12B). Four days after immunisation, sera obtained from mice receiving miR-125b over-expressing B cells contains similar titres of anti-NP IgG compared to sera from mice receiving control infected B cells. However eight days after immunisation, sera from mice that received miR-125b over-expressing B cells contains significantly less anti-NP IgG compared to mice receiving control B cells ($p=0.0383$ decrease=61%). To test for development of higher affinity, NP-specific IgG, binding to NP_2-BSA coated ELISA plates was tested. NP-specific high affinity IgG molecules become detectable eight days after immunisation (Figure 6.12C). These are produced at similar titres in mice receiving control B cells and mice receiving miR-125b over-expressing B cells. When the ratio of high affinity IgG to total IgG is compared there is no significant difference in affinity when miR-125b is over-expressed in B cells on day 8 (Figure 6.12D). Therefore transfection of B cells which leads to IRF4 inhibition and AID reduction does not vastly affect affinity maturation.

CSR is tested by measuring the ratio of IgG to IgM. Whilst this ratio is not significantly altered four days post-immunisation, the ratio is significantly decreased in the sera of mice receiving miR-125 infected B cells eight days post-immunisation (Figure 6.12E). Increased titres of anti-NP IgM were not due to higher numbers of miR-125b infected B cells being transferred into wild-type hosts as four and eight days after immunisation, numbers of NP-binding GFP^+ cells in the spleens detected by FC are similar between the two groups (Figure 6.12F). Likewise eight days after immunisation numbers of NP-specific infected cells are not significantly different between the two groups.

To exclude NP-specific antibodies produced by endogenous plasma cells, ELISAs were repeated with QM allotype specific antibodies. IgM^a, expressed by QM B cells, becomes detectable four days after immunisation. Until eight days after immunisation, IgM^a titres are similar between the two groups (Figure 6.12G). $IgG1^a$ titres in contrast are significantly reduced at both four ($p=0.0407$ decrease=52%) and eight days ($p=0.05$ decrease=70%) post-immunisation in sera from mice that received miR-125b infected B cells (Figure 6.12H). The reduced ratio of $IgG1^a$ to IgM^a fig. 6.12I shows that there is reduced switching until eight days after immunisation. Interestingly IgG1 switch transcripts are induced normally when B cells are infected with miR-125b (Figure 6.12J).

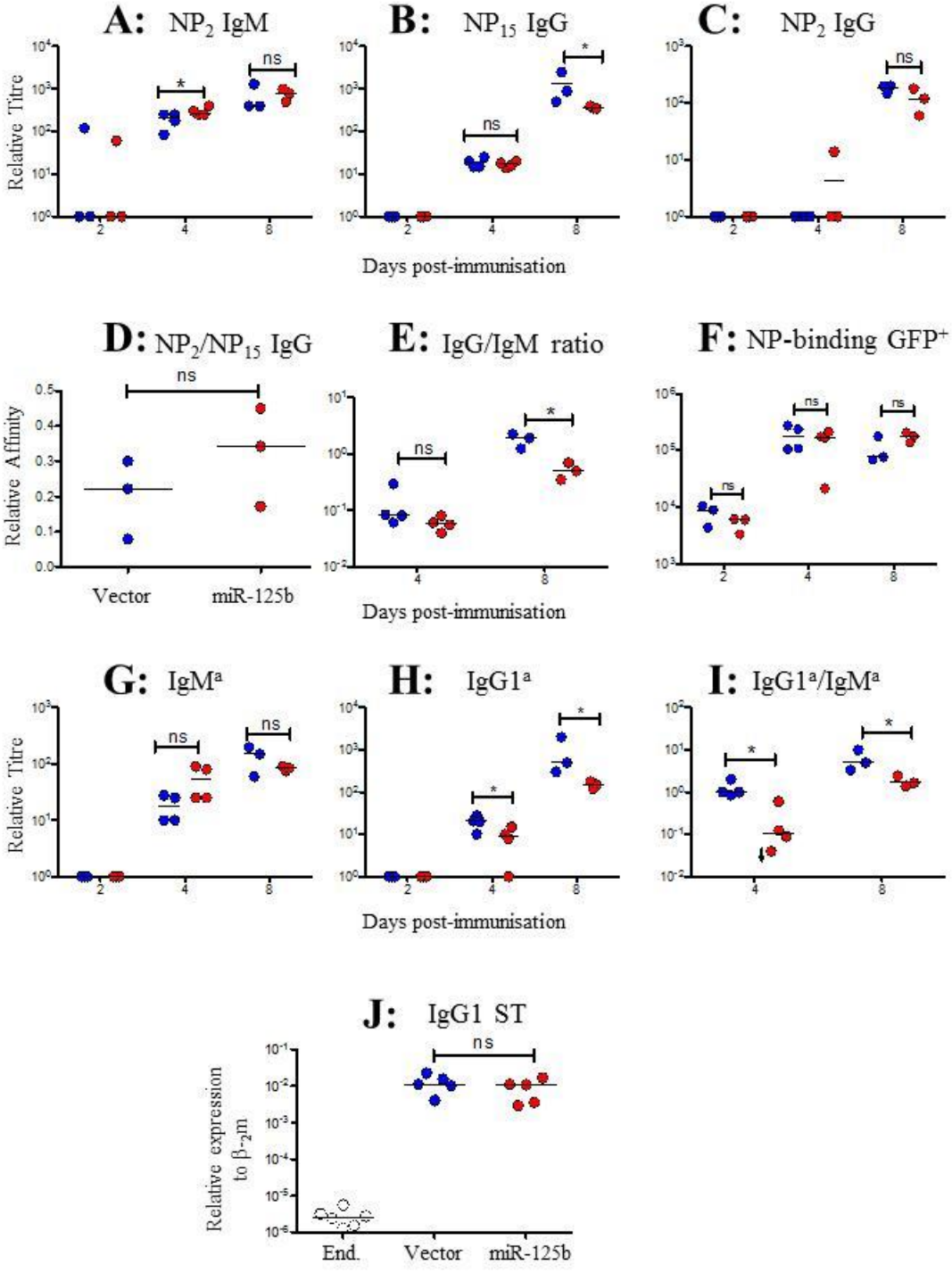

A: NP2 IgM
B: NP15 IgG
C: NP2 IgG
Relative Titre
Days post-immunisation
D: NP2/NP15 IgG
E: IgG/IgM ratio
F: NP-binding GFP+
Relative Affinity
Vector
miR-125b
G: IgMa
H: IgG1a
I: IgG1a/IgMa
J: IgG1 ST
Relative expression to β-2m
End.
ns

Figure 6.12 *Class switching is impaired when B cells over-express miR-125b.* Control (blue) or miR-125b infected B cells (red) were transferred into CGG primed hosts and immunised with NP-CGG. **A:** IgM binding to NP_2-BSA. **B**: IgG binding to NP_{15}-BSA. **C**: IgG binding to NP_2-BSA, **D**: Relative affinity: ratio of IgG binding to NP_2/IgG binding to NP_{15}. **E:** Ratio of IgG binding to NP_{15}-BSA compared to IgM binding to NP_2-BSA. **F:** Numbers of GFP^+ NP-Binding B cells were quantified by FC. **G**: IgM^a binding to NP_{15}-BSA, **H:** $IgG1^a$ binding to NP_{15}-BSA, **I:** Switching ratio of IgM^a to $IgG1^a$. **J**: IgG1 heavy chain germline transcripts in sorted control vector or miR-125b infected B cells two days after immunisation. Each data point represents one mouse. Data are representative of x2 experiments.

6.2.8 Inhibition of IRF4 and AID protein induction leads to reduced Ig class switching in situ

To test whether reduced AID protein leads to a reduction in Ig class switching in situ, spleen sections were analysed by IF. Control infected or miR-125b over-expressing plasmablasts within the extrafollicular foci were tested for expression of IgM or IgG five days after immunisation. During the carrier primed NP-CGG response, IgG1 (Toellner et al., 1996) is the major isotype which B cells switch to. Sections were stained with antibodies against IgM, IgG and the QM BCR to identify transferred cells that had switched to IgG production (Figure 6.13A). The proportion of IgM expressing cells was significantly increased in miR-15b over-expressing B cells compared to control infected B cells (increase=1.5%, p=0.05) (Figure 6.13B). In addition, the proportion of IgG expressing cells is significantly reduced when mice over-express miR-125b (p=0.0129, decrease=40%) (Figure 6.13C). This leads to a decreased ratio of Ig class switching when miR-125b is over-expressed (p=0.05 decrease=75%) (Figure 6.13D).

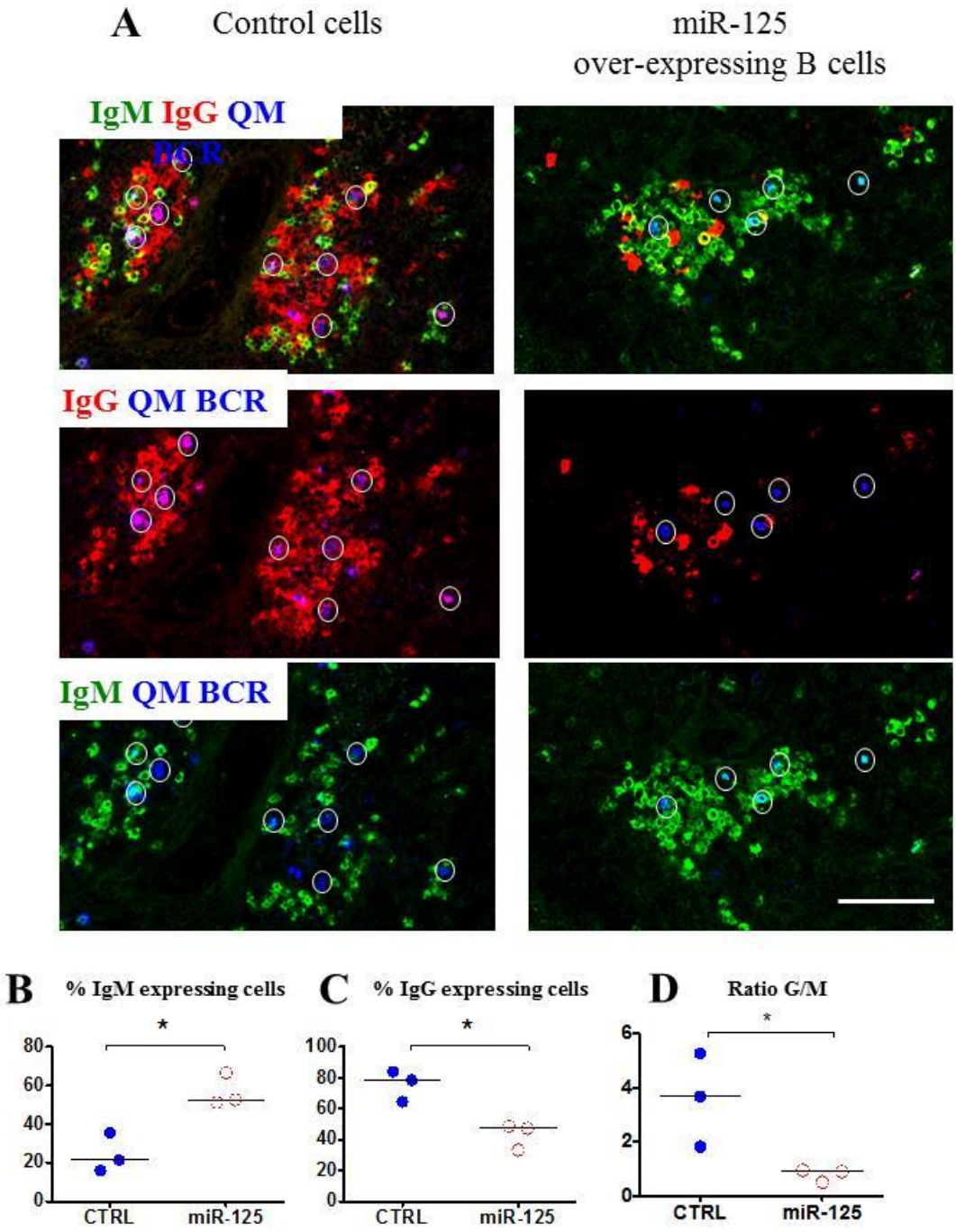

A
Control cells
miR-125
over-expressing B cells
IgM IgG QM
BCR
IgG QM BCR
IgM QM BCR
B
% IgM expressing cells
80
60
40
20
0
CTRL
miR-125
C
% IgG expressing cells
100
80
60
40
20
0
CTRL
miR-125
D
Ratio G/M
6
4
2
0
CTRL
miR-125

Figure 6.13 *Class switching is impaired when B cells over-express miR-125b*. Control or miR-125b infected B cells were transferred into CGG primed hosts and immunised with NP-CGG. **A:** Five days later spleens from mice receiving control cells (top) or miR125b over-expressing cells (bottom) were stained with labelled antibodies against IgM (green), IgG (red), and the QM BCR (blue). **B and C:** Ig class switching within control (blue) or miR-125b infected B cells (red) was quantified from fluorescently stained sections and expressed as the percentage of cells expressing IgM (**B**) and the percentage of cells expressing IgG (**C**). Circles indicate QM BCR expressing transferred cells. Scale bar indicates 50μm.

6.2.9 IRF4 does not regulate AID expression within the germinal centre

AID protein expression is also required for affinity maturation and Ig class switching within the germinal centre reaction. We found that affinity maturation was unaffected when B cells over-express miR-125b (Figure 6.12D) yet AID mRNA was reduced in the germinal centre population at all timepoints tested (Figure 6.11A). Therefore we tested induction of AID at the protein level. Spleen sections were tested for expression of AID protein within germinal centre B cells. Four days after immunisation germinal centres derived from miR-125b infected B cells express similar levels of AID protein (Figure 6.14A). Therefore IRF4 intermediate level expression is dispensable for the regulation of AID within the germinal centre. The few IRF4int cells in the light zone (Figure 6.14B) are not likely to be the cells that go on to express AID.

6.3 Summary

We found that overexpression of miR-125b lead to a reduction in early plasma-cell generation four and five days after immunisation. In contrast, numbers of germinal centre B cells were increased eight days after immunisation in mice overexpressing miR-125b. Additionally numbers of memory-type cells within the marginal zone of the spleen accumulated with time after immunisation and memory cells within the blood were increased. Furthermore, overexpression of miR-125b in B cells was found to repress all stages of IRF4 expression at the mRNA and protein level. This leads to reduced *Aicda* transcripts and reduced AID protein levels in B cells. Moreover, reduced AID protein levels resulted in reduced CSR frequency within the extrafollicular plasmablast population.

In contrast, affinity maturation was not affected by IRF4 repression and AID protein levels appeared normal within the GC compartment. This indicates that AID protein is not regulated by IRF4 within the GC compartment, indeed the majority of GC B cells do not express IRF4 protein.

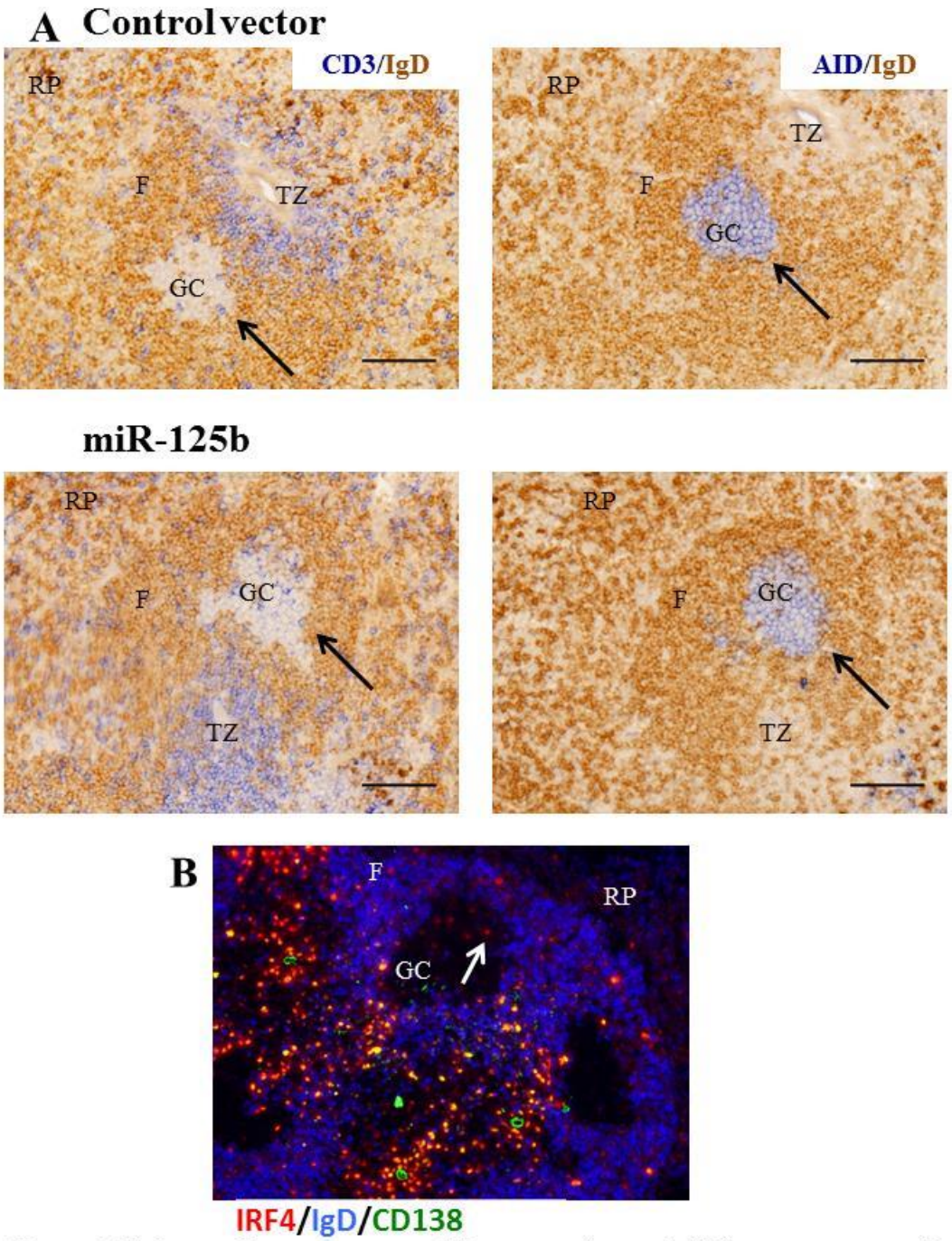


Figure 6.14 *Germinal centres express AID protein when miR-125b is over-expressed.* **A:** Control or miR-125b infected B cells were transferred into CGG primed hosts and immunised with NP-CGG. **A:** Four days later spleens from mice receiving control cells (top) or miR125b over-expressing cells (bottom) were stained with antibodies against IgD (brown) in conjunction with CD3 (left) or AID (right). Scale bar indicates 100μm. **B:** IRF4^{int} cells in the light zone of a WT mouse, image courtesy of Yang Zhang. GC: Germinal centre, TZ: Tzone, F: follicle, RP: red pulp.

7: DISCUSSION

7.1 Divergent GC and extrafollicular responses

Following binding of antigen via their surface Ig, B cells migrate from the marginal zone or follicles to the border between follicles and T zone where they are able to interact with antigen-primed T cells (Liu et al., 1991; Toellner et al., 1996). This induces proliferation of B blasts and later migration to different regions of the spleen occurs (Maclennan et al., 2003). B blasts differentiate into germinal centre cells within the follicles whereas extrafollicular B blasts differentiate within the extrafollicular regions of the spleen. Whilst extrafollicular foci produce fast, generally non-mutated antibody, the slower, GC pathway is important for the long-term production of affinity-matured antibody. It is largely unknown which signals decide these divergent cell fates. This thesis is largely concerned with differentiation via the extrafollicular pathway and Ig CSR within this population.

7.2 Extrafollicular Ig CSR

CSR is a process which occurs at a high frequency within the GC (Liu et al., 1996). However class switched antibody is generated during TI-II responses that do not induce GC (García De Vinuesa et al., 1999) and in responses which produce non-productive GC (Vinuesa et al., 2000), also during the carrier-primed response to TD antigen γ1 switch transcripts are detectable within 12 h of secondary immunisation, while B cells still reside in the outer T zone and before GC are formed (Toellner et al., 1996) and during a primary T-dependent response, switch transcripts are detectable within three days of immunisation while GCs appear after day 5 (Toellner et al., 1998). The slightly later induction of Ig switch transcripts during the primary T-dependent response is due to the requirement for T-cell priming, which delays the B-cell response. Marshall *et al* (Marshall et al., 2011) have shown directly that Ig

CSR occurs before formation of the GC in activated B blasts . These B blasts express *Aicda* in the absence of Bcl6 and are the precursors of switched plasmablasts produced during the extrafollicular T-independent response (Marshall et al., 2011). In this manner *Aicda* induction within activated B blasts may be responsible for the small amount of extrafollicular Ig gene hypermutation that occurs in some autoimmune responses (William et al., 2002) as well as during the QM TI-II response (Toellner et al., 2002).

T-independent class switching can be induced not only by polysaccharide antigens, but also by viral proteins such as the latent membrane protein 1 (LMP1) of Epstein-Barr virus. LMP1 induces class switching to C_γ, C_α, and C_ε via upregulation of BAFF and APRIL on B cells (He et al., 2003). These TNF family members and their receptors have been shown previously to be crucial for T-independent responses (Bülow et al., 2001; Stein et al., 2002). DCs and monocytes express BAFF and APRIL and this induces Ig CSR to C_γ, and/or C_α in the presence of IL-10 or TGF-β (Litinskiy et al., 2002). T-independent class switching to IgA is also common within the intestine (Fagarasan and Honjo, 2003) and class switching to IgA can be induced *in vitro* in the absence of T cells if lamina propria stromal cells are present (Fagarasan et al., 2001) This process requires secretion of TGF-β (Cazac and Roes, 2000; Coffman et al., 1989; Sonoda et al., 1989; van Ginkel et al., 1999) which may originate from lamina propria stromal cells.

7.3 IRF4 for B-cell differentiation

7.3.1 Regulation of AID, GC response and Ig CSR

IRF4 is a transcription factor which binds DNA with high affinity when interacting with members of the Ets family (PU.1 or SpiB) in B cells (Brass et al., 1999; Eisenbeis et al., 1995; Nagulapalli and Atchison, 1998). IRF4 has been proposed to have multiple functions in B cells. A role for IRF4 in Ig CSR was first proposed when it was noted that stimulation of B cells with CD40 and IL-4, which induces CSR rather than plasma-cell differentiation, lead to

upregulation of IRF4 protein (Gupta et al., 1999). Mice lacking IRF4 failed to upregulate AID and had defective production of class switched antibody (Sciammas et al., 2006; 2011). Mixed bone marrow chimeras of $IRF4^{WT}$ and $IRF4^{KO}$ cells demonstrated that $IRF4^{KO}$ B cells failed to form $BCL6^{+}$ GC cells (Ochiai et al., 2013). In addition, mice which lacked IRF4 in the B-cell compartment (CD19-cre) had impaired GC responses but normal B-cell maturation (Ochiai et al., 2013).When $IRF4^{KO}$ hen egg lysozyme (HEL)-specific mice were immunised with HEL-SRBC, they failed to form GC and mRNA transcripts of *BCL6*, *Obf1*, which encodes Bob-1, and *Aicda* were reduced, whilst PAX5 mRNA was unaffected (Ochiai et al., 2013). Interestingly Bob-1 is important in B-cell signalling as its direct target is Btk, required for signalling via the BCR (Brunner, 2006). IRF4 bound DNA with high affinity when interacting with members of the Ets family (PU.1 or SpiB) or E2A, however the PU.1/SpiB interaction surface was dispensable for class switching and AID did not appear to be a direct target of IRF4 (Sciammas et al., 2006). This is in contrast to chicken cells where PU.1 and IRF4 in conjunction have been shown to directly regulate AID (Luo and Tian, 2010). In addition, whilst partial rescue of CSR occurred with retroviral AID expression, retroviral expression of IRF4 was more efficient at normalising CSR (Sciammas et al., 2006) indicating that this effect is not direct. This defect in CSR was not due to locus accessibility as germline switch transcripts for Cγ1 were induced normally but Iμ-Cγ1 post switch transcripts were reduced in $IRF4^{KO}$ B cells (Klein et al., 2006; Sciammas et al., 2006).

7.3.2 Regulation of terminal plasma-cell differentiation

IRF4 has also been shown to be crucial for plasma-cell differentiation and was associated with downregulation of BCL6 and activation of BLIMP-1 (Klein et al., 2006; Saito et al., 2007; Sciammas et al., 2006). Immunising $IRF4^{KO}$ hen egg lysozyme (HEL) specific mice with HEL-SRBC lead to a failure to form PC and mRNA transcripts of *Prdm1* were reduced (Ochiai et al., 2013). Immunisation with HEL of varying affinities for the HyHEL10 BCR demonstrates that higher affinity drives B cells to $IRF4^{high}$ plasma cells (Ochiai et al., 2013).

Strong signalling via the BCR has been shown previously to induce higher numbers of extrafollicular plasmablasts (Paus et al., 2006). This was found to be due to greater proliferation of this B-cell population (Chan et al., 2009)

These functions of IRF4 appear contradictory, since AID and IRF4 are components of mutually antagonistic gene regulatory networks. AID protein is expressed within the GC (Muramatsu et al., 1999), the site of B-cell somatic hypermutation and Ig class switching (Liu et al., 1996). With the exception of a few cells in the light zone (Falini et al., 2000), germinal centre B cells do not express IRF4 protein or mRNA. Conversely, AID is downregulated in plasma cells that express high levels of IRF4 (Marshall et al., 2011).

This apparent discrepancy can be explained by the dual expression pattern of IRF4. We have shown that following immunisation of QMxC57BL/6 mice with NP-Ficoll, IRF4 mRNA levels peak within all blasts within one hour. Furthermore B blasts within the follicles and marginal zone express *intermediate* levels of IRF4 protein within one hour of activation. Within 4 hours, B blasts migrate to the border between the follicle and the T zone. This level of IRF4 protein is lower than the expression detected within plasmablasts and precedes the first expression of AID mRNA and protein. Furthermore, BCR stimulation alone is sufficient for this rapid upregulation of IRF4, as purified B cells stimulated *in vitro* upregulate IRF4 mRNA and protein within 30 minutes of stimulation. Interestingly AID mRNA and protein are detectable within 48 hours of activation, before germinal centres or plasma cells have formed, whilst B cells are located in the inner/outer follicle. Furthermore we detected co-expression of IRF4 and AID protein in activated B blasts two days after activation. As these B blasts do not express *Bcl-6* mRNA (Marshall et al., 2011) this suggests that Ig class switching is regulated in a different manner in B blasts compared to GC B cells. Cells expressing lower

levels of IRF4 protein than found in plasma cells have been reported previously *in vitro* following BCR stimulation (Sciammas et al., 2011) and this was associated with CSR. However Sciammas et al (2013) proposed that the intermediate phase of IRF4 induction is important for GC B-cell differentiation and CSR within the GC. We show that intermediate level IRF4 is upregulated in all B blasts regardless of whether they go on to differentiate into germinal centre or extrafollicular plasma cells and this occurs immediately after activation, long before GC are formed.

Two models have been proposed for the regulation of BLIMP1 and AID by IRF4. These are the basic bistability model, and the kinetic control model. In the former the initial IRF4 production rate does not affect the time spent in a CSR state. In the latter model, all cells pass through a transient CSR state, the duration of which is set by the initial IRF4 production rate. This in turn is set by the strength of the BCR signal. Stronger stimulation through the BCR leads to greater IRF4 protein expression and less time spent in the transient CSR state (Sciammas et al., 2011). Experiments manipulating the strength of signaling through the BCR indicate that stronger BCR signaling does induce a higher concentration of IRF4 (Sciammas et al., 2011). We were unable with our model to test whether the production rate of IRF4 affected the time spent in CSR, this will be an interesting question to address in future. This could be tested by using mice with different BCR affinities for antigen, such as B1-8^{hi} and B1-8^{low} B cells (Shih et al., 2002), these have BCR with a 40-fold difference in affinity for NP. Activated antigen-specific cells could be sorted within the first hours of activation and IRF4 mRNA and protein levels tested for differences in production rate. This could be followed by testing of the timing of AID induction which is required for extrafollicular CSR. With the first model, any variation in B-cell IRF4 production betwen the two mouse strains should not affect the timing of AID induction or the frequency of CSR. In the latter model

B1-8^{hi} B cells would induce AID earlier than B1-8^{low} cells and/or demonstrate higher CSR frequency.

To further test the role of intermediate-level IRF4 protein for AID induction and Ig class switching, we used a number of approaches. We used mice lacking signalling molecules or over-expressing the micro-RNA miR-125b. Within these models we measured induction of both early intermediate-level IRF4 and late high-level IRF4. This was correlated with induction of AID protein and Ig CSR.

7.4 Induction of high-level IRF4 protein and plasma-cell differentiation and GC formation via NFκB1

NFκB1/p50 had been shown to facilitate B-cell signalling, both via the BCR, and by ligation of CD40 (Francis et al., 1998). In addition, NFκB1 was involved in the induction of IRF4 protein expression (Berberich et al., 1994; Grumont and Gerondakis, 2000; Saito et al., 2007). We found that blocking signalling through NFκB1 inhibits plasma-cell generation and germinal centre formation in NFκB1 deficient mice. Four days after immunisation of NFκB1^{KO} mice, antigen-specific B cells are located in the follicles and express intermediate-high levels of IRF4 protein, whereas wild-type B cells differentiate into germinal centre IRF4-low cells or form IRF4-high plasma-cell foci.

The absence of low-level IRF4 expression four days after immunisation of NFκB1^{KO} mice is due to a defect in germinal centre formation. Germinal centre B cells do not express IRF4, with the exception of some individual IRF4^{high} cells located in the light zone. The majority of these light zone IRF4^{high} cells express BLIMP-1. These are probably positively-selected plasmablast precursors which are exiting the germinal centre (De Silva et al., 2012). IRF4^{high} cells could also be identified on the border between the GC dark-zone and the T zone, and

these cells express plasmablast markers and probably represent migration of plasma cells out of the GC (Zhang, Y unpublished).

Cell transfer experiments reveal that inhibition of germinal centre formation is partly due to a defect in the stroma, and partly due to a B-cell defect. FDC are essential for the formation of germinal centres due to their production of chemokines such as CXCL13 (Cyster et al., 2000) and their capture and display of opsonised antigen via complement and antibody receptors (Sukumar et al., 2008). Defects in these cells are most likely responsible for the lack of germinal centres in NFκB1 mice (Ferguson and Corley, 2005; Victoratos et al., 2006), indeed we found that FDC networks fail to differentiate following immunisation and that immune complex trapping by FDC is reduced in the absence of NFκB1. In addition, NFκB1^{KO} B cells produce smaller germinal centres when transferred into wild-type hosts. Taken together with the failure of NFκB1^{KO} B cells to downregulate IRF4, this may indicate that NFκB1 is required for the transcriptional network leading to IRF4 downregulation in the germinal centre. Homodimers of the NFκB1 subunit p50 act as transcriptional repressors (Zhong et al., 2002) due to their lack of a transactivation domain. Homodimers of p50 have been shown to form a complex with the histone deacetylase HDAC-1 and these complexes bound DNA to supress expression of NFκB target genes (Zhong et al., 2002). NF-κB1 deficient mice have been reported to form small germinal centres ten days after SRBC immunisation (Weih et al., 2001). Although we did not observe germinal centre formation following immunisation of QM NFkB1 deficient mice with NP-Ficoll, small non-specific germinal centres are present pre-immunisation, indicating that NFκB1^{KO} B cells are capable of germinal centre formation. These may develop more slowly, or may only differentiate in response to TD antigen. CD40L signalling is crucial for generation of GC during the TD response (Castigli et al., 1994; Chirmule et al., 2000; Kawabe et al., 1994; Xu et al., 1994) whereas in the absence of T-cell derived signals during the TI-II response, signalling via FDC may be of more

significance. CD40 has two other ligands which are C4BP, a protein which binds to the activated complement component C4b (Brodeur et al., 2003) and the heat shock protein (Hsp70) from Mycobacterium tuberculosis (Lazarevic et al., 2003). Germinal centre formation during the TI-II response has been shown to require CD40 but not T-cell derived CD40L and blocking interactions between complement C4 and FDC lead to decreased GC volumes (Gaspal et al., 2006). Therefore due to decreased immune complex trapping on FDC, a lack of C4BP is the most likely reason that TI-II antigen induced GC do not develop in NF-κB1 deficient mice. Reduced C4BP in NFκB1^{KO} spleens could be confirmed by IHC and real time RT-PCR. In addition, an agonistic anti-CD40 antibody could be administered following immunisation of NFκB1^{KO} mice, in order to test whether the defect in GC formation could be rescued by signaling through CD40.

The lack of IRF4^{high} cells, in contrast, is due to the absence of plasma cells. NFκB1 deficient B cells arrest at an intermediate/intermediate-high level of IRF4 expression, which seems insufficient for further differentiation towards plasma cells, except in a very few B cells. Lack of plasma-cell differentiation leads to reduced titres of both non-switched and switched antibodies. This is similar to previous studies following immunisation of NFκB1 deficient animals with NP_{15}-CG (Sha et al., 1995).This defect is B-cell intrinsic. However, the ratio of switched to non-switched antibody and therefore the efficiency of CSR is not reduced in the absence of NFκB1.

7.5 NFκB1^{KO} B cells display impaired proliferation and survival

NFκB1^{KO} B cells transferred into wild-type hosts undergo higher levels of apoptosis two days after activation. This is supported by previous studies which show that NFκB1^{KO} B cells had impaired survival (Sha et al., 1995; Snapper et al., 1996). This may occur via a number of pathways; signalling via NFκB1 was required for the degradation of the pro-apoptotic protein Bim (Banerjee et al., 2008). In addition, signalling through BAFF-R activated NFκB1 and

increased protein levels of the anti-apoptotic protein Bcl-X_L (Hatada et al., 2003). In contrast, apoptosis is not increased in NFκB1^{KO} B cells, three days after activation. Signalling via NFκB1 may not be required for B-cell survival at this phase of B-cell differentiation but does appear to be required for IRF4 induction and plasma-cell differentiation. Conversely, proliferation of NFκB1^{KO} B cells is decreased only at three days after immunisation. Although there are fewer cells derived from the transferred NFκB1^{KO} population, the percentage these transferred cells which become plasma cells is less.

B cells require a number of factors to differentiate into antibody secreting cells. B cells from NFκB1 deficient mice do not migrate from the follicles. Positioning of B cells within the white pulp is required for plasma-cell differentiation, as demonstrated by studies with EBI2 deficient B cells. Lack of migration to the extrafollicular region lead to a failure to form plasmablasts (Gatto et al., 2009) in EBI2^{KO} mice. Extrafollicular plasmablasts produced in a GC-independent manner are usually located around the bridging channels connecting T zones with the red pulp. Migration to this location may be essential for access to DC derived factors such IL-6 and APRIL, which promoted plasma-cell differentiation and survival (Mohr et al., 2009).

The absence of high-level IRF4 expression in NFκB1^{KO} B cells led us to investigate whether induction of early, intermediate level IRF4 was normal when NFκB1 was absent.

7.6 Intermediate level IRF4 expression is sufficient of Ig class switching in mice lacking NFkB1.

We found no significant difference in this phase of IRF4 induction both at the mRNA and the protein level. This is supported by previous studies (Grumont and Gerondakis, 2000). In addition, NFκB1 deficient B cells migrate normally to the border with the T zone and express activation markers to a similar or greater degree than NFκB1 wild-type cells. This indicates

that NFκB1 is not required for the early phase of IRF4 induction and that these two phases are regulated differently.

As high-level IRF4 is blocked when NFκB1 was absent, but intermediate level IRF4 was unaffected, we tested whether this was sufficient for AID induction and Ig class switching. Following differentiation in a wild-type environment, two days post-activation we found that NFκB1^{KO} B cells locate within the follicles and upregulate nuclear AID protein to the same level as found in NFκB1^{WT} B cells. This was confirmed with FC staining of AID protein.

Within the few NFκB1^{KO} cells that become plasma cells, we found that Ig class switching is normal. There is no reduction in the IgG3/IgM ratio by ELISA, ELispot or within extrafollicular plasma cells differentiating in situ on tissue sections. In addition IgG3 germline switch transcripts are normally induced within two days of immunisation in NFκB1^{KO} B cells, as tested following cell transfer and immunisation. In summary, whilst deficiency of NFκB1 leads to a selective defect in plasma-cell differentiation and high-level IRF4, the early intermediate phase of IRF4 expression is unaffected and this leads to normal expression of nuclear AID protein and Ig class switching.

7.7 C-REL^{KO} mice recapitulate the phenotype of NFκB1^{KO} mice

We found that C-REL^{KO} mice have impaired responses to SRBC, with reduced numbers of responding B cells two days after immunisation. Furthermore, five days after immunisation there is a complete absence of germinal centres and a dramatic reduction in plasma-cell numbers. We then went on to test induction of intermediate level IRF4 in response to NP-Ficoll and found that six hours after immunisation, IRF4 protein levels are normally expressed in C-REL^{KO} mice. This is in contrast to a previous study which showed that CREL^{KO} lymphocytes failed to upreguate IRF4 2 h post-stimulation with LPS or anti-IgM *in vitro* (Grumont and Gerondakis, 2000). It is possible that ligation of surface BCR by anti-IgM stimulated a different signalling pathway to that induced by NP-Ficoll and that C-REL is not

required following NP-Ficoll stimulation. Stimulation of B cells with anti-IgM lead to inhibition of terminal differentiation and Ig secretion, regardless of the specificity of the anti-IgM antibody (Grandien et al., 1993), whereas NP-Ficoll immunisation induces differentiation of plasmablasts. We have also found that stimulation of B cells *in vivo* with anti-IgD-dextran leads to initial B-cell activation and migration but an inhibition of terminal differentiation of plasma cells (data not shown).

Alternatively our results may be due to the mouse knockout model used. Grumont *et al* (2000) used an earlier C-REL^{KO} model whereby the region encoding amino acids 145-588 was replaced with a PGK neo cassette. This resulted in a truncated protein that lacks DNA binding, dimerization, nuclear transport and transcriptional transactivation (Köntgen et al., 1995). In contrast our C-REL^{KO} mice lack the exon encoding amino acids 102-130 which encodes the Rel-homology domain (RHD) only, thereby leaving an intact transactivation domain (Liou et al., 1999). The RHD contains sequences required for DNA binding, dimerization, association with IκB family members and the nuclear localisation sequence (Perkins, 2007). Carrasco *et al* (1998) produced a mouse lacking only the transcriptional activation domain of C-REL. This mouse exhibited more severe defects than mice lacking the entire C-REL protein, possibly due to decreased compensation from other family members (Carrasco et al., 1998).

Responding B blasts downregulate CD62L normally and upregulate CD86, yet CD69 induction is reduced. CD69 is upregulated within four hours of stimulation in B cells (Arvå and Andersson, 1999) and has been shown to possess a negative regulatory role in T cells (la Fuente et al., 2012). Upregulation of CD69 has been reported following interaction with latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV) and this required signalling via the NFκB family(Ishikawa and Mori, 2013). CD69 was required for downregulation of

sphingosine 1-phosphate receptor -1 ($S1P_1$) following activation of B cells with IFN-α/β and this prevented lymphocyte egress from lymphoid organs thereby retaining lymphocytes at the site of activation (Shiow et al., 2006). Therefore C-REL^{KO} B cells may demonstrate impaired retention within the spleen following activation. Indeed we observed reduced numbers of activated B cells following immunisation. This would need to be tested by measuring the number of antigen-specific B cells in the blood following immunisation, as within 6 h of immunisation antigen-specific lymphocyte numbers in the blood and lymph decrease when CD69 is normally upregulated (Shiow et al., 2006).

C-REL has an important role in a number of cell types. In DCs this signalling molecule was required for the efficient production of IFN-γ and IL-4, and thereby the efficient activation of T cells (Boffa et al., 2003). C-REL^{KO} mice were defective in their production of cytokines such as Il-2, IL-3 and granulocyte macrophage colony stimulating factor (Köntgen et al., 1995; Liou et al., 1999). In addition C-REL absence can lead to lymphoid hyperplasia (Carrasco et al., 1998). Due to the diverse roles of C-REL in numerous cell types, we tested the differentiation of C-REL^{KO} B cells in a wild-type environment. Following normal upregulation of IRF4 protein and mRNA 2 h post-activation we find that AID protein levels by FC and in situ differentiating B blasts are normal. However plasma cells and germinal centre differentiation are inhibited. Defects in plasma-cell differentiation are B-cell intrinsic but not due to a failure to upregulate *Prdm1* levels. Furthermore, the few plasma cells which successfully differentiate express normal levels of IRF4 protein. Whilst antibody titres produced by CREL^{KO} B cells are reduced, Ig CSR is not. . Furthermore the induction of both IgG3 germline switch transcripts and the induction of post-switched Iμ-Cγ3 transcripts are normal indicating that the heavy chain locus is accessible and CSR occurs normally to IgG3.

7.8 Summary NFκB family members in B-cell activation

The active subunit of NFκB1 is p50. This is processed from a larger 105KDa subunit. Homodimers of p50 are transcriptional repressors as p50 contains a DNA binding domain but not a transactivation domain. Expression of p50 may therefore be required for the down-regulation of IRF4 in germinal centre cells. p50 can also form heterodimers with RelA, RelB and C-REL. As these binding partners contain a transactivation domain, they are capable of acting as transcriptional activators. Neither NFκB1 or C-REL are required for intermediate level IRF4 expression in the first hours following immunisation. However high level IRF4 expression associated with plasma-cell differentiation is blocked when NFκB is absent (Figure 7.1). Expression of intermediation level IRF4 is sufficient for AID upregulation and Ig class switching, even in the absence of plasma-cell differentiation. To test the factors which regulate intermediate level IRF4 induction, IF staining for NFκB family members could be performed to test which members are located within the nucleus immediately after activation via the BCR. In addition, gene silencing via small interfering RNA (siRNA) could be utilised to test each NFκB member separately. To test a wide range of signaling molecules at the RNA level, gene arrays could be utilised on sorted activated QM B cells.

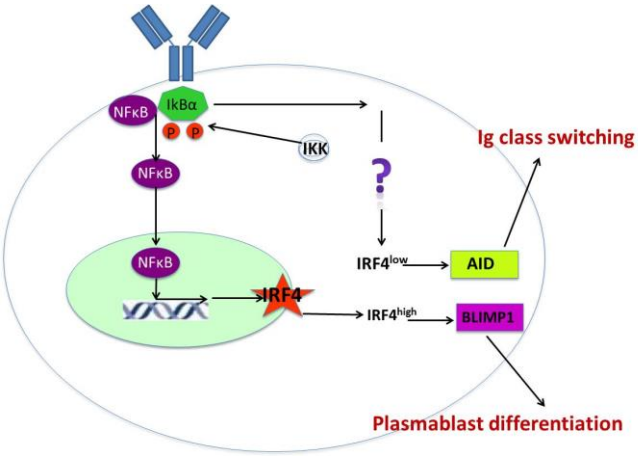


Figure 7 1 *Signalling leading to IRF4 intermediate and IRF4 high expression*

Following BCR stimulation, IKK phosphorylates IκBα and targets it for degradation leading to the release of NFκB family members. These translocate to the nucleus where they induce IRF4 protein. High levels of which lead to BLIMP1 expression and plasma-cell differentiation. Signals mediating the upregulation of intermediate level IRF4 are currently unknown.

Naive B cells expressed p50, C-REL, p52 and RelB. All of these were detected by electrophoretic mobility shift assay (EMSA) 2 hours after BCR stimulation, or following CD40L treatment. In addition, p65 was detected after activation, but not in resting B cells (Francis et al., 1998). This contradicts an earlier study which shows that pre-B cells and naïve B cells expressed p65 (Grumont and Gerondakis, 1994). This indicates that all these family members are part of DNA-binding complexes. There may be redundant roles for different NF-kappaB family members in IRF4 induction following stimulation through the BCR (Francis et al., 1998) and redundancy within the NF-kappaB family has been observed for NFκB1/p50 and NFκB2/p52 (Franzoso et al., 1997). Interestingly 24h after stimulation RelB activity was undetectable (Figure 7.2). Other studies have shown that p50/CREL heterodimers were the main complexes formed in activated B cells (Grumont and Gerondakis, 1994). NFκB1/p50^{KO} mice did not increase their levels of other NF-κB family members in a compensatory manner (Sha et al., 1995), in fact levels of Rel, RelB and p65 but not p52 were found to be lower in

mice lacking p50. However the frequency of different NF-κB complexes in the absence of p50 may still have a role in compensatory functions (Sha et al., 1995).

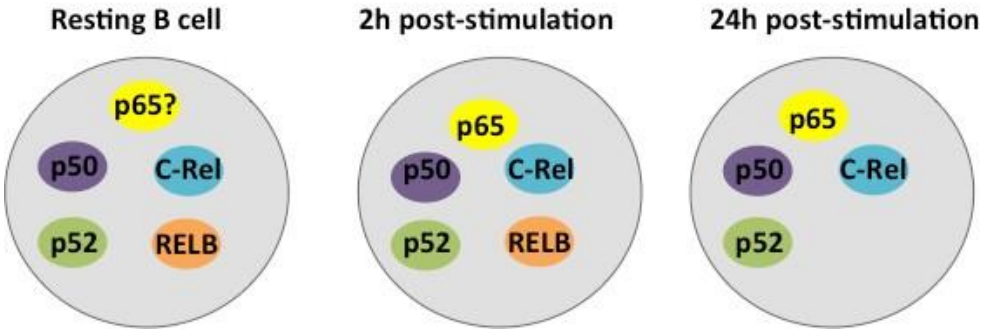


Figure 7 2 NFκB family members involved in initial B-cell activation and differentiation

Resting B cells express p50, C-REL, p52, RELB and possibly p65. In the first hours following activation expression of NFκB family members are similar whereas 24 hours following stimulation expression of RELB is lost.

7.9 Overexpression of miR-125b leads to expansion of the splenic red pulp largely due to an increase in NK and CD11c+-cell numbers.

miR-125b was found to be highly expressed within haematopoietic stem cells (O'Connell et al., 2010a; Ooi et al., 2010) and its role in these cells is controversial. Ooi *et al* (2010) found that myeloid cells were expanded when miR-125b was over-expressed. In contrast O'Connell *et al* (2010) found an expansion in the lymphoid compartment, specifically $CD8^+$ T cells. We found that numbers of immature B cells within the bone marrow are reduced, however this experiment would need to be repeated to confirm the result and numbers of splenic B cells are similar whether mice were infected with control cells or cells over-expressing miR-125b. In addition, there is no increase in T-cell numbers in spleens from miR-125b infected mice. However there are significantly more $CD11c^+$ and NK cells in the spleens of mice that overexpress miR-125b, this represents an increase in both the lymphoid and myeloid compartment. Contradictory reports on the effect of miR-125b overexpression within haematopoietic stem cells may be due to the different expression systems used in each study.

The study by O'Connell *et al* used a murine stem-cell virus to transfect haematopoetic cells whereas Ooi *et al* (2010) used a lentivirus expression vector, which resulted in overexpression by 35 fold. O'Connell *et al* (2010) also found that the myeloproliferative disorder was miR-125b dose dependent.

7.10 Overexpression of miR-125b leads to expanded GC volume later during the response and decreased plasma-cell differentiation

Within eight days of immunisation, GCs in mice overexpressing miR-125b are significantly larger. As miR-125b has been shown to be highly expressed within centroblasts in the germinal centre (Gururajan et al., 2010) and IRF4 and BLIMP1 are targets of this micro-RNA, this may be due to a failure of plasma cells to exit the germinal centre (Gururajan et al., 2010; Malumbres et al., 2009). In addition, germinal centre cells may be less sensitive to apoptosis. It has been shown that miR-125b had a role in apoptosis via blockage of caspase activation (Puissegur et al., 2012) and inhibition of the pro-apoptotic gene *Trp53inp1* (Enomoto et al., 2011).

Extrafollicular plasma-cell numbers are reduced four and five days after immunisation, however, 8 d after immunisation, plasma-cell numbers have normalised despite miR-125b overexpression. During the splenic antibody response, extrafollicular plasmablasts proliferate for about four days (Sze et al., 2000). After this plasmablasts differentiate into plasma cells that stop further proliferation. In responses where large numbers of plasma cells develop, most plasma cells will die via apoptosis within the next day, until numbers sustainable by the niches available in the red pulp have been reached (Sze et al., 2000). The fact that WT and miR-125b overexpressing B cells yield similar numbers of plasma cells 8 d post immunisation, although plasmablasts proliferation and differentiation are seriously affected, shows that plasma-cell survival and the ability to be accommodated by survival niches is not dependent on IRF4.

7.11 Blocking intermediate level IRF4 leads to a defect in AID protein and mRNA and Ig CSR

A luciferase reporter assay was used to test whether IRF4 is a direct binding target of miR-125b. We found that IRF4 protein levels were reduced by miR-125a, miR-125b-1 and miR-125b-2 overexpression. MiR-125-1 and miR-125b-2 are located on different chromosomes and possess different pre-miRNA sequences, however following processing by DICER, the mature sequences are identical (O'Connell et al., 2010a).

To confirm this effect *in vivo* within murine cells, we tested expression of IRF4 mRNA two days after immunisation in control B cells and miR-125b overexpressing B cells. miR-125b overexpressing B cells were found to express significantly less IRF4 mRNA and this leads to a decrease in translation to IRF4 protein. IRF4 protein levels were found to be further supressed four days after immunisation in mice overexpressing miR-125b. Repression of IRF4 by miR-125b has been reported previously (Gururajan et al., 2010; Malumbres et al., 2009).

Reduced intermediate level IRF4 expression in B cells overexpressing miR-125b lead to decreased levels of *Aicda* mRNA and AID protein. This resulted in reduced Ig class switched antibodies and reduced Ig class switching in situ within the extrafollicular foci of the splenic red pulp.

In contrast, miR-125b overexpressing B cells formed AID^+ germinal centres within B-cell follicles and these expressed normal levels of AID protein. AID is also required for somatic hypermutation and Ig CSR within GC B cells that do not express IRF4. As miR-125b is highly expressed in centroblasts (Gururajan et al., 2010), this may explain why GC cells do

not express IRF4 protein. Therefore it is likely that AID is regulated differently within the GC. One candidate for this regulation with the GC is IRF8. IRF8 is highly expressed by centroblasts within the dark zone. Using small interfering RNA knockdowns of IRF8 expression, the targets of this transcription factor were found to be *Aicda* and *Bcl6.* In mouse and human cells, both genes demonstrated binding to IRF8 at their 5' sequences following chromatin immunoprecipitation (CHIP) assay (Lee et al., 2006). In addition, mice lacking IRF8 had poorly defined GC structures that expressed reduced *Aicda* and *Bcl6* transcripts. However another study (Feng et al., 2011) showed that CD19-cre IRF8^{KO} mice seem to have no defect in antibody production following immunisation, however they do exhibit increased numbers of marginal zone and follicular B cells. A higher frequency of responding cells may therefore compensate for reduced CSR.

7.12 miR-125b overexpressing B cells locate to the marginal zone of the spleen eight days after immunisation

We found that eight days after immunisation miR-125b overexpressing B cells accumulated in the marginal zone of the spleen in significantly higher numbers. Memory B cells locate to this region during the TD response (Anderson et al., 2007). We found that these cells are IgD^{-} and IgM^{+} which is typical for marginal zone cells. To test whether this increase in marginal zone B cells reflects stronger splenic differentiation towards memory cells we tested the expression of memory-cell markers in blood five and eight days after immunisation. Memory B cells can be detected in the blood as early as one week after immunisation (Blink et al., 2005) and they express higher protein levels of FAS, CD80 and CD62L. We detected GFP+ NP-binding cells in the blood of mice which had received control infected or miR-125b infected B cells at five days after immunisation. These cells expressed higher levels of CD80, FAS and CD62L than non-antigen specific B cells in the blood. However there was no difference in the expression of CD80 or CD62L between control infected B cells and miR-125b overexpressing B cells. However, expression of FAS was significantly higher in mice

over-expressing miR-125b. Signalling events leading to the production of memory cells remain elusive. Whilst memory cells do express IRF4 (Cattoretti et al., 2006), IRF4 does not appear to be required for the generation of memory cells (Klein et al., 2006). However it may be required for their maintenance and subsequent differentiation to plasma cells (Klein et al., 2006). Repressing IRF4 protein in our system may drive cells which should have differentiated into plasma cells into alternative cell fate decisions.

7.13 Micro-RNA regulation of genes

micro-RNAs can regulate protein levels via different mechanisms: miRNAs not only lead to degradation of the messenger RNA of the target protein but binding of micro-RNA to the messenger sequence can also block translation. Indeed in some cases, mRNA levels can be increased whilst protein levels are decreased in an apparently compensatory mechanism (Malumbres et al., 2009). We found that overexpression of miR-125b did lead to a reduction in both mRNA transcripts and protein levels of IRF4. IRF4 has been shown previously to be a direct target of miR-125b (Gururajan et al., 2010; Malumbres et al., 2009). This also occurs in macrophages where miR-125b is highly expressed and represses IRF4, this potentiates macrophage activation with increased macrophage responsiveness to IFN-γ and higher levels of CD80 expressed (Chaudhuri et al., 2011). However, it is possible that the phenotype observed may be due to effects on other genes, as micro-RNAs can regulate many genes (Vigorito et al., 2007). miR-125b has also been shown to target BLIMP-1 in addition to IRF4 (Gururajan et al., 2010; Malumbres et al., 2009). Defects in plasma-cell generation may therefore be due to repression of BLIMP-1.

Ig class switching by extrafollicular plasma blasts – the main focus of this study – is initiated within two days of immunisation (Marshall et al., 2011). AID induction in T zone B blasts two days after immunisation is long before high levels of IRF4 and Blimp1 are appearing in

emerging plasmablasts, or before GC B cells appear. Therefore we propose that the effects of mir125b on Ig CSR are due to repression of intermediate levels of IRF4 and extrafollicular AID and not on the following downstream differentiation events.

7.14 General discussion

We have provided evidence that intermediate levels of IRF4 are expressed rapidly after activation, and that these are required for extrafollicular AID protein expression and Ig class switch recombination within extrafollicular B blasts. This is a question that has been difficult to test experimentally. Although this does not appear to be a direct effect as an earlier CHIP-PCR experiments found no IRF4 binding in the *Aicda* gene (Sciammas et al., 2006). However, this study tested only small parts of the Aicda gene for IRF4 binding. Therefore, it is possible that IRF4 binding was missed. More recent genome wide CHIP experiments found evidence of at least three IRF4 binding sites in the *Aicda* gene (Kent et al., 2002) Alternatively, IRF4 may form complexes with other transcription factors (Shindo et al., 2011) and may regulate a gene/genes which regulates AID.

AID is likely to be differently regulated within GC B cells, as most do not express IRF4 and a number of factors have been proposed to have a role here. AID facilitates DNA cleavage and deregulation of AID is implicated in malignancies (Feldhahn et al., 2007). Therefore AID expression and function is a tightly regulated process.

7.15 Conclusion

In summary, many different cross-interacting mechanisms of AID regulation exist, and dependent at the differentiation stage of the B-cell, different mechanisms may be active. We

have mainly focused on regulation of extrafollicular Ig class switching. This showed that intermediate level expression of IRF4, which has been implied to regulate AID expression and Ig class switching in the GC (Sciammas et al., 2013), correlate with and is essential for extrafollicular Ig class switching. However, although this was not the focus of this study, it seems that IRF4, although expressed in some GC centrocytes, does not regulate follicular CSR in the GC.

We have correlated IRF4 expression with different phases of B blast differentiation. Furthermore we have shown evidence of the role of the graded expression of IRF4 in Ig class switching via the induction of AID protein. This data complements research from other groups which have used knock-out models (Klein et al., 2006; Ochiai et al., 2013) and *in vitro* methodology (Sciammas et al., 2006; 2011) to test this relationship.

REFERENCES

Adkins, B., Leclerc, C., and Marshall---Clarke, S. (2004). Neonatal adaptive immunity comes of age. Nat Rev Immunol *4*, 553–564.

Allen, C.D.C., and Cyster, J.G. (2008). Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. Seminars in Immunology *20*, 14–25.

Allen, C.D.C., Okada, T., and Cyster, J.G. (2007). Germinal---center organization and cellular dynamics. Immunity *27*, 190–202.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350–355.

Anderson, S.M., Tomayko, M.M., Ahuja, A., Haberman, A.M., and Shlomchik, M.J. (2007). New markers for murine memory B cells that define mutated and unmutated subsets. Journal of Experimental Medicine *204*, 2103–2114.

Ansel, K.M., McHeyzer---Williams, L.J., Ngo, V.N., McHeyzer---Williams, M.G., and Cyster, J.G. (1999). In vivo---activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J. Exp. Med. *190*, 1123–1134.

Aoufouchi, S., Faili, A., Zober, C., D'Orlando, O., Weller, S., Weill, J.---C., and Reynaud, C.---A. (2008). Proteasomal degradation restricts the nuclear lifespan of AID. Journal of Experimental Medicine *205*, 1357–1368.

Arvå, E., and Andersson, B. (1999). Kinetics of cytokine release and expression of lymphocyte cell---surface activation markers after in vitro stimulation of human peripheral blood mononuclear cells with Streptococcus pneumoniae. Scand. J. Immunol. *49*, 237–243.

Bajénoff, M., Glaichenhaus, N., and Germain, R.N. (2008). Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. The Journal of Immunology *181*, 3947–3954.

Bajpai, U.D., Zhang, K., Teutsch, M., Sen, R., and Wortis, H.H. (2000). Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. J. Exp. Med. *191*, 1735–1744.

Baldassarre, F., Mallardo, M., Mezza, E., Scala, G., and Quinto, I. (1995). Regulation of NF---kappa B through the nuclear processing of p105 (NF---kappa B1) in Epstein---Barr virus---immortalized B cell lines. J Biol Chem *270*, 31244–31248.

Banerjee, A., Grumont, R., Gugasyan, R., White, C., Strasser, A., and Gerondakis, S. (2008). NF---kappaB1 and c---Rel cooperate to promote the survival of TLR4---activated B cells by

neutralizing Bim via distinct mechanisms. Blood *112*, 5063–5073.

Basso, K., Schneider, C., Shen, Q., Holmes, A.B., Setty, M., Leslie, C., and Dalla---Favera, R. (2012). BCL6 positively regulates AID and germinal center gene expression via repression of miR---155. Journal of Experimental Medicine *209*, 2455–2465.

Basso, K., Sumazin, P., Morozov, P., Schneider, C., Maute, R.L., Kitagawa, Y., Mandelbaum, J., Haddad, J., Chen, C.---Z., Califano, A., et al. (2009). Identification of the human mature B cell miRNome. Immunity *30*, 744–752.

Basu, U., Wang, Y., and Alt, F.W. (2008). Evolution of phosphorylation---dependent regulation of activation---induced cytidine deaminase. Mol. Cell *32*, 285–291.

Baumann, I., Kolowos, W., Voll, R.E., Manger, B., Gaipl, U., Neuhuber, W.L., Kirchner, T., Kalden, J.R., and Herrmann, M. (2002). Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis Rheum. *46*, 191–201.

Baumgarth, N. (2011). The double life of a B---1 cell: self---reactivity selects for protective effector functions. Nat Rev Immunol *11*, 34–46.

Berberich, I., Shu, G.L., and Clark, E.A. (1994). Cross---linking CD40 on B cells rapidly activates nuclear factor---kappa B. J Immunol *153*, 4357–4366.

Betz, B.C., Jordan---Williams, K.L., Wang, C., Kang, S.G., Liao, J., Logan, M.R., Kim, C.H., and Taparowsky, E.J. (2010). Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. Journal of Experimental Medicine *207*, 933–942.

Blink, E., Light, A., Kallies, A., Nutt, S., Hodgkin, P., and Tarlinton, D. (2005). Early appearance of germinal center---derived memory B cells and plasma cells in blood after primary immunization. Journal of Experimental Medicine *201*, 545–554.

Boffa, D.J., Feng, B., Sharma, V., Dematteo, R., Miller, G., Suthanthiran, M., Nunez, R., and Liou, H.---C. (2003). Selective loss of c---Rel compromises dendritic cell activation of T lymphocytes. Cell. Immunol. *222*, 105–115.

Bollig, N., Brüstle, A., Kellner, K., Ackermann, W., Abass, E., Raifer, H., Camara, B., Brendel, C., Giel, G., Bothur, E., et al. (2012). Transcription factor IRF4 determines germinal center formation through follicular T---helper cell differentiation. Proceedings of the National Academy of Sciences *109*, 8664–8669.

Bonizzi, G., and Karin, M. (2004). The two NF---kappaB activation pathways and their role in innate and adaptive immunity. Trends in Immunology *25*, 280–288.

Brass, A.L., Zhu, A.Q., and Singh, H. (1999). Assembly requirements of PU.1---Pip (IRF---4) activator complexes: inhibiting function in vivo using fused dimers. Embo J *18*, 977–991.

Brodeur, S.R., Angelini, F., Bacharier, L.B., Blom, A.M., Mizoguchi, E., Fujiwara, H., Plebani, A., Notarangelo, L.D., Dahlback, B., Tsitsikov, E., et al. (2003). C4b---binding protein (C4BP) activates B cells through the CD40 receptor. Immunity *18*, 837–848.

Brunner, C. (2006). Btk expression is controlled by Oct and BOB.1/OBF.1. Nucleic Acids

Research *34*, 1807–1815.

Bülow, von, G.U., van Deursen, J.M., and Bram, R.J. (2001). Regulation of the T---independent humoral response by TACI. Immunity *14*, 573–582.

Caamano, J., and Hunter, C.A. (2002). NF---kappaB family of transcription factors: central regulators of innate and adaptive immune functions. Clin. Microbiol. Rev. *15*, 414–429.

Calame, K. (2008). Activation---dependent induction of Blimp---1. Current Opinion in Immunology *20*, 259–264.

Cariappa, A., Tang, M., Parng, C., Nebelitskiy, E., Carroll, M., Georgopoulos, K., and Pillai, S. (2001). The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. Immunity *14*, 603–615.

Carrasco, D., Cheng, J., Lewin, A., Warr, G., Yang, H., Rizzo, C., Rosas, F.R., Snapper, C.M., and Rodrigo, B. (1998). Multiple Hemopoietic Defects and Lymphoid Hyperplasia in Mice Lacking the Transcriptional Activation Domain of the c---Rel Protein. J. Exp. Med. *187*, 973–984.

Cascalho, M., Ma, A., Lee, S., Masat, L., and Wabl, M. (1996). A Quasi---Monoclonal Mouse. *272*, 1649–1652.

Castigli, E. (2004). Impaired IgA class switching in APRIL---deficient mice. P Natl Acad Sci Usa *101*, 3903–3908.

Castigli, E., Alt, F.W., Davidson, L., Bottaro, A., Mizoguchi, E., Bhan, A.K., and Geha, R.S. (1994). CD40---deficient mice generated by recombination---activating gene---2---deficient blastocyst complementation. P Natl Acad Sci Usa *91*, 12135–12139.

Cattoretti, G., Shaknovich, R., Smith, P.M., Jäck, H.---M., Murty, V.V., and Alobeid, B. (2006). Stages of Germinal Center Transit Are Defined by B Cell Transcription Factor Coexpression and Relative Abundance. The Journal of Immunology 6930–6939.

Cazac, B.B., and Roes, J. (2000). TGF---beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity *13*, 443–451.

Cerchietti, L.C., Ghetu, A.F., Zhu, X., Da Silva, G.F., Zhong, S., Matthews, M., Bunting, K.L., Polo, J.M., Farès, C., Arrowsmith, C.H., et al. (2010). A small---molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. Cancer Cell *17*, 400–411.

Cesta, M. (2006). Normal Structure, Function, and Histology of the Spleen. Toxicologic Path. *34*, 455–465.

Chahwan, R., Edelmann, W., Scharff, M.D., and Roa, S. (2012). AIDing antibody diversity by error---prone mismatch repair. Seminars in Immunology *24*, 293–300.

Chan, T.D., Gatto, D., Wood, K., Camidge, T., Basten, A., and Brink, R. (2009). Antigen Affinity Controls Rapid T---Dependent Antibody Production by Driving the Expansion Rather than the Differentiation or Extrafollicular Migration of Early Plasmablasts. The Journal of Immunology *183*, 3139–3149.

Chappell, C.P., Draves, K.E., Giltiay, N.V., and Clark, E.A. (2012). Extrafollicular B cell

activation by marginal zone dendritic cells drives T cell---dependent antibody responses. Journal of Experimental Medicine *209*, 1825–1840.

Chaudhuri, A.A., So, A.Y.L., Sinha, N., Gibson, W.S.J., Taganov, K.D., O'Connell, R.M., and Baltimore, D. (2011). MicroRNA---125b Potentiates Macrophage Activation. The Journal of Immunology *187*, 5062–5068.

Chaudhuri, J.J., and Alt, F.W.F. (2004). Class---switch recombination: interplay of transcription, DNA deamination and DNA repair. Nat Rev Immunol *4*, 541–552.

Chaudhuri, J., Khuong, C., and Alt, F.W. (2004). Replication protein A interacts with AID to promote deamination of somatic hypermutation targets. Nature *430*, 992–998.

Chen, C.---Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science *303*, 83–86.

Chen, J., Trounstine, M., Alt, F.W., Young, F., Kurahara, C., Loring, J.F., and Huszar, D. (1993a). Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. International Immunology *5*, 647–656.

Chen, J., Trounstine, M., Kurahara, C., Young, F., Kuo, C.C., Xu, Y., Loring, J.F., Alt, F.W., and Huszar, D. (1993b). B cell development in mice that lack one or both immunoglobulin kappa light chain genes. Embo J *12*, 821–830.

Chirmule, N., Tazelaar, J., and Wilson, J.M. (2000). Th2---dependent B cell responses in the absence of CD40---CD40 ligand interactions. J Immunol *164*, 248–255.

Chu, V.T., Enghard, P., Riemekasten, G., and Berek, C. (2007). In vitro and in vivo activation induces BAFF and APRIL expression in B cells. J Immunol *179*, 5947–5957.

Cinamon, G., Zachariah, M.A., Lam, O.M., Foss, F.W., and Cyster, J.G. (2008). Follicular shuttling of marginal zone B cells facilitates antigen transport. Nature Publishing Group *9*, 54–62.

Coffman, R.L., Lebman, D.A., and Shrader, B. (1989). Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide---stimulated murine B lymphocytes. J. Exp. Med. *170*, 1039–1044.

Cyster, J.G., and Goodnow, C.C. (1995). Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. J. Exp. Med. *182*, 581–586.

Cyster, J.G., Ansel, K.M., Reif, K., Ekland, E.H., Hyman, P.L., Tang, H.L., Luther, S.A., and Ngo, V.N. (2000). Follicular stromal cells and lymphocyte homing to follicles. Immunol. Rev. *176*, 181–193.

Dautigny, N., Chabre, H., Garcia, C., and Ezine, S. (1996). Marked depletion at the late pro---B cell stage in the bone marrow of lpr mice correlates with the development of lymphadenopathy but not autoimmunity. Eur J Immunol *26*, 2087–2092.

De Silva, N.S., Simonetti, G., Heise, N., and Klein, U. (2012). The diverse roles of IRF4 in late germinal center B---cell differentiation. Immunol. Rev. *247*, 73–92.

de Yébenes, V.G., Belver, L., Pisano, D.G., González, S., Villasante, A., Croce, C., He, L., and

Ramiro, A.R. (2008). miR---181b negatively regulates activation---induced cytidine deaminase in B cells. Journal of Experimental Medicine *205*, 2199–2206.

Dedeoglu, F.F., Horwitz, B.B., Chaudhuri, J.J., Alt, F.W.F., and Geha, R.S.R. (2004). Induction of activation---induced cytidine deaminase gene expression by IL---4 and CD40 ligation is dependent on STAT6 and NFkappaB. International Immunology *16*, 395–404.

Dengler, H.S., Baracho, G.V., Omori, S.A., Bruckner, S., Arden, K.C., Castrillon, D.H., DePinho, R.A., and Rickert, R.C. (2008). Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. Nature Publishing Group *9*, 1388–1398.

Di Bernardo, M.C., Crowther---Swanepoel, D., Broderick, P., Webb, E., Sellick, G., Wild, R., Sullivan, K., Vijayakrishnan, J., Wang, Y., Pittman, A.M., et al. (2008). A genome---wide association study identifies six susceptibility loci for chronic lymphocytic leukemia. Nat. Genet. *40*, 1204–1210.

Ding, Q., Yeung, M., Camirand, G., Zeng, Q., Akiba, H., Yagita, H., Chalasani, G., Sayegh, M.H., Najafian, N., and Rothstein, D.M. (2011). Regulatory B cells are identified by expression of TIM---1 and can be induced through TIM---1 ligation to promote tolerance in mice. J. Clin. Invest. *121*, 3645–3656.

Drutman, S.B., Kendall, J.C., and Trombetta, E.S. (2012). Inflammatory spleen monocytes can upregulate CD11c expression without converting into dendritic cells. The Journal of Immunology *188*, 3603–3610.

Eisenbeis, C.F., Singh, H., and Storb, U. (1995). Pip, a novel IRF family member, is a lymphoid---specific, PU.1---dependent transcriptional activator. Genes & Development *9*, 1377–1387.

Enomoto, Y., Kitaura, J., Hatakeyama, K., Watanuki, J., Akasaka, T., Kato, N., Shimanuki, M., Nishimura, K., Takahashi, M., Taniwaki, M., et al. (2011). Eμ/miR---125b transgenic mice develop lethal B---cell malignancies. Leukemia *25*, 1849–1856.

Enomoto, Y., Kitaura, J., Shimanuki, M., Kato, N., Nishimura, K., Takahashi, M., Nakakuma, H., Kitamura, T., and Sonoki, T. (2012). MicroRNA---125b---1 accelerates a C---terminal mutant of C/EBPα (C/EBPα---C(m))---induced myeloid leukemia. Int. J. Hematol. *96*, 334–341.

Facchetti, F., Appiani, C., Salvi, L., Levy, J., and Notarangelo, L.D. (1995). Immunohistologic analysis of ineffective CD40---CD40 ligand interaction in lymphoid tissues from patients with X---linked immunodeficiency with hyper---IgM. Abortive germinal center cell reaction and severe depletion of follicular dendritic cells. J Immunol *154*, 6624–6633.

Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K., and Honjo, T. (2001). In situ class switching and differentiation to IgA---producing cells in the gut lamina propria. Nature *413*, 639–643.

Fagarasan, S., and Honjo, T. (2003). Intestinal IgA synthesis: regulation of front---line body defences. Nat Rev Immunol *3*, 63–72.

Falini, B., Fizzotti, M., Pucciarini, A., Bigerna, B., Marafioti, T., Gambacorta, M., Pacini, R., Alunni, C., Natali---Tanci, L., Ugolini, B., et al. (2000). A monoclonal antibody (MUM1p)

detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. Blood *95*, 2084–2092.

Fan, C.M., and Maniatis, T. (1991). Generation of p50 subunit of NF---kappa B by processing of p105 through an ATP---dependent pathway. Nature *354*, 395–398.

Feldhahn, N., Henke, N., Melchior, K., Duy, C., Soh, B.N., Klein, F., Levetzow, von, G., Giebel, B., Li, A., Hofmann, W.---K., et al. (2007). Activation---induced cytidine deaminase acts as a mutator in BCR---ABL1---transformed acute lymphoblastic leukemia cells. J. Exp. Med. *204*, 1157–1166.

Feng, J., Wang, H., Shin, D.---M., Masiuk, M., Qi, C.---F., and Morse, H.C. (2011). IFN regulatory factor 8 restricts the size of the marginal zone and follicular B cell pools. The Journal of Immunology *186*, 1458–1466.

Ferguson, A.R., and Corley, R.B. (2005). Accumulation of marginal zone B cells and accelerated loss of follicular dendritic cells in NF---kappaB p50---deficient mice. BMC Immunol. *6*, 8.

Francis, D.A., Sen, R., Rice, N., and Rothstein, T.L. (1998). Receptor---specific induction of NF---kappaB components in primary B cells. International Immunology *10*, 285–293.

Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E.W., Brown, K.D., Leonardi, A., Tran, T., Boyce, B.F., and Siebenlist, U. (1997). Requirement for NF---kappa B in osteoclast and B---cell development. Genes & Development *11*, 3482–3496.

García De Vinuesa, C., Gulbranson---Judge, A., Khan, M., O'Leary, P., Cascalho, M., Wabl, M., Klaus, G.G., Owen, M.J., and MacLennan, I.C. (1999). Dendritic cells associated with plasmablast survival. Eur J Immunol *29*, 3712–3721.

Garnotel, R., Rittié, L., Poitevin, S., Monboisse, J.C., Nguyen, P., Potron, G., Maquart, F.X., Randoux, A., and Gillery, P. (2000). Human blood monocytes interact with type I collagen through alpha x beta 2 integrin (CD11c---CD18, gp150---95). J Immunol *164*, 5928–5934.

Garraud, O., Borhis, G., Badr, G., Degrelle, S., Pozzetto, B., Cognasse, F., and Richard, Y. (2012). Revisiting the B---cell compartment in mouse and humans: more than one B---cell subset exists in the marginal zone and beyond. BMC Immunol. *13*, 63–63.

Gaspal, F.M.C., McConnell, F.M., Kim, M.---Y., Gray, D., Kosco---Vilbois, M.H., Raykundalia, C.R., Botto, M., and Lane, P.J.L. (2006). The generation of thymus---independent germinal centers depends on CD40 but not on CD154, the T cell---derived CD40---ligand. Eur J Immunol *36*, 1665–1673.

Gatto, D., and Brink, R. (2013). B cell localization: regulation by EBI2 and its oxysterol ligand. Trends in Immunology *34*, 336–341.

Gatto, D., Paus, D., Basten, A., Mackay, C.R., and Brink, R. (2009). Guidance of B cells by the orphan G protein---coupled receptor EBI2 shapes humoral immune responses. Immunity *31*, 259–269.

Geisberger, R., Rada, C., and Neuberger, M.S. (2009). The stability of AID and its function in class---switching are critically sensitive to the identity of its nuclear---export sequence. Proceedings of the National Academy of Sciences *106*, 6736–6741.

Goldfarb, A.N., Flores, J.P., and Lewandowska, K. (1996). Involvement of the E2A basic helix---loop---helix protein in immunoglobulin heavy chain class switching. Molecular Immunology *33*, 947–956.

Grandien, A., Modigliani, Y., Coutinho, A., and Andersson, J. (1993). Suppression of B cell differentiation by ligation of membrane---bound IgM. Eur J Immunol *23*, 1561–1565.

Grosschedl, R., Weaver, D., Baltimore, D., and Costantini, F. (1984). Introduction of a mu immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. Cell *38*, 647–658.

Grumont, R.J., and Gerondakis, S. (1994). The subunit composition of NF---kappa B complexes changes during B---cell development. Cell Growth Differ. *5*, 1321–1331.

Grumont, R.J., and Gerondakis, S. (2000). Rel induces interferon regulatory factor 4 (IRF---4) expression in lymphocytes: modulation of interferon---regulated gene expression by rel/nuclear factor kappaB. J. Exp. Med. *191*, 1281–1292.

Gupta, S., Jiang, M., Anthony, A., and Pernis, A.B. (1999). Lineage---specific modulation of interleukin 4 signaling by interferon regulatory factor 4. J. Exp. Med. *190*, 1837–1848.

Gururajan, M., Haga, C.L., Das, S., Leu, C.M., Hodson, D., Josson, S., Turner, M., and Cooper, M.D. (2010). MicroRNA 125b inhibition of B cell differentiation in germinal centers. International Immunology *22*, 583–592.

Hannedouche, S., Zhang, J., Yi, T., Shen, W., Nguyen, D., Pereira, J.P., Guerini, D., Baumgarten, B.U., Roggo, S., Wen, B., et al. (2011). Oxysterols direct immune cell migration via EBI2. Nature *475*, 524–527.

Hardy, R.R., and Hayakawa, K. (2012). Positive and negative selection of natural autoreactive B cells. Adv. Exp. Med. Biol. *750*, 227–238.

Hargreaves, D.C., Hyman, P.L., Lu, T.T., Ngo, V.N., Bidgol, A., Suzuki, G., Zou, Y.R., Littman, D.R., and Cyster, J.G. (2001). A coordinated change in chemokine responsiveness guides plasma cell movements. J. Exp. Med. *194*, 45–56.

Hatada, E.N., Do, R.K.G., Orlofsky, A., Liou, H.---C., Prystowsky, M., Maclennan, I.C.M., Caamano, J., and Chen---Kiang, S. (2003). NF---kappa B1 p50 is required for BLyS attenuation of apoptosis but dispensable for processing of NF---kappa B2 p100 to p52 in quiescent mature B cells. J Immunol *171*, 761–768.

Hauser, A.E., Shlomchik, M.J., and Haberman, A.M. (2007). In vivo imaging studies shed light on germinal---centre development. Nat Rev Immunol *7*, 499–504.

Hayden, M.S. (2004). Signaling to NF--- B. Genes & Development *18*, 2195–2224.

He, B., Raab---Traub, N., Casali, P., and Cerutti, A. (2003). EBV---encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell---independent Ig heavy chain class switching. J Immunol *171*, 5215–5224.

Hikida, M., Casola, S., Takahashi, N., Kaji, T., Takemori, T., Rajewsky, K., and Kurosaki, T. (2009). PLC---gamma2 is essential for formation and maintenance of memory B cells. Journal of Experimental Medicine *206*, 681–689.

Hodgkin, P.D., Lee, J.H., and Lyons, A.B. (1996). B cell differentiation and isotype switching is related to division cycle number. J. Exp. Med. *184*, 277–281.

Honjo, T., Kinoshita, K., and Muramatsu, M. (2002). Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu. Rev. Immunol. *20*, 165–196.

Hsia, C.Y., Cheng, S., Owyang, A.M., Dowdy, S.F., and Liou, H.---C. (2002). c---Rel regulation of the cell cycle in primary mouse B lymphocytes. International Immunology *14*, 905–916.

Huber, M., Brüstle, A., Reinhard, K., Guralnik, A., Walter, G., Mahiny, A., Löw, von, E., and Lohoff, M. (2008). IRF4 is essential for IL---21---mediated induction, amplification, and stabilization of the Th17 phenotype. P Natl Acad Sci Usa *105*, 20846–20851.

Huong, L.T., Kobayashi, M., Nakata, M., Shioi, G., Miyachi, H., Honjo, T., and Nagaoka, H. (2013). In Vivo Analysis of Aicda Gene Regulation: A Critical Balance between Upstream Enhancers and Intronic Silencers Governs Appropriate Expression. PLoS One *8*, e61433.

Ihanus, E., Uotila, L.M., Toivanen, A., Varis, M., and Gahmberg, C.G. (2007). Red---cell ICAM---4 is a ligand for the monocyte/macrophage integrin CD11c/CD18: characterization of the binding sites on ICAM---4. Blood *109*, 802–810.

Iida, S., Rao, P.H., Butler, M., Corradini, P., Boccadoro, M., Klein, B., Chaganti, R.S., and Dalla---Favera, R. (1997). Deregulation of MUM1/IRF4 by chromosomal translocation in multiple myeloma. Nat. Genet. *17*, 226–230.

Inamine, A., Takahashi, Y., Baba, N., Miyake, K., Tokuhisa, T., Takemori, T., and Abe, R. (2005). Two waves of memory B---cell generation in the primary immune response. International Immunology *17*, 581–589.

Ingalls, R.R., and Golenbock, D.T. (1995). CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J. Exp. Med. *181*, 1473–1479.

Ise, W., Kohyama, M., Schraml, B.U., Zhang, T., Schwer, B., Basu, U., Alt, F.W., Tang, J., Oltz, E.M., Murphy, T.L., et al. (2011). The transcription factor BATF controls the global regulators of class---switch recombination in both B cells and T cells. Nature Publishing Group *12*, 536–543.

Ishikawa, C., and Mori, N. (2013). Epstein---Barr virus latent membrane protein 1 induces CD69 expression through activation of nuclear factor---κB. Int. J. Oncol. *42*, 1786–1792.

Ito, S., Nagaoka, H., Shinkura, R., Begum, N., Muramatsu, M., Nakata, M., and Honjo, T. (2004). Activation---induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. P Natl Acad Sci Usa *101*, 1975–1980.

Jabara, H.H., Weng, Y., Sannikova, T., and Geha, R.S. (2009). TRAF2 and TRAF3 independently mediate Ig class switching driven by CD40. International Immunology *21*, 477–488.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp---1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science *325*, 1006–1010.

Kalampokis, I., Yoshizaki, A., and Tedder, T.F. (2013). IL---10---producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res. Ther. *15 Suppl 1*, S1.

Karban, A.S., Okazaki, T., Panhuysen, C.I.M., Gallegos, T., Potter, J.J., Bailey---Wilson, J.E., Silverberg, M.S., Duerr, R.H., Cho, J.H., Gregersen, P.K., et al. (2004). Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. Hum. Mol. Genet. *13*, 35–45.

Katakai, T., Suto, H., Sugai, M., Gonda, H., Togawa, A., Suematsu, S., Ebisuno, Y., Katagiri, K., Kinashi, T., and Shimizu, A. (2008). Organizer---like reticular stromal cell layer common to adult secondary lymphoid organs. The Journal of Immunology *181*, 6189–6200.

Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T., and Kikutani, H. (1994). The immune responses in CD40---deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity *1*, 167–178.

Kelly, L.M., Pereira, J.P., Yi, T., Xu, Y., and Cyster, J.G. (2011). EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues. The Journal of Immunology *187*, 3026–3032.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, D. (2002). The human genome browser at UCSC. Genome Res. *12*, 996–1006.

Kenter, A.L. (2004). Mapping of a Functional Recombination Motif that Defines Isotype Specificity for ---> 3 Switch Recombination Implicates NF--- B p50 as the Isotype---specific Switching Factor. Journal of Experimental Medicine *199*, 617–627.

Kim, S.---W., Ramasamy, K., Bouamar, H., Lin, A.---P., Jiang, D., and Aguiar, R.C.T. (2012). MicroRNAs miR---125a and miR---125b constitutively activate the NF---κB pathway by targeting the tumor necrosis factor alpha---induced protein 3 (TNFAIP3, A20). Proceedings of the National Academy of Sciences *109*, 7865–7870.

Klein, U., and Dalla---Favera, R. (2008). Germinal centres: role in B---cell physiology and malignancy. Nat Rev Immunol *8*, 22–33.

Klein, U., Casola, S., Cattoretti, G., Shen, Q., Lia, M., Mo, T., Ludwig, T., Rajewsky, K., and Dalla---Favera, R. (2006). Transcription factor IRF4 controls plasma cell differentiation and class---switch recombination. Nat Immunol *7*, 773–782.

Kool, M., Soullié, T., van Nimwegen, M., Willart, M.A.M., Muskens, F., Jung, S., Hoogsteden, H.C., Hammad, H., and Lambrecht, B.N. (2008). Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. Journal of Experimental Medicine *205*, 869–882.

Koralov, S.B., Muljo, S.A., Galler, G.R., Krek, A., Chakraborty, T., Kanellopoulou, C., Jensen, K., Cobb, B.S., Merkenschlager, M., Rajewsky, N., et al. (2008). Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell *132*, 860–874.

Köntgen, F., Grumont, R.J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., and Gerondakis, S. (1995). Mice lacking the c---rel proto---oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin---2 expression. Genes & Development *9*,

1965–1977.

Kraal, G., and Janse, M. (1986). Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. Immunology *58*, 665–669.

Kranich, J., Krautler, N.J., Heinen, E., Polymenidou, M., Bridel, C., Schildknecht, A., Huber, C., Kosco---Vilbois, M.H., Zinkernagel, R., Miele, G., et al. (2008). Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. Journal of Experimental Medicine *205*, 1293–1302.

Krop, I., de Fougerolles, A.R., Hardy, R.R., Allison, M., Schlissel, M.S., and Fearon, D.T. (1996). Self---renewal of B---1 lymphocytes is dependent on CD19. Eur J Immunol *26*, 238–242.

la Fuente, de, H., Cibrián, D., and Sánchez---Madrid, F. (2012). Immunoregulatory molecules are master regulators of inflammation during the immune response. FEBS Lett. *586*, 2897–2905.

Lang, G.A., Johnson, A.M., Devera, T.S., Joshi, S.K., and Lang, M.L. (2011). Reduction of CD1d expression in vivo minimally affects NKT---enhanced antibody production but boosts B---cell memory. International Immunology *23*, 251–260.

Lazarevic, V., Myers, A.J., Scanga, C.A., and Flynn, J.L. (2003). CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol M. tuberculosis infection. Immunity *19*, 823–835.

Lee, C.H., Melchers, M., Wang, H., Torrey, T.A., Slota, R., Qi, C.---F., Kim, J.Y., Lugar, P., Kong, H.J., Farrington, L., et al. (2006). Regulation of the germinal center gene program by interferon (IFN) regulatory factor 8/IFN consensus sequence---binding protein. J. Exp. Med. *203*, 63–72.

Lenschow, D.J., Sperling, A.I., Cooke, M.P., Freeman, G., Rhee, L., Decker, D.C., Gray, G., Nadler, L.M., Goodnow, C.C., and Bluestone, J.A. (1994). Differential up---regulation of the B7---1 and B7---2 costimulatory molecules after Ig receptor engagement by antigen. J Immunol *153*, 1990–1997.

Lenz, G., Nagel, I., Siebert, R., Roschke, A.V., Sanger, W., Wright, G.W., Dave, S.S., Tan, B., Zhao, H., Rosenwald, A., et al. (2007). Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell---like diffuse large B cell lymphoma. J. Exp. Med. *204*, 633–643.

Lesinski, G.B., and Westerink, M.A. (2001). Novel vaccine strategies to T---independent antigens. J. Microbiol. Methods *47*, 135–149.

Leuenberger, M., Frigerio, S., Wild, P.J., Noetzli, F., Korol, D., Zimmermann, D.R., Gengler, C., Probst---Hensch, N.M., Moch, H., and Tinguely, M. (2010). AID protein expression in chronic lymphocytic leukemia/small lymphocytic lymphoma is associated with poor prognosis and complex genetic alterations. Mod. Pathol. *23*, 177–186.

Li, P., Spolski, R., Liao, W., Wang, L., Murphy, T.L., Murphy, K.M., and Leonard, W.J. (2012). BATF–JUN is critical for IRF4---mediated transcription in T cells. Nature *490*, 543–546.

Li, S.C., Rothman, P.B., Zhang, J., Chan, C., Hirsh, D., and Alt, F.W. (1994). Expression of I mu---C gamma hybrid germline transcripts subsequent to immunoglobulin heavy chain class switching. International Immunology *6*, 491–497.

Lin, L., DeMartino, G.N., and Greene, W.C. (1998). Cotranslational biogenesis of NF---kappaB p50 by the 26S proteasome. Cell *92*, 819–828.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL---21 acts directly on B cells to regulate Bcl---6 expression and germinal center responses. Journal of Experimental Medicine *207*, 353–363.

Liou, H.C., Jin, Z., Tumang, J., Andjelic, S., Smith, K.A., and Liou, M.L. (1999). c---Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. International Immunology *11*, 361–371.

Liou, H.C., Sha, W.C., Scott, M.L., and Baltimore, D. (1994). Sequential induction of NF---kappa B/Rel family proteins during B---cell terminal differentiation. Mol. Cell. Biol. *14*, 5349–5359.

Litinskiy, M.B., Nardelli, B., Hilbert, D.M., He, B., Schaffer, A., Casali, P., and Cerutti, A. (2002). DCs induce CD40---independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol *3*, 822–829.

Liu, C., Yang, X.V., Wu, J., Kuei, C., Mani, N.S., Zhang, L., Yu, J., Sutton, S.W., Qin, N., Banie, H., et al. (2011). Oxysterols direct B---cell migration through EBI2. Nature *475*, 519–523.

Liu, W., Meckel, T., Tolar, P., Sohn, H.W., and Pierce, S.K. (2010). Intrinsic properties of immunoglobulin IgG1 isotype---switched B cell receptors promote microclustering and the initiation of signaling. Immunity *32*, 778–789.

Liu, Y.J., Malisan, F., de Bouteiller, O., Guret, C., Lebecque, S., Banchereau, J., Mills, F.C., Max, E.E., and Martinez---Valdez, H. (1996). Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. Immunity *4*, 241–250.

Liu, Y.J., OLDFIELD, S., and MacLennan, I.C. (1988). Memory B cells in T cell---dependent antibody responses colonize the splenic marginal zones. Eur J Immunol *18*, 355–362.

Liu, Y.J., Zhang, J., Lane, P.J., Chan, E.Y., and MacLennan, I.C. (1991). Sites of specific B cell activation in primary and secondary responses to T cell---dependent and T cell---independent antigens. Eur J Immunol *21*, 2951–2962.

LIU, Y., OLDFIELD, S., and MACLENNAN, I. (1988). Memory B---Cells in T---Cell---Dependent Antibody---Responses Colonize the Splenic Marginal Zones. Eur J Immunol *18*, 355–362.

Loike, J.D., Sodeik, B., Cao, L., Leucona, S., Weitz, J.I., Detmers, P.A., Wright, S.D., and Silverstein, S.C. (1991). CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A alpha chain of fibrinogen. P Natl Acad Sci Usa *88*, 1044–1048.

Lu, R. (2008). Interferon regulatory factor 4 and 8 in B---cell development. Trends in Immunology *29*, 487–492.

Lu, T.T., and Cyster, J.G. (2002). Integrin---mediated long---term B cell retention in the splenic marginal zone. Science *297*, 409–412.

Luo, H., and Tian, M. (2010). Transcription factors PU.1 and IRF4 regulate activation induced cytidine deaminase in chicken B cells. Molecular Immunology *47*, 1383–1395.

Mackay, F., and Schneider, P. (2009). nri2572. Nat Rev Immunol *9*, 491–502.

Maclennan, I.C.M., Toellner, K.---M., Cunningham, A.F., Serre, K., Sze, D.M.Y., Zúñiga, E., Cook, M.C., and Vinuesa, C.G. (2003). Extrafollicular antibody responses. Immunol. Rev. *194*, 8–18.

Malhotra, S., Kovats, S., Zhang, W., and Coggeshall, K.M. (2009). B cell antigen receptor endocytosis and antigen presentation to T cells require Vav and dynamin. J Biol Chem *284*, 24088–24097.

Malumbres, R., Sarosiek, K.A., Cubedo, E., Ruiz, J.W., Jiang, X., Gascoyne, R.D., Tibshirani, R., and Lossos, I.S. (2009). Differentiation stage---specific expression of microRNAs in B lymphocytes and diffuse large B---cell lymphomas. Blood *113*, 3754–3764.

Marshall, J.L., Flores---Langarica, A., Kingsley, R.A., Hitchcock, J.R., Ross, E.A., López---Macías, C., Lakey, J., Martin, L.B., Toellner, K.---M., MacLennan, C.A., et al. (2012). The capsular polysaccharide Vi from Salmonella typhi is a B1b antigen. The Journal of Immunology *189*, 5527–5532.

Marshall, J.L., Zhang, Y., Pallan, L., Hsu, M.---C., Khan, M., Cunningham, A.F., Maclennan, I.C.M., and Toellner, K.---M. (2011). Early B blasts acquire a capacity for Ig class switch recombination that is lost as they become plasmablasts. Eur J Immunol *41*, 3506–3512.

Martin, F., and Kearney, J.F. (2001). B1 cells: similarities and differences with other B cell subsets. Current Opinion in Immunology *13*, 195–201.

Maseda, D., Smith, S.H., DiLillo, D.J., Bryant, J.M., Candando, K.M., Weaver, C.T., and Tedder, T.F. (2012). Regulatory B10 cells differentiate into antibody---secreting cells after transient IL---10 production in vivo. The Journal of Immunology *188*, 1036–1048.

McCoy, C.E., Sheedy, F.J., Qualls, J.E., Doyle, S.L., Quinn, S.R., Murray, P.J., and O'Neill, L.A.J. (2010). IL---10 inhibits miR---155 induction by toll---like receptors. J Biol Chem *285*, 20492–20498.

Mebius, R.E., and Kraal, G. (2005). Structure and function of the spleen. Nat Rev Immunol *5*, 606–616.

Metzger, H. (1992). Transmembrane signaling: the joy of aggregation.

Mishima, Y., Fukao, A., Kishimoto, T., Sakamoto, H., Fujiiwara, T., and Inoue, K. (2012). Translational inhibition by deadenylation---independent

mechanisms is central to microRNA---mediated

silencing in zebrafish. Pnas *109*, 1104–1109.

Mohr, E., Cunningham, A.F., Toellner, K.---M., Bobat, S., Coughlan, R.E., Bird, R.A., Maclennan, I.C.M., and Serre, K. (2010). IFN---{gamma} produced by CD8 T cells induces

T---bet---dependent and ---independent class switching in B cells in responses to alum---precipitated protein vaccine. Proceedings of the National Academy of Sciences *107*, 17292–17297.

Mohr, E., Serre, K., Manz, R.A., Cunningham, A.F., Khan, M., Hardie, D.L., Bird, R., and Maclennan, I.C.M. (2009). Dendritic cells and monocyte/macrophages that create the IL---6/APRIL---rich lymph node microenvironments where plasmablasts mature. The Journal of Immunology *182*, 2113–2123.

Moisini, I., and Davidson, A. (2009). BAFF: a local and systemic target in autoimmune diseases. Clin. Exp. Immunol. *158*, 155–163.

Montecino---Rodriguez, E., and Dorshkind, K. (2012). B---1 B cell development in the fetus and adult. Immunity *36*, 13–21.

Morita, S., Kojima, T., and Kitamura, T. (2000). Plat---E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. *7*, 1063–1066.

Morrison, V.L., Barr, T.A., Brown, S., and Gray, D. (2010). TLR---mediated loss of CD62L focuses B cell traffic to the spleen during Salmonella typhimurium infection. The Journal of Immunology *185*, 2737–2746.

Muppidi, J.R., Arnon, T.I., Bronevetsky, Y., Veerapen, N., Tanaka, M., Besra, G.S., and Cyster, J.G. (2011). Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone. Journal of Experimental Medicine.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation---induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell *102*, 553–563.

Muramatsu, M., Sankaranand, V.S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N.O., and Honjo, T. (1999). Specific expression of activation---induced cytidine deaminase (AID), a novel member of the RNA---editing deaminase family in germinal center B cells. J Biol Chem *274*, 18470–18476.

Muto, T., Okazaki, I.---M., Yamada, S., Tanaka, Y., Kinoshita, K., Muramatsu, M., Nagaoka, H., and Honjo, T. (2006). Negative regulation of activation---induced cytidine deaminase in B cells. P Natl Acad Sci Usa *103*, 2752–2757.

Nagulapalli, S., and Atchison, M.L. (1998). Transcription factor Pip can enhance DNA binding by E47, leading to transcriptional synergy involving multiple protein domains. Mol. Cell. Biol. *18*, 4639–4650.

Ngo, V.N., Cornall, R.J., and Cyster, J.G. (2001). Splenic T zone development is B cell dependent. J. Exp. Med. *194*, 1649–1660.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcgamma receptors as regulators of immune responses. Nat Rev Immunol *8*, 34–47.

Nutt, S.L., Taubenheim, N., Hasbold, J., Corcoran, L.M., and Hodgkin, P.D. (2011). The genetic network controlling plasma cell differentiation. Seminars in Immunology.

O'Connell, R.M., Chaudhuri, A.A., Rao, D.S., Gibson, W.S.J., Balazs, A.B., and Baltimore, D.

(2010a). MicroRNAs enriched in hematopoietic stem cells differentially regulate long---term hematopoietic output. Proceedings of the National Academy of Sciences *107*, 14235–14240.

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., and Baltimore, D. (2010b). Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol *10*, 111–122.

O'Neill, L.A., Sheedy, F.J., and McCoy, C.E. (2011). MicroRNAs: the fine---tuners of Toll---like receptor signalling. Nat Rev Immunol *11*, 163–175.

Ochiai, K., Maienschein---Cline, M., Simonetti, G., Chen, J., Rosenthal, R., Brink, R., Chong, A.S., Klein, U., Dinner, A.R., Singh, H., et al. (2013). Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. Immunity *38*, 918–929.

Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). Antigen---engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS Biol. *3*, e150.

Ooi, A.G.L., Sahoo, D., Adorno, M., Wang, Y., Weissman, I.L., and Park, C.Y. (2010). MicroRNA---125b expands hematopoietic stem cells and enriches for the lymphoid---balanced and lymphoid---biased subsets. Proceedings of the National Academy of Sciences *107*, 21505–21510.

Park, S.---R., Zan, H., Pal, Z., Zhang, J., Al---Qahtani, A., Pone, E.J., Xu, Z., Mai, T., and Casali, P. (2009). HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class---switch DNA recombination and somatic hypermutation. Nature Publishing Group *10*, 540–550.

Patenaude, A.---M., Orthwein, A., Hu, Y., Campo, V.A., Kavli, B., Buschiazzo, A., and Di Noia, J.M. (2009). Active nuclear import and cytoplasmic retention of activation---induced deaminase. Nat. Struct. Mol. Biol. *16*, 517–527.

Paun, A., and Pitha, P.M. (2007). The IRF family, revisited. Biochimie *89*, 744–753.

Paus, D., Phan, T.G., Chan, T.D., Gardam, S., Basten, A., and Brink, R. (2006). Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. J. Exp. Med. *203*, 1081–1091.

Peng, S.L., Szabo, S.J., and Glimcher, L.H. (2002). T---bet regulates IgG class switching and pathogenic autoantibody production. P Natl Acad Sci Usa *99*, 5545–5550.

Pereira, J.P., Kelly, L.M., Xu, Y., and Cyster, J.G. (2009). EBI2 mediates B cell segregation between the outer and centre follicle. Nature *460*, 1122–1126.

Perkins, N.D. (2007). Integrating cell---signalling pathways with NF---kappaB and IKK function. Nat. Rev. Mol. Cell Biol. *8*, 49–62.

Petro, J.B., Rahman, S.M., Ballard, D.W., and Khan, W.N. (2000). Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. J. Exp. Med. *191*, 1745–1754.

Pierce, S.K., and Liu, W. (2010). The tipping points in the initiation of B cell signalling: how small changes make big differences. Nat Rev Immunol *10*, 767–777.

Polo, J.M., Dell'Oso, T., Ranuncolo, S.M., Cerchietti, L., Beck, D., Da Silva, G.F., Prive, G.G., Licht, J.D., and Melnick, A. (2004). Specific peptide interference reveals BCL6 transcriptional and oncogenic mechanisms in B---cell lymphoma cells. Nat. Med. *10*, 1329–1335.

Puissegur, M.P., Eichner, R., Quelen, C., Coyaud, E., Mari, B., Lebrigand, K., Broccardo, C., Nguyen---Khac, F., Bousquet, M., and Brousset, P. (2012). B---cell regulator of immunoglobulin heavy---chain transcription (Bright)/ARID3a is a direct target of the oncomir microRNA---125b in progenitor B---cells. Leukemia *26*, 2224–2232.

Quong, M.W., Martensson, A., Langerak, A.W., Rivera, R.R., Nemazee, D., and Murre, C. (2004). Receptor editing and marginal zone B cell development are regulated by the helix---loop---helix protein, E2A. J. Exp. Med. *199*, 1101–1112.

Ramiro, A.R., Jankovic, M., Eisenreich, T., Difilippantonio, S., Chen---Kiang, S., Muramatsu, M., Honjo, T., Nussenzweig, A., and Nussenzweig, M.C. (2004). AID is required for c---myc/IgH chromosome translocations in vivo. Cell *118*, 431–438.

Randall, T.D., Heath, A.W., Santos---Argumedo, L., Howard, M.C., Weissman, I.L., and Lund, F.E. (1998). Arrest of B lymphocyte terminal differentiation by CD40 signaling: mechanism for lack of antibody---secreting cells in germinal centers. Immunity *8*, 733–742.

Rawlings, D.J., Schwartz, M.A., Jackson, S.W., and Meyer---Bahlburg, A. (2012). Integration of B cell responses through Toll---like receptors and antigen receptors. Nat Rev Immunol *12*, 282–294.

Reif, K., Ekland, E.H., Ohl, L., Nakano, H., Lipp, M., Förster, R., and Cyster, J.G. (2002). Balanced responsiveness to

chemoattractants from adjacent

zones determines B---cell position. Nature *416*, 94–99.

Renshaw, B.R., Fanslow, W.C., Armitage, R.J., Campbell, K.A., Liggitt, D., Wright, B., Davison, B.L., and Maliszewski, C.R. (1994). Humoral immune responses in CD40 ligand---deficient mice. J. Exp. Med. *180*, 1889–1900.

Rickert, R.C., Jellusova, J., and Ana, M., V (2011). Signaling by the tumor necrosis factor receptor superfamily in B---cell biology and disease. Immunol. Rev. *244*, 115–133.

Rothman, P., Lutzker, S., Gorham, B., Stewart, V., Coffman, R., and Alt, F.W. (2004). Structure and expression of germline immunoglobulin gamma 3 heavy chain gene transcripts: implications for mitogen and lymphokine directed class---switching. International Immunology *2*, 621–627.

Rubtsov, A.V., Rubtsova, K., Fischer, A., Meehan, R.T., Gillis, J.Z., Kappler, J.W., and Marrack, P. (2011). Toll---like receptor 7 (TLR7)---driven accumulation of a novel CD11c^{+} B---cell population is important for the development of autoimmunity. Blood *118*, 1305–1315.

Ruland, J., Duncan, G.S., Elia, A., del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D.G., Bouchard, D., Wakeham, A., Ohashi, P.S., et al. (2001). Bcl10 is a positive regulator of

antigen receptor---induced activation of NF---kappaB and neural tube closure. Cell *104*, 33–42.

Rush, J.S., Liu, M., Odegard, V.H., Unniraman, S., and Schatz, D.G. (2005). Expression of activation---induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division---linked class switch recombination. P Natl Acad Sci Usa *102*, 13242–13247.

Sadhu, C., Ting, H.J., Lipsky, B., Hensley, K., Garcia---Martinez, L.F., Simon, S.I., and Staunton, D.E. (2007). CD11c/CD18: novel ligands and a role in delayed---type hypersensitivity. Journal of Leukocyte Biology *81*, 1395–1403.

Saijo, K., Mecklenbräuker, I., Santana, A., Leitger, M., Schmedt, C., and Tarakhovsky, A. (2002). Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha. J. Exp. Med. *195*, 1647–1652.

Saito, M., Gao, J., Basso, K., Kitagawa, Y., Smith, P.M., Bhagat, G., Pernis, A., Pasqualucci, L., and Dalla---Favera, R. (2007). A Signaling Pathway Mediating Downregulation of BCL6 in Germinal Center B Cells Is Blocked by BCL6 Gene Alterations in B Cell Lymphoma. Cancer Cell *12*, 280–292.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K., et al. (2003). Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity *18*, 675–685.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. *182*, 389–400.

Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Tanaka, Y., Kumagai, Y., Miyake, T., Matsushita, K., Okazaki, T., Saitoh, T., et al. (2010). The Jmjd3---Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nature Publishing Group *11*, 936–944.

Sayegh, C.E., Quong, M.W., Agata, Y., and Murre, C. (2003). E---proteins directly regulate expression of activation---induced deaminase in mature B cells. Nat Immunol *4*, 586–593.

Schenten, D., Kracker, S., Esposito, G., Franco, S., Klein, U., Murphy, M., Alt, F.W., and Rajewsky, K. (2009). Pol zeta ablation in B cells impairs the germinal center reaction, class switch recombination, DNA break repair, and genome stability. Journal of Experimental Medicine *206*, 477–490.

Schiemann, B., Gommerman, J.L., Vora, K., Cachero, T.G., Shulga---Morskaya, S., Dobles, M., Frew, E., and Scott, M.L. (2001). An essential role for BAFF in the normal development of B cells through a BCMA---independent pathway. Science *293*, 2111–2114.

Scholzen, T., and Gerdes, J. (2000). The Ki---67 protein: from the known and the unknown. J. Cell. Physiol. *182*, 311–322.

Schrader, C.E., Guikema, J.E.J., Linehan, E.K., Selsing, E., and Stavnezer, J. (2007). Activation---induced cytidine deaminase---dependent DNA breaks in class switch

recombination occur during G1 phase of the cell cycle and depend upon mismatch repair. J Immunol *179*, 6064–6071.

Sciammas, R., Li, Y., Warmflash, A., Song, Y., Dinner, A.R., and Singh, H. (2011). An incoherent regulatory network architecture that orchestrates B cell diversification in response to antigen signaling. Mol Syst Biol *7*, 1–15.

Sciammas, R., Shaffer, A.L., Schatz, J.H., Zhao, H., Staudt, L.M., and Singh, H. (2006). Graded expression of interferon regulatory factor---4 coordinates isotype switching with plasma cell differentiation. Immunity *25*, 225–236.

Sen, R., and Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer---binding protein Nf---kappa B by a posttranslational mechanism. Cell *47*, 921–928.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF---kappa B leads to multifocal defects in immune responses. Cell *80*, 321–330.

Shaffer, A.L., Yu, X., He, Y., Boldrick, J., Chan, E.P., and Staudt, L.M. (2000). BCL---6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity *13*, 199–212.

Shaffer, A.L., Emre, N.C.T., Lamy, L., Ngo, V.N., Wright, G., Xiao, W., Powell, J., Dave, S., Yu, X., Zhao, H., et al. (2008). IRF4 addiction in multiple myeloma. Nature *454*, 226–231.

Shah, H.B., Joshi, S.K., Rampuria, P., Devera, T.S., Lang, G.A., Stohl, W., and Lang, M.L. (2013). BAFF--- and APRIL---Dependent Maintenance of Antibody Titers after Immunization with T---Dependent Antigen and CD1d---Binding Ligand. The Journal of Immunology.

Shapiro---Shelef, M., Lin, K.---I., McHeyzer---Williams, L.J., Liao, J., McHeyzer---Williams, M.G., and Calame, K. (2003). Blimp---1 is required for the formation of immunoglobulin secreting plasma cells and pre---plasma memory B cells. Immunity *19*, 607–620.

Shenoy, G.N., Chatterjee, P., Kaw, S., Mukherjee, S., Rathore, D.K., Bal, V., Rath, S., and George, A. (2012). Recruitment of memory B cells to lymph nodes remote from the site of immunization requires an inflammatory stimulus. The Journal of Immunology *189*, 521–528.

Shih, T.---A.Y., Roederer, M., and Nussenzweig, M.C. (2002). Role of antigen receptor affinity in T cell---independent antibody responses in vivo. Nat Immunol *3*, 399–406.

Shindo, H., Yasui, K., Yamamoto, K., Honma, K., Yui, K., Kohno, T., Ma, Y., Chua, K.J., Kubo, Y., Aihara, H., et al. (2011). Interferon regulatory factor---4 activates IL---2 and IL---4 promoters in cooperation with c---Rel. Cytokine *56*, 564–572.

Shiow, L.R., Rosen, D.B., Brdicková, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon---alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540–544.

Siegert, S., and Luther, S.A. (2012). Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes. Front Immunol *3*, 285.

Singh, H., and Sciammas, R. (2006). Shedding B cell identity. Immunity *24*, 239–240.

Smith, B.A., Gartner, S., Liu, Y., Perelson, A.S., Stilianakis, N.I., Keele, B.F., Kerkering, T.M., Ferreira---Gonzalez, A., Szakal, A.K., Tew, J.G., et al. (2001). Persistence of infectious HIV on follicular dendritic cells. J Immunol *166*, 690–696.

Snapper, C.M., and Paul, W.E. (1987). Interferon---gamma and B cell stimulatory factor---1 reciprocally regulate Ig isotype production. Science *236*, 944–947.

Snapper, C.M., Zelazowski, P., Rosas, F.R., Kehry, M.R., Tian, M., Baltimore, D., and Sha, W.C. (1996). B cells from p50/NF---kappa B knockout mice have selective defects in proliferation, differentiation, germ---line CH transcription, and Ig class switching. J Immunol *156*, 183–191.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N., and Takatsu, K. (1989). Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production. J. Exp. Med. *170*, 1415–1420.

Soyer, O.U., Akdis, M., Ring, J., Behrendt, H., Crameri, R., Lauener, R., and Akdis, C.A. (2013). Mechanisms of peripheral tolerance to allergens. Allergy *68*, 161–170.

Spain, L.M., and Mulligan, R.C. (1992). Purification and characterization of retrovirally transduced hematopoietic stem cells. P Natl Acad Sci Usa *89*, 3790–3794.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Stamm, C., Barthelmann, J., Kunz, N., Toellner, K.---M., Westermann, J., and Kalies, K. (2013). Dose---Dependent Induction of Murine Th1/Th2 Responses to Sheep Red Blood Cells Occurs in Two Steps: Antigen Presentation during Second Encounter Is Decisive. PLoS One *8*, e67746.

Stavnezer, J., Guikema, J.E.J., and Schrader, C.E. (2008). Mechanism and Regulation of Class Switch Recombination. Annu. Rev. Immunol. *26*, 261–292.

Stein, J.V., López---Fraga, M., Elustondo, F.A., Carvalho---Pinto, C.E., Rodríguez, D., Gómez---Caro, R., De Jong, J., Martínez---A, C., Medema, J.P., and Hahne, M. (2002). APRIL modulates B and T cell immunity. J. Clin. Invest. *109*, 1587–1598.

Su, T.T., Guo, B., Kawakami, Y., Sommer, K., Chae, K., Humphries, L.A., Kato, R.M., Kang, S., Patrone, L., Wall, R., et al. (2002). PKC---beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. Nat Immunol *3*, 780–786.

Sukumar, S., Shikh, El, M.E., Tew, J.G., and Szakal, A.K. (2008). Ultrastructural study of highly enriched follicular dendritic cells reveals their morphology and the periodicity of immune complex binding. Cell Tissue Res. *332*, 89–99.

Sze, D.M., Toellner, K.M., García De Vinuesa, C., Taylor, D.R., and MacLennan, I.C. (2000). Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. J. Exp. Med. *192*, 813–821.

Ta, V.---T., Nagaoka, H., Catalan, N., Durandy, A., Fischer, A., Imai, K., Nonoyama, S., Tashiro, J., Ikegawa, M., Ito, S., et al. (2003). AID mutant analyses indicate requirement for class---switch---specific cofactors. Nat Immunol *4*, 843–848.

Tan, L.P., Wang, M., Robertus, J.---L., Schakel, R.N., Gibcus, J.H., Diepstra, A., Harms, G., Peh, S.---C., Reijmers, R.M., Pals, S.T., et al. (2009). miRNA profiling of B---cell subsets: specific miRNA profile for germinal center B cells with variation between centroblasts and centrocytes. Lab. Invest. *89*, 708–716.

Tang, M.L., Steeber, D.A., Zhang, X.Q., and Tedder, T.F. (1998). Intrinsic differences in L---selectin expression levels affect T and B lymphocyte subset---specific recirculation pathways. J Immunol *160*, 5113–5121.

Tanigaki, K., Han, H., Yamamoto, N., Tashiro, K., Ikegawa, M., Kuroda, K., Suzuki, A., Nakano, T., and Honjo, T. (2002). Notch---RBP---J signaling is involved in cell fate determination of marginal zone B cells. Nat Immunol *3*, 443–450.

Taylor, J.J., Pape, K.A., and Jenkins, M.K. (2012). A germinal center---independent pathway generates unswitched memory B cells early in the primary response. Journal of Experimental Medicine *209*, 597–606.

Taylor, P.R., Pickering, M.C., Kosco---Vilbois, M.H., Walport, M.J., Botto, M., Gordon, S., and Martinez---Pomares, L. (2002). The follicular dendritic cell restricted epitope, FDC---M2, is complement C4; localization of immune complexes in mouse tissues. Eur J Immunol *32*, 1888–1896.

Teng, G., Hakimpour, P., Landgraf, P., Rice, A., Tuschl, T., Casellas, R., and Papavasiliou, F.N. (2008). MicroRNA---155 is a negative regulator of activation---induced cytidine deaminase. Immunity *28*, 621–629.

Terrier, B., Joly, F., Vazquez, T., Benech, P., Rosenzwajg, M., Carpentier, W., Garrido, M., Ghillani---Dalbin, P., Klatzmann, D., Cacoub, P., et al. (2011). Expansion of functionally anergic CD21---/low marginal zone---like B cell clones in hepatitis C virus infection---related autoimmunity. The Journal of Immunology *187*, 6550–6563.

Tew, J.G., Wu, J., Fakher, M., Szakal, A.K., and Qin, D. (2001). Follicular dendritic cells: beyond the necessity of T---cell help. Trends in Immunology *22*, 361–367.

Tew, J.G., Wu, J., Qin, D., Helm, S., Burton, G.F., and Szakal, A.K. (1997). Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. Immunol. Rev. *156*, 39–52.

Toellner, K.M.K., Gulbranson---Judge, A.A., Taylor, D.R.D., Sze, D.M.D., and MacLennan, I.C.I. (1996). Immunoglobulin switch transcript production in vivo related to the site and time of antigen---specific B cell activation. J. Exp. Med. *183*, 2303–2312.

Toellner, K.M., Luther, S.A., Sze, D.M., Choy, R.K., Taylor, D.R., MacLennan, I.C., and Acha---Orbea, H. (1998). T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J. Exp. Med. *187*, 1193–1204.

Toellner, K.---M., Jenkinson, W.E., Taylor, D.R., Khan, M., Sze, D.M.Y., Sansom, D.M., Vinuesa, C.G., and Maclennan, I.C.M. (2002). Low---level hypermutation in T cell---independent

germinal centers compared with high mutation rates associated with T cell---dependent germinal centers. J. Exp. Med. *195*, 383–389.

Toyama, H., Okada, S., Hatano, M., Takahashi, Y., Takeda, N., Ichii, H., Takemori, T., Kuroda, Y., and Tokuhisa, T. (2002). Memory B cells without somatic hypermutation are generated from Bcl6---deficient B cells. Immunity *17*, 329–339.

Tran, T.H., Nakata, M., Suzuki, K., Begum, N.A., Shinkura, R., Fagarasan, S., Honjo, T., and Nagaoka, H. (2009). B cell–specific and stimulation---responsive enhancers derepress Aicda by overcoming the effects of silencers. Nature Publishing Group *11*, 148–154.

Turner, C.A., Mack, D.H., and Davis, M.M. (1994). Blimp---1, a novel zinc finger---containing protein that can drive the maturation of B lymphocytes into immunoglobulin---secreting cells. Cell *77*, 297–306.

Turner, M., and Vigorito, E. (2008). Regulation of B--- and T---cell differentiation by a single microRNA. Biochem. Soc. Trans *36*, 531.

van Engeland, M., Ramaekers, F.C., Schutte, B., and Reutelingsperger, C.P. (1996). A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry *24*, 131–139.

van Ginkel, F.W., Wahl, S.M., Kearney, J.F., Kweon, M.N., Fujihashi, K., Burrows, P.D., Kiyono, H., and McGhee, J.R. (1999). Partial IgA---deficiency with increased Th2---type cytokines in TGF---beta 1 knockout mice. J Immunol *163*, 1951–1957.

van Kooten, C., and Banchereau, J. (2000). CD40---CD40 ligand. Journal of Leukocyte Biology *67*, 1–17.

Victoratos, P., Lagnel, J., Tzima, S., Alimzhanov, M.B., Rajewsky, K., Pasparakis, M., and Kollias, G. (2006). FDC---specific functions of p55TNFR and IKK2 in the development of FDC networks and of antibody responses. Immunity *24*, 65–77.

Vigorito, E., Perks, K.L., Abreu---Goodger, C., Bunting, S., Xiang, Z., Kohlhaas, S., Das, P.P., Miska, E.A., Rodriguez, A., Bradley, A., et al. (2007). microRNA---155 Regulates the Generation of Immunoglobulin Class---Switched Plasma Cells. Immunity *27*, 847–859.

Vikstrom, I., Carotta, S., Luthje, K., Peperzak, V., Jost, P.J., Glaser, S., Busslinger, M., Bouillet, P., Strasser, A., Nutt, S.L., et al. (2010). Mcl---1 Is Essential for Germinal Center Formation and B Cell Memory. Science *330*, 1095–1099.

Vinuesa, C.G., Cook, M.C., Ball, J., Drew, M., Sunners, Y., Cascalho, M., Wabl, M., Klaus, G.G.B., and Maclennan, I.C.M. (2000). germinal centres without t cells. J. Exp. Med. *191*, 485–493.

Vinuesa, C.G., Sze, D.M.Y., Cook, M.C., Toellner, K.---M., Klaus, G.G.B., Ball, J., and Maclennan, I.C.M. (2003). Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens. Eur J Immunol *33*, 297–305.

Vinuesa, C., Sunners, Y., Pongracz, J., Ball, J., Toellner, K., Taylor, D., MACLENNAN, I., and Cook, M. (2001). Tracking the response of Xid B cells in vivo: Tl---2 antigen induces migration and proliferation but Btk is essential for terminal differentiation. Eur J Immunol *31*, 1340–1350.

Vuong, B.Q., and Chaudhuri, J. (2012). Combinatorial mechanisms regulating AID---dependent DNA deamination: Interacting proteins and post---translational modifications. Seminars in Immunology *24*, 264–272.

Wakabayashi, C., Adachi, T., Wienands, J., and Tsubata, T. (2002). A distinct signaling pathway used by the IgG---containing B cell antigen receptor. Science *298*, 2392–2395.

Watanabe, K., Sugai, M., Nambu, Y., Osato, M., Hayashi, T., Kawaguchi, M., Komori, T., Ito, Y., and Shimizu, A. (2010). Requirement for Runx proteins in IgA class switching acting downstream of TGF---beta 1 and retinoic acid signaling. The Journal of Immunology *184*, 2785–2792.

Weih, D.S., Yilmaz, Z.B., and Weih, F. (2001). Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. J Immunol *167*, 1909–1919.

Wieder, R., Cornetta, K., Kessler, S.W., and Anderson, W.F. (1991). Increased efficiency of retroviral---mediated gene transfer and expression in primate bone marrow progenitors after 5---fluorouracil---induced hematopoietic suppression and recovery. Blood *77*, 448–455.

William, J., Euler, C., Christensen, S., and Shlomchik, M.J. (2002). Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. Science *297*, 2066–2070.

Xu, J., Foy, T.M., Laman, J.D., Elliott, E.A., Dunn, J.J., Thomas J Waldschmidt, I.J.E.T.R.J.N.S., Elesmore, J., Noelle, R.J., and Flavellt, R.A. (1994). Mice Deficient for the CD40 Ligand. Immunity *1*, 423–431.

Xu, Z., Fulop, Z., Wu, G., Pone, E.J., Zhang, J., Mai, T., Thomas, L.M., Al---Qahtani, A., White, C.A., Park, S.---R., et al. (2010). 14---3---3 adaptor proteins recruit AID to 5"---AGCT---3---"rich switch regions for class switch recombination. Nat. Struct. Mol. Biol. *17*, 1124–1135.

Yamane, A., Robbiani, D.F., Resch, W., Bothmer, A., Nakahashi, H., Oliveira, T., Rommel, P.C., Brown, E.J., Nussenzweig, A., Nussenzweig, M.C., et al. (2013). RPA accumulation during class switch recombination represents 5"---3" DNA---end resection during the S---G2/M phase of the cell cycle. Cell Rep *3*, 138–147.

Yoshizaki, A., Miyagaki, T., DiLillo, D.J., Matsushita, T., Horikawa, M., Kountikov, E.I., Spolski, R., Poe, J.C., Leonard, W.J., and Tedder, T.F. (2012). Regulatory B cells control T---cell autoimmunity through IL---21---dependent cognate interactions. Nature *491*, 264–268.

Zhang, J., Jima, D.D., Jacobs, C., Fischer, R., Gottwein, E., Huang, G., Lugar, P.L., Lagoo, A.S., Rizzieri, D.A., Friedman, D.R., et al. (2009). Patterns of microRNA expression characterize stages of human B---cell differentiation. Blood *113*, 4586–4594.

Zhao, A., Zeng, Q., Xie, X., Zhou, J., Yue, W., Li, Y., and Pei, X. (2012). MicroRNA---125b induces cancer cell apoptosis through suppression of Bcl---2 expression. J Genet Genomics *39*, 29–35.

Zhong, H., May, M.J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF---kappa B determines its association with CBP/p300 or HDAC---1. Mol. Cell *9*, 625–636.