Project one: The Role Of Lyp Phosphatase in Primary Human Macrophages

Project two: cRel and its effect on class switch recombination, IRF4 induction and plasma cell differentiation during a T-Independent immune response

By

LEWIS BLAIR CAWKWELL

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College of Medical and Dental Sciences

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Abstract

PTPN22 encodes the protein tyrosine phosphatase Lyp. Lyp is a known negative regulator of TCR signalling where it has been shown to dephosphorylate Src family kinases. Certain mutations in *PTPN22* are associated with increased risk of autoimmune disease such as rheumatoid arthritis. This includes the well documented C1858T substitution. Previous studies into understanding the role of *PTPN22* in health and disease have focused on lymphocytes, primarily T cells. Despite this, myeloid cells have a higher expression of Lyp. Lyp can also interact with other signalling proteins, including the Rho GTPase, Vav, which has multiple roles in cell signalling pathways. This project therefore looked at the role of Lyp in primary human macrophages by using a competitive reversible inhibitor of Lyp protein activity. It was shown that Lyp is a negative regulator of reactive oxygen species (ROS) production in unstimulated cells and in response to PMA. We also show that primary macrophages heterozygous for the autoimmune-associated C1858T variant produce high levels of pro-inflammatory cytokines in apparently unstimulated conditions. These novel findings provide insight into how C1858T is associated with inflammatory disease such as rheumatoid arthritis.

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Abbreviations

BCR	B cell receptor
DAG	Diamino glycerol
ERK	Extracellular-signal-regulated kinases
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motiff
LAT	Linker for activation of T cells
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ	phospholipase C γ
SH2	Src homology 2
SH3	Src homology 3
SNP	single nucleotide polymorphism
TCR	T cell receptor
WASP	Wiskott-Aldrich syndrome protein
ZAP-70	zeta chain associated protein kinase 70

Introduction

Many autoimmune diseases, including rheumatoid arthritis are thought to have genetic predispositions and many genome-wide association studies have identified genetic loci which correlate with autoimmunity. A large number of genes may be involved in susceptibility to particular diseases with, for example, at least 46 contributing to the population risk of developing rheumatoid arthritis [1].

Many of the strongest associations are among the MHC loci [2] but the strongest susceptibility loci outside the MHC [3-7] is a single-nucleotide-polymorphism (SNP) within *PTPN22*. *PTPN22* encodes Lyp phosphatase, which is a known negative regulator of TCR signalling and so studies into Lyp and autoimmunity have concentrated on lymphocytes. This is despite reports that Lyp expression is actually relatively low within T cells and high within myeloid cells [8]. Myeloid cells, particularly macrophages are the predominant cell type within the inflamed synovium in rheumatoid arthritis (RA) [9].

For this reason it would be of interest to investigate the possible roles of Lyp in macrophages. In order to do this, we must first understand the role of Lyp in T cells, as discussed below.

Lyp phosphatase

Lyp, encoded by *PTPN22*, is a protein tyrosine kinase which is a member of the PEST non-receptor classical class I protein tyrosine phosphatase (PTP) family [10]. Structurally, Lyp contains an N-terminal PTP domain and a non-catalytic C-terminal domain. Within the C-terminal are four proline-rich motifs followed by a C-terminal homology domain. PEP, the murine homolog shares 89% amino acid identity within the PTP domain and 61% within the non-catalytic regions [10]. Lyp and PEP are expressed in cells of the haematopoietic system [10,11] with both of these PTP's having been implicated in negative regulation of TCR signalling [10,12,13]. Lyp phosphatase came to prominence after an SNP

variant was found be linked to the autoimmune disease type-1 diabetes [3]. This variant arises from a C1858T base substitution, resulting in an R620W amino acid change. The same R620W variant has since been identified as a risk factor in RA [4,5] and systemic lupus erythematous [6,7].

The pathways involved in TCR signalling have been reviewed elsewhere [14] and are summarised in figure 1. In brief, upon TCR ligation, Src family kinases such as Lck and Fyn are recruited to the TCR complex. Src family kinases are capable of intracellular phosphorylation of ITAMs found on CD3 and the ζ chains within the TCR complex. Zap-70, a Syk kinases family member, is recruited to double phosphorylated ITAMs via its two SH2 domains. Lck can then phosphorylate Zap70 within its activation tyrosines Y319 and Y343.

Adaptor proteins are essential in linking the early signalling events with downstream molecules. LAT is a membrane bound adaptor recruited to the TCR complex. Zap-70 can phosphorylate LAT at several tyrosines. LAT can then recruit multiple further molecules via their SH2 domains including PLCγ, PI3K, SLP76 and Grb2. This allows formation of signalling microclusters where localised protein-protein interactions occur. These molecules are important in the downstream signalling pathways typical of T cell activation. Lck associates with CD8 and CD4 co-receptors. Under steady state, Lck is phosphorylated at the negative regulatory tyrosine Y505 by Csk, keeping it inactive [15,16]. Phosphorylated Y505 causes the inhibitory 'tail bite' effect where Lck's internal SH2 domain binds to Y505 [16]. Under steady state, Csk associates through its SH2 domain with phosphorylated PAG at the plasma membrane, where it can negatively regulate Lck [17]. Lck is also a substrate of Lyp [12]. It is believed Lyp keeps the activation tyrosine Y394 dephosphorylated, thereby contributing to Lck inactivation [17].

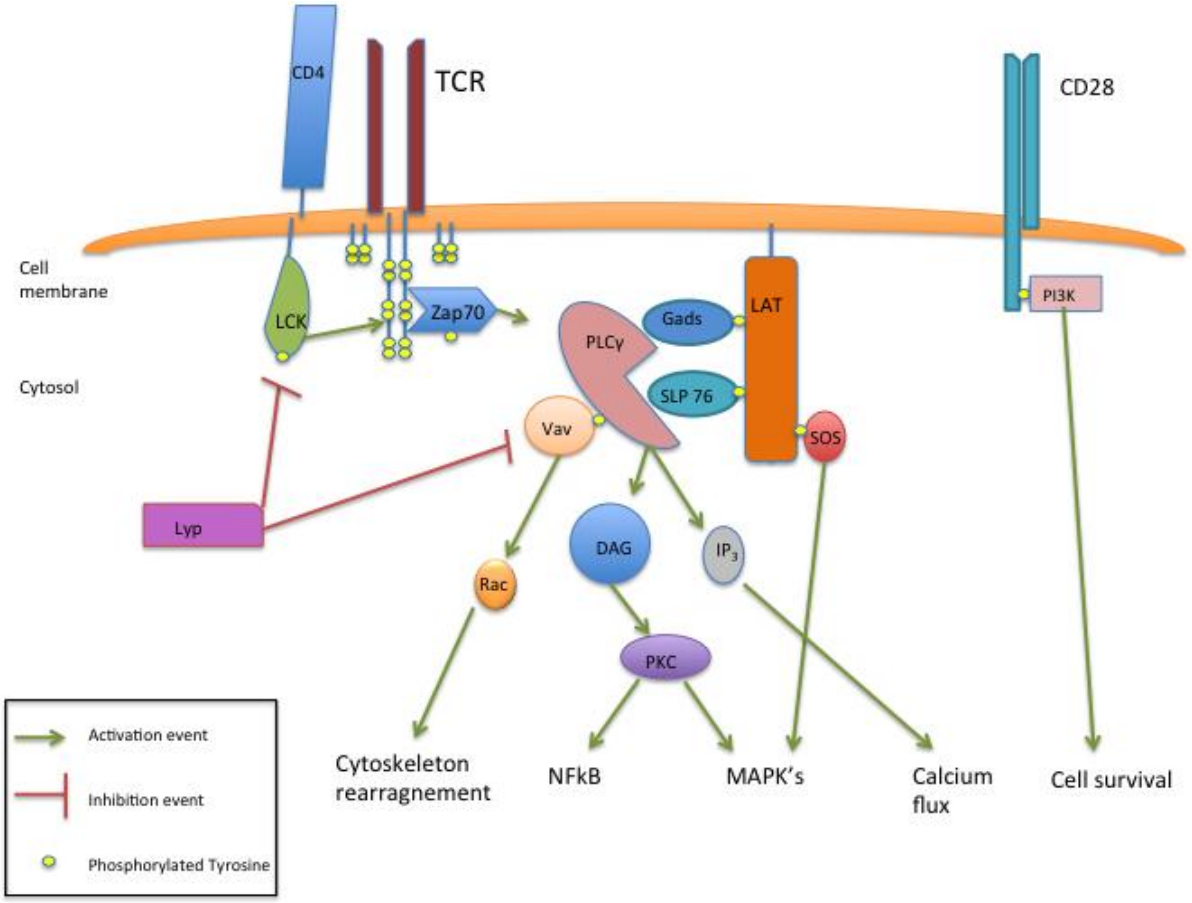

Figure 1: Summary of CD4 T cell TCR signalling and Lyp interaction. After antigen stimulation of the TCR, the CD4 co-receptor comes into proximity, bringing with it Lck. Lck phosphorylates the ITAMs of the CD3 chains and zeta chains associated with the TCR. Zap70 is recruited to the ITAMs and phosphorylated and activated by Lck. Zap70 can then phosphorylate LAT and the several molecules that it recruits, forming a signalling complex. PLCγ can cleave membrane bound phosphoinositol biphsophate (not showing), generating IP_3 and DAG. IP_3 acts as a secondary messenger facilitating calcium flux. DAG can activate isoforms of PKC without calcium, and isoforms with calcium. The MAPK's ERK, JNK and P38 are important in many cell signalling cascades and processes. TCR, T cell receptor; PLCγ, phospholipase C gamma; DAG, diacylglycerol; LAT, linker for activation of T cells; SOS, son of sevenless; P13K, phosphoinositide 3 kinase; IP3, inositol triphosphate; PKC, phosphokinase C; ZAP-70, zeta chain associated protein kinase 70; NFκB, nuclear factor Kb.

Furthermore, Lyp/PEP can interact with Csk [13,18,19]. This interaction is mediated through the Csk SH3 domain and the Proline-rich 1 (P1) domain of Lyp. One model proposes this interaction to be essential for Lyp function [17]. While Csk is bound to PAG, its recruitment of Lyp to the lipid raft would bring it into the proximity of its substrates, allowing a synergistic inhibition of Lck under steady-state conditions. Upon TCR stimulation, Lck can be activated and PAG is dephosphorylated by an unknown phosphatase. Csk can then no longer bind and is released [20]. Lck is then dephosphorylated at Y505 by an unknown phosphatase. This is followed by Lck autophosphorylation at Y394 and its full activation.

The R620W mutation and the role of Lyp in T cells

The R620W mutation occurs within the P1 domain motif, the site of the Csk interaction. It is proposed this mutation prevents Lyp from binding Csk [3]. A cell line transfection study with the W620 variant suggested it to cause a loss of phosphatase function [21]. The variant could also be a less stable protein [22]. The PEP variant R619W compares to the Lyp R620W mutation. When knocked into mice, PEP protein levels were largely reduced. The authors argue that a reduced Lyp protein availability results in a reduced TCR negative regulation causing increased T cell activation. Supporting this, knock-in mice had increased activated/memory lymphocytes. Yet mice did not exhibit signs of autoimmunity despite the well identified correlation between Lyp and autoimmunity. It should also be noted PEP and Lyp are much less conserved within the catalytic domain, so it is possible that protein interactions and any mechanisms of degradation could differ.

Multiple other studies have found the variant to have a gain of phosphatase function. Primary T cells homozygous and heterozygous for W620 or transfection of primary human T cells with the variant have been found to have greater inhibition of TCR signalling. This includes reductions in calcium flux; phosphorylation of ITAMS, ERK and LAT; IL2 and IL10 production; activation markers; and proliferation [23] [24] [25].

The general consensus from these results is that the R620W mutation results in a gain of function; i.e. increased inhibition of TCR signalling. There is some difficulty in understanding how this concept fits into the model of Lyp and TCR signalling. It may be that the poor interaction with Csk actually impedes Lyp negative regulation. Lck is capable of phosphorylating Lyp at inhibitory tyrosine Y536 [26]. Phosphorylation here is important in TCR negative regulation. The W620 variant shows less phosphorylation here and it's proposed a Csk interaction is required to allow Lck to act on Lyp. Lyp can also be negatively phosphorylated by PKCδ at serine 35 [27]. It is possible that failure to interact with Csk could also impede this negative regulation.

Evidence has accumulated against the requirement for Lyp/Csk interaction at the plasma membrane for Lyp function. When Csk is bound to PAG, it was found not to co-immunoprecipitate with Lyp or PEP [25,28]. Vang and colleagues provide evidence for a model where Lyp and Csk are in a cytosolic complex within resting T cells, with Lyp in an inhibited state. Upon TCR ligation, the complex dissociates and Lyp can be recruited to the lipid raft. Here Lyp can function as a phosphatase and negatively regulate T cell signalling.[25]. So Lyp could function in negative feedback of TCR stimulation.

No matter which model is correct, it is still not fully understood how a gain in Lyp phosphatase function could relate to an increased risk in autoimmunity. One theory arose from the SKG mouse strain [22]. These mice spontaneously develop a chronic arthritis similar to RA. This is due to a ZAP-70 mutation causing poor association with ITAMs, therefore reducing its phosphorylative capacity. This results in poor signal transduction upon TCR ligation. An altered thymocyte selection was found in SKG mice, due to the lowered TCR signalling threshold in autoreactive thymocytes that would otherwise have been deleted. The concept of an increased positive selection of autoreactive thymocytes due to an increased negative regulation by Lyp variants is therefore possible.

As mentioned above Lyp has a number of target substrates among proteins involved in TCR signalling, but it has a broader range of substrates than these, as observed from substrate trapping experiments with a Lyp variant without phosphatase activity. In these experiments Lyp has also been found to associate with Vav proteins [12]. These have a wide range of functions in activation and adhesion of leukocytes and the roles of Vav are discussed below.

Vav

The Vav family of proteins are a conserved group found from nematodes to humans where they act as guanine exchange factors (GEFs) for Rho GTPases [29]. Rho GTPases are molecular switches which are either in the 'active' GTP-bound or 'inactive' GDP-bound form. Vav proteins catalyse the activation of the Rho GTPase via exchange of GDP to GTP. In this active state, Rho GTPases can

interact with effectors, playing essential roles in the regulation of actin polymerisation; cell cycle; gene expression; and cell adhesion [30]. Vav1, Vav2 and Vav3 are all found in mammals, where Vav1 is restricted mainly to haematopoietic cells while Vav2 and Vav3 have a much broader expression [29].

Vav Structure

Vav protiens are highly conserved with the same basic structure. The central Dbl homology (DH) domain exhibits GEF activity to Rho GTPases. To the carboxyl side lie the pleckstrin homology (PH) and C1 domains which have been implicated with the regulation of GEF activity. Amino to DH is the calponin homology (CH) and the acidic domains which can inhibit GEF activity. Also found at the carboxyl terminus are the two SH3 and single SH2 domains. SH3 and SH2 domains function in protein-protein interactions and therefore it is believed that Vav can also function as an adaptor during signalling, a function not found in other GEFs [31].

Vav Activation

Vav GEF activity depends on Tyrosine phosphorylation [32]. Upon TCR and BCR stimulation, Vav is rapidly phosphorylated [33,34] by Src family kinases [31]. Phosphorylation of the three Tyrosine residues within the acidic domain allows binding of GTPase substrates [35]. Phosphorylation within the carboxyl terminal can also regulate GEF activity [36]. Phosphoinisitols which bind the PH domain can also regulate GEF activity and assist in Vav membrane recruitment [37]. Vav1 has been shown to favour its GEF activity towards Rac1, Rac2 and RhoG [32,38]. Vav2 and Vav3 favour RhoA, RhoB and RhoG [39,40].

Vav functions

Since Vav is activated following surface receptor stimulation, Vav proteins may act as links between the cell surface receptor and signal transduction, particularly cytoskeletal rearrangements. For example, in TCR signalling and the immunological synapses [41]; macrophage adhesion [42]; neutrophil activation after integrin [43]; and neutrophil and macrophage chemotaxis to receptor ligands [44 45].

Interestingly, Vav also plays an important role in other cellular activities as best exemplified in TCR signalling. T cells lacking Vav1 show defects in proliferation, reduced activation marker expression, reduced calcium flux and cytokine release after TCR stimulation [31]. Furthermore, Vav1 deficient mice have defects in positive and negative selection of thymocytes [31]. The functions of Vav1 in T cell signalling are discussed below.

Vav1 GEF activity is required in some T cell signalling pathways involving Rac, Akt and integrins. However GEF activity is not required in calcium flux, ERK MAP kinase activation, cell polarisation and NFAT activation [36,46]. The SH3-SH2-SH3 cassette and acidic domain tyrosines can interact with several proteins involved in TCR signalling such as Shc, Csk, Sap, NCK, SLP76, ZAP-70 Grb2 [31,36] and Lck, PI3K and PLCγ1 [47]. Together, this indicates Vav to have adaptor functions.

Vav complex formation can lead to many downstream effector functions, including PLCγ1 activation [48]. A Vav1/SLP76 complex can recruit and activate PLCγ1 [48,49], essential in several downstream functions, including calcium flux, proliferation, NFAT activation, and ERK activation [50-52]. Vav can lead to Ras GEF activation through several mechanisms, including cytoskeletal rearrangements [53] and Grb2 recruitment, which recruits the Ras GEF SOS [54]. The same SH3 domain allows a Vav1/NCK/SLP76 complex, which controls actin rearrangement at the immunological synapse through WASP [55,56].

Therefore, Vav has several roles in T cell signalling and activation and Lyp could potentially regulate these activities through Vav by directly dephosphorylating essential Tyrosine residues. Since Lyp is expressed highest within myeloid cells such as macrophages it's important to understand these pathways.

Macrophage FcγR signalling

Fcγ receptors (FcγR) bind to the Fc portion of IgG antibodies. There are three FcγR classes in humans: RI (CD64) which binds with high affinity to IgG; RII (CD32) and RIII (CD16) which bind with low affinity

to IgG [57]. Each of these receptors express or are associated with an intracellular signalling domain. The inhibitory receptor, (only FcγRIIb) contains an ITIM and can have negative effects on cell activation. The activating receptors contain/associate with an ITAM and can facilitate cell activation signals and phagocytosis. FcγRs belong to the same immunoglobulin superfamily as the TCR. Therefore, both signalling pathways have considerable overlap.

FcγR Initiation of signalling

ITAM phosphorylation is carried out by the Src family kinases Lyn, Hck and Fgr and Syk kinase is recruited and phosphorylated [58-60] [61,62]. Syk and Lyn can phosphorylate LAT, allowing downstream signalisomes [63,64]. Despite SLP76 being highly expressed in macrophages, it appears not to be required in signal transduction [65-67].

The downstream pathways

Just like TCR signalling, there is downstream activation of PLCγ, PI3K, MAPKs and transcription factors [60]. Details of some of these pathways are described below.

As seen in TCR signalling, PLCγ can be recruited to LAT and activated by Syk kinase activity, resulting in IP3 generation and subsequent calcium release [68] [69]. This calcium flux may not be required for FcγR-mediated phagocytosis [70,71] but is required for phagosomal maturation [72]. During FcγR-mediated phagocytosis, PLCγ is recruited to the phagocytic cup. Its inhibition results in poor phagocytosis in murine macrophages [73]. Since calcium may not be required initially, the PLCγ generation of DAG may be important here. DAG is capable of activating the novel and classic PKC isoforms which have a role in ERK activation in macrophage phagocytosis

After FcγR stimulation, PKC can activate Raf-1 which can initiate the ERK MAPK pathway. Inhibition of ERK can also impair phagocytosis in macrophages [74,75]. [76]. ERK's role here could possibly be the activation of PLA2 which mediates production of Arachidonic acid which facilitates FcγR-mediated phagocytosis [77]. PI3K is recruited to LAT where it is activated by Syk [57]. Inhibition of PI3K can prevent

FcγR-mediated phagocytosis due to a failure of phagosome closure in murine macrophages [78,79] [80]. PI3K can also initiate ERK activation after FcγR ligation in monocytes and macrophages [81,82].

In macrophages, the rho family GTPases Rac and cdc42 are essential in actin polymerisation and ingestion of IgG opsonised substrates [83,84]. Vav is required in FcγR-mediated phagocytosis in murine macrophage-like cell lines[85] but not in murine primary macrophages [86]. Similarly, FcγR-mediated Rac activation did not require Vav in these primary macrophages [86]

Macrophage ROS production

ROS production requires the NADPH oxidase modular enzyme. The classical model of NADPH oxidase activation involves a calcium-dependent activation of PKC [87]. PKC then phosphorylates NADPH subunits within the cytosol. A complex of p47phox/p67phox/p40phox with Rac1 or Rac2 then forms. Vav is required for Rac activation. This complex translocates to the plasma membrane where it assembles with the flavocytochromes gp91phox and p22phox forming the full enzyme. The oxidase is then active within the membrane of phagosomes. NADPH is utilised as a substrate to reduce O2 to $O2^-$, initiating ROS production.

ROS production accompanies many macrophage downstream signalling events. For example, FcγR-mediated phagocytosis and production of ROS are mediated by related pathways in macrophages. Here, the Syk, PI3K, PKC activation pathway leads to both phagocytosis and ROS production after FcγR ligation [76].

Macrophage Cytokine signalling

Macrophages are major producers of pro-inflammatory cytokines during inflammation, including macrophages in the synovium during RA. The regulation of these cytokines depends on transcription factors that are typically activated in several receptor signal transduction pathways. NFκB is an obvious example [88] and this has been shown in macrophage TNFα regulation [89,90]. MAPKs are also implicated in the regulation of TNFα. P38 MAPK can regulate TNFα mRNA stability [91] and regulate

several transcription factors, including NFκB [92-94]. In primary human macrophages, p38 MAPK has been shown to regulate TNFα transcription via NFκB [91]. Another study described p38 MAPK regulation of NFκB in conjunction with ERK [95].

NFAT is another important transcription factor in cytokine regulation, including TNFα [96]. Most of the NFAT family are activated through calcium signalling. NFAT acts synergistically with AP-1. AP-1 activation depends on heavily on MAPK pathways [97]. Therefore, multiple points where Lyp and Vav could influence cytokine production exist.

This project

The aim of this project is to investigate whether Lyp is involved in the three major macrophage signalling pathways discussed: FcγR; ROS production; and cytokine production. We believe that based the molecules involved in these pathways, Lyp is likely to be involved, either directly, or through Vav regulation. The use of a Lyp inhibitor will be used to investigate the effect found within these pathways. We hypothesized that Lyp inhibition will cause decreased negative regulation of these pathways, in other words, these pathways will be more active. By using a gold-based compound capable of competitive reversible inhibition of PTP activity, with a 10-fold selectivity for Lyp over other PTP's [98] we showed that Lyp is involved in Macrophage signalling. This was most evident In ROS production. We also show that Lyp might regulate Vav phosphorylation.

Materials and Methods

Primary macrophage generation from human blood

From healthy donor from the lab

300µl of EDTA (Sigma) per 10ml of blood was added to a 50ml tube. 30 to 40mls of blood was then drawn and poured into the tube. Blood was made up to 50ml with RPMI (Sigma). 15ml of ficoll (GE healthcare) was added each to two 50ml tubes. 25ml of blood were then pipetted on top of the ficoll layers. These tubes were centrifuged for 30mins at 1500RPM with no brake. The PBMC layer was removed and added to separate tubes. These tubes were topped up to 50ml with RPMI then centrifuged for 10mins at 1500RPM. Supernatant was aspirated, cells resuspended in RPMI and counted.

Cells were then centrifuged at 300g for 10mins. Supernatant was aspirated then cells resuspended in 80µl of macs buffer (PBS (Oxoid); 0.5% BSA (Sigma); 2mM EDTA) per $10x10^7$ cells. CD14 microbeads (Militenyl Biotech) were then used to positively select CD14 expressing PBMCs using a MACs separator according to manufacturer's instructions. Selected cells were resuspended in RPMI and counted. Cells were then washed in RPMI.

From blood apheresis cone

Blood was made up to 50ml with RPMI. PBMCs were purified as above. PBMC's were washed with HBSS media (Sigma) at 1400RPM for 5 minutes at 20°C three times. Cells resuspended in 40ml of RPMI with 10% fetal calf serum (fcs) (Sigma). 20mls of cell suspension was layered on top of a 15ml Percoll solution (GE healthcare). This was then centrifuged at 500g with acceleration and deceleration set low at 1 for 30minutes at 20°C. The middle monolayer of cells was extracted and made up to 50ml with RPMI, then centrifuged at 1800RPM for 5 minutes.

Cells were resuspended to $4x10^6$ per ml in RPMI. 5mls of cells were plated onto petri dishes and incubated for 1 hour at 37°C to allow monocytes to adhere. RPMI was thrown off, 5mls of media

(RPMI; 10% fcs; Glutamine, streptavidin, penicillin (Sigma)) was then added and cells were scraped off the plate. Media with scraped cells was transferred to a fresh tube and centrifuged for 10 minutes at 1500RPM.

Macrophage differentiation

Cells were resuspended to $1x10^6$/ml in media. GM-CSF (Invitrogen) was added at 1µl/ml of suspension. Cells were plated in 6 well plates at $2x10^6$/well. Plates were incubated at 37°C for six days.

Macrophage removal

Adherent macrophages had to be removed from the bottom of plate wells. Medium was taken off and added to a 15ml tube. 0.5ml of Hanks enzyme-free cell dissociation buffer (Gibco) was added to each well. Plates were incubated for 15minutes at 37°C. Plates were then vigorously tapped to help removal. A cell scraper was then used to scrape of remaining cells. Solution was then added to the falcan tube. The tube was centrifuged for 10minutes at 15000RPM. Cells were washed in fresh media twice.

Western blot

Preparation of samples:

$5x10^6$ cells per treatment were removed from media. Cells were washed in media and then resuspended to $5x10^6$ cells per ml of media. $5x10^6$ cells were then incubated with PTP Lyp inhibitor [98] (Calbiochem) at a final concentration of 0.6 or 1.25µM and $5x10^6$ control cells incubated without Lyp inhibitor for 40 minutes at 37°C. Cells were then washed in PBS before resuspension in 500ml of PBS and transferring to eppendorfs. Cells were washed twice in PBS at 3000RPM for 5 minutes before resuspension in 40µl of PBS. 10µl of 5x SDS loading buffer was then added to cells

Sample running and membrane transfer:

SDS containing samples were heated on a 100°C heating block for 10 minutes. 10ul of sample was then pipetted into the appropriate well. 10ul of prestained marker loading control (Invitrogen) was then added. The gel was then run until proteins reached the bottom of the gel. Proteins on gel were transferred onto membrane using the turbo where the membrane is soaked in methanol for one minute; gel placed on the membrane, with seven pieces of filter paper on top and bottom.

Sample development:

For antibodies used, see tables 1 and 2. Membrane was blocked in 5% BSA in PBSTT (PBS; 0.1% Tween (Fischer Scientific) for one hour on a shaker at room temperature (RT). 10ml of primary antibody solution in 5% BSA in PBSTT was added then membrane was left overnight at 4°C on a shaker. Membrane was washed for 10 minutes on a shaker at RT in PBSTT three times. 10ml of secondary antibody solution in PBSTT was added. Membrane was left on a shaker for one hour at RT. Membrane was washed for 10 minutes on as shaker at RT in PBSTT three times. Membrane was developed in ECL solution A and B (GE healthcare) for 3 minutes. Membrane was then run on a chemidoc.

Table 1: western blot primary antibody

Antibody	**Dilution**	**Company**
Rabbit anti-human Vav	1/1000	Milipore
Rabbit anti-human Phospho-Vav	1/1000	Santa Cruz Biotech
Mouse anti-phospho-Tyrosine	1/1000	Milipore

Table 2: western blot secondary antibody

Antibody	**Conjugation**	**Dilution**	**Company**
Goat anti-mouse	HRP	1/10000	GE healthcare
Donkey anti-rabbit	HRP	1/10000	GE healthcare

FACs DHR

Cells were resuspended in fresh media at $1x10^6$ cells per ml. Cells were treated with and without Lyp inhibitor at $1x10^6$ cells per treatment group. 200µl of cells from each Lyp inhibitor concentration and control cells were added to FACS tubes. For each concentration, one tube is left without dye. For the rest of tubes, 4µl of Dihydrorhodamine 1,2,3 (DHR) solution (invitrogen) was added to give a final concentration of 10µM. Each tube is then incubated for 5 minutes at 37°C. One tube from each concentration then has the appropriate agonist added: LPS at 10ng/ml or PMA at 200nM (both Sigma). One tube from each concentration is left untreated. 200µl of PBS was then added to each tube. Tubes were run and fluorescence measured using FL1 of a Dako Cyan ADP flow cytometer. Summit v4.3 was used to analyse the data.

Calcium signalling

Cells were resuspended in fresh media at $1x10^6$ cells per ml. $1.5x10^6$ cells were required for each treatment group, Lyp inhibitor was added to give desired concentrations. To prepare the dye, 110µl of DMSO (Sigma) was added to a fresh tube of Indo-1 AM (Invitrogen). Dye was added to cells at 7µl per ml of cell suspension. Cells were then incubated at 37°C for 40 minutes. Indo-1 AM passively diffuses into cells. Cellular esterases cleave this leaving the cell impermeant calcium indicator Indo-1. The binding of Indo-1 to calcium causes a shift in fluorescence emission. The ratio of bound to unbound fluorescence is then used to indicate intracellular free calcium levels.

Cells were then centrifuged for 10minutes at 15000RPM at 20°C and supernatant flicked off. Cells then washed twice in HBSS. Cells were then resuspended in HBSS (Gibco) to $1x10^6$ cells per ml with 1.5ml of cell suspension added to the appropriate cuvette.

Each cuvette was placed in a 37°C water bath for 5 minutes before running in the fluorometer machine with a magnetic stirrer. Agonists were added at appropriate times. Agonists added were 8µl of primary mouse anti-human CD64 (Serotec) followed by 16 µl goat anti-mouse (Sigma) to cause

crosslinkage. 5µl Ionomycin (Sigma) was added once calcium levels were basal as a positive loading control.

Cytokine ELISA

For each donor, macrophages were differentiated as before, but in a 96 well plate, with $2.5x10^4$ cells per well. After 6 days differentiation, lipopolysaccharide (LPS) was added added to appropriate wells at a concentration of 10ng/ml. Lyp inhibitor was also added to appropriate wells to give a final concentration of 1µM. Each plate was then incubated overnight at 37°C.

All ELISA's were performed using respective eBioscinces Ready Set Go kits. ELISA plates were coated with 100µl per well of capture antibody diluted in coating buffer. Plate was incubated overnight at 4°C then washed 4 times in wash buffer. Plate was blocked with 200µl per well of assay diluent (1:5 in distilled water) for 1 hour at RT. Plate was washed 4 times in wash buffer. 100µl of standard was added to the top of appropriate wells, which was then serially diluted 2-fold in assay diluent (1:5 in distilled water) with the last well at a concentration of zero. Macrophage culture plates were centrifuged at 1400RPM for 10 minutes at 20°C. 50µl of macrophage supernatant from each donor was then added neat into wells in replicates of four for each condition. Plate was then incubated at RT for 2 hours, then washed four times in wash buffer.

100µl of detection antibody diluted in assay diluent (1:5 in distilled water) was added to each well and the plate was incubated for one hour at room temperature before washing four times in wash buffer. 100µL of Avidin-HRP diluted in assay diluent (1:5 in distilled water) to the same concentration as respective detection antibody was added and plate was incubated for 30 minutes at room temperature. Plate was then washed six times in wash buffer. 100µl of Substrate solution was added to each well, plates were incubated at room temperature for 15 minutes before 50µl of Stop solution was added. Plates were then read at 450nm in a plate reader.

Results

Peripheral blood was obtained from healthy donors either from volunteers from the lab or from a blood cone obtained from the national blood bank. After purification, GM-CSF was added to media to induce differentiation of M1 macrophages for 6 days. After this, differentiated macrophages had to be scraped off plates for use in the various experiments, with the exception of the cytokine ELISA, which used the supernatants.

Lyp negatively regulates ROS production

Both Lyp and Vav could potentially regulate the molecules involved in the ROS production. In order to study this, we looked at the effect of Lyp inhibition on ROS production. We hypothesised that if Lyp does regulate pathways involved in ROS production, its inhibition should result in an increased ROS production. To investigate ROS production, we used Dihydrorhodamine 1,2,3, (DHR). DHR diffuses into cells where it can be oxidised to fluorescent Rhodamine 1,2,3. This fluorescence is therefore a marker of superoxide presence. First, cells were incubated with increasing concentrations of Lyp inhibitor for 40 minutes at 37°C. Control cells were incubated without Lyp inhibitor. Cells were next incubated with DHR for five minutes before PMA or LPS was added. 15 minutes later, cells were then analysed for fluorescence by flow cytometry. This experiment was repeated three times and the results are plotted below (figure 2).

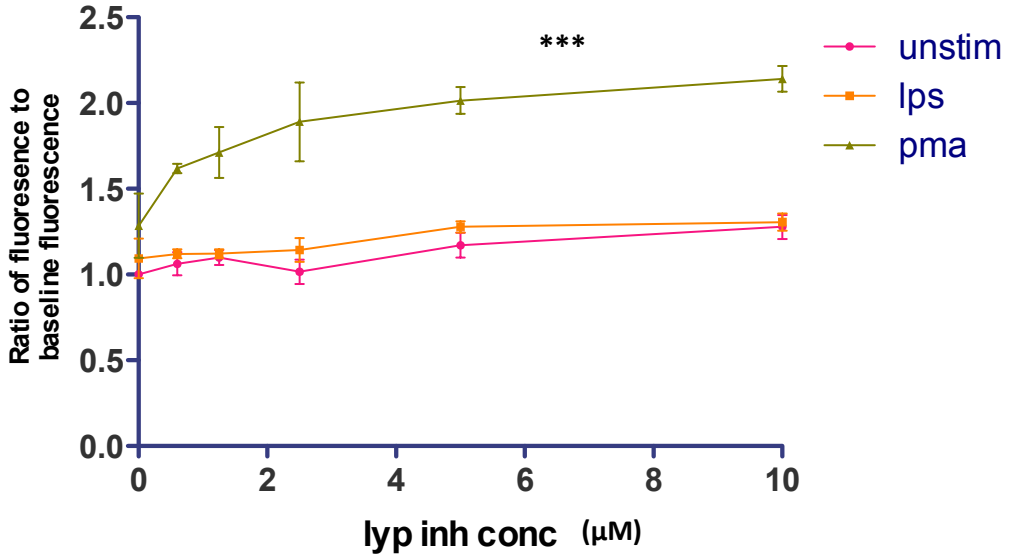

Figure 2: Lyp inhibition and ROS production. Macrophage were pre incubated with various concentrations of Lyp inhibitor (Lyp inh conc µM) were loaded with DHR, then left unstimulated or stimulated with either LPS or PMA. Cells were then run on the flow cytometer, with the mean fluorescence of ROS sensitive DHR measured for each sample. The experiment was repeated two more times with different donor cells. The baseline fluorescence was noted as the fluorescence for unstimulated cells without Lyp inhibitor. The fluorescence for each sample was then compared to the baseline fluorescence from the respective experiment and plotted as a ratio. Error bars show standard deviation. Statistical analysis performed using graphpad prism 5. A Two-Way ANOVA was performed between the PMA treated cells and unstimulated cells. p< 0.001 = *.**

The level of ROS was found to increase with increasing concentration of Lyp inhibitor. Surprisingly, LPS addition did not increase ROS compared to unstimulated cells. PMA addition did significantly increase ROS compared to unstimulated cells. These results suggest that Lyp can function to reduce basal ROS in resting cells and induced ROS after cell activation by PMA.

Lyp and calcium flux after FcγR crosslinkage

We wanted to investigate the role of Lyp in signal transduction after FcγR crosslinkage. For this we decided to use calcium flux as an indicator of signalling, since an intracellular calcium increase is associated with FcγR ligation. We hypothesised that Lyp inhibition will cause a decreased negative regulation of FcγR signal transduction, and therefore greater calcium flux. Cells were incubated with Lyp inhibitor at a low concentration of 0.6µm and the higher concentration of 1.25µm.

Control cells were incubated without any Lyp inhibition. At the same time cells were incubated with indo-1 dye, an indicator of free calcium for 40 minutes. While in the fluorometer machine,

anti-CD64 was first added, followed by a secondary antibody to crosslink and induce signalling. This experiment was repeated three times, with calcium flux presented as a ratio of calcium before crosslinkage compared to peak calcium after crosslinkage (figure 3). The results from each experiment were non consistent and on the whole no significant difference in calcium flux after CD64 crosslinking at either Lyp inhibitor concentration compared to control cells.

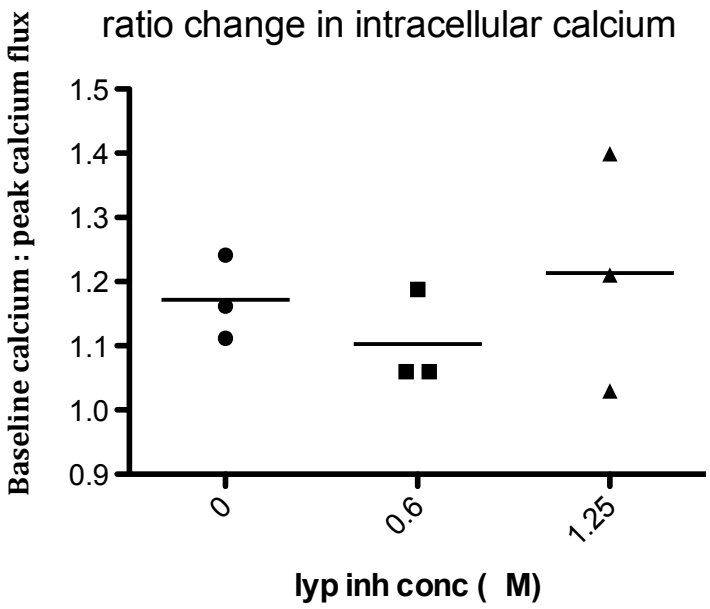

Figure 3: Lyp inhibitor and calcium flux after CD64 ligation. Macrophages were pre-incubated with Lyp inhibitor at concentrations of 0.6µM or 1.25µM, with control cells incubated without Lyp inhibitor. Cells were incubated with the calcium sensitive dye Indo-1 then run in a fluorometer machine in a cuvette, which measures fluorescent output. Anti-CD64 antibody was added to coat CD64, 100 seconds later the secondary goat anti-mouse antibody was added to crosslink the CD64 receptor and induce signalling. After the calcium flux reached basal levels again, ionomycin was added as a positive loading control. B) The experiment was repeated two more times with different donor cells. Samples have been plotted as a ratio of the peak fluorescence during calcium flux compared to the baseline calcium level before antibody addition.

Lyp and cytokine production

Primary human T cells expressing the W620 variant of Lyp have altered cytokine production [25]. Based on this, and the multiple points where Lyp could affect cytokine signalling pathways, we decided to investigate the effect of Lyp inhibition on pro-inflammatory cytokine production. We hypothesised that Lyp inhibition will result in a decreased negative regulation of pro-inflammatory cytokine production. Macrophages where obtained from three separate donors and cells were incubated in four conditions: with or without Lyp inhibitor, and with or without LPS. ELISA's were set up for detection of TNFα, IL1β and IL6 release into supernatant. Each cytokine ELISA was performed in replicates of four for each condition for each donor.

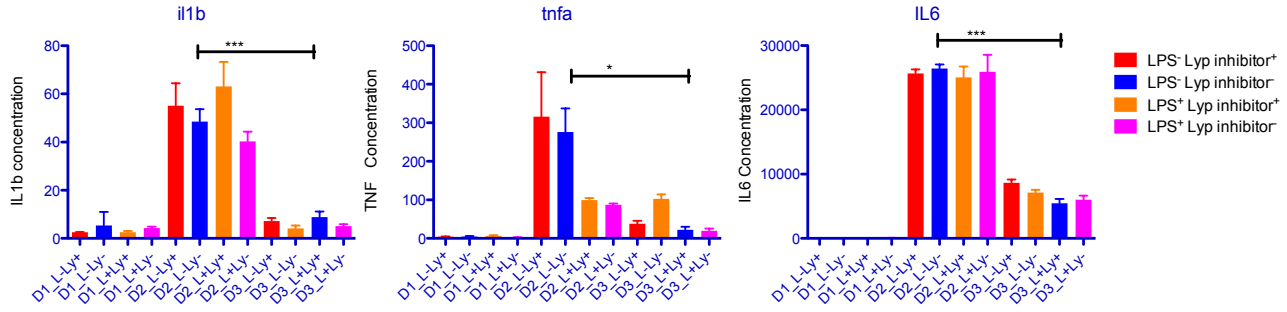

Figure 4: Cytokine productions by macrophages. The three donor macrophages are labelled from D1, D2 and D3. Macrophages were incubated overnight in one of four conditions: no LPS, with Lyp inhibitor (red/L-Ly+); no LPS, no Lyp inhibitor (blue/L-Ly-); with LPS, with Lyp inhibitor (orange/L+Ly+); with LPS, no Lyp inhibitor (pink/L+Ly-). The following day, supernatants were analysed by ELISA for IL1β, TNFα and IL6. Statistical analysis was performed for the no LPS, no Lyp inhibitor groups. A repeated measurements one-way ANOVA was performed where $p < 0.05$ = * and $p < 0.001$ = *.**

Surprisingly, the presence of Lyp inhibitor and/or LPS did not seem to stimulate cytokine release, with LPS actually decreasing TNFα production (figure 4). One donor was found to have a significantly greater cytokine release than the other two donors figure 4. We later learned that this donor is heterozygous for the W620 variant (data not shown). This result would suggest that expression of

the W620 variant correlates with a decreased negative regulation of cytokine production. Donor 1 also responded particularly poorly in terms of cytokine production.

Regulation of Vav by Lyp

Since Vav is a substrate of Lyp and since Vav could have a role in the investigated pathways, we wanted to see whether Lyp could regulate Vav activity. In order to investigate the role of Lyp in Vav regulation, we performed a western blot for phosphorylated Vav in the presence of Lyp inhibition. Macrophages were initially incubated with 0.6µm and 1.25µm of Lyp inhibitor. Control cells were incubated without Lyp inhibitor. SDS loading buffer was added directly to whole cells to allow lysis, before cells were run. After running and transferring, the blot was initially probed for phosphotyrosine (data not shown). However, after getting hold of anti-phosphorylated Vav antibody, the blot was stripped and re-probed for this. Thus antibody binds specifically to the Tyrosine 174 residue of Vav proteins, phosphorylation here is required for Vav activation and GEF activity [35]. As a loading control, the blot was re-stripped and re-probed for anti-Vav to allow a comparison of Vav protein levels between samples.

From figure 5, we can see that the sample of macrophages with a higher Lyp inhibition (1.25µm) has a less intense stain for phosphorylated Vav than control and the low Lyp inhibitor concentration (0.6µm) cell samples. This is suggestive that a higher inhibition of Lyp activity results in less phosphorylation of Vav at Tyr 174. However, the sample of macrophages with the high inhibitor concentration has a less intense stain for Vav, suggesting that there was less Vav protein present in this sample anyway. It is therefore difficult to interpret whether the higher inhibition results in less phosphorylation of Vav. There is no other obvious difference in band intensities between samples. There is clear shift in molecular weight between the Vav bands at around 95kDA and phosphorylated

Vav bands at around 60kDA. This is surprising giving that both antibodies are supposed to detect Vav at its known molecular weight of 95kDA and this further reflects the non-conclusiveness of this result.

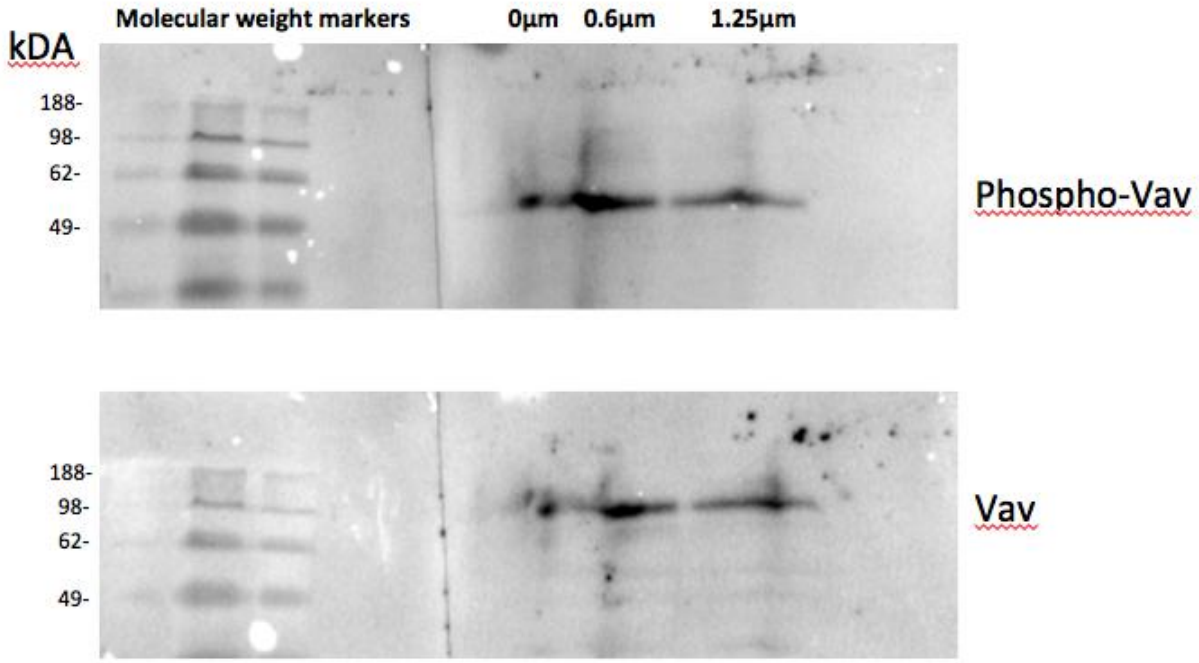

Figure 5: Lyp inhibition and Vav phosphorylation. Macrophages were incubated with Lyp inhibitor at 0.6µM and 1.25µM with control cells (0µM) incubated without Lyp inhibitor. Cells were lysed directly in SDS buffer, then western blot was performed. Cells were probed first with anti-phosphorylated Tyr-174 Vav antibody as shown in the upper blot (Phospho-Vav). The blot was the stripped and re-probed with anti-Vav antibody as shown in the lower blot (Vav).

Discussion

Variants of Lyp phosphatase are linked to autoimmune diseases such as rheumatoid arthritis. Studies of Lyp have previously only focused on lymphocytes; this is despite the fact that myeloid cells have a higher Lyp expression. The purpose of this study was to investigate the role of Lyp in primary human macrophages. We used an established Lyp inhibitor and looked at the effects of Lyp inhibition on macrophage signalling pathways.

We found that Lyp inhibition increased the level of ROS in unstimulated and PMA stimulated cells. An important cellular source of ROS after PMA treatment is from NADPH oxidase enzymes . Primary human macrophages have previously been shown to produce ROS in response to PMA [99]. PMA can activate conventional and novel PKC isoforms which can phosphorylate and induce translocation of p47phox. In neutrophils, after PMA treatment, the conventional PKCβ isoform is required for NADPH activation [100]. The isoform(s) activated after PMA treatment in macrophages is unknown. However, primary human monocytes activated by opsonised zymosan use PKCδ to phosphorylate $p47^{phox}$ [87]. PMA treatment of these cells also caused PKCδ activation, which could then phosphorylate $p47^{phox}$ when purified from cells. PKCα is also activated and can then positively regulate NADPH function by cPLA2 generation in these cells. Further experiments could utilise various PKC inhibitors to deduce which isoforms are important in human macrophages.

The results suggest that Lyp negatively regulates ROS production. Lyp may negatively regulate DAG-mediated activation of PKC, and thereby ROS production. There are several non-exclusive pathways where this could happen. Lyp can dephosphorylate Src family kinases [12,13] and may be capable of negatively regulating Vav. Src family kinases can lead to DAG generation. Vav can activate PLCγ [48] thereby also linking Vav to DAG-mediated PKC activation. Both of these pathways could facilitate an increased basal ROS production. PKCδ can negatively phosphorylate Lyp, inhibiting its activity in T cells [27]. PKC can also negatively regulate PYPN22 expression in human chronic lymphoid leukemic B cells [101] Whether Lyp can interact with PKCδ, or other isoforms, directly inhibiting them, is unknown.

A direct PKC inhibition by Lyp could explain the large increase in ROS with Lyp inhibition of PMA treated cells.

Vav is also involved later in the ROS pathway. Vav has been implicated with roles on ROS production through Rac activation [102-105]. Vav deficient murine primary macrophages fail to produce ROS after LPS induction [102]. It was shown Vav GEF activity is required here to activate Rac2. In primary murine neutrophils, Vav1 and 3 are required in ROS production after FcγR ligation [103]. Again, Vav was required for Rac activation here. In human neutrophils, Vav1 can directly interact with the NDAPH subunit $P67^{phox}$ where it activates Rac2 after fLMP stimulation[106]. Rac activation is essential in allowing Rac binding to $P67^{phox}$ as well as membrane-bound cytochrome b, which is required in NADPH oxidase activation [37].

Vav is not required for PMA induced ROS production by murine macrophages [102], suggesting Lyp function through Vav activation is not responsible for the increased ROS with Lyp inhibited PMA treated cells in this study. Interestingly, FcγR signalling in DC's activates NADPH oxidase, allowing ROS production important in cross presentation [105]. This pathway also requires Vav. With the requirement of Src family kinases in FcγR signalling, it is possible Lyp could regulate Vav in DC's. It would have been interesting to investigate the FcγR pathway and ROS in this study. This area can be addressed in future studies.

We found LPS to have no effect on ROS production compared to unstimulated cells. This is despite previous studies reporting rapid and detectable ROS levels for hours after LPS stimulation at similar concentrations in murine macrophages [107-109] as well as primary human monocytes [110] and macrophages [104]. These reports used different methods of detecting ROS, including different dyes and using plate readers to measure fluorescence. These different methods could account for the different results.

We also found that the addition of LPS failed to effect pro-inflammatory cytokine production by macrophages. Again this was surprising, especially since LPS has been shown numerous times to induce cytokine production, including TNFα, in primary human macrophages [111]. Together, this indicates that macrophages may have been previously been activated by LPS, possibly during the differentiation incubation, and therefore no longer responsive to this signalling pathway. This pathway may even have begun to downregulate itself, which could explain the decreased TNFα we found with LPS stimulation. The use of antibiotics in the culture medium during macrophage differentiation could have resulted in release of LPS from any present gram-negative bacteria. Antibiotics could be avoided during the incubation, but it is likely any present gram-negative bacteria will multiply and shed LPS into the media anyway. Future experiments involving LPS could utilise monocytes that can be used right after purification, avoiding the differentiation incubation.

We were surprised to find no significant difference in calcium flux after CD64 ligation with Lyp inhibition. Experiments using another Lyp inhibitor in Jurkat T cells have found an increased calcium flux with increased Lyp inhibition after TCR ligation [112]. Our lab using the same inhibitor in this study has also found an increased calcium flux with increased Lyp inhibition in jurkat T cells (unpublished data, R. Bayley). We therefore expected an inhibition of Lyp activity to cause a decreased negative regulation of Src family kinases, thereby allowing a stronger CD64 signal transduction, as indicated by calcium flux.

Due to variation between experiments, results are inconclusive. In one experiment, a high Lyp inhibitor concentration did result in a greater calcium flux than control cells. In another experiment, the high Lyp inhibitor concentration gave a lower calcium flux than control cells. This may have been caused by differences in genetic polymorphisms between donor cells. This experiment needs to be repeated to investigate if any of these trends are continued. A greater concentration range would also allow a more in depth analysis.

This is the first report of macrophages expressing the W620 variant. These macrophages displayed a much greater production of the pro-inflammatory cytokines TNFα, IL1β and IL6. This lab has also seen that CD4 T cells from W620 carriers have an increased TNFα and IFNγ production (unpublished data, R. Bayley). It has previously been reported that primary CD4 T cells expressing the W620 variant have decreases in IL2 [23,24] and IL10 [24] production after TCR stimulation, other cytokines tested, including TNFα showed no difference between PTPN22 genotypes [24], A decreased IL10 expression has also been described in unstimulated, un-purified peripheral leukocytes from donors with the W620 variant with Anti-neutrophil cytoplasmic antibody disease [113]. This decrease correlated with an increased basal W620 Lyp phosphatase activity and decreased ERK phosphorylation. In contrast another study found stimulated or unstimulated PBMC's from W620 homozygotes and carriers showed no difference in cytokine production, including IL10, compared to healthy controls [25].

Our results from primary macrophages expressing the W620 variant differ from these previous published reports of primary cells. The unpurified leukocytes and the PBMC's from the previous reports [25,113] would have lacked macrophages since peripheral blood was used, with no macrophage differentiation stage. It is interesting that from the unpurified leukocyte report, the increased basal W620 activity also correlated with an increased P38 MAPK phosphorylation [113].

As mentioned earlier, P38 MAPK is important at least in TNFα regulation in macrophages. If P38 MAPK is also increased in variant expressing macrophages, this could explain our unique results and would highlight the importance of macrophages in driving the inflammatory response seen in the synovium of RA patients. Interestingly, the knockdown of Lyp expression in the human monocytic cell line THP1 resulted in a greater MAPK phosphorylation with increased IL6 and IL17 [22]. Our experiment needs to be repeated with more W620 variant donors and controls and to compare the phosphorylation levels of different MAPKs and also look into regulatory cytokine production such as IL10.

It should be noted that our donor was healthy, despite Lyp W620 expression, demonstrating that other factors can contribute to autoimmune risk with Lyp W620. It would be interesting to find out what differs between healthy and autoimmune prevalent carriers of Lyp W620, be it environmental or further genetic predispositions. Donor 1 from this experiment did not appear to produce a detectable amount of cytokine with the exception of small amounts of IIβ in some of the replicates. This may only have been an experimental artefact and I would suggest donor 1 cells to have been dead.

There are several possible reasons why Lyp inhibitor had no effect on cytokine production. Firstly, Lyp may not be a negative regulator of pro-inflammatory cytokines. Though the exceedingly high cytokine production seen with the W620 variant expression would argue against this, other genetic polymorphisms from this donor could be responsible for the higher cytokine production. Yet the report of increased IL6 and IL17 from THP1 cells after Lyp knockdown would dispute this [22]. The Lyp inhibitor is a reversible competitive inhibitor [98]. Hence a more likely explanation would be that the inhibitor could have been out-competed and possibly degraded during the overnight incubation.

We hypothesised that Lyp can regulate Vav activity by directly dephosphorylating it. We therefore expected Lyp inhibition to result in an increased Vav phosphorylation at Tyr-174, but this was not seen. Although it looked like a higher inhibitor concentration resulted in less Vav phosphorylation, it is possible there was less Vav protein present in the sample as suggested by the subtle difference Vav in band intensities. A decreased phosphorylation could indicate Lyp to be a positive regulator of Vav activity, where it can directly dephosphorylate inhibitory Tyrosine residues, thereby allowing Vav phosphorylation on activation tyrosines such as Tyr-174.

On top of the many technical issues with the blot as discussed below, the reliability of this experiment is further questioned giving that the phosphorylated Vav antibody gave a bands at around 50kDA instead of the expected 95kDA. It is unlikely due to Vav degradation since the Vav

antibody only gave bands at Vav's full molecular weight of around 95kDA. I have no reasonable explanation for this phenomenon.

Despite the stripping process, the original phosphotyrosine stain is still visible within the lanes, thus questioning the efficiency of the stripping. Poor stripping could have resulted in the different band intensities between samples. Ideally, an immune-precipitation for Vav should be performed after cell lysis. In order to further investigate Lyp regulation of Vav, the western blot process will need to be optimised and repeated. Again, a greater range of Lyp inhibitor concentrations could give more in depth results.

The Lyp inhibitor used is a competitive reversible inhibitor of PTPs with a ten-fold selectivity for Lyp. The problem with such an inhibitor is therefore the specificity, especially at higher concentrations. Ideally, the lab would like to specifically knockdown expression of PTPN22 mRNA in macrophages. If this technique can be mastered then experiments could be repeated with PTPN22 knock-down cells. This would be a more effective way of showing that Lyp does have a role in macrophage signalling pathways.

In conclusion, this is the first report that Lyp does have a role in primary human macrophage signalling pathways. By using a Lyp inhibitor, we have shown that Lyp is a negative regulator of ROS production in unstimulated and PMA stimulated cells. The role of Lyp in calcium signalling after CD64 ligation and in Vav activation remains unknown. We also provide evidence that the Lyp W620 variant expression in macrophages allows a greater production of pro-inflammatory cytokines. This has implications in understanding how the W620 risk allele can function in autoimmune diseases such as RA.

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cRel and its effect of class switch recombination, IRF4 induction and plasma cell differentiation during a T-Independent immune response

This project is submitted in partial fulfilment of the requirements for the award of the MRes

Lewis Blair Cawkwell

UNIVERSITY OF BIRMINGHAM

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Abstract

During the B cell immune response, extrafollicular foci form after an initial proliferative burst which become a site of early plasma cell production. The NP-Ficoll immune response is a T-independent reaction, which results in significant extrafollicular foci formation. The NFκB member, cRel, is important in activated B-cell proliferation and survival. It's also been shown *in vitro* to be important in class switch recombination (CSR) and induction of IRF4. IRF4 is essential in plasma cell differentiation as well as CSR.

We showed that after NP-Ficoll immunisation, *cRel* $^{-/-}$ (KO) B cells produce far less plasma cells. And this coincided with low NP-specific antibody production, particularly IgG isotypes. Despite this low plasma cell production, WT mice receiving KO or WT B-cells had similar splenic *Irf4* levels. Splenic levels of immunoglobulin switch and *Aid* transcripts, both essential in CSR, were similar. Fluorescent staining also showed a similar frequency of WT and KO plasma cells had switched to IgG expression. Together, this project shows that despite the defect in plasma cell production with the B-cell intrinsic loss of *cRel*, the few plasma cells which are produced are still capable of CSR. However, the low numbers mean actual antibody production is low.

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Introduction

B cells are an essential component of the adaptive immune system. Antigen recognition through the B cell receptor (BCR) can facilitate B cell activation and differentiation into short or long-lived antibody-secreting plasma cells or into memory B cells. The B cell immune response may be thymus dependant (TD) and require cognate T cell help or be thymus independent (TI), not requiring T cells. TI type 1 (TI-1) reactions involve B cell activation through mitogenic signals such as TLR signalling. TI type 2 (TI-2) involves extensive BCR crosslinking through repetitive epitopes, typical of polysaccharides.

The mature naïve B cell pool is heterogenic in phenotype as well as function [1]. The majority are recirculating follicular B2 B cells (FO B cells), which circulate the secondary lymphoid organs (SLO), homing into primary B cell follicles. Here, networks of follicular dendritic cells (FDCs) can present antigen. FDC production of the chemokine CXCL13 attracts the $CXCR5^{hi}CCR7^{lo}$ FO B cells into the follicles [2]. T cells, in contrast are $CXCR5^{lo}CCR7^{hi}$, allowing accumulation into CCL19 and CCL21 rich T cell zones.

T zones are found next to follicles and this arrangement of lymphocyte rich zones make up the white pulp in the spleen, the cortex and paracortex of lymph nodes and appear in organized mucosal associated tissues. FO B cells typically respond in a TD manner. A marginal zone (MZ) consisting of B cells and macrophages surrounds the white pulp edge between the marginal sinus and the red pulp [3]. The red pulp is a red blood cell, reticular cell, and macrophage rich zone important in blood filtration.

MZ B cells are another B2 cell subset [3]. These cells show a pre-activated phenotype, can self renew and do not recirculate. They can however capture immune complex via CD21 and transport it into follicles transferring it to FDC. Positioned next to the marginal sinus, these cells are important in rapid TI responses to blood-borne antigen. B cells of a similar phenotype have been described in lymph nodes, Peyer's patches and tonsils [4]. B1 B cells as described in mice include the $CD5^+$ B1a cells and the $CD5^-$ B1b cells [5]. These cells are the main B cell population in the peritoneal and pleural cavities while rare in the spleen and lymph nodes. They are the major producers of natural antibody, an important first line of defence to infection. They may be activated with or without BCR stimulation to form plasma cells that produce IgM or IgA.

The B cell immune response

FO B cells enter the follicles where they reside for around 24 hours before recirculating to another secondary lymphoid organ. Should they encounter specific antigen within the follicle, or even free antigen in the blood or lymph, FO B cells will migrate to the follicular-T cell border. Likewise, should MZ B cells recognise antigen, they will also migrate to the T cell border [4 6]. An upregulation of CCR7 by antigen activated B cells allows this migration [2]. Likewise, during a TD response, dendritic cell (DC) primed CD4 T cells upregulate CXCR5 to meet cognate B cells.

At this border, the B cells receive proliferation and differentiation signals. During a TD response, B cells present processed antigen via MHC class II molecules to cognate CD4 T cells by 12 hours [4]. This cognate interaction initiates reciprocal survival signals, including B cell CD40 engagement [7]. $CD11^{hi}$ DCs are also present at the border and can provide BAFF or APRIL survival signals [8]. This could be particularly important in TI B cell survival. Innate helper cells including neutrophils [9], NK cells [10] and iNKT cells [11] can also provide help in TI and TD responses as can microbial TLR signals.

After 1 to 2 days here, B cells begin proliferation at the border [8,4]. Clones may undergo as little as two cell cycles [4] although reports include at least three divisions two days into a TD response [12]. Class switch recombination (CSR) from IgM to other Ig heavy chain classes can also occur during this proliferation between days 2 and 3, but is restricted to B blasts [12 46]. After this short proliferative burst, B cells move into the splenic red pulp or lymph node subcapsular interfollicular areas then form extrafollicular foci, or move into follicles and initiate germinal centres [12,4].

The extrafollicular foci is a site of early and rapid plasma cell differentiation. This starts with B blasts differentiation into proliferating and low antibody producing plasmablasts, followed by terminal differentiation into plasma cells [4]. Early recirculating memory cells can also form from these foci [12]. Plasma cells produced here are typically short lived. The germinal centre (GC) is a dedicated site for cycles of further B cell proliferation, affinity maturation and CSR of the BCR with selection of high affinity clones. These clones can then contribute to

plasma cell and memory cell differentiation [4] [13]. GC's are classically thought of as TD, but TI non-productive GC's can occur under certain circumstance [14].

Plasma cell differentiation

Differentiation of plasma cells requires a complex transcriptional network including Blimp1, XBP1 and IRF4. Blimp1 has been described as the master regulator of plasma cell differentiation. Transfection of this zinc-finger transcriptional repressor into B cell lines can induce differentiation into a plasma cell phenotype [15]. Blimp1 was further clarified as a master regulator when mice whose B cells lacked Blimp1 failed to produce plasma cells or produce antibody sufficiently [16].

Blimp1 inhibition of c-myc [17] halts the cell cycle allowing terminal differentiation. Blimp1 can inhibit genes involved in B cell function and GC differentiation including Pax5 and Bcl6 [18]. Pax5 is expressed throughout B cell subsets but not in plasma cells [18]. It can activate genes involved in B cell function, including BCR signalling, while repressing genes associated with plasma cell differentiation, including XBP1 [18].

XBP1 activates genes involved in the secretory apparatus, essential in antibody secretion [18]. Inhibition of Blimp1[19] therefore allows XBP1 activation and function [20] while helping shut down B cell function. Therefore downregulation of Pax5 is necessary for plasma cell differentiation. However one paper describes how the initial downregulation of Pax5 is independent of Blimp1 with B cell's

lacking Blimp1 capable of differentiating into a low antibody secreting pre-plasmablast stage [21].

Bcl6 is highly expressed in GC B cells. Here it acts as a master regulator allowing high levels of proliferation and repressing DNA damage response to allow BCR affinity maturation, while repressing Blimp1 expression and preventing plasma cell differentiation [18]. Blimp1 can reciprocally repress Bcl6 [22] to prevent GC B cell state. It also represses genes involved in CSR, which are highly expressed in GC's [22]. The repression of Bcl6 is important in preventing GC B cell differentiation and a downregulation of Bcl6 is particularly important in exiting the GC state. IRF4 is another transcription factor now appreciated to be essential in plasma cell development as discussed in the next section.

IRF4 in B cells

IFR4 is a transcription factor capable of interacting with a variety of co-factors [23]. The best described cofactor interaction is that of PU.1 and also the closely related SPI-B. Dimer interactions with these co-factors are important in the transcription of IgH and IgL-κ loci. The role in immunoglobulin production is further exemplified in *Irf4* KO mice [24]. Despite a normal B cell development, these mice exhibit extremely reduced serum immunoglobulin levels and fail to produce a detectable antibody response to immunisation.

Human and mouse lymphoid tissue histology later showed how IRF4 expression was high in plasma cells but largely absent from GC B cells [25,26]. The IRF4

expressing GC B cells appear to be centrocytes in the light zone and most co-express Blimp1 [27]. This is suggestive that these cells are precursors of plasma cells. There is now good evidence to show IRF4 is required in plasma cell differentiation. It was shown *in vivo* that plasma cell differentiation from GC's required IRF4 expression [28]. Despite lack of IRF4, Blimp1 was still upregulated, but not sufficient for plasma cell development, suggesting IRF4 to work in parallel to Blimp1. Another study found that B cells lacking IRF4 activated *in vitro* also failed to differentiate into plasma cells [29]. These cells failed to upregulate Blimp1 suggesting IRF4 is upstream of Blimp1.

This difference in where IRF4 functions with Blimp1 is likely due to the different experimental systems used. There is also evidence that Blimp1 can activate IRF4 expression [30]. Negative regulation of IRF4 by MITF occurs in naïve B cells [31] and can be upregulated during B cell activation via NFκB [3233]. Both studies also described how CSR was inhibited, due to a failure to upregulate AID, which as described next, is essential in CSR.

AID, CSR and IRF4

CSR, as reviewed elsewhere [34], is the process of switching to another heavy chain constant region, thereby producing antibody of different effector functions. This process occurs through a recombination event between switch regions, located upstream of each heavy chain constant gene loci, with deletion of the intervening DNA. AID is responsible for de-aminating cytosines to uracil bases in each switch region. Uracils are subsequently removed and nicks are incorporated into the

DNA by further proteins. Mismatch repair proteins then facilitate end join recombination.

Transcription is required through the switch region, beginning at the *I* exon upstream of the switch region and continuing through the C_H locus. This transcript, known as the switch or germline transcript must then be spliced to bring the *I* exon to the first C_H exon to allow productive CSR [35,36]. The transcript is not thought to code a protein and its functional role is not known, but may form DNA-RNA hybrids to open the switch region and allow CSR. [34].

AID is positively regulated by Pax5 [37] and negatively regulated by Blimp1 [22]. IRF4 is also required for AID expression during CSR [28,29]. AID is expressed in GC founder cells and GC B cells. AID is also expressed in some B blasts of the extrafollicular response, allowing CSR before it is repressed in plasmablasts and plasma cells [6]. Based on IRF4 transduction rescue experiments with IRF4 KO B cells, its been proposed a low/intermediate IRF4 level allows CSR but not plasma cell differentiation [29]. As Blimp1 and IRF4 levels increase, plasma cell differentiation is initiated with AID and CSR is inhibited.

cRel

The NFκB transcription factor family consists of five proteins: p65-RelA; RelB; cRel; p105/p50; and p100/p52 [38]. These proteins form various dimers capable of regulating the transcription if genes involved in an array of processes such as cell proliferation, differentiation and survival. Dimers are usually found in the

inactive state bound to inhibitory κ B (IκB) within the cytosol. Cell signalling events can result in phosphorylation of IκB's by IκB kinase (IKK) followed by IκB degradation, allowing NFκB translocation and target gene regulation.

One interesting NFκB family member is cRel, which commonly forms homodimers and dimers with p50 and less often with p65-RelA and p52 [39]. cRel has a more promiscuous DNA target sequence recognition and can bind different residues than the other NFκB members [40]. In immature B cells, cRel is low but becomes the main dimer, in the p50-cRel form in mature B cells. cRel knock out mice have revealed much about cRels requirement for T and B cell immune functions. In particular, KO mice have reduced B cell proliferation and survival to stimulation with LPS, anti-IgM, anti-CD40 and antigen. Antibody production is also reduced, and GC's are found to be small/irregular [39].

cRel allows proliferations through *cMyc* activation in the G1 phase and allows movement to the S phase. cRel also prevents apoptosis through activation of the *Bcl-2* anti-apoptotic gene family. There is also evidence cRel allows B cell isotype switching by initiating and enhancing transcription through S regions [4142,43]. Intriguingly, in cRel KO mice, B and T cells fail to upregulate IRF4 in response to various mitogens [33]. Thus cRel regulation of IRF4 could represent another way it might facilitate CSR.

This project

The Toellner lab is interested in the early events of the B cell immune response. In this project, we are investigating the B cell immune response to the TI antigen NP-Ficoll. By using *cRel*$^{-/-}$ mice and adoptive transfer of *cRel*$^{-/-}$ B cells, we hypothesised that the absence of *cRel* will result in defects in the production of NP-specific plasma cells. We also hypothesised that the absence of *cRel* will result in poor induction of *Irf4* and *Aid* transcripts as well poor induction of CSR.

Materials and methods

Mice and immunisations

All mice used were males aged 6 to 8 weeks old and maintained in specific pathogen-free conditions at the Biomedical Services Unit at the University of Birmingham, UK and treated according to The Home Office guidelines.

In the first experiment, C57/BL6 mice were used. *c-Rel*$^{+/+}$ wild type and *c-Rel*$^{-/-}$ knock out mice were immunised via intraperatoneal (i.p) injection of NP-Ficoll (Bioscearch Technologies, CA) dissolved in PBS at 30µg/200µl per mouse. NP-Ficoll gives a T-independent response useful for specifically studying a B cell response. Cohorts of mice were then sacrificed at day 0 (before immunisation) and day 5 and 7 after immunisation.

QM (quasi-monoclonal) mice have the NP-specific rearranged 17.2.25 V_HDJ_H segment on one allele, with a J_H deletion on the other allele and deletion of the J_k loci [44]. This results in around 95% of the B cells expressing NP-specific B cell receptors. QM mice used here also express enhanced yellow fluorescent protein (eYFP) in every cell [45]. These mice were crossed onto the wild type (*c-Rel*$^{+/+}$) or knock-out (KO) (*c-Rel*$^{-/-}$) C57/BL6 background. Naïve mice were then used to provide naïve B cells for the cell transfer experiment.

Wild type C57/BL6 mice received cell transfer by intravenous (i.v) injection. The 2 hour and day 2 cohort received 1x10^6 cells, with the day 5 cohort receiving 4x10^5 cells from the wild type or knock-out QM donors. KO C57/BL6 recipient

mice also received $4x10^5$ wild type QM donor cells. 24 hours later, all mice received an NP-Ficoll immunisation by i.p injection at 30µg/200µl per mouse. The respective cohorts were then sacrificed at 2 hours, 2 days and 5 days after immunisation. The KO recipient mice were also sacrificed at day 5.

Cell transfer

Spleens were removed from four wild type and four knock out QM mice. Spleens were mashed in media (RPMI 1640, penicillian streptomycin and 10% fetal calf serum (all sigma)) then centrifuged at 1200RPM for 5 minutes at 24°C. Supernatant was removed and cells were pooled together into wild type and knock out splenocytes. Red blood cells were lysed by addition of 1ml of ACK lysing buffer (Invitrogen) per spleen for one minute before topping up to 5mls with media then centrifuging as before. Splenocytes were then resuspended in MACs buffer then counted in 10% Trypan Blue to differentiate live and dead cells. Splenocytes were then sorted using CD43 beads (Miltenyi Biotech) according to manufacturers instructions. CD43 is expressed on non-B cells and activated B cells, allowing a negative selection of naïve B cells that pass through the MACs column.

Flow cytometry

For antibodies, see appendix table 1.

Mice were sacrificed at the indicated times. All steps performed on ice or at 4°C. Sections of spleen were mashed in media into a single cell suspension, then

filtered through a 70µl cell strainer then washed twice in media by centrifugation at 1200RPM for 5 minutes. $1x10^6$ cells from each splenic sample as well as some to represent single colour/unstained controls were resuspended in 200µl of FACS buffer (sterile PBS with 0.5% FCS and 0.2mg EDTA). Cells were first stained with Hoechst nuclear dye (1/3000) in FACs buffer which stains high in dead/dying cells. After quickly washing in FACS buffer, cells were stained with FC receptor II/III block at 1/200 in FACS buffer for 20 minutes. After washing, fluorescently conjugated antibodies were added to samples and appropriate antibody added to single stain controls, diluted in FACS buffer to the cells for 30 minutes. After washing in FACS buffer twice, cells were resuspended in FACS buffer, transferred to FACS tubes then run in a Dako Cyan analyser and FlowJo software (FlowJo, Ashland, OR). Around $5x10^5$ events were collected for each sample

Immunohistochemistry

For all antibodies, buffers and substrates see appendix tables 2, 3 and 4.

6µm spleen sections were cut onto glass slides then fixed in acetone for 20 minutes at 4°C. Slides were washed in Tris pH 7.6 for 10 minutes. Slides were then stained with unconjugated primary antibodies diluted in Tris pH 7.6 for one hour then washed in Tris pH 7.8 for 5 minutes. Secondary antibodies were incubated with 10µl normal mouse serum (Dako, UK) in 90µl Tris pH 7.6 for 30 minutes at RT before adding more Tris pH 7.6 to make final dilution. Secondary antibodies were added to slides for 45 minutes before washing again for 5

minutes. The tertiary Vectastain ABC-AP kit (Vector Laboratories, USA), with solution A and B added to Tris pH 7.6 (1/100) was added to the slides for 30 minutes before another 5 minute wash. Slides were then stained with peroxidase substrate until colour was present. Slides were washed for 5 minutes again then stained with Alkaline-phosphate substrate until colour was present. Slides were then washed one final time before rinsing twice in distilled water. Slides were air dried then cover slips were secured on top with gelatine (Sigma). Pictures were taking with a Leica CTR6000 microscope with micropublisher 5.ORTV camera using Q Capture software.

Quantification

Slides were viewed under a light microscope. NP positive cells present within the red pulp were classified as NP-specific plasma cells for quantification. At early time points, single cells were counted. At later time points, a point counting technique was used where plasma cell foci that crossed the bottom right corner of each square were counted. The number of these intersects were then multiplied by the average number of plasma cells present within three typical foci. For each mouse, half the spleen area was used for quantification.

Fluorescent staining for immunoglobulin switching

For antibodies, see appendix table 5

Slides were cut and fixed as above. All wash steps were performed in PBS and all staining/blocking was performed with reagent diluted in a 10%BSA in PBS buffer. First slides were washed for 5 minutes then slides were blocked in 10% normal horse serum. The primary Rat anti-mouse IgG was then added at 1/200, with a control section receiving buffer only for 1 hour. After washing for 5 minutes, slides were stained with the secondary donkey anti-rat-Cy3 for 45 minutes. Slides were then washed for 30 minutes in PBS then blocked for 20 minutes in normal rat serum. Then slides were stained with anti-IgD-PB; rat idiotype-Cy5 and goat anti mouse IgM-FITC for 45 minutes (antibodies were first incubated with 10% normal rat serum for 30 minutes). Slides were then washed for 5 minutes before coverslips were attached using ProLong Gold antifade reagent (Invitrogen). Slides were stored at -20°C until required.

Pictures were taking with a Leica CTR6000 microscope. For quantification, ten plasma cell foci were photographed at x20. For each foci, 10 to 15 idiotype plasma cells were counted. It was then determined whether or not these cells co-stained for IgG.

Taqman real time PCR

For reverse transcription mix and for primers and probes, and primer array details, see appendix tables 6 and 7.

8µm spleen sections were cut on the cryostat. Sections were disrupted and homogenised using the QIAshredder columns and mRNA was prepared using

RNeasy mini kits according to manufactures instructions (both Qiagen). RNA was stored at -80°C if not used straight away. To generate cDNA, 3µl of random primer (Promega) was added to 30µl of the mRNA. Sample was then denatured at 70°C for 10 minutes before cooling on ice. 27µl of a reverse transcription mix was then added to each sample before a one hour incubation at 41°C followed by 10 minutes at 90°C.

cDNA was stored at -20°C if not used straight away. 0.5µl of each cDNA sample was added into a 384 well plate with 5µl of the appropriate probes and primers specific to the target gene or the housekeeping gene (β2-microglobulin) and Taqman PCR master mix. The plate was run in an ABI 7900 real-time PCR machine (Applied Biosystems). The PCR cycle was 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Data was analysed using the SDS 2.2.2 software (Applied Biosystems) to set the thresholds. The quantification cycle (Cq) for each samples target and housekeeping gene was noted, then the target gene was quantified by removing the housekeeping Cq from the target Cq then taking the square root of this value.

ELISA

For all antibodies and serum dilutions, see appendix tables 8 and 9.

Blood was removed from mice at the respective times by cardiac puncture. After centrifuging at 13000RPM for 10 minutes at 24°C, serum was removed then

stored at -20°C until required. Coating buffer (15mM Na_2CO_3; NaHCO 35mM) with NP_2BSA or $NP_{15}BSA$ (both made in house) at a concentration of 5µg per ml was added to 96 welled plates (Nunc, Thermo Scientific) at 100µl per well. Plates were incubated at 4°C overnight before washing three times in wash buffer (PBS 100mM; 0.05% Tween 20). Plates were then blocked in blocking buffer (PBS with 1% BSA), at 200µl per well for one and a half hours at 37°C before washing again three times. For detection, mouse serum, a negative control serum and a positive control serum were diluted in diluting buffer (blocking buffer plus 0.05% Tween 20). The negative control was an unimmunised Cγ1 CRE mouse aged 2-6 weeks. The positive control was day 5 of a CGG primed NPCGG boosted BALB/C mouse.

Serum was serially diluted in blocking buffer down the plate. The plate was incubated for one hour at 37°C before washing three times. Isotype specific detection antibody diluting buffer was added at 100µl per well for one hour at 37°C. For non-AP conjugated detection antibody, after washing three times, Alkaline-phosphatase streptavidin (Vector) in dilution buffer was added (1/500) for one hour at 37°C. After washing three times, 100µl of p-nitrophenyl phosphate (N2770, Sigma) was added to wells for 30 minutes at 37°C. Plates were then read at 405nm using Synergy HT Microplate Reader (Bio-Tek, USA.

Statistics

The Mann-Whitney test was performed using graphpad prism 5 where appropriate.

Results

cRel knock-out mice produce less NP specific plasma cells

In the first experiment, wild type (WT) and cRel knock out (KO) mice of the C57/BL6 background were immunised with NP-Ficoll with the exception of the day 0 cohort as an unimmunised control. The respective cohorts were then sacrificed at day 0, day 5 and day 7 for analysis.

Immunohistology of the spleen was performed. NP positive cells that were present in the red pulp were classified as red pulp plasma cells/plasmablasts. By day 5, foci of large numbers of NP-specific plasma cells were prevalent in WT spleens and were of similar size by day 7 (figure 1). In contrast, day 5 KO spleens had fewer foci containing less cells. By day 7, foci were still rare, but more cells were present in the clusters, suggesting a low level of proliferation has occurred.

NP-specific red pulp plasma cells were quantified from labelled tissue sections (figure 2). Plasma cells were significantly higher than background and KO mice by day 5 in WT mice with a median around 50-fold greater than in KO mice. NP-specific plasma cells were not above unimmunised background level until day 7, although the difference is non-significant. However in KO mice the median is now only about 5-fold lower than the WT day 7 mice. There is some variation at day 7 in the KO group, with one mouse still at background levels and one mouse at a similar level to the WT day 7 group.

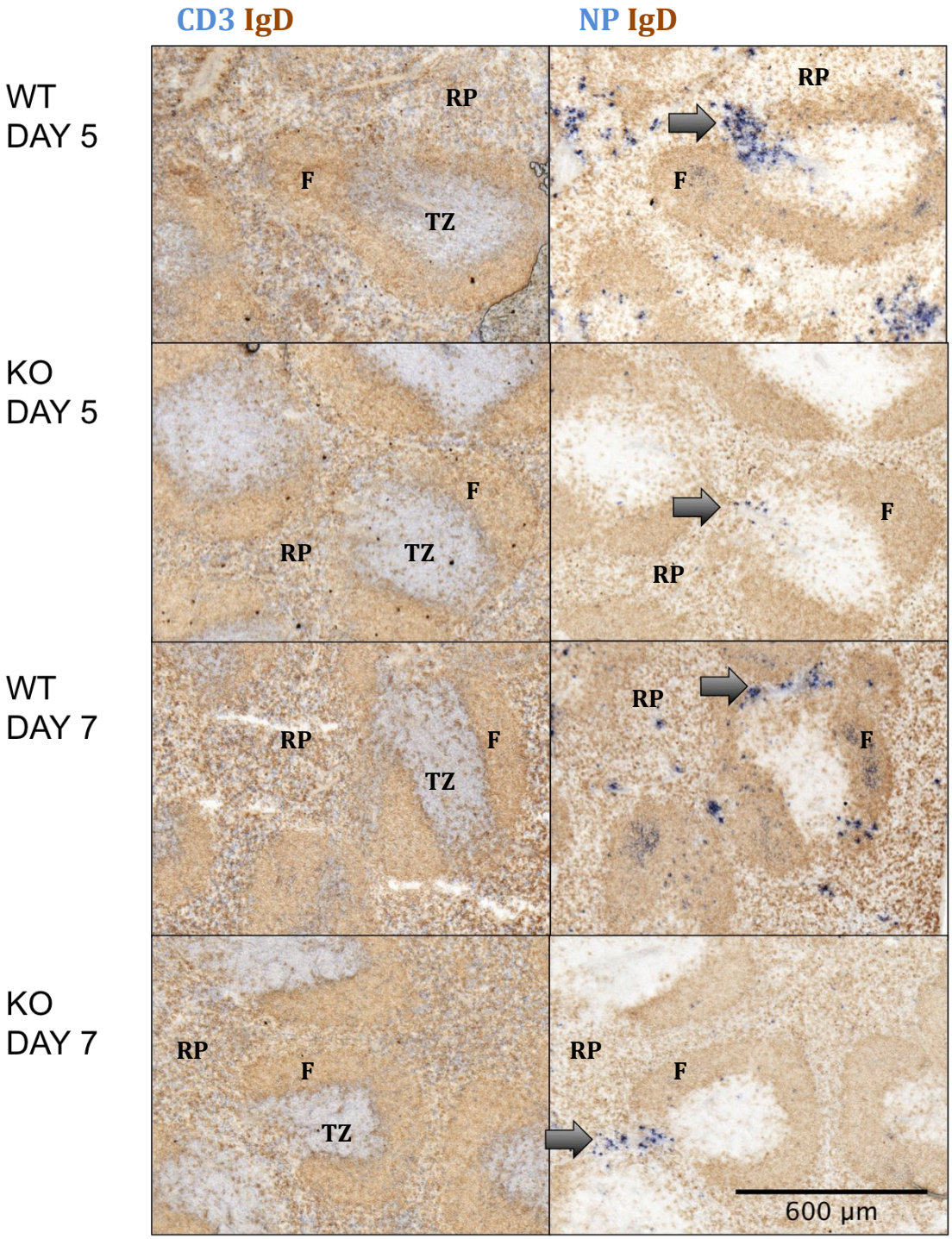

Figure 1: NP-specific plasma cells of the splenic red pup. WT and *cRel* $^{-/-}$ (KO) mice immunised with NP-Ficoll then sacrificed at the indicated time points. Spleens were sectioned and Immunohistology was performed. Sections on the left were stained for CD3 (blue) and IgD (brown); sections on the right were stained for NP binding (blue) and IgD (brown). Arrows indicate clusters of NP-specific plasmablasts/plasma cells. Also indicated is the red pulp (RP), follicle (F) and the T cells zone (TZ). All images taking at x20 and the scale is representative for all images.

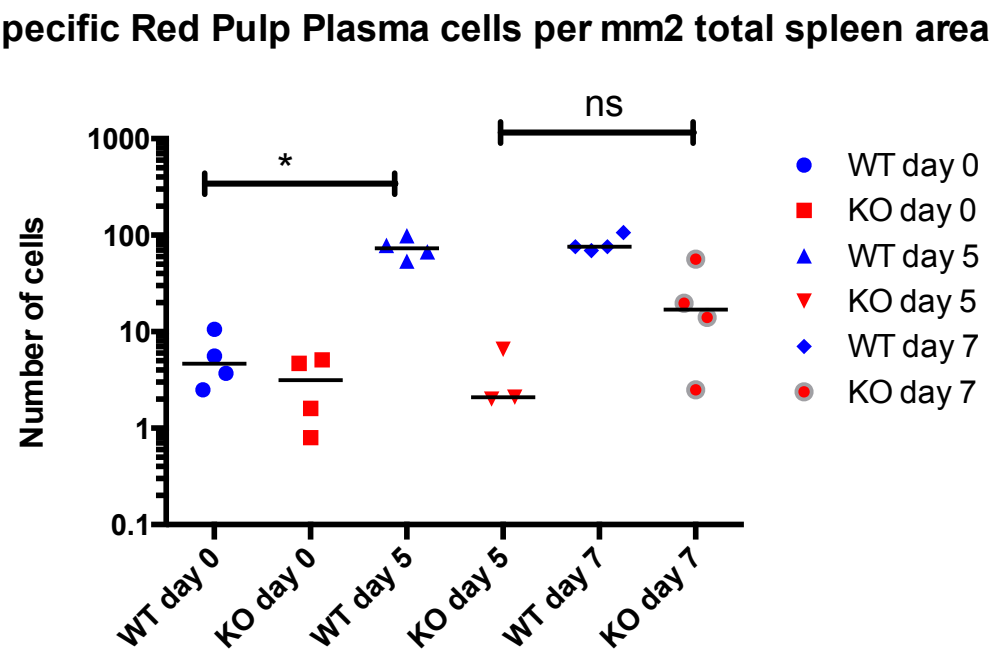

Figure 2: splenic red pulp NP specific plasma cells/blasts. WT (blue) and *cRel* $^{-/-}$ (KO) (red) mice immunised with NP-Ficoll, with the exception of the day 0 cohort, left as an unimmunised control. Immunohistology was performed on splenic sections at the indicated time points. Red pulp NP specific plasma cells/blasts were quantified from half the spleen area. Data shows the individual mouse values for NP plasma cells per mm^2. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where $p<0.05$ = * and ns = non significant.

cRel knock-out mice produce a lower NP-specific antibody response

Serum was taken from sacrificed mice and ELISA was performed for NP-specific antibody as shown in figure 3. NP-specific antibody is present in the unimmunised control, for WT and some KO mice. This is likely to be low affinity natural antibody and not specific to an NP immune response. In WT mice high IgM levels are seen by day 5, which increase to day 7. Low but significant titres of IgG are detectable by day 5 in WT mice, with a large increase seen at day 7. WT mice also produced detectable IgG1 from day 5. IgG2a is only detectable at low levels, in two WT mice and one KO at day 7.

In contrast, KO mice show a consistently lower NP-specific antibody level. KO mice have significantly reduced IgM levels at day 5 and 7 compared to WT,

although levels have increased in some KO mice at day 7. KO Total IgG levels are also significantly reduced compared to WT and similar to background levels at day 5. Again, some KO mice have increased levels by day 7. KO IgG3 is actually similar to WT at day 5, except from one mouse with non-detectable levels. By day 7, levels have increased although the group is still below WT. IgG1 is also undetectable in KO mice at day 5. Only one KO mouse gave IgG1 by day 7.

Although antibody levels do seem to increase a little from day 5 to day 7 in the KO groups, it is still lower than WT. The exception to this is one KO mouse from day 7, which was consistently high in antibody titre and NP-specific red pulp plasma cells (figure 2). This genotype of this mouse was confirmed to be *cRel*-/- (personal communication, Laura George).

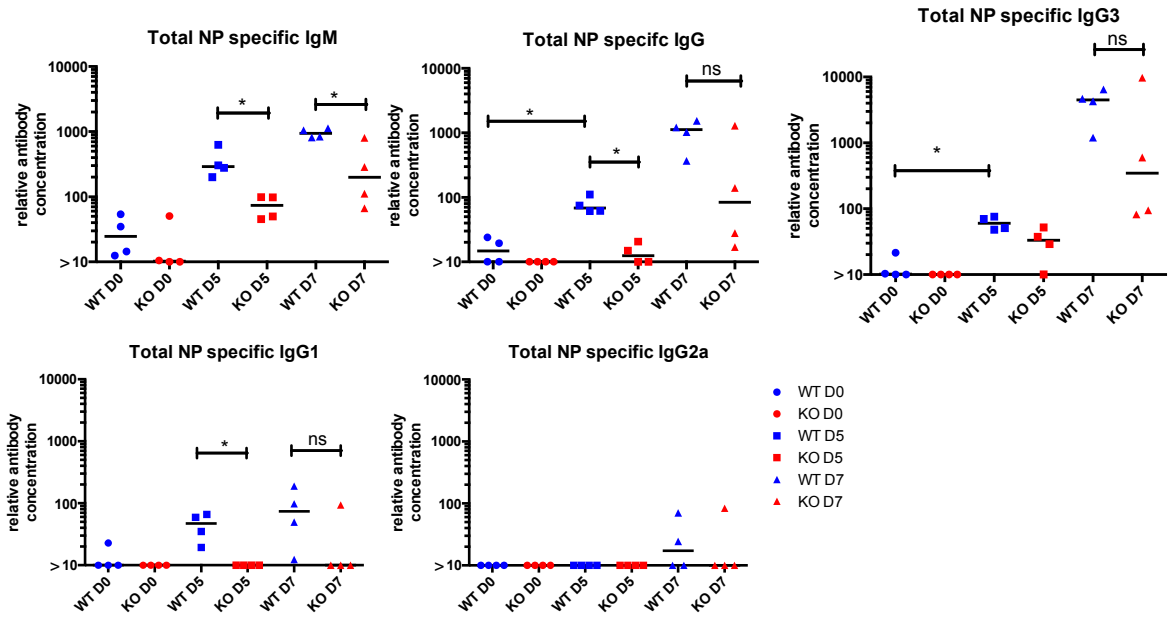

Figure 3: NP-specific antibody ELISA. WT and *cRel* $^{-/-}$ (KO) mice immunised with NP-Ficoll, with the exception of the day 0 cohort, left as an unimmunised control. Mice were sacrificed at the indicated time points. Serum was taking at the indicated time points and ELISA performed for various antibody isotypes. Each data point represents a single individual mouse value. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where $p<0.05$ = * and ns = non significant.

This experiment shows that in the absence of cRel, there are defects in extrafollicular plasma cell production and defects in specific antibody production in response to NP-Ficoll. However, it is unknown whether this is due to defects in the B cells themselves (B cell intrinsic) or of the supporting stromal environment. In order to investigate this, an adoptive transfer experiment was performed. QM mice have a B cell repertoire with around 95% BCR specific to NP [44]. Naïve splenic B cells (CD43 negative) from WT QM mice and *cRel* KO QM mice were adoptively transferred into WT C57/BL6 mice. Mice were immunised with NP-Ficoll then sacrificed for analysis at 2 hours, 2 days and 5 days after immunisation. As a control, WT QM cells were also transferred to *cRel* KO C57/BL6 mice, before immunisation as above then analysis at day 5 only.

cRel KO transferred B cells form less extrafollicular foci of plasma cells

Immunohistology of the spleens was performed as before. Anti-idiotype was used to specifically identify transferred cells. Extrafollicular foci of idiotype plasma cells was not evident until day 5, at which point, mice that received WT cells had a greater number of splenic red pulp plasma cells, with the median around 6 fold greater than in KO>WT mice (figure 4 and 5). WT cells gave the same number of plasma cells whether in a WT or KO environment (figure 5), suggesting that the main role of cRel is B cell intrinsic.

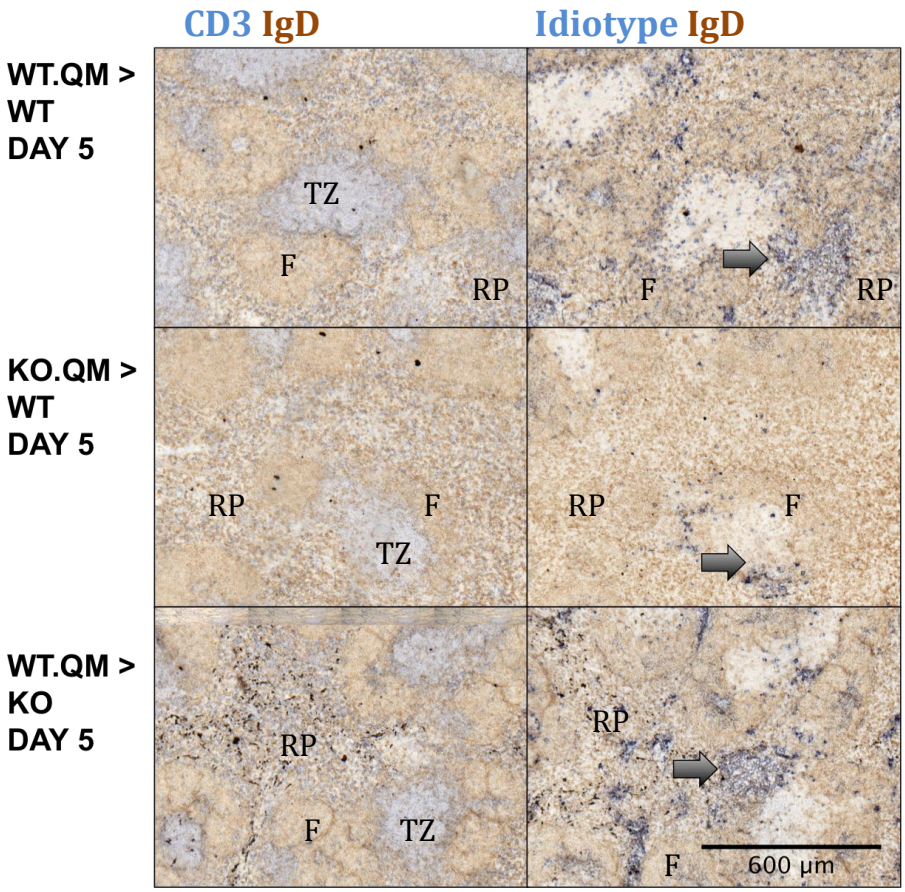

Figure 4: idiotype specific plasma cells of the splenic red pup. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT (WT.QM>WT), (KO.QM>WT) or KO hosts (WT.QM>KO). Mice were then immunised with NP-Ficoll. Immunohistology was performed on splenic sections 5 days later. Sections on the left were stained for CD3 (blue) and IgD (brown); sections on the right were stained for transferred cell idiotype (blue) and IgD (brown). Arrows indicate clusters of NP-specific plasmablasts/plasma cells. Also indicated is the red pulp (RP), follicle (F) and the T cells zone (TZ). All images taking at x20 and the scale is representative for all images.

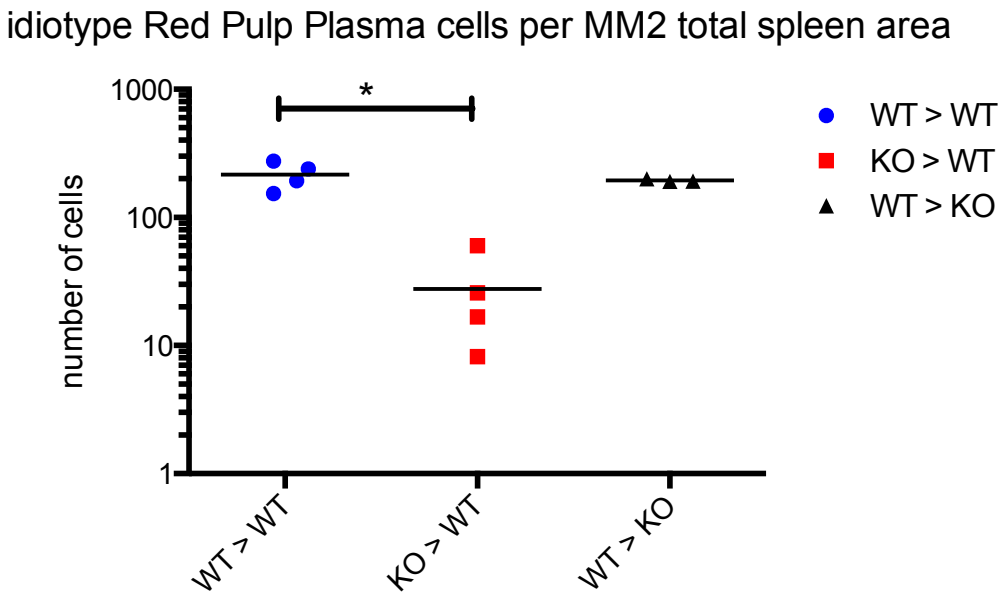

Figure 5: idiotype NP-specific plasma cells. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll. Immunohistology was performed on splenic sections at the indicated time points. Red pulp NP specific plasma cells/blasts were quantified from half the spleen area. Data shows the individual mouse values for NP plasma cells per mm^2. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where $p<0.05$ = *

There are less KO transferred B cells present at day five compared to WT transferred B cells

Flow cytometry was performed on splenocytes. This was not performed on the WT>KO group. Gating analysis is summarised in figure 6A, and results in 6B. Transferred cells were identifiable from the eYFP, detectable in the FITC channel. WT transferred cells have increased significantly by day 5, where they are also significantly greater in frequency than KO transferred cells. Of these transferred cells at day 5, a greater frequency express the plasmablast/plasma cell marker CD138, although this was not significant. A similar frequency of the CD138$^-$ transferred cells were found to express the GC marker Fas. The greater number of WT transferred cells agrees with the quantification data above.

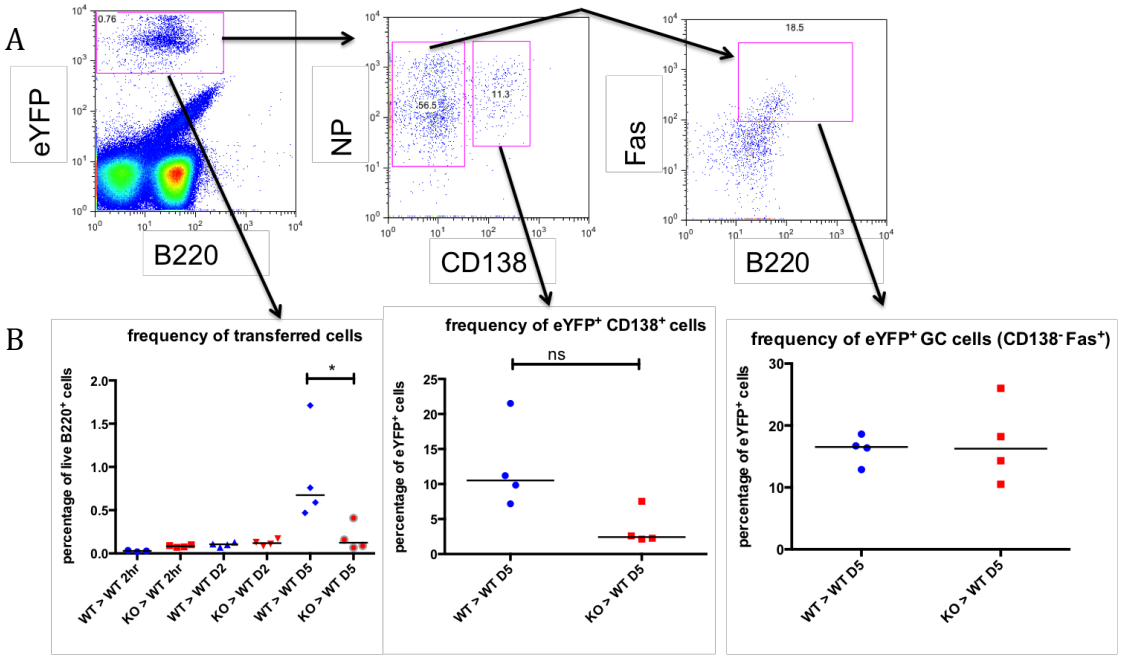

Figure 6: flow cytometry of splenocytes. eYFP +Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and spleens analysed at the indicated time points. Flow cytometry was performed on splenocytes and representative gating analysis is showing in A, where arrows to the right indicate gate analysis in the next FACS plot. Splenocytes were gated for lymphocytes, single cells then live cells (not shown). Live cells were then gated for transferred cells, which stained positive for eYFP. Transferred cells were gated for ability to bind NP (NP^+) and from day 5, whether they were plasmablasts/plasma cells ($CD138^+$) or not ($CD138^-$). The non-plasmablast/plasma cell gate was then gated for GC B cells (Fas^+). In B, data from flow cytometry is shown. Downwards arrows indicate quantification data from the gating analysis of part A. Data shows the individual mouse values. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where $p<0.05$ = * and ns = non-significant.

B cells that lack cRel have poor antibody production

Serum was obtained from sacrificed mice and analysed for NP-specific antibody as seen in figure 7. WT>WT NP-specific IgM levels are still low by day 2, but by day 5 high levels are seen. When ELISA is performed for detection of only NP specific IgM^a, which allows detection of only the antibody produced by transferred cells, we again see high levels in WT>WT. NP-specific IgG is undetectable until day 5, at which point transferred WT cells are capable of producing some IgG1 in WT>WT. Small levels of high affinity IgG are also detectable in WT>WT mice by day 5.

KO>WT mice also produce similar levels IgM by day 2. NP-specific IgM is slightly, but significantly lower in KO>WT mice by day 7. However, when we look at IgM^a its apparent that the KO transferred cells give only barely detectable levels by day 5. This suggests the KO transferred cells give minimal contribution to the IgM response. KO>WT mice did not give a detectable NP-specific IgG response.

WT>KO mice gave similar antibody levels at day 5 to WT>WT mice. This included IgM^a and IgG^a produced only by the transferred cells. This suggests NP-specific WT cells in the KO environment are differentiating in absence of competition from the cRel deficient host cells.

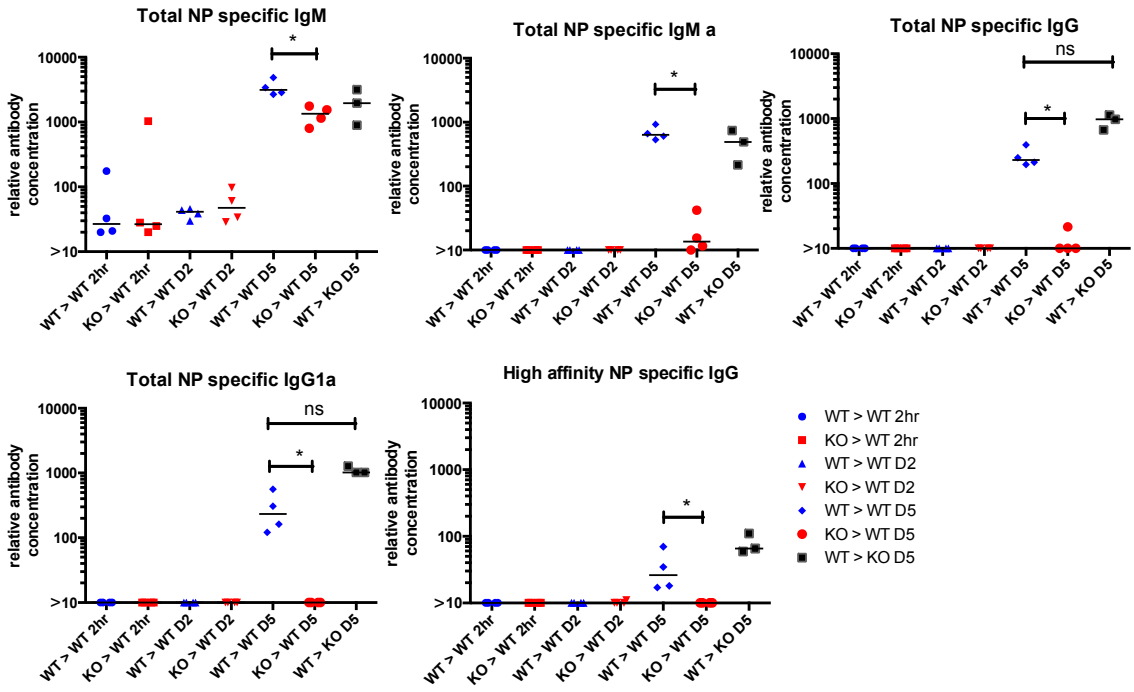

Figure 7: NP-specific antibody ELISA. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll. Mice were sacrificed and serum was taking at the indicated time points and ELISA performed for various antibody isotypes. Each data point represents a single individual mouse value. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where $p<0.05$ = * and ns = non significant.

The absence of cRel from B cell or from environment does not effect IRF4 transcription

IRF4 is upregulated in activated B cells and a high expression is required for plasma cell differentiation [29]. Since cRel is implicated in IRF4 induction [33] and since IRF4 is high in plasma cells we expected that, giving the reduction in plasma cells in the KO>WT mouse groups, IRF4 will be reduced, by day 5 compared to WT.QM groups. For this experiment, an unimmunised control group was required to compare background IRF4 levels. For this, the day 0 WT cohort from the first experiment was used. Spleen sections were cut and cDNA prepared from whole sections. PCR was then performed for IRF4 transcripts as shown in figure 8.

In both groups *Irf4* expression from the whole spleen has increased compared to our background control by 2 hours, as seen in figure. Neither the WT>WT or KO>WT groups at 2 hours were significantly greater than background (not shown). However, taking both groups into a single 2 hour group did give a significant increase compared to background. *Irf4* transcripts then decrease to background levels at day 2 in both groups. This agrees with previous data from the Toellner lab (Laura George, unpublished data). Surprisingly, *Irf4* did not increase to day 5 in any group, despite the high numbers of plasma cells in the WT>WT and WT>KO groups.

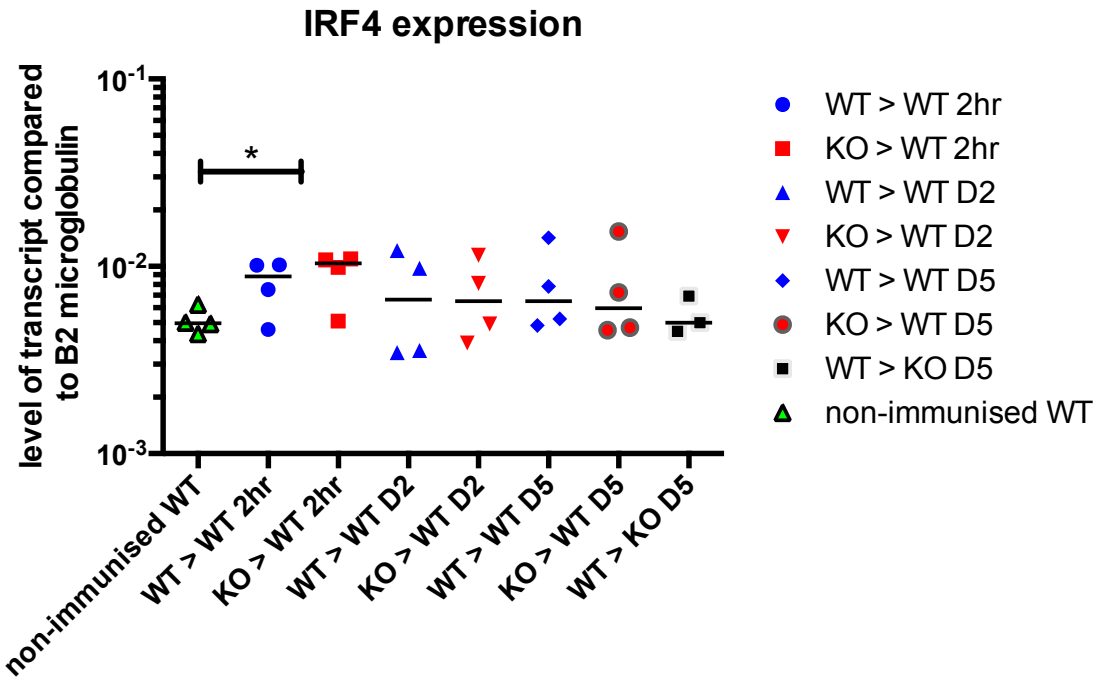

Figure 8: splenic *irf4* transcripts levels compared to housekeeping gene transcripts (β2m). Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and sacrificed at the indicated time points. A non-immunised WT group was also included as a background control. cDNA was prepared from spleen sections. PCR was performed for detection of *irf4* and *β2m* transcripts. Each data point shows individual values from single mice. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the non immunised group, and the two 2 hour groups as a whole, as shown by the bar, where p<0.05 = *

Mice receiving WT or KO B cells have a similar ability to initiate CSR

The appearance of immunoglobulin heavy chain germline transcripts (IgH switch transcripts) correlates with induction of CSR. The low titres of IgG seen in KO>WT mice could be due to lack of transcription through the Ig heavy chain switch regions. PCR of whole spleen sections was performed as before, with detection of the IgG1 switch transcript; IgG3 switch transcript; and Recombined IgM – IgG3 heavy chain transcript which indicates successful class switch recombination to IgG3 as shown in figure 9.

The IgG1 switch transcript is required for switching to IgG1. By day 2, the WT>WT group produce high levels of this transcript, significantly higher than

background, yet this does not increase to day 5. The KO>WT group is still at background by day 2, although one mouse gave a high level. By day 5, the KO>WT group has largely caught up to the WT>WT, although the median is slightly lower.

The IgG3 switch transcript is required for switching to IgG3. Surprisingly, the non-immunised control group has a very high transcript level. All immunised groups at each time point show great variation, but in terms of the median, they increase from the 2 hour time point and are all similar levels by day 2 and 5.

The recombined IgG3 heavy chain transcript shows the level of IgG3 that is being transcribed. Similarly, the non-immunised control is higher than expected. At 2 hours and 2 days, the WT>WT groups are slightly higher than the KO>WT groups. Both groups have increased by day 5 above background level and to a similar level. The same mouse that had low day 5 IgG3 switch transcripts from the KO>WT group was well below background IgG3 heavy chain transcripts at day 5. The WT>KO control group is also slightly lower. Together, results suggest the lack of IgG detection by day 5 in KO>WT mice is not due to an inability produce switch transcripts across the whole spleen.

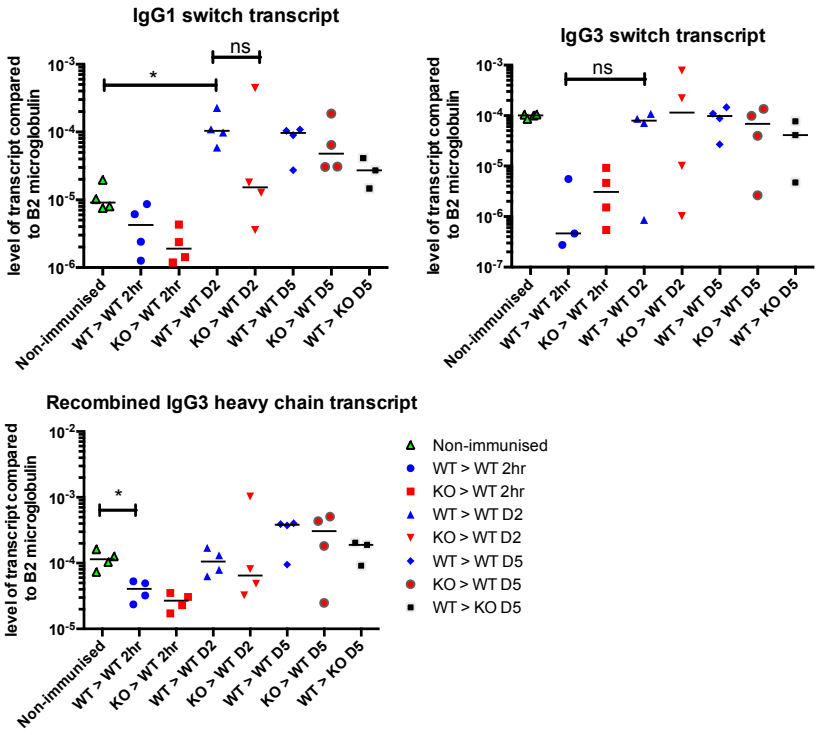

Figure 9: splenic switch and recombined heavy chain transcript levels compared to housekeeping gene transcripts (β2m). Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and sacrificed at the indicated time points. A non-immunised WT group was also included as a background control. cDNA was prepared from spleen sections. PCR was performed for detection of *igg1 switch transcript; igg3 switch transcript; recombined igg3 heavy chain transcript;* and *β2m* transcripts. Each data point shows individual values from single mice. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where p<0.05 = * and ns = non significant.

Splenic AID transcript levels are similar between WT and KO groups

AID is a key factor of CSR and is regulated by IRF4, we therefore investigated its induction throughout the immune response. PCR was performed as before with detection of *aid* transcripts. Previous studies have found *aid* transcript expression in B blasts from the follicle-T zone border from 2 days after NP-Ficoll immunisation (Marshall 2011). Coinciding with the induction of CSR, *Aid* transcripts are above background 2 days after immunisation in both the WT>WT and KO>WT groups as shown in figure 10. Although KO>WT level is slightly lower than WT>WT, this difference was not significant. Similar levels of

transcript are also found at day 5. Since AID is not expressed in plasmablasts or plasma cells [6], this probably reflects AID expressed in GC's.

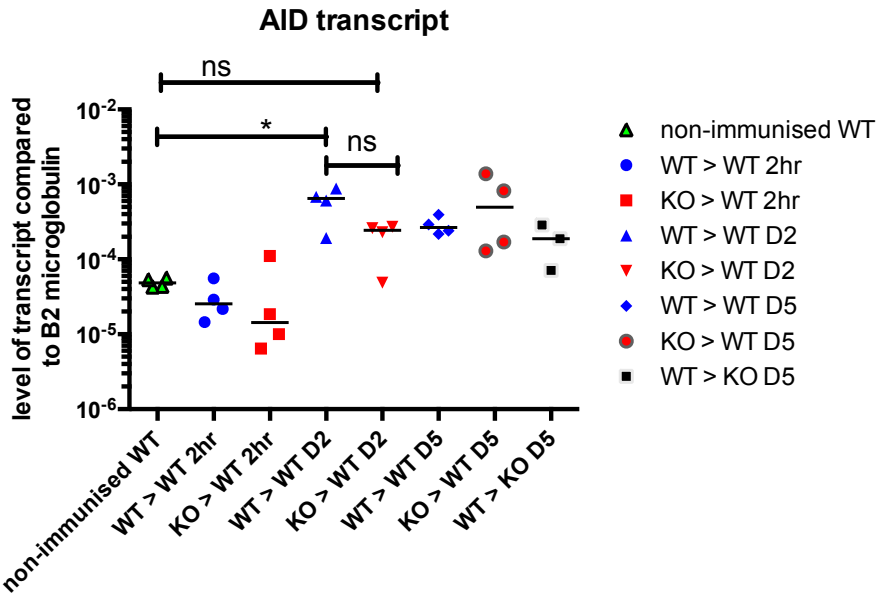

Figure 10: splenic *aid* transcript levels compared to housekeeping gene transcripts (β2m). Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and sacrificed at the indicated time points. A non-immunised WT group was also included as a background control. cDNA was prepared from spleen sections. PCR was performed for detection of *aid* and *B2m* transcripts. Each data point shows individual values from single mice. The black lines within each group represent the group median. Statistical analysis involved the Mann-Whitney test between the two indicated groups as shown by the bar, where p<0.05 = * and ns = non significant.

The absence of cRel does not affect the ability of plasma cells to switch to IgG

The previous PCR experiments show there is little defect at a global level in *Irf4* transcripts, switch transcripts and *Aid* transcripts. Since ELISA data showed transferred cells were poor producers of IgG, these cells may not have actually switched to IgG. In order to investigate the ability the specific transferred cells to switch from IgM to IgG at a cellular level, splenic sections from day 5 only were placed onto slides as before. Slides were then fluorescently stained for the QM IgH idiotype, IgD to indicate follicular areas, and IgM and IgG to analyse class switching. The percentage of transferred plasma cells that costained for IgG were

then quantified and presented in figure 11. Typical images of foci are shown in figure 12.

Despite the variation in the WT.QM>WT group, there is no difference in the ability of Idiotype cells to switch to IgG. Of course, there are much less transferred cells/plasma cells present at day 5 in the KO.QM group (figure 6B and 13). Only small numbers of idiotype cells are involved in foci of KO>WT spleens compared to the majority seen in WT>WT and WT>KO (figures 12 and 13).

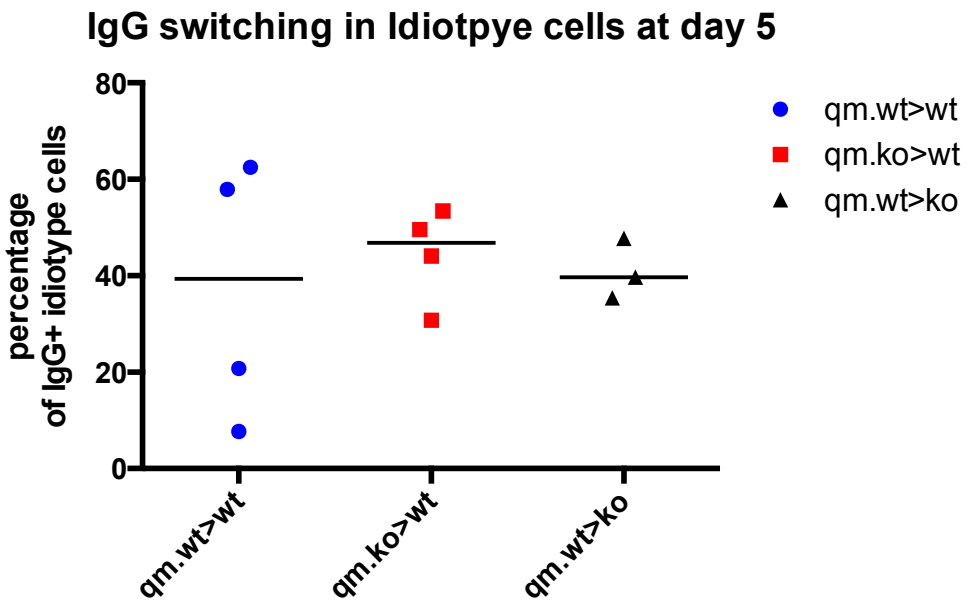

Figure 11: percentage of IgG⁺ transferred cells. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and at day 5 spleens were removed for analysis. Splenic sections were cut onto slides, which were fluorescently stained for transferred cells, IgM and IgG. The percentage of transferred cells which costained for IgG were quantified.

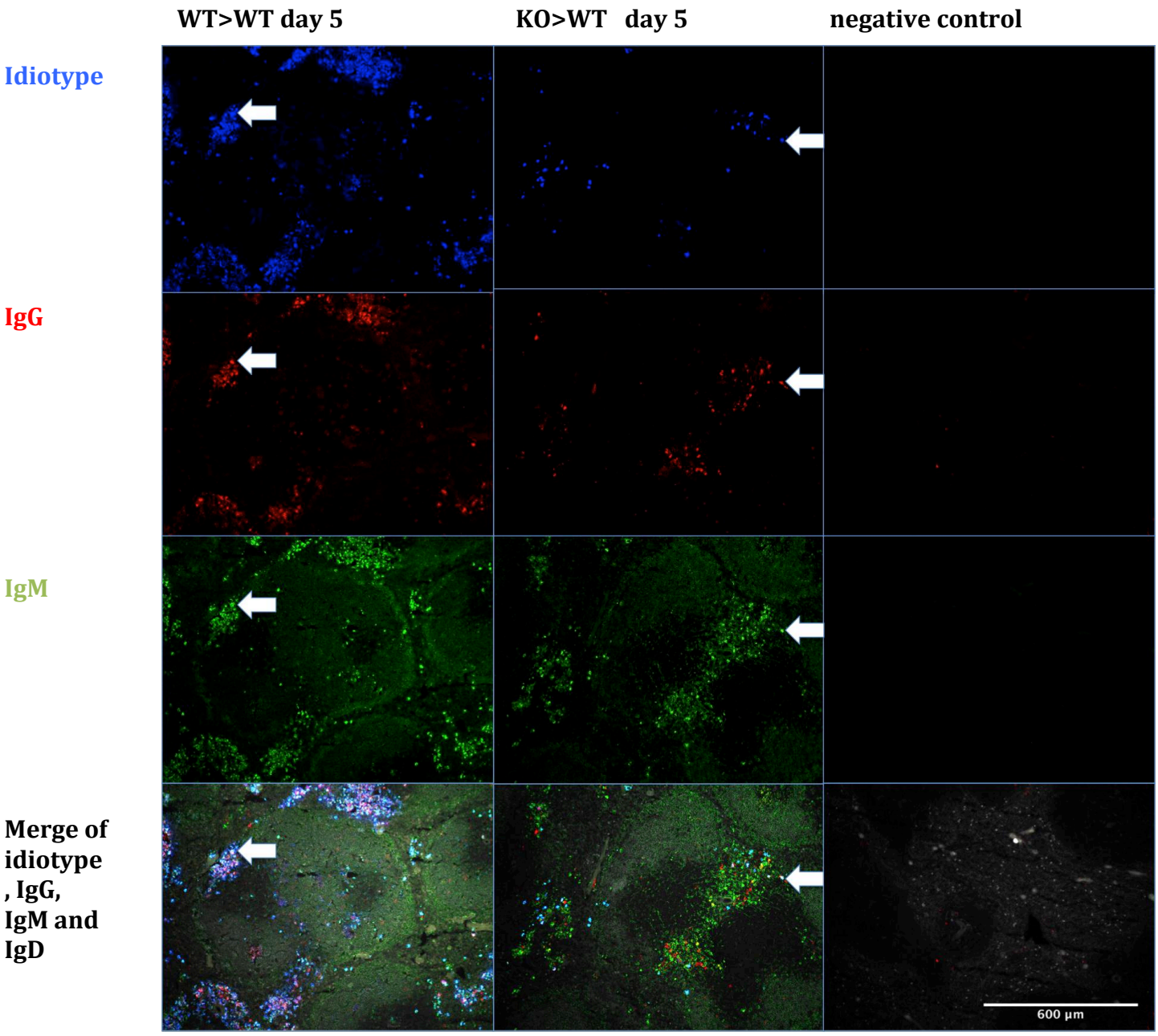

Figure 12: Fluorescent images and merges from spleen sections. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT hosts (WT>WT), (KO>WT). Mice were then immunised with NP-Ficoll and 5 days later, spleens were sectioned and fluorescent staining was performed. Each column shows a representative section from a WT>WT (left), KO>WT (middle) mouse and a negative control section (right). The negative control received no primary antibody. Staining used was anti-idiotype (blue) to show transferred cells; anti-IgG (red); anti-IgM (green) and anti-IgD (grey). Each column shows a single colour stain for idiotype, IgG and IgM as indicated. The last row shows the merge of these single stains all together, with IgD. Arrows indicate a particular idiotype plasma cells that also expresses IgG as well as IgM. These cells show up as white staining in the merge.

WT>WT KO>WT WT>KO

Idiotype
IgG
IgD

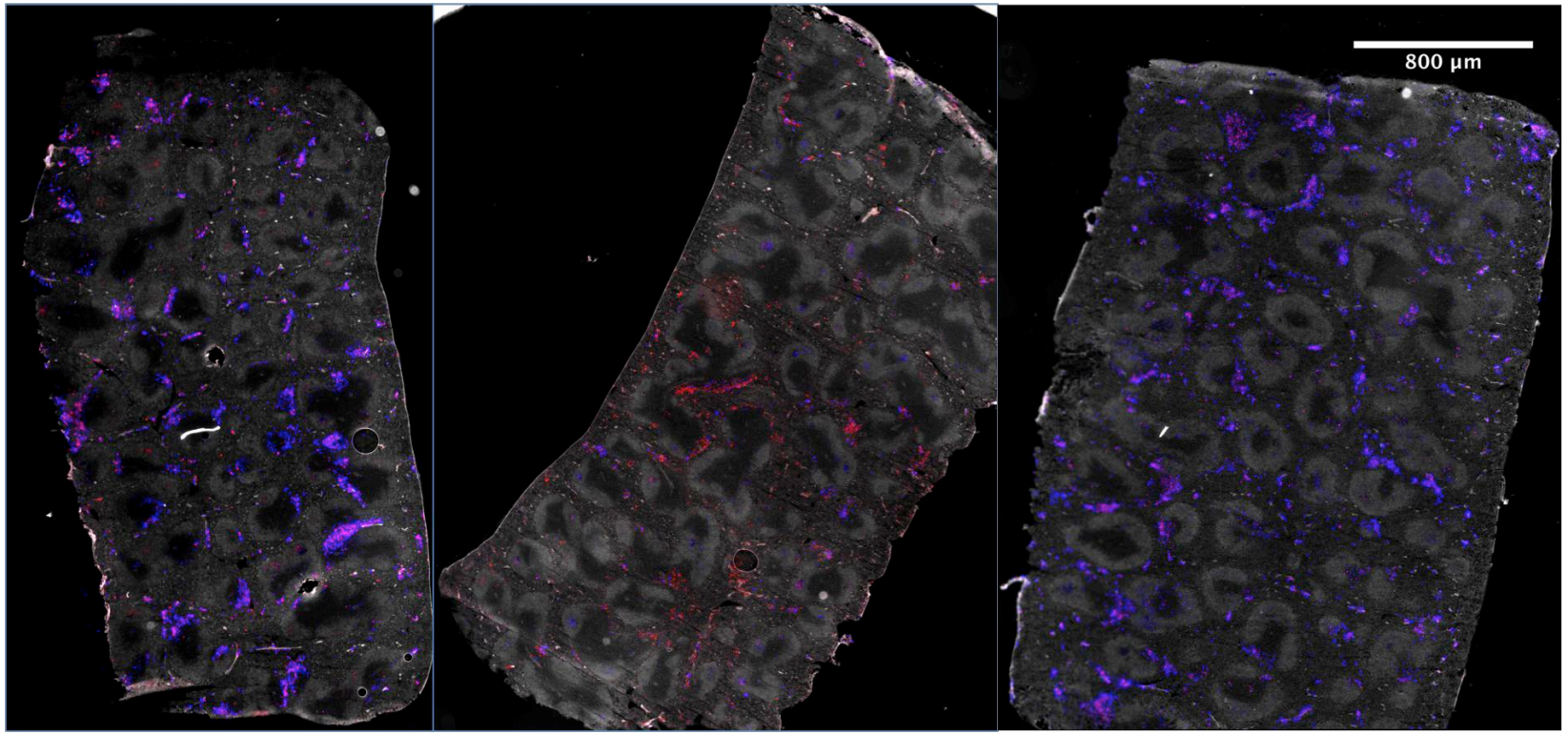

Figure 13: Tilescans of typical whole spleen sections. Naïve B cells from WT QM and cRel-/- (KO) QM mice were transferred into WT or KO hosts. Mice were then immunised with NP-Ficoll and at day 5 spleens were removed for analysis. Splenic sections were cut onto slides, which were fluorescently stained for transferred cells with idiotype (blue), IgG (red) and IgD (grey). WT>WT spleen is on the left; KO>WT in middle; WT>KO on right. Costaining of idiotype and IgG is seen as purple staining. Tilescans were made from individual images at 10x and pieced together. Scale is representative for all figures

Discussion

Immunisation of both WT C57/BL6 mice and C57/BL6 mice transferred with WT.QM B cells resulted in a strong extrafollicular immune response with high numbers of splenic red pulp NP-specific plasma cells. These plasma cells peaked at day 5 after immunisation. From the first experiment, we see the numbers stay constant to day 7. This is consistent with previous studies which found NP-Ficoll immunisation of C57BL6 mice resulted in foci first appearing at day 3 and peaking at day 5 before decreasing in week two after immunisation [46].

We found that in cRel KO mice, NP-specific plasma cells do not increase above background until after day 5. This defect was found to be B cell intrinsic, since cRel absence in only B cells still resulted in small plasma cell numbers compared to WT>WT mice, whereas a B cell extrinsic absence had no effect, at least at day 5. Flow cytometry data also showed significantly less transferred cells by day 5 in KO>WT mice. This difference is transferred cell/plasma cell numbers likely reflects a decreased proliferation and increased apoptosis in the absence of intrinsic cRel.

cRel KO B cells also show increased apoptosis to mitogenic stimuli, including BCR ligation [474849]. Members of the Bcl2 pro-survival gene family, Bcl_{XL} and A1 have been found to be cRel target genes in B cells [50]. Transgenic expression of either of these in cRel KO B cells can provide partial protection from apoptosis upon BCR ligation [50,51]. It is therefore likely that the strong BCR crosslinkage

caused by NP results in a high level of apoptosis in cRel KO B cells. Hence, many of the responding NP-specific cells do not survive, so never contribute to plasma cell and antibody production.

Previous studies describe how cRel KO B Cells show poor proliferation [4847] [49] and its been shown these B cells fail to progress from the G1 to S phase of the cell cycle [52]. The cRel target gene E2F3a is important in transition to the S phase [53]. E2F3a activity allows induction of cyclin-E. Cyclin-CDK complexes are formed upon BCR ligation and allow hyperphosphorylation and inactivation of Rb. Rb can then no longer bind and repress E2F proteins, allowing them to facilitate cell cycle progression. Therefore, any responding cRel KO B cells that do survive are likely to be unable to proliferate and contribute to high plasma cell numbers. Future study could confirm *in vivo* proliferation defects through CFSE labelling with flow cytometry, or BrdU staining with immunohistology.

Despite this, NP-specific plasma cells increased between day 5 and 7 in some cRel KO mice, with a group median increase of around 6 fold. cRel mRNA has been found to be downregulated in plasma cells compared to mature B cells [54,55]. It is therefore possible that responding NP-specific cells that do survive and make it to the plasmablast stage, will be less affected by the cRel deficiency and capable of some clonal proliferation before terminal differentiation to plasma cells.

Even before immunisation, NP-specific IgM was detectable at low titres, in some mice from both the WT and KO groups. This will most likely be natural antibody,

produced by B1 B cells and MZ B cells. The higher titres detected from day 5 will reflect antibody produced during the NP-specific immune response. NP-specific IgM peaks at day 7, which is similar to previous studies which found significant titres of NP-specific IgM by day 5, peaking at day 7 and remaining high thereafter [46].

IgG3 was reported to be the major switched antibody produced, along with high some IgG1 during an NP-Ficoll response [46]. Likewise, during the WT response, we found IgG3 to give the highest IgG titre and significant titres of IgG1 were also detected. The absence of cRel results in poor NP-specific antibody detection. From the first experiment, we see that KO mice have reduced antibody production of IgM and IgG. KO antibody production does increase however at day 7, and this correlates with the increase in NP-specific plasma cells.

The adoptive transfer experiment shows that, despite high IgM titres, KO B cells do not contribute much to IgM production. Again, this likely reflects the very low numbers of KO transferred cells/plasma cells by day 5. Endogenous cells must then be responsible for this IgM, but surprisingly, even endogenous cells fail to produce much detectable IgG. The high levels of antibody of QM idiotype from the QM cRel WT cells suggest endogenous cells may not be the major antibody producers in these mice. The high frequency of NP-specific transferred cells may be able to outcompete endogenous cells for an accessory cell, such as $CD11^+$ DC's, for class switching signals. Thus, low frequencies of NP-specific endogenous cells in KO>WT mice may be initially outcompeted and possibly miss out on switching signals. However the KO>WT tilescan did show endogenous cells have switched

to IgG. A lot of these foci lacked idiotype cells, so it's possible that they were not NP-specific.

Previous studies have described serum levels of IgM, IgG1, IgG2a, IgG2b and IgG3 to be reduced in cRel KO mice [48]. IgG3 was shown to be marginally reduced at day 7 post NP-LPS immunisation in cRel knock out mice, though other isotypes were not tested, IgG1 was largely reduced after TD NP-KLH immunisation [48]. However, this study did not attribute whether this was due to an actual defect in antibody production/switching or simply reduced plasma cell numbers.

It has been described *in vitro* that cRel KO B cells fail to switch to IgG3, IgG1 or IgE in response to the appropriate stimuli [5643]. This correlated with lack of induction of IgG1 and IgG3 switch transcripts. A cRel binding site has been described in the *I* promoter of the IgG1 switch transcript [57]. cRel has also been proposed to bind to the 3'IgH enhancer downstream of the C_H locus, another way cRel could regulate CSR [42]. On top of this, cRel KO B cells fail to switch to IgE *in vitro* despite normal induction of the E switch transcript [56], suggesting other roles for cRel in CSR and antibody production.

We investigated the ability for CSR at a global level from the spleens from the second experiment. During the NP-Ficoll response, IgG3 switch transcripts increase above background within 24 hours [46]. Likewise, we found in WT>WT mice, IgG1 and IgG3 switch transcripts are expressed to high levels by day 2. The median level of these transcripts did not increase by day 5, despite the high numbers of plasma cells within the extrafollicular foci at day 5.

It was also noted that the non-immunised mouse group did have rather high levels of the IgG3 switch transcript especially. We think its unlikely this group represent the background Cγ3 switch transcripts which would have been found in the immunised mouse groups before immunisation, since IgG3 is the dominant antibody response yet switch transcripts were never above background and have apparently decreased significantly within two hours. These mice may have had a background immune response resulting in high IgG3 (not specific to NP). This control group was not part of the original experimental set up, and therefore there may have been a higher background response in this cohort due to a non-related infection.

IgG1 switch transcripts were reduced at day 2 in most KO>WT mice compared to WT>WT mice, but by day 5, transcripts were equivalent, as were IgG3 switch transcripts. The ability to complete CSR and transcribe a recombined IgG3 antibody was also equivalent in the KO>WT and WT>WT groups by day 5. A similar relationship was found with *Aid* transcription. Although this did not show that our cRel KO B cells are capable of CSR, it shows that at a global level, B cells in the spleen should be capable of switching and producing a detectable IgG response.

We did however investigate the ability of the transferred cells to switch to IgG expression at day 5 by fluorescent staining with costaining for idiotype and IgG. Perhaps surprisingly, there was no difference in the proportion of transferred cells that were now expressing IgG. Although there were a lot less transferred

cells that became plasma cells, similar proportions of transferred cells had switched to IgG in both the WT>WT and KO>WT as well as the WT>KO mice. This would also agree with the earlier possibility that transferred cells may outcompete endogenous cells for switching signals. While the subclass of IgG was not determined, this result taking together with the PCR data does suggest that the absence of cRel does not affect CSR and IgG expression during an NP-Ficoll response.

Splenic *Irf4* transcripts increased within the first 2 hours. It has been shown that MITF negatively regulates IRF4 in naïve B cells [31]. After *in vitro* B cell activation, *Mitf* transcripts decrease while *Irf4* increases. This lab has previously found *Irf4* transcripts to increase in the first 4 hours after immunisation before falling to low levels again (Laura George, personal communication).

Giving that high IRF4 levels are thought to be required for plasma cell differentiation [29], we expected to find high *irf4* levels by day 5. The lack of this may be due to *Irf4* transcripts being transiently expressed at high levels before day 5, and this phase of initiation of plasma cell differentiation may have been missed. It is also possible that PCR of the whole spleen is not sensitive enough to pick up small changes in B cell *Irf4* expression, particularly if $IRF4^-$ GC cells are present by day 5. The data is compatible with the absence of cRel allowing low level IRF4 expression required for AID induction and CSR, but not the high levels of IRF4 required for plasma cell differentiation [29].

Ideally, PCR should be performed on sorted cells, such as the transferred cells. This would allow an accurate and sensitive analysis of transcription in the transferred B cells without the influence of other cells. For example, *Aid* transcripts should not be seen by day 5 in transferred cells that have become plasma cells, but *Aid* is also expressed in GC B cells, and is under different regulation. This could explain why we still see *Aid* transcripts at this time point. Antigen-specific B cell populations have been FACS sorted during this study, and future analysis should focus on gene expression in isolated cells.

Despite what was found in previous studies, we found *cRel* KO to have no effect on IRF4 induction or CSR. Since these previous studies involved *in vitro* work, it is possible that different signals and the local environment can overcome potential defects in IRF4 and CSR. However, cRel deficiency leads to a reduction in the amount of NP-specific plasma cells found, and subsequently, the level of antibody detected was much lower. As discussed above, it is likely that the RT-PCR from whole tissue sections is not sensitive enough and future studies on isolated cells may be more accurate. We also hypothesise that a high level expression of IRF4 (present in WT cells, and possibly absent in *cRel* KO cells), was missed because an earlier time point should have be chosen for the analysis of high level IRF4 expression triggering plasma cell differentiation, and future studies could address this.

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Appendix

Table 1: Antibody for flow cytometry

Antibody specificity	**Conjugation**	**Dilution**	**Company**
B220	PE-Cy5.5	1/300	BD pharmingen
NP	PE	1/30,000	Gift from Prof Peter Lane
Fas	PE-Cy7	1/100	BD pharmingen
CD138	APC	1/100	BD pharmingen

Table 2: Unconjugated immunohistochemistry antibody

Specificity	**Dilution**	**Company**
Rat anti-mouse CD3	1/1000	AbD serotec
Sheep anti-mouse IgD	1/1000	Abcam
Sheep anti-NP	1/3000	In house
Rat anti-mouse IgD	1/1000	BD pharm
Rat anti-idiotype	1/100	Gift from Dr Imanish-Kari

Table 3: Conjugated immunohistochemistry secondary antibody

Specificity	**Conjugation**	**Dilution**	**Company**
Rabbit anti-rat	biotin	1/600	Dako
Donkey anti-sheep	peroxide	1/100	The Binding Site
Donkey anti-sheep	biotin	1/100	The Binding Site
Rabbit anti-rat	peroxide	1/80	Dako

Table 4: Buffers and substrates for immunohistology

Tris pH 7.6	1.5L Physiological NaCl 1.5L 0.1M Hcl 1.0L 0.2M Tris
Tris pH 9.2	As for pH 7.6 but made to pH 9.2
Peroxidase substrate	One DAB tablet (sigma) dissolved in 15ml of Tris pH 7.6. 10ml was then filtered with the addition of one drop of hydrogen peroxide
Alkaline phosphate substrate	8mg of Levamisole (Sigma) is dissolved in 10ml of Tris pH 9.2. Naphtol AS-MX phosphate was dissolved in 370µl of dimethyl formamide (sigma). This was added to the Levamisole solution, before 10mg of fast blue BB salt (Sigma) was added and the final solution was filtered.

Table 5: Fluorescent antibody staining

Specificity	conjugation	Dilution	Company
Rat anti-mouse IgG	-	1/200	AbD serotec
Donkey anti-rat	Cy3	1/200	Milipore
Anti-IgD	PB	1/50	eBiosciences
Rat anti-idiotype	Cy5	1/100	Gift from Dr Imanish-Kari
Goat anti-mouse IgM	FITC	1/100	Southern Biotech

Table 6: PCR reverse transcription mix

Reverse transcription mix	Volumes required per sample	Company
5x first strand buffer	12µl	Invitrogen
0.1M DTT	6µl	Invitrogen
10mM dNTP's	3µl	Invitrogen
MMLV reverse transcriptase	3µl	Invitrogen
RNasin RNase inhibitor	1.5µl	Promega
RNase free water	1.5µl	Quigon

Table 8: Primers and probes required for real time PCR

Name of target sequence		Nucleotide sequence
β2-Microglulin	Forward primer	CATACGCCTGCAGAGTTAAGCA
	Reverse primer	ATCACATGTCTCGATCCCAGTAGA
	TaqMan probe	CAGTATGGCCGAGCCCAAGACCG
IRF4	Forward primer	GGAGGACGCTGCCCTCTT
	Reverse primer	TCTGGCTTGTCGATCCCTTCT
	TaqMan probe	AGGCTTGGGCATTGTTTAAAGGCAAGTTC
IgG1 switch transcript	Forward primer	CGAGAAGCCTGAGGAATGTGT
	Reverse primer	GGAGTTAGTTTGGGCAGCAGAT
	TaqMan probe	TGGTTCTCTCAACCTGTAGTCCATGCCA
recombined IgG3 heavy chain transcript	Forward primer	TCTGGACCTCTCCGAAACCA
	Reverse primer	ACCGAGGATCCAGATGTGTCA
	TaqMan probe	CTGTCTATCCCTTGGTCCCTGGCTGC
IgG3 heavy chain germline transcript	Forward primer	GACCAAATTCGCTGAGTCATCA
	Reverse primer	ACCGAGGATCCAGATGTGTCA
	TaqMan probe	CTGTCTATCCCTTGGTCCCTGGCTGC
AID	Forward primer	GTCCGGCTAACCAGACAACTTC
	Reverse primer	GCTTTCAAAATCCCAACATACGA
	TaqMan probe	TGCATCTCGCAAGTCATCGACTTCGT

Table 8: Experiment 2 ELISA serum dilutions and antibodies

ELISA type	**Antibody detection**	**Conjugation**	**Antibody company**	**Serum and negative standard dilution**	**Positive standard serum dilution**
NP15	IgM	AP	Southern Biotech	1/30	1/50
NP15	Total IgG	AP	Southern Biotech	1/10	1/100
NP15	IgG1	AP	Southern Biotech	1/10	1/100
NP15	IgG2a	AP	Southern Biotech	1/10	1/20
NP15	IgG3	AP	Southern Biotech	1/10	1/20

Table 9: Experiment 2 ELISA serum dilutions and antibodies

ELISA type	**Antibody detection**	**Conjugation**	**Antibody company**	**Serum and negative standard dilution**	**Positive standard serum dilution**
NP15	IgM	AP	Southern Biotech	1/20	1/30
NP15	Total IgG	AP	Southern Biotech	1/10	1/200
NP15	IgG1[a]	-	Biolegend	1/10	1/10
NP15	IgM[a]	-	Biolegend	1/10	1/10
NP2	IgG	AP	Southern Biotech	1/10	1/50