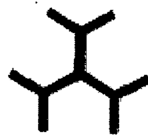
STUDY OF ANTIGEN PRESENTATION IN B CELLS

Thesis submitted to **Jawaharlal Nehru University** in partial fulfillment of requirement for the degree of
Doctor of Philosophy



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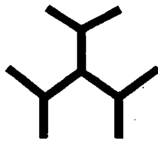

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CERTIFICATE

This is to certify that the thesis titled "**Study of Antigen Presentation in B Cells**" submitted by **Priyadarshini Chatterjee** in partial fulfilment of the degree of **Doctor of Philosophy** from Jawaharlal Nehru University, embodies the work done by the candidate under my guidance at the National Institute of Immunology, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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.... to my family

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Abbreviations

-	negative
-/-	knockout
%	percent
α	alpha
β	beta
γ	gamma
μCi	micro Curie
μg/ml	microgram per milliliter
μl	microlitre
μM	micromolar
mM	millimolar
+	positive
° C	degree celcius
AID	activation induced cytidine deaminase
APC	antigen presenting cell
ASC	antibody secreting cell
bg/bg	Beige mice
BLNK	B cell linker
BMDM	Bone marrow derived-macrophages
BCR	B cell receptor
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
Cat	cathepsin
CD	cluster of differentiation
CFA	Complete Freunds Adjuvant
CFSE	5-carboxyfluorescein succinimidyl ester
CHS	Chediak Higashi syndrome
CIIV	class II containing vesicles
CLIP	class II associated invariant chain peptide
CLP	commom lymphoid progenitor

CO_2	carbondioxide
CPM	counts per minute
CPRG	chlorophenolred-β-D-galactopyranoside
CSR	class-switch recombination
CTL	cytotoxic T lymphocyte
CHX	cycloheximide
DC	dendritic cell
DLN	draining lymph node
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxynucleotriphosphate
DTT	dithiothriol
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
ER	endoplasmic reticulum
Erk	extracellular regulated kinase
FCS	fetal calf serum
FDCs	follicular dendritic cells
Fig.	Figure
g/l	gram per litre
GL	germ line
GC	germinal center
H2O2	hydrogen peroxide
h / hr	hours
hi	high
HRP	horse radish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intercellular cell adhesion molecule
ICOS	inducible co-stimulatory molecule
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain

IL	interleukin
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibition motif
IU	international units
JNK	c-jun N-terminal kinase
kDa	kilodalton
KO	knockout
Lamp-1	Lysosome Associated Membrane Protein-1
LDA	limiting dilution assay
LFA	lymphocyte function associated antigen
lo	low
LPS	lipopolysaccharide
LYST	lysosomal trafficking regulator
mAb	monoclonal antibody
MACS	magnetic cell sorting
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
mg	milligram
MIICs	MHC class II containing compartments
MHC	major histocompatibility complex
min	minutes
mAmp	milliampere
ml	millilitre
mM	millimolar
mOVA	maleylated ovalbumin
MyD	myeloid differentiation factor
NF-κB	nuclear factor k of B cells
NF-AT	nuclear factor of activated T cells
ng/ml	nanogram per millilitre
NK	natural killer
nM	nanomolar
OPD	orthophenyl diamine
OVA	ovalbumin
PBMCs	peripheral blood mononuclear cells

PBS	phosphate buffered saline
PE	phycoerythrin
PEC	peritoneal exudate cell
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphatidyl-inositol-3kinase
PKA	protein kinase A
PKC	protein kinase C
PLC γ	phospholipase C-gamma
PLN	peripheral lymph node
pMHC	peptide-MHC
PMSF	phenylmethylsulphonyl fluoride
PNA	peanut agglutinin
PTK	protein tyrosine kinase
rads	radiation absorbed dose
RAG	recombination activating genes
rpm	rotations per minute
RT	room temperature
SA	streptavidin
s.c.	subcutaneous
SH2	Src homology 2 domain
SHM	somatic hypermutation
SLP-76	SH2-domain containing leukocyte protein of 76 kDa
SR	scavenger receptor
TCR	T cell receptor
TD	T dependent
TdT	terminal deoxynucleotidyl transferase
TI	T independent
TLR	toll like receptors
TNF	tumor necrosis factor
UPR	unfolded protein response
WT	wildtype

Introduction

Adaptive immune responses are mediated by the T and the B lymphocytes with help from dendritic cells (DCs) and macrophages and are characterized by features such as antigenic memory, clonal diversity and stringent self - non self discrimination. Both T and B lymphocytes express clonotypic receptors on their surface for specific recognition of target antigen. B cell receptors (BCR) can recognize specific antigenic epitopes directly on an intact circulating antigen while T cell receptors (TCR) recognize antigen that has been processed into peptides and presented in association with molecules of the major histocompatibility complex (MHC). The receptors are generated by germ-line gene rearrangements in the bone marrow that results in a diverse repertoire of clones, a vital strategy adopted by the immune system to fight the extremely high diversity observed in pathogenic organisms. During development these receptors get screened for their reactivity against self and only those clones which do not bind self antigens with high affinity survive over time and constitute the peripheral pool of lymphocytes. To accommodate the huge repertoire of lymphocytes within the limited space of the lymphoid tissue, each of the clones is represented in very small numbers in the periphery. Thus one crucial step following engagement of the receptors by cognate antigen is the triggering of the antigen specific clones for expansion. Clonal expansion is followed by differentiation either into relatively short-lived effector cells that help in the immediate clearance of infection, or into resting memory cells which survive for longer periods of time and can respond to a subsequent antigen encounter by rapid proliferation and differentiation.

The BCR is a complex which consists of an antigen binding membrane immunoglobulin (mIg) that is non-covalently associated with two polypeptide chains, CD79a and CD79b, which are responsible for initiation of downstream signaling (Gauld *et al.*, 2002; Harwood

and Batista, 2008; Hasler and Zouali, 2001; Jumaa *et al.*, 2005). Ligation of the BCR by its cognate antigen is followed by phosphorylation of certain motifs in the cytoplasmic tails of the CD79a and the CD79b molecules, called the immuno-tyrosine receptor activation motifs or the ITAMs. Phosphorylation of these ITAMs results in formation of a signaling complex which contains 3 classes of activated protein tyrosine kinases (the Src family kinase Lyn, the Syk kinase, and the Tec family kinase Btk) and various adaptor molecules. The downstream events include mitogen activated protein kinase (MAPK) activation and increase in intracellular Ca^{2+} levels, which eventually lead to the activation and subsequent nuclear translocation of transcription factors, NF-kB and NF-AT, as well as to the activation of other factors like c-jun and fos, that alter their DNA binding ability. B cell activation is an outcome of signals transduced through the BCR along with signals generated from certain co-receptors such as, CD19, CD21, CD22, CD32 and CD72 that are expressed on the B cell surface and can either positively or negatively modulate BCR signal transduction (Danzer *et al.*, 2003; Ono *et al.*, 1997; Shoham *et al.*, 2006; Tedder, 1998).

Apart from recognition of its target through the BCR, B cells can also employ other receptors, such as the toll-like receptors (TLR) for recognizing pathogen associated molecular patterns and undergo activation. For example, binding of lipopolysaccharide (LPS) to TLR4 on B cells can activate B cells resulting in its proliferation, differentiation into plasma cells and death in a T-independent (TI) fashion (Akira and Takeda, 2004; Fagarasan and Honjo, 2000). Plasma cells represent the final stage of B cell differentiation and are devoted to the production of large amounts of Ig. Another class of TI antigen are generally repeating polymers like dextran, present on the surface of the pathogens, which can crosslink a number of BCRs on a B cell surface resulting in B cell activation. The TI

responses usually take place in the marginal zone of the spleen, but TI antigens can also stimulate B cells at other locations, such as, in the peritoneal cavity (Fagarasan and Honjo, 2000; McHeyzer-Williams, 2003). The plasma cells in case of a TI response have short half-lives and can secrete IgM and low levels of IgG1 and IgG3. There is little affinity maturation of antibodies or generation of antigenic memory in case of such responses (Fagarasan and Honjo, 2000; Macpherson *et al.*, 2000; McHeyzer-Williams, 2003; Montecino-Rodriguez and Dorshkind, 2006).

For eliciting effective humoral responses to most protein antigens, a B cell requires help from antigen specific T cells. Such antigens are referred to as T-dependent (TD) antigens. The T cell help may be either in the form of cognate cell to cell interactions or in the form of cytokines secreted by the T cells. During a TD immune response, naïve B cells carrying antigen specific receptors are activated within the extra-follicular T cell areas of the secondary lymphoid organs and initiate formation of foci of proliferating B cells. Here, they can undergo class switching and show inter-clonal competition due to differences in their relative affinity for the antigen and undergo differentiation into plasma cells with short half-lives (Liu *et al.*, 1991; Slifka *et al.*, 1998; Toellner *et al.*, 1996). A few B cells do not undergo terminal differentiation in the foci, but move into the follicles where they interact with CD4 T cells and follicular dendritic cells (FDCs) to establish germinal centres (GCs) (Klein and Dalla-Favera, 2008). In the GCs, the B cells undergo extensive proliferation coupled to somatic hypermutation in the form of random mutations introduced in the variable region of the BCR, producing receptors with varying affinities for the antigen (Papavasiliou and Schatz, 2000; Wagner and Neuberger, 1996). A significant proportion of these B cells undergo apoptosis while competing for the limited amount of antigen

presented on the FDCs in the GCs and only the B cells expressing BCRs with high affinity for the antigen are selected over time. Such affinity-based selection of responding B cells over time, especially under steadily decreasing amounts of antigen in the milieu result in affinity maturation of the antibody response when the mutated B cells differentiate into plasma cells (Han *et al.*, 1997). Class switching also takes place in the GCs where there is switching of isotype from IgM to any of the downstream isotypes, such as, IgG, IgA or IgE, that have diverse physiological functions. As only a few B cells from the extrafollicular foci form the founder GC B cells, there is majorly an intra-clonal competition among the B cells in the GCs. The selected high affinity clones then exit the cell-cycle and undergo differentiation either into memory B cells or into plasma cells. Some of the plasma cells that find niche in the bone marrow are retained there and can continue secreting antibodies for long periods of time (Arpin *et al.*, 1995).

A B cell needs to present antigen derived peptides in association with MHCII molecules in order to recruit help from antigen specific T cells. B cells along with DCs and macrophages are known as professional antigen presenting cells (APCs) that can provide continual surveillance against extracellular pathogens and present pathogen derived peptides to CD4 T cells in the context of MHC class II molecules. Unlike macrophages and dendritic cells, B cells are relatively inefficient at uptake of antigens through phagocytosis or pinocytosis (Trombetta and Mellman, 2005; Watts, 1997). They are, however, highly efficient at internalizing antigens through their surface BCR into endosomes. Internalized antigens move through the different compartments of the endocytic pathway before they reach the lysosomes where receptor antigen uncoupling and antigen degradation occur. In APCs, there are specialized compartments known as the MHC class II containing compartments (MIICs)

that have late endosomal / lysosomal characteristics (Neefjes, 1999; Stern *et al.*, 2006; Tulp *et al.*, 1994) and are highly efficient at processing antigens and generating peptide MHCII complexes (pMHCII). The efficient sorting of the internalized BCR to early endosomal and late endosomal compartments requires association of the BCR with certain adaptor proteins and kinases like BLNK and Syk respectively, that have been known to play a role in BCR signal transduction (Lankar et al., 1998; Siemasko and Clark, 2001; Siemasko *et al.*, 2002). The decreasing pH and the presence of several proteases in the endocytic compartments helps in degradation of the antigen and generation of peptide fragments of the exact size that can fit into the peptide binding groove of the MHC II molecules (Trombetta and Mellman, 2005; Watts, 1997).

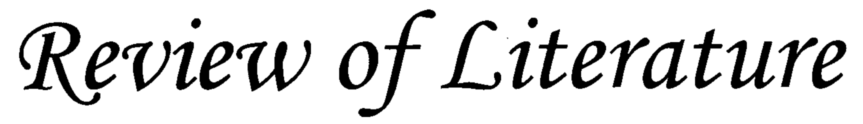
The peptide binding groove in MHCII molecules remain occupied by the invariant chain (Ii) when the newly synthesized molecule moves from the trans-golgi network to the endocytic pathway (Cresswell, 1994; Kvist *et al.*, 1982). Sequential degradation of Ii in the endocytic compartments finally leaves a small fragment that stays associated with the peptide binding groove of the MHCII molecule and is called the class II associated invariant chain peptide (CLIP). Removal of CLIP and loading of peptide fragments onto MHCII molecules involves a chaperone protein called H-2M (Alfonso and Karlsson, 2000; Karlsson, 2005; Miyazaki *et al.*, 1996; Morris *et al.*, 1994). Another chaperone H-2O, present in B cells is known to inhibit the function of H-2M in a pH dependent manner (Alfonso *et al.*, 2003a; Alfonso *et al.*, 2003b) with the inhibitory effect being greatest at pH above 5.5. Thus, in B cells this favours presentation of antigens from the late endosomal and lysosomal compartments. The pMHCII complexes transported to the surface of the B cells can then recruit antigen specific T cell help. The initial contact between a T cell and a B cell

is antigen independent and depends on adhesion mediated by ICAM-1/LFA-1 and CD2/CD48 molecules while the TCR scans the peptide-MHC complexes on the B cell surface (Lee *et al.*, 2002). Following establishment of antigen specific contact, members of the B7 family of receptors like CD80, CD86 (Azuma *et al.*, 1993; Chambers and Allison, 1997; Kariv *et al.*, 1996; Linsley and Ledbetter, 1993), ICOS (McAdam *et al.*, 2000), PD-L1 (Freeman *et al.*, 1989), PD-L2 (Latchman *et al.*, 2001) as well as the tumor necrosis family receptors on the B cells like CD40 (Grewal and Flavell, 1998), CD27 (Akiba *et al.*, 1998; Kobata *et al.*, 1995), CD30 (Cerutti *et al.*, 2000; Cerutti *et al.*, 1998), and CD95 (Catlett and Bishop, 1999; Krammer, 2000) are known to modify the T cell – B cell interactions (Bishop and Hostager, 2001) and modulate B cell proliferation, class switching, somatic hypermutation, differentiation and death.

There are two factors that regulate the outcome of B cell activation, namely, the strength and duration of the signal generated from the BCR and the quality of the T cell help available. To dissect which of these factors play a dominant role in controlling different aspects of B cell activation, we used the Beige (bg/bg) mouse. This is a mouse homologue for the Chediak-Higashi syndrome (CHS) in humans with mutation in the lysosomal trafficking regulator (*lyst*) gene (Barbosa *et al.*, 1996; Shiflett *et al.*, 2002; Spritz, 1998). LYST has been shown to be associated with microtubules (Faigle *et al.*, 1998) and exhibits a cytosolic distribution (Perou *et al.*, 1997). Mutation in this gene affects lysosomal morphology and functioning in many cell types like the melanosomes, platelets, cytotoxic T cells, natural killer cells and neutrophils (Baca *et al.*, 1989; Bahadoran *et al.*, 2001; Haliotis *et al.*, 1980; Paigen *et al.*, 1990; Salles *et al.*, 2008). Efficient functioning of the endosomal / lysosomal pathway is an important parameter that will determine the time for which a BCR

ligand complex remains intact before it gets degraded in the lysosomes. Since it is known that the BCR remains associated with different signaling intermediates while it gets sorted to the compartments of the endocytic pathway, it is possible that the BCR may retain its signaling potency in the early endosomes and that in bg/bg B cells the signaling competence may be maintained for a longer period of time as compared to wild type (WT) B cells. The other outcome of a defective endo-lysosomal pathway might be the inability of B cells to efficiently process and present antigenic peptides to T cells. In support of this hypothesis, B cells from CHS patients show delayed peptide loading onto MHCII molecules as well as a delay in the transport of the pMHCII complexes to the cell surface (Faigle *et al.*, 1998). These may translate into decreased or delayed responsiveness on the part of the T cells.

Thus, this study has attempted to assess the effect of *lyst* mutation on B cell antigen presentation following antigen uptake through different endocytic routes and the duration of BCR signaling following its internalization. Since the strength and duration of signaling from the BCR and the ability of B cells to present antigens to T cells, both contribute towards modulating B cell responses, we used bg/bg mice as a tool to dissect the relative contribution of these two factors in regulating humoral immune responses.



Review of Literature

Following natural infection or immunization, antigens are captured and processed by the innate immune cells, such as DCs, following which they migrate from the site of antigen encounter to the T cell zones of the local lymph nodes. In this zone the DCs make contact with re-circulating naïve T cells, whose receptors are specific for peptides presented by the DCs. This interaction results in the activation of these T cells and they subsequently move towards the zone in-between the T cell rich area and the B cell rich follicular zone. Here, they make contact with antigen-primed B cells by recognizing antigenic peptides in association with MHCII molecules presented on these B cells. This leads to formation of a primary focus of clonal expansion at the border between T-cell and B-cell zones, where both types of lymphocytes interact and proliferate for several days (Garside *et al.*, 1998; Jacob and Kelsoe, 1992; Liu *et al.*, 1991). Some of the proliferating B cells can exit cycle and differentiate into effector cells. This results in induction of the first phase of the humoral immune response. The primary focus involutes after several days, and many of the lymphocytes comprising the focus undergo apoptosis. The effector cells formed in this first phase survive only for about a week and during this period they help in lowering the antigen load. However, since antibodies have life spans of only a few days (Sigounas *et al.*, 1994), these short lived effector cells are unable to sustain the humoral response. Generation of long lived effector cells take place in specialized structures that are formed in the follicular zone of the secondary lymphoid organs called germinal centers (GCs). Here, the cells undergo extensive proliferation, acquire mutations in their BCRs and the clones that exhibit high affinity for the antigen get selected over time. Thus, the GC reaction produces high affinity variants that can eventually differentiate to effector cells or re-stimulable memory cells that help in long term maintenance of protective immunity.

B cell development:

B-lymphopoeisis begins in the bone marrow from pluripotent hematopoeitic stem cells and progresses through highly regulated distinctive stages. The common lymphoid progenitor (CLP), characterized by the expression of c-kit and IL-7 receptor α chain, is the precursor of B and T cells (Hardy and Hayakawa, 2001). In these early progenitor cells, the enzymes recombination activating genes (RAG)1, RAG2 (Igarashi *et al.*, 2002; Oettinger *et al.*, 1990; Schatz *et al.*, 1989) and terminal deoxynucleotidyl transferase (TdT) (Gore *et al.*, 1991) are induced. While RAG1 and RAG2 play an important role in Ig and TCR gene rearrangements, TdT is essential for insertion of non-template encoded nucleotides during the recombination event for the generation of antigen receptor diversity (N-region insertions). The importance of RAG is evident from null mutations in this gene that at an early stage completely blocks B and T cell development (Mombaerts *et al.*, 1992) while mice bearing a targeted deletion of the TdT gene lack N region insertions (Gilfillan *et al.*, 1993; Komori *et al.*, 1993). B cell progenitors that arise from the CLPs in the bone marrow are called pre-pro B cells and can be identified by the expression of the B cell associated marker B220, CD43 and AA4.1. The progenitor cells at this stage do not express molecules like CD19 or HSA (Allman *et al.*, 1999; Li *et al.*, 1996; Rolink *et al.*, 1999). These B lineage committed cells undergo joining of the D and the J gene segments on the heavy chain chromosome with the help of RAG genes to become early pro B cells. Differentiation to the pro-B cell stage depends critically on *Pax5* gene that codes for the transcription factor BSAP and affects expression of CD19, CD79a, λ5 and other genes of the B lineage (Busslinger and Urbanek, 1995; Nutt *et al.*, 1997). Joining of a V segment to the D-J_H completes the late pro-B cell stage. Pairing of the μ heavy chain with the surrogate light chain complex containing VpreB and λ5 chains, results in formation of the pre-BCR

(Melchers, 2005) that is expressed on the cell surface, and the resultant cells are called the pre B cells. Pre-BCR signaling is triggered possibly by binding of these cells to ligands on stromal cells (Gauthier *et al.*, 2002) and a clone of B cells expressing the same heavy chain is generated. The importance of the pre-BCR in B cell development is apparent in mice that have targeted deletions of different pre-BCR components. For example, in $\mu mt^{-/-}$ mice, deletion of the exon that encodes the transmembrane region of the μ chain prevents expression of the membrane-bound μ chain during B cell development (Kitamura *et al.*, 1991). This results in a two-fold enrichment of pro-B cells and a complete block of B cell development beyond this stage. Signaling through the pre-BCR causes the cells to enter into cycle and downregulate RAG1/RAG2 expression (Grawunder *et al.*, 1995) so as to avoid V to DJ rearrangement on the second DJ recombined heavy chain allele (Geier and Schlissel, 2006). The pre-BCR signaling negatively regulates the FOX class O family of transcription factors (FOXO), which play an important role in induction of RAG expression (Amin and Schlissel, 2008; Herzog *et al.*, 2008). This signaling also downregulates the expression of surrogate light chain genes (Parker *et al.*, 2005; Thompson *et al.*, 2007), thereby creating a scenario where lesser and lesser pre-BCRs can form. At this stage, with gradual decrease in pre-BCR signaling the RAG genes are turned on again and rearrangement at the light chain loci takes place (Reth *et al.*, 1987). The rearrangement takes place initially at the κ chain locus, and if unsuccessful on both the alleles, it proceeds to the λ light chain locus (Tiegs *et al.*, 1993). Once the light chain has been successfully synthesized, it is expressed with the μ chain on the cell membrane to form the B cell receptor complex (BCR) and the cell is now called an immature B cell. Immature B cells are very sensitive to antigen binding, and if they bind self antigen with high affinity in the bone marrow they undergo apoptosis. Those that bind self antigen with lower affinity either

become anergic or undertake receptor editing with another round of light chain gene rearrangement (Benschop *et al.*, 1999; Goodnow *et al.*, 1988; Hardy and Hayakawa, 2001; Hartley *et al.*, 1991; Lang *et al.*, 1996; Tiegs et al., 1993). BCR signaling is important for the final cessation of RAG expression. The immature B cells that exit the bone marrow and come to the periphery are termed Transitional (T)-1 cells. These cells enter the spleen to undergo further maturation to T2 and T3 stages before they finally mature to naïve B cells (Allman *et al.*, 2001; Carsetti *et al.*, 1995; Loder *et al.*, 1999). The autocrine secretion of IFNγ by immature B cells, which downregulates the expression of integrin on these cells, helps in their exclusion from lymph nodes and from sites of infection after their exit from the bone marrow (Flaishon *et al.*, 2000). The T1, T2 and T3 subsets can be identified by different cell surface markers (Allman *et al.*, 2001; Su *et al.*, 2004; Su and Rawlings, 2002). T1 cells are CD21-CD23-, CD62L-, CXCR5lo, IgM^{hi}, IgD^{lo}, whereas T2 cells are CD21+, CD23+, CD62L+, IgM^{hi}, IgD^{hi} and T3 cells are CD21+, CD23+, CD62L+, IgM^{lo}, IgD^{hi}. T1 stage has been shown to be an important deletional checkpoint where BCR crosslinking has been shown to induce exaggerated apoptosis (Loder *et al.*, 1999; Petro *et al.*, 2002; Su and Rawlings, 2002). The tumor necrosis family (TNF) member, BAFF has been implicated to play a vital role in T1 to T2 transition during B cell development (Batten *et al.*, 2000). BAFF-deficient mice have a developmental block in the T1 stage, and in contrast, BAFF-transgenic mice have enhanced transitional and marginal zone B cell populations (Batten *et al.*, 2000; Thien *et al.*, 2004). Differentiation into the different naïve B cell subsets, viz. the follicular (B2) B cell (FO), marginal zone B cell (MZ) and the B1 B cells is dependent on the strength of the signal generated from the BCR. Using a BCR transgenic system specific for the antigen, Thy-1 (Wen *et al.*, 2005), it has been shown that high levels of soluble self-antigen leads to B1 B cell development (preventing at the same time MZ and FO B cell differentiation) while lower doses of the same antigen promote preferential differentiation of

the MZ B cells. Interestingly, the absence of self antigen led to the development of FO B cells, suggesting that "basal" BCR signaling can drive differentiation of this B cell subset, in principle (Casola *et al.*, 2004; Levine *et al.*, 2000; Wen *et al.*, 2005). Thus, according to this 'signal strength' hypothesis, the strongest BCR signals initiate B1 B cell development, followed by the MZ B cells and the FO B cells with decreasing strength of the BCR signal (Casola *et al.*, 2004). MZ B cells, as the name suggests are present in the MZ of the spleen and have high levels of expression of IgM, CD21, CD1d, CD38, CD9, CD25, B7, MHCII and low levels of IgD as compared to FO B cells (Pillai *et al.*, 2005; Su and Rawlings, 2002). The B1 B cells which develop from the fetal liver, are largely self replenishing and are principally located in the peritoneal and the pleural cavities (Montecino-Rodriguez and Dorshkind, 2006).

T cell - dependent B cell responses:

Upon antigen engagement, T and B cells undergo changes in their migratory status and are found to move from their respective locations to a zone between the T cell and the B cell rich areas in the secondary lymphoid organs (Garside *et al.*, 1998; Hargreaves *et al.*, 2001; Ho *et al.*, 1986; Jacob *et al.*, 1991). Cognate interaction between these cells at the T–B junction can support the generation of extrafollicular foci of antibody producing plasma cells and can also lead to the formation of germinal centers. The physiological cues responsible for directing B cells through one pathway or the other are currently poorly understood. Some workers are of the opinion that responding cells stochastically follow either response pathway such that the original specificities recruited to the response are represented equally in both the extrafollicular and early GC populations (Blink *et al.*, 2005; Dal Porto *et al.*, 1998), while others are of the opinion that it is the affinity and avidity of

BCR ligation that guides this decision (Paus *et al.*, 2006). Activation of the B cell brings about a lot of changes in the surface phenotype of these cells. Naïve B cells have an IgD^{hi} IgM^{lo} $CD38^{hi}$ $MHCII^{int}$ phenotype. On activation, they rapidly downregulate surface expression of IgD and CD38, and upregulate levels of MHCII, CD80, CD86, CD44, CD71 and CD24 (Futran *et al.*, 1989; Oliver *et al.*, 1997; Shinall *et al.*, 2000). Activation is followed by B cell proliferation. Clusters of proliferating B cells can be seen as discrete foci in the outer periarteriolymphoid sheath (PALS) of the spleen by 2-3 days following i.p. immunization (Jacob *et al.*, 1991). In the extrafollicular foci, some cells can exit cycle and undergo differentiation into antibody secreting plasma cells. The differentiation to plasma cells is regulated by the expression of the transcription factor, Blimp-1 (Mock *et al.*, 1996; Shaffer *et al.*, 2002), which suppresses genes like c-myc (Lin *et al.*, 1997) that are involved in B cell proliferation. On the other hand, those cells which are committed to form GCs upregulate the B cell lymphoma protein 6 (Bcl-6), which in turn suppresses the plasmablast program of differentiation by repressing Blimp-1 (Fearon *et al.*, 2001). The plasma cells that are generated in the foci have a short half-life, of about 5-7 days (Slifka *et al.*, 1998) and secrete antibodies that may either be of the IgM isotype or may have undergone class switching to other isotypes (Liu *et al.*, 1991; Toellner *et al.*, 1996). Class switch recombination (CSR) involves induction of double stranded breaks and a DNA recombination event involving a non-homologous end-joining pathway between switch (S) regions located at the 5' end of all heavy chain constant region genes except IgD. This results in the excision of the intervening region between the two S sites and generates an extra-chromosomal circular DNA called the switch circle (Stavnezer *et al.*, 2008). Cytokines released by T cells and DCs determine the isotype to which B cells will switch by inducing transcription from germ line (GL) promoters situated upstream to each S region. GL promoters have cytokine-responsive elements, such as $GL\gamma 1$ and ε promoters that are

induced by IL-4 and GLγ2b and α promoters that are induced by TGFβ. The resulting GL transcripts are sterile RNAs and do not encode proteins but help to direct enzymes that are important in CSR to specific S regions by generating single-stranded DNA templates (Stavnezer *et al.*, 2008). B cells do not undergo somatic hypermutation before or during the extrafollicular response and only germ-line encoded antibodies are generated in this case (Hargreaves *et al.*, 2001; Ho *et al.*, 1986). Some B cells which do not undergo terminal differentiation in the extrafollicular foci can move into the follicles and establish germinal centers (Klein and Dalla-Favera, 2008).

Germinal centres:

The development of GCs enables the adaptive immune system to produce an antibody response with high affinity, sustain the response over long periods, and rapidly recall the same set of high affinity antibodies upon subsequent exposure to the pathogen (Leanderson *et al.*, 1992; Liu and Arpin, 1997; Liu *et al.*, 1997). In the GCs, B cells acquire novel phenotypic characteristics. They bind peanut agglutinin (PNA), downregulate CD38, and become positive for the activation marker recognized by the GL-7 monoclonal antibody (Kelsoe, 1996; Oliver *et al.*, 1997). Initially, GCs are evident within the secondary lymphoid follicles as blast cell aggregates that progressively displace primary B cells into a mantle zone around the follicle (Klein and Dalla-Favera, 2008). The rapidly proliferating B cells in the GCs, namely, the centroblasts congregate towards the basal end of the GC, where FDCs are sparse and compose the GC dark zone (Klein and Dalla-Favera, 2008). The actively proliferating centroblasts accumulate mutations in the form of single nucleotide exchanges at DNA strand breaks, along with small deletions and duplications in the variable region at the rate of 10^{-3} mutations per base pair per generation (Bross *et al.*, 2000; Papavasiliou and

Schatz, 2000; Wagner and Neuberger, 1996). This phenomenon is termed somatic hypermutation (SHM). Centroblasts upregulate genes associated with cell proliferation (Golay *et al.*, 1998; Ranuncolo *et al.*, 2007) and telomerase expression (Hu *et al.*, 1997), while genes for the proteins that can sense DNA damage are specifically silenced (Phan and Dalla-Favera, 2004; Ranuncolo *et al.*, 2007). A transcription factor that plays an important role in the establishment of GCs and in the ability of the centroblasts to undergo rapid proliferation without sensing genotoxic stress or undergoing differentiation is Bcl-6 (Golay *et al.*, 1998; Phan and Dalla-Favera, 2004; Ranuncolo *et al.*, 2007; Shaffer *et al.*, 2000). As the GCs mature, a proportion of the centroblasts fall out of cycle and migrate towards the FDC rich apical side of the GC. These non-cycling cells are termed centrocytes, and constitute the GC light zone (Klein *et al.*, 2003; Shaffer *et al.*, 2001). The expression of different chemokine receptors regulate the cycling of B cells between the dark zone and the light zone. Centroblasts express CXCR4, the ligand for which is CXCL12 present on the stromal cells in the dark zone. Centrocytes on the other hand express CXCR5, the ligand for which is CXCL13, present on the FDCs that are rich in the light zone (Allen *et al.*, 2004). The cells in the light zone expressing the mutated Ig, undergo affinity based selection through intra-clonal competition. The mutants with low affinity BCR die by apoptosis due to their failure to bind to antigens trapped by the FDCs (Shokat and Goodnow, 1995) whereas, the high affinity clones bind antigen, which acts as the first survival signal. The FDCs can act as depots for antigen (Schriever and Nadler, 1992; Tew *et al.*, 1980) and present immune complexes to B cells that are captured on its surface due to high levels of expression of the complement receptor CD21 and the FcγRIIB (Camacho *et al.*, 1998; Cozine *et al.*, 2005; Tew *et al.*, 1997; Wu *et al.*, 1996) B cells can also endocytose antigen in the form of iccosomes (Burton *et al.*, 1991), that are immune complex coated bodies released from the FDCs. B cells receive the second survival signal when they process and

present these antigens and recruit help from antigen specific T cells (Vinuesa *et al.*, 2005). Since antigen in the GC becomes limiting with time, this offers the high affinity clones a selective advantage (Batista and Neuberger, 1998). These clones then differentiate into memory cells and plasma cells and result in an increase in the quality of the serum antibody, a process known as affinity maturation (Han *et al.*, 1997). In the light zone of the GCs, B cells also undergo isotype switching (Liu *et al.*, 1996; Rooney *et al.*, 2004). An enzyme that plays a vital role in both SHM as well as CSR is the activation induced cytidine deaminase (AID) (Muramatsu *et al.*, 2000; Revy *et al.*, 2000). AID expression is induced in B cells (Muramatsu *et al.*, 2000) through ligation of CD40 and is further enhanced by stimulation through other receptors, such as, IL-4R and TLR (Xu *et al.*, 2007). It acts on single stranded DNA during transcription and removes amino groups from cytidine bases (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003), which subsequently get acted upon by enzymes such as uracil DNA glycosylases and DNA polymerases that help in error prone DNA repair (Li *et al.*, 2003). It has been reported that mutations in different domains of AID can differentially affect its role in CSR and SHM, possibly due to different set of co-factors that interact with these domains during the two processes (Barreto *et al.*, 2003; Imai *et al.*, 2003; Ta *et al.*, 2003). The high affinity variants that are selected in the GC reaction then undergo differentiation either into terminally differentiated plasma cells or into re-stimulable resting memory cells. Until recently, the factors that guide GC B cell differentiation were largely unknown, although some transcription factors crucial for plasma cell commitment had been identified. Transcriptional factors like Blimp-1, XBP-1, IRF-4 identified in plasma cells have been suggested to play a role in secretory activity of these cells. Blimp-1 is considered the 'master regulator' of plasma cell differentiation (Turner *et al.*, 1994) and has been considered both necessary and sufficient for this process (Shapiro-Shelef and Calame, 2005). It blocks the proliferative program in the cells by directly repressing genes like c-

myc (Lin *et al.*, 1997) and also inhibits a number of genes required for, induction of GC (Bcl-6, Pax-5), BCR signaling (lyn,Btk), SHM and CSR (AID, DNA-PKCs), interaction with T cells (MHCII, CD86), and homing to the follicles (CXCR5) (Shaffer *et al.*, 2002). IRF-4 positively regulates plasma cell generation by promoting Blimp-1 expression (Sciammas *et al.*, 2006), whereas, Pax-5 (Neurath *et al.*, 1994; Rinkenberger *et al.*, 1996), Bach-2 (Ochiai *et al.*, 2006) and Mitf (Lin *et al.*, 2004) either directly or indirectly represses Blimp-1 expression. Blimp-1 turns on the protein X-box binding protein, XBP-1 that helps in the formation of the secretory form of IgM (Shaffer *et al.*, 2004). More recently, microarray analysis of naïve, memory and plasma cells isolated from immunized mice have revealed sets of genes that are specifically induced in these three cell populations (Bhattacharya *et al.*, 2007). In memory cells, transcription factors like *Ets1*, *Klf2*, *Klf3*, *Hhex*, *FoxP1*, and *Notch2* have been identified that are also expressed in naïve B cells and are likely to be involved in survival and quiescence of these cells. Others such as, *Klf9, Ski*, *Mll3*, *Pml*, *Tcf4*, and *Bmi1* expressed in the memory B cells are also expressed in hematopoeitic stem cells and have been suggested to be important in self-renewal (Bhattacharya *et al.*, 2007). These studies also revealed expression of AID in memory B cells. Although the transcript levels were lower than that in GC B cells, they were much higher than that observed in naïve B cells, which indicates that these cells remain poised to undergo further rounds of SHM and CSR on antigen re-encounter (Bhattacharya *et al.*, 2007).

T cell - independent B cell responses:

B cell responses to some pathogen associated antigens can be achieved without the involvement of T cell help. Such antigens are called T-independent (TI) antigens. TI

antigens can be grouped into 2 categories, TI-1 and TI-2 antigens. The TI-1 antigens are directly mitogenic like LPS, and can bind to receptors on the B cell surface other than the BCR and induce polyclonal B cell activation. On the other hand, the TI-2 antigens like dextrans and other repeating polymers on the bacterial surface can crosslink a number of BCRs on a B cell, resulting in B cell activation. The effector cells in case of a TI response produce antibodies of low affinity and short half-lives. The two major B cell subsets that are responsible for antigen specific TI responses are the B1 B cells and the marginal zone (MZ) B cells (Fagarasan and Honjo, 2000; McHeyzer-Williams, 2003). The B1 B cells home predominantly to the peritoneal and the pleural cavities and can recognize common bacterial antigens. They differ from the conventional B-2 B cells with respect to their development, surface phenotype and a BCR repertoire that is skewed towards the recognition of TI-2 antigens. Stimulation of B2 B cells by BCR engagement can lead to the activation of different MAPKs and transcription factors NF-AT and NF-κB. Unlike these cells, the ERK-MAPK pathway and NF-AT activation pathways are constitutively active in the B1 B cells (Wong *et al.*, 2002). However, they exhibit a lack of p38 MAPK activation as well as NF-κB induction, and a delay in the activation of JNK upon BCR cross-linking (Wong *et al.*, 2002). Thus, it has been seen that BCR stimulation of the B1 B cells does not lead to their complete activation as indicated by the lack of maximal upregulation of specific markers such as, CD25, CD69 and CD86 (Wong *et al.*, 2002). However, these cells can be stimulated by ligation of receptors other than the BCR, such as the TLRs, and it has been found that stimulation through the TLRs, 4, 7 and 9, can induce extensive proliferation in B1 B cells (Gururajan *et al.*, 2007). They are capable of self-renewal and can differentiate spontaneously into plasma cells that secrete IgM and IgG3 antibodies. These 'natural serum antibodies' display extensive poly-reactivity and can serve as an early defense to many

pathogens. However, many of these natural antibodies can also bind self antigens, and hence B1 B cells have been associated with the development of several autoimmune diseases (Viau and Zouali, 2005). Activation of B1 B cells results in upregulation of different chemokine receptors on their surface, following which they migrate to the gut associated lymphoid tissues (GALT) and the spleen (Macpherson *et al.*, 2000; Montecino-Rodriguez and Dorshkind, 2006). In the GALT, they function as major contributors in mucosal IgA responses to commensal bacteria (Macpherson et al., 2000).

The MZ of the spleen is lined by the marginal sinus, into which the blood drains from the circulation before moving into venous sinuses of the organ. The location of the MZ B cells thereby helps them gain easy access to blood-borne pathogens (Fagarasan and Honjo, 2000; Pillai *et al.*, 2005). MZ B cells have been known for long to be important in responses against polysaccharide antigens. The importance of MZ B cells in TI responses has been demonstrated by using mice deficient for the Pyk-2 tyrosine kinase. These mice have no MZ B cells and exhibit severe reduction in IgM as well as IgG3 antibody levels in response to Ficoll, a TI-2 antigen (Guinamard *et al.*, 2000). Due to high levels of CD21 expression on MZ B cells, cross-linking of CD21 and BCR by complement coated antigens reduces the threshold of activation of these cells to low affinity antigens (Guinamard *et al.*, 2000; Martin *et al.*, 2001). MZ B cells have higher levels of Blimp-1 and this contributes to generation of rapid and massive waves of IgM producing plasmablasts upon their activation (Martin *et al.*, 2001). Eventual differentiation of the MZ B cells into plasma cells requires additional interactions with $CD11c^{lo}$ DCs that move from the blood into the bridging channels and the T cell – B cell boundary in the spleen following antigenic stimulation (Balazs *et al.*, 2002). Recently it has been shown that MZ B cells can also respond to TD antigens (MacLennan *et al.*, 2003). The MZ B cells can continuously shuttle between the marginal zone and the

follicular zone in the spleen, and while migration to the follicle requires the chemokine receptor CXCR5, returning to the marginal zone requires the sphingosine-1-phosphate receptors, $S1P_1$ and $S1P_3$ (Cinamon *et al.*, 2004; Cinamon *et al.*, 2008). This movement facilitates the interaction of MZ B cells with T cells at the boundary between the T cell zone and the B cell follicles (MacLennan *et al.*, 2003; William *et al.*, 2002). Migration of the MZ B cells to the follicles also facilitates delivery of blood borne antigens to the follicular dendritic cells (FDCs) which eventually results in follicular B cell activation (Cinamon *et al.*, 2008).

Signaling through Toll-like receptor 4:

LPS is a major component in the cell wall of gram negative bacteria and is the primary target for recognition by B cells. Stimulation of B cells by LPS induces proliferation, differentiation to plasma cells, and also promotes their antigen presenting function by enhancing expression of MHC class II and co-stimulatory molecules (Barrachina *et al.*, 1999; Lenschow *et al.*, 1994). TLR4 is the signal-transducing receptor for LPS, and requires interaction with the glycoprotein MD-2 for LPS recognition (Shimazu *et al.*, 1999). It was later found that the expression of TLR4–MD-2 complex in mature B cells is much lower than that in macrophages (Akashi *et al.*, 2000) and B cells have another TLR family receptor, RP105 which is important in LPS recognition (Miura *et al.*, 1996; Miyake *et al.*, 1994; Miyake *et al.*, 1995). Surface expression of RP105 is dependent on its co-expression with a MD-2 homolog protein, MD-1 (Miura *et al.*, 1998; Miyake *et al.*, 1998). More recently it has been shown that RP105 can be expressed on DCs and macrophages, although in these cells it has been found to negatively regulate LPS mediated responses (Divanovic *et al.*, 2005). LPS bound to the LPS-binding protein (LBP) is recognized by a molecule called

CD14 (Jiang *et al.*, 2005), that can exist either in a membrane-bound or a soluble form and helps in bringing the LPS-LBP complex closer to the TLR4/MD-2 and RP105/MD-1 receptors on the B cell surface. Binding of LPS to the receptors leads to the recruitment of the cytosolic adaptor proteins MyD88 and Mal, which in turn activates IL-1R-associated kinase (IRAK)-4 and facilitates phosphorylation of the downstream molecule, IRAK-1 (Akira and Takeda, 2004; Peng, 2005; Takeda *et al.*, 2003). IRAK-1 dissociates from the receptor complex to interact with the tumor necrosis family receptor-associated factor 6 (TRAF6) and this IRAK-1/TRAF6 complex then initiates subsequent signaling steps leading to the activation of the I-κB kinase complex, which phosphorylates and causes degradation of I-κB. NF-κB which is released as a result of degradation of I-κB then translocates to the nucleus initiating gene transcription (Akira and Takeda, 2004; Covert *et al.*, 2005; Doyle and O'Neill, 2006). The IRAK-1/TRAF6 complex also activates the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways and the transcription factor AP-1 (Akira and Takeda, 2004; Doyle and O'Neill, 2006; Gay and Gangloff, 2007; Peng, 2005; Takeda *et al.*, 2003). Engagement of TLR4 can also induce activation of a MyD88-independent signaling pathway involving the adaptor proteins, Toll/Interleukin-1 receptor domain-containing adaptor protein inducing interferon-γ (TRIF) and TRIF-related adaptor molecule (TRAM). This signaling pathway results in the activation of the interferon responsive factor 3, which mediates transcription of interferon-inducible genes and activates a delayed wave of NF-κB transcription (Akira and Takeda, 2004; Covert *et al.*, 2005; Peng, 2005; Takeda *et al.*, 2003). A recent study has shown that the MyD88 dependent and independent pathways are initiated sequentially, with the former pathway being initiated at the plasma membrane, and the latter from the early endosomes following endocytosis of TLR4 (Kagan *et al.*, 2008). Following ligation, TLR4 is endocytosed in a dynamin-

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dependent process, and inhibition of endocytosis resulted in the TRAM-TRIF dependent signaling cascade being completely abrogated. Eventually, these two signaling pathways downstream of LPS that are operative at two distinct sub-cellular locations results in the generation of pro-inflammatory cytokines and type I interferons respectively (Kagan *et al.*, 2008).

B cell receptor signaling:

The B-cell antigen receptor (BCR) is composed of membrane immunoglobulin molecule (mIg) that helps in antigen recognition and associated signaling molecules CD79a and CD79b (Keegan and Paul, 1992; Reth, 1992). Ligand aggregated BCR molecules have a greater propensity to partition into glycosphingolipid-rich microdomains of the plasma membrane called the lipid rafts as opposed to monomeric BCR (Sproul *et al.*, 2000). These lipid rafts have increased concentrations of different protein tyrosine kinases such as Lyn which causes phosphorylation of conserved motifs, the ITAMs (Reth, 1989) in the cytoplasmic tails of CD79a and CD79b molecules (Simons and Ikonen, 1997). Clustering of BCRs into lipid rafts along with Lyn results in progressive amplification of the ITAM phosphorylation (Gold *et al.*, 1991), and promotes subsequent recruitment and activation of the SH2 domain containing cytosolic protein tyrosine kinase, Syk. The protein tyrosine kinases Blk, Fyn and Lck are also activated by ligation of mIg. The rafts exclude phosphatases such as, CD45R, which could essentially dampen the BCR induced signaling events (Cheng *et al.*, 1999a; Simons and Ikonen, 1997). Syk is essential to couple the BCR to distal signal transduction elements and it executes this function by interaction and phosphorylation of the adaptor molecule, B cell linker protein (BLNK) (Hayashi *et al.*, 2000), which then acts as a molecular scaffold for the nucleation of several different

signaling pathways. The signalosome that assembles on BLNK contains molecules such as, Vav, Growth factor receptor bound protein 2 (Grb2), SOS (a guanine nucleotide exchange factor), Bruton's tyrosine kinase (Btk), phosphoinositide 3-kinase (PI3K), and phospholipase C-γ2 (PLCγ2) (Dal Porto *et al.*, 2004; Kurosaki, 1999). PI3K activates phosphoinositide-dependent protein kinase 1 (PDK1), which in turn phosphorylates Akt resulting in its nuclear translocation, along with the activation of a number of substrate molecules like the pro-apoptotic molecule Bad, NF-κB and transcription factors like E2F and CREB (Suzuki *et al.*, 2003). Phosphorylated PLC-γ hydrolyzes phosphatidylinositol-1,4,5-bisphosphate (PIP2) into two potent second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 interacts with receptors on the endoplasmic membrane, resulting in an efflux of Ca^{2+} (Kurosaki, 1999). Increased intracellular Ca^{2+} levels result in the activation of calmodulin and calcineurin, which in turn activates and induces nuclear translocation of nuclear factor of activated T cells (NFAT). DAG is an activator of several members of the serine-threonine protein kinase C (PKC) family. Activation of PKC also leads to the activation of transcription factors including c-jun, c-fos and of IκB kinase (IKK). The latter phosphorylates IκB and releases NFκB, which then translocates to the nucleus and results in the induction of numerous genes that are involved in immune responses. The Grb2-SOS protein complex catalyzes the activation of Ras, followed by Raf, a serine-threonine kinase which subsequently triggers the MAPK signaling cascade (Pawson, 1995). The MAPK family consists of three members, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK. Following activation, these kinases phosphorylate different sets of transcription factors like Elk-1, c-Myc, c-Jun, ATF-2 and others, thereby altering their DNA binding ability and downstream patterns of gene expression (Tedder, 1998).

B cell co-receptors:

Signals transduced by the BCR can be further modified by co-receptors which can either have a positive effect on BCR signaling, such as, CD19, CD21, CD23, CD35, CD81 (TAPA), PIR-A and the LEU-13 complex (Carter and Fearon, 1992; Tedder *et al.*, 1994), while others like CD22, CD32 and PIR-B have negative effects. CD19 is a B cell specific co-receptor whose expression is initiated at early stages of B cell development and is found predominantly in a protein complex with CD21, CD81 and leu-13 (Carter and Fearon, 1992). B cells are hypo-responsive in the absence of CD19 and hyper responsive when it is over-expressed (Fujimoto *et al.*, 1999; Sato *et al.*, 1997; Sato *et al.*, 1995). Through the binding of complement tagged antigens, CD21 mediates the cross-linking of the BCR with CD19. This co-ligation allows CD19 to function as an adaptor protein recruiting signaling molecules like Vav, PI3K, Lyn and activating the PLCγ2 and the MAPK signaling pathways (Fearon and Carroll, 2000). Another complement receptor present on B cells is CD35, which also helps in B cell responses to complement coated antigens. Different domains of the protein CD81 have been implicated to have roles in the processing, intracellular trafficking and the membrane functions of CD19 (Shoham *et al.*, 2006).

The receptor for sialic acid, CD22, is unique in possessing both ITAMs and ITIMs (Immunoreceptor based tyrosine inhibitory motifs) (Geisberger *et al.*, 2003; Kurosaki, 2002; Ono *et al.*, 1997). While the ITAMs serve as docking sites for the Src-family kinases, the ITIMs serve as docking sites for phosphatases such as SHP1 and SHIP. It has been shown that CD22 supresses MAPK activation in B cells when cross-linked to Ig alone or to the co-ligated complex of Ig and CD19 (Tooze *et al.*, 1997). Consistent with this negative role, $CD22^{-/-}$ B cells show an enhanced $[Ca^{2+}]_i$ levels following BCR ligation. It has been found

that CD22 is mostly bound to ligands on the B cell surface and a small population remains in the unbound or "unmasked" state. The unmasked state has been linked to an activated phenotype in B cells with lesser ITIM phosphorylation and SHP1 recruitment (Danzer *et al.*, 2003). The proportion of unmasked CD22 is higher in transitional B cells, MZ B cells and peritoneal B1 B cells and can be linked to a more activated phenotype observed in these cells as compared to follicular B cells. Another co-receptor, CD72 has been indicated to have a dual role in B cell activation. CD72 cross-linking induces B cell proliferation and increases MHCII expression. However, an ITIM on its tail has been reported to recruit SHP-1, thereby suggesting a negative role in BCR signaling (Wu *et al.*, 1998). The paired Ig-like receptor A, PIR-A pairs with the FcγR, forming an activating complex, whereas PIR-B inhibits BCR induced tyrosine phosphorylation of Btk and Syk (Maeda *et al.*, 1999).

FcγRIIB (CD32), a member of the Ig gene superfamily, has an ITIM in its cytoplasmic tail. It blocks B cell proliferation on cross-linking with antigen receptors (Phillips and Parker, 1983; Phillips and Parker, 1984). Involvement of CD32 in antigen receptor signaling also leads to premature termination of the BCR induced Ca^{2+} flux (Wilson *et al.*, 1987). The negative functions of FcRIIB appear to be primarily mediated by SHIP alone and does not depend on SHP1 activity (Ono *et al.*, 1996; Ono *et al.*, 1997; Tridandapani *et al.*, 1997).

Another class of receptors, the tumor necrosis family receptors (TNFRs) has been shown to be crucial in the regulation of immune responses by influencing many physiological processes such as, lymphocyte development, activation, proliferation, differentiation and death (Gommerman and Browning, 2003; Gravestein and Borst, 1998; Nishikawa *et al.*, 2003). It includes members like CD40, which on interaction with CD154 expressed on activated T cells can promote GC formation (Clark *et al.*, 1996; Raman *et al.*, 2003), isotype

switching (Armitage *et al.*, 1993; Jabara *et al.*, 1990; Rousset *et al.*, 1991), inhibit plasma cell generation and induce memory B cell differentiation (Arpin *et al.*, 1995; Raman *et al.*, 2003). The importance of CD40 in regulating B cell responses is evident from studies which show that mice deficient in CD40 exhibit compromised TD antibody responses and GC formation (Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994). Another TNFR, CD27, can help in memory B cell development (Hendriks *et al.*, 2000) and inhibits generation of plasma cells following ligation (Raman *et al.*, 2000). Other TNFRs with death domain in their cytoplasmic tail, like CD95, APO-3 can induce apoptosis (Gravestein and Borst, 1998).

Antigen uptake by antigen presenting cells (APCs):

B cells and the other professional APCs, namely, the macrophages and the DCs, take up extracellular antigens and present antigen derived peptides on MHC class II molecules for scrutiny by CD4 T cells. Different APCs can capture, process and present antigens at vastly differing efficiencies (Unanue and Askonas, 1968). DCs and macrophages are efficient at pinocytic and phagocytic uptake of macromolecules and pathogens, while B cells are very poor at internalizing antigens except through their surface BCRs.

Phagocytosis: Phagocytosis involves the ingestion or engulfment of large particles or cells with the help of cell membrane extensions called pseudopods in an actin dependent phenomenon (Aderem and Underhill, 1999). DCs and macrophages are capable of phagocytosing small particles as well as large particles or cells > 1μm in diameter, while B cells are relatively inefficient at phagocytosis (Aderem and Underhill, 1999; Beron *et al.*, 1995; Desjardins *et al.*, 1994; Oh and Swanson, 1996; Rabinowitz *et al.*, 1992). It has been

proposed that the ER fuses with the cell surface in order to supply the extra membrane required for this event, although this suggestion awaits direct support (Gagnon *et al.*, 2002). Binding of particles to cell surface receptors can trigger the process of phagocytosis by causing actin polymerization and pseudopod extension (Trombetta and Mellman, 2005; Watts, 1997). The resultant vesicle that forms is called a phagosome. Newly formed phagosomes undergo progressive remodelling, acquiring and losing endosomal markers before they finally fuse with the lysosomes, resulting in the formation of phago-lysosomes (Duclos *et al.*, 2000). The early phagosomes actively exchange materials with early endosomes before active membrane exchange with the late endosomes and then with the lysosomes. Thus, the phagosomes progressively acquire the degradative enzymes and the acidification machinery present in the endocytic compartments (Beron *et al.*, 1995; Oh and Swanson, 1996; Roberts *et al.*, 2006; Vieira *et al.*, 2003). Among enzymes delivered to the macrophage phagosomes from the endosomes and the lysosomes are glycosidases such as galactosidase, mannosidase, hexosaminidase and proteases such as cathepsins B, L, H and S (Rabinowitz *et al.*, 1992; Savina and Amigorena, 2007). In DCs the levels of different proteases in the phagosomes is found to be much lower than those in macrophages (Delamarre *et al.*, 2005). Also, DCs have been found to express different members of the cystatin family of protease inhibitors (El-Sukkari *et al.*, 2003). It has therefore been suggested that the degradative efficiency of the phagosomes in the DCs is lower than that in the macrophages. Phagosome maturation into phagolysosomes is precisely controlled by small GTPases of the Rab family. Rab5 controls the early phases of phagosome maturation, while Rab7 determines later fusion events with the late endosoems and the lysosomes (Roberts *et al.*, 2006; Vieira *et al.*, 2003). Phagosome maturation also requires retrieval of certain membrane components, a process that involves Rab11 (Leiva *et al.*, 2006). The continuous interaction with lysosomes and the ability to replenish hydrolases ensures

complete degradation of the phagocytosed material in the phagolysosomes (Aderem and Underhill, 1999; Beron *et al.*, 1995; Desjardins *et al.*, 1994; Trombetta and Mellman, 2005; Watts, 1997).

Pinocytosis or fluid phase endocytosis: This is a form of endocytosis in which small particles are brought into the cell, suspended in fluid within small vesicles which move through the endocytic compartments, and finally fuse with the lysosomes resulting in hydrolysis of the cargo (Swanson and Watts, 1995). Although pinocytosis is constitutive in different APCs, the pinocytic rate exhibited by B cells is about 20-30 fold lower than that seen in DCs (Lutz *et al.*, 1996; Sallusto *et al.*, 1995). On LPS activation, however, the pinocytic ability of B cells has been found to be enhanced by about 10 fold (Krieger *et al.*, 1985). Peritoneal macrophage cells treated with IFN-γ show suppression of the pinocytic ability and simultaneous stimulation of phagocytosis associated with changes in the cytoskeleton (Wang *et al.*, 1984). Thus, different conditions can modulate the endocytic ability of the APCs. Macropinocytosis is a modification of pinocytosis for uptake of large volumes of extracellular fluid and fluid dissolved antigens in an actin dependent process. It is associated with membrane ruffling activity which results from the bending of a single lamellipodium extended from the cell surface (Falcone *et al.*, 2006; Swanson and Watts, 1995; Trombetta and Mellman, 2005; Watts, 1997), eventually leading to the formation of discrete vacuoles called macropinosomes (Falcone et al., 2006). The macropinosome matures through interaction with different endocytic compartments and finally fuses with the lysosomes (Racoosin and Swanson, 1993). The activity of different Src-family kinases, including c-Src have been implicated to play a role in macropinosome biogenesis and trafficking (Kasahara *et al.*, 2007). In macrophages and immature DCs, treatment of the

cells with growth factors has been found to increase the efficiency of macropinocytosis (Krieger *et al.*, 1985; Lutz *et al.*, 1996; Racoosin and Swanson, 1993; Sallusto *et al.*, 1995; Swanson and Watts, 1995).

Receptor-mediated endocytosis: Receptors on the cell surface can be endocytosed following ligation, with the help of molecules such as clathrin (McCormick *et al.*, 2005; Vargas *et al.*, 2002) and caveolin (Vargas *et al.*, 2002). The clathrin molecule has 3 heavy chains forming triskelia, each with a tightly associated light chain. Clathrin triskelia, together with additional coat components and adaptor proteins, assemble into a polygonal lattice at the plasma membrane resulting in the formation of coated pits (Ford *et al.*, 2001). Assembly of further monomers causes invagination of these coated pits to form vesicles that are cleaved by the action of GTPases like Dynamin (Loerke *et al.*, 2009). Caveolae on the other hand are plasmalemmal invaginations, rich in cholesterol and abundant in the scaffolding protein caveolin, both of which function to concentrate receptor molecules (Rothberg *et al.*, 1992). Of the three caveolins known till date, caveolin 3 is exclusively present in muscle cells (Tang *et al.*, 1996), while caveolin-1 and caveolin-2 are co-expressed in many different cell types (Scherer *et al.*, 1997). Some receptors can also be concentrated in detergent resistant lipid microdomains in the plasma membrane and can undergo endocytosis, independent of clathrin or caveolin (Trombetta and Mellman, 2005; Watts, 1997). B cells are most efficient at internalizing antigens bound to the BCR, although some other receptors like the complement receptors (Croix *et al.*, 1996; Dempsey *et al.*, 1996) and scavenger receptors (Bansal *et al.*, 1999; Mukherjee *et al.*, 2001) may also play a role in antigen uptake. Of the different molecules found to be associated with receptor-mediated endocytosis, BCR internalization has been found to involve clathrin. It has been

demonstrated that BCR ligation induces Src kinase dependent phosphorylation of lipid-raft associated clathrin heavy chain, which leads to receptor internalization. B cells conditionally deficient in clathrin heavy chain expression show a 70% reduction in BCR internalization and the residual clathrin independent internalization process can be blocked by actin and raft antagonists (Anderson *et al.*, 2000; McCormick *et al.*, 2005; Stoddart *et al.*, 2002; Stoddart *et al.*, 2005). Such antagonists can also affect the clathrin dependant internalization process. Thus, along with clathrin, association with lipid rafts and actin cytoskeleton also appears to be essential for BCR internalization. Internalization of scavenger receptors following ligation, requires segregation of the ligated receptors into clathrin-coated pits (Kosswig *et al.*, 2003). The di-leucine motif in the cytoplasmic tail of these receptors has been found to be important in their targeting to the clathrin pit associated adaptor proteins (Chen *et al.*, 2006).

Trafficking through the endocytic compartments:

Endocytic cargo that has been internalized is either sorted from early endosomes to late endosomes and then to lysosomes, or it may reach the recycling endosomes (Trombetta and Mellman, 2005; Watts, 1997; York and Rock, 1996). Each of these compartments is characterized by specific marker molecules. Early endosomes express early endosomal antigen 1 (EEA1), Rab4, Rab5 and transferrin receptor (TfR). Recycling endosomes express Rab11. Late endosomes express Rab7, lysobisphosphatidic acid (LBPA), and cation-dependent as well as cation-independent mannose-6-phosphate receptors (M6PR), while lysosomes express lysosome associated membrane protein 1 (LAMP1), LAMP2, CD63, CD82 and β-hexosaminidase. Cross-linking of the BCR by antigen results in rapid targeting of the ligated BCR to the late endosomal / lysosomal compartments and this accelerated

delivery of antigen may be essential *in vivo* during periods of rapid T cell-dependent selection processes (Cheng *et al.*, 1999b). CD79a and CD79b are both required for efficient trafficking of internalized BCR-ligand complexes to late endosomes (Li *et al.*, 2002; Siemasko *et al.*, 1999). Chimeric molecules of human platelet derived growth factor engineered to contain cytoplasmic domains of either CD79a or CD79b are both internalized but they are trafficked at different rates to the endosomal compartments; those with the CD79b cytoplasmic tail rapidly moves through the endocytic pathway to reach the lysosomes, while the chimera with the CD79a tail moves at a much slower rate , (Bonnerot *et al.*, 1995; Li et al., 2002; Patel and Neuberger, 1993; Siemasko *et al.*, 1999). It is only when the two are co-expressed that there is proper kinetics and specificity of internalized BCR targeting. ITAM tyrosines in CD79a along with Igβ and Syk (Lankar *et al.*, 1998; Siemasko and Clark, 2001) help in the early endosomal targeting of internalized cargo, whereas binding of adaptors like BLNK (Siemasko and Clark, 2001; Siemasko *et al.*, 2002) and Vav to CD79a (Siemasko and Clark, 2001) help in sorting and entry of the BCR containing vesicles to the late endosomes.

The MHC II molecule is made up of α and β polypeptide chains, which engage a third molecule called the invariant chain (Ii) immediately after their translation in the ER (Cresswell, 1994; Kvist *et al.*, 1982). Trimerization of Ii brings in 3 MHC II molecules together to form a nonameric complex (Cresswell, 1994; Wolf and Ploegh, 1995). This association of Ii with nascent MHCII molecules serves multiple functions. Ii functions as a guardian of the peptide binding groove in MHC II, and prevents premature binding of peptides onto it (Roche and Cresswell, 1990; Roche and Cresswell, 1991; Teyton *et al.*, 1990). It also guides folding and egress of the MHCII molecules from the ER (Anderson and Miller, 1992). The di-leucine motif in the cytoplasmic tail of Ii helps to target MHCII

molecules to the endosomal compartments (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Pieters *et al.*, 1993).

It was observed that B cells harbor a major pool of their intracellular class II molecules that have exited the trans-golgi network, in endocytic compartments morphologically similar to late endosomes and lysosomes, collectively termed the MHC class II containing compartments (MIICs) (Amigorena *et al.*, 1994; Neefjes, 1999; Peters *et al.*, 1991). There are reports which suggests that newly synthesized MHCII molecules can enter compartments that share some similarities with the early endosomes, contain very few vesicles and are abundant in Ii (Geuze, 1998). These are called the early or the pre- MIICs, from where the MHCII molecules move into the MIICs. The MIICs have negligible amounts of TfRs, but are enriched in late endosomal / lysosomal markers like CD63, CD82, LAMP1 and LAMP2. They are highly acidic and contain lysosomal enzymes like β-hexosaminidase thereby providing a milieu that is conducive for peptide loading onto MHC class II molecules. MIICs may be of multivesicular, intermediate or multilaminar types, with the multivesicular MIICs being positioned earlier in the endocytic pathway than the multilaminar MIICs, and gradually maturing into the latter type (Stern *et al.*, 2006; Tulp *et al.*, 1994). The multivesicular MIICs essentially have a limiting membrane enclosing characteristic internal membrane vesicles called the intra-luminal multivesicular bodies (IVBs) and both the internal vesicles as well as the limiting membrane have been found to contain MHCII molecules.

Processing cargo in the endocytic compartments:

The endocytic pathway exposes the internalized cargo to progressively lower pH and to higher concentrations of lysosomal hydrolases which helps in unfolding and degradation of the proteins. The amount of proteases that is found in macrophages is much higher than that encountered in DCs or B cells. Thus, while macrophages are highly efficient at rapid degradation of internalized proteins, DCs and B cells show a more limited capacity in lysosomal degradation, a strategy that favours antigen presentation (Delamarre et al., 2005). The pH decreases from 6.5-6.8 in the early endosomes, to 5.0-6.0 in the late endosomes and finally to about 4.5 in the lysosomes (Trombetta and Mellman, 2005; Watts, 1997). The composition and concentration of hydrolytic enzymes also differ in the different compartments of the endosomal-lysosomal pathway. Proteases prevalent in the APCs are the aspartate protease, cathepsin (Cat) D, the cysteine proteases, Cat B, F, H, L, S and Z, and asparagine endopeptidase (AEP) (Hsing and Rudensky, 2005; Riese and Chapman, 2000). Cat S plays an important role both in Ii degradation as well as in antigen processing in the peripheral lymphoid tissues, while in the thymic epithelial cells Cat L exhibits a similar function. During proteolysis, protein is first unfolded by the enzyme GILT (gamma interferon induced lysosomal thiol reductase), after which AEP catalyzes its unlocking (Hsing and Rudensky, 2005; McGrath, 1999; Trombetta and Mellman, 2005; Watts, 1997). Other proteases then carry out subsequent degradation, attacking either the amino-terminal end or the carboxy-terminal end of the protein depending on their mode of action, finally generating peptides that can fit into the peptide binding groove of the MHC II molecules. Peptide loading onto MHC II occurs only after proteasomal degradation of Ii and this is found to intersect with degradation of the antigenic molecules in the different compartments of the endocytic pathway (Blum and Cresswell, 1988; Pieters, 1997; Pieters *et al.*, 1991).

Removal of Ii occurs in an ordered proteolytic reaction forming p31, p22 and p10 intermediates starting from the carboxy terminal of the molecule. The p10 intermediate is then transformed into CLIP, a portion of the Ii that remains associated with the peptide binding groove of MHCII molecules, by the action of Cat S in B cells and DCs (Hsing and Rudensky, 2005; McGrath, 1999; Riese and Chapman, 2000; Trombetta and Mellman, 2005; Watts, 1997).

Loading of peptides on MHCII requires the removal of CLIP since it physically blocks the peptide binding cleft. Although CLIP can spontaneously dissociate from some MHCII alleles at the low pH that exists in late endosomal and lysosomal compartments (Kropshofer *et al.*, 1995; Urban *et al.*, 1994), most MHCII molecules rely on H-2M (in mouse; HLA-DM in humans) (Morris et al., 1994), a non-classical MHCII molecule present in the endocytic compartments (Pierre *et al.*, 1996; Sanderson *et al.*, 1994), for its function. It was seen that APCs from H-2M deficient mice present most peptides poorly, and accumulate CLIP-MHCII complexes on the cell surface (Miyazaki *et al.*, 1996; Sette *et al.*, 1992). H-2M exerts three important functions - it acts as a chaperone and binds to empty MHCII molecules which helps in stabilizing the latter till it gets loaded with a peptide (Kropshofer *et al.*, 1997), acts as a peptide exchanger, whereby it releases CLIP from the MHCII groove and finally it acts as a peptide editor by ensuring that only high affinity peptides get loaded onto the MHCII molecules so that long-lived MHCII peptide complexes are expressed on the cell surface (Kropshofer *et al.*, 1996). The H-2M mediated peptide loading activity has an acidic optimum. A vital factor that restricts antigen presentation in B cells is the presence of H-2O (in mouse; HLA-DO in humans) in close association with H-2M (Alfonso and Karlsson, 2000; Karlsson, 2005). H-2O-H-2M complexes are distributed throughout different endocytic compartments but are mostly concentrated in late endosomes and

lysosomes. They constitutively recycle between the plasma membrane and the endosomal compartments (Liljedahl *et al.*, 1996; van Lith *et al.*, 2001). Targeting of H-2O-H-2M complexes to the endosomal compartments is mediated by a tyrosine-based targeting motif in the cytoplasmic tail of H-2M (Lindstedt *et al.*, 1995). Like H-2M alone, the complexes have also been seen to undergo substantial reversible conformational change upon acidification (Alfonso *et al.*, 2003a; Denzin *et al.*, 2005). The influence of H-2O on H-2M mediated peptide exchange is pH-dependent with the inhibitory effect being most noticeable when the pH is over 5.5 (Alfonso *et al.*, 2003a; Denzin *et al.*, 2005) indicating that DO preferentially inhibits DM activity in the early endocytic compartments where the pH is greater than 5.5, while continuing to allow efficient peptide loading in the late endosomes and the lysosomes. Initially, functional analysis of antigen presentation by H-2O$^{-/-}$ B cells compared to wild type B cells showed that exogenous proteins internalized by fluid-phase endocytosis were presented more efficiently in the absence of H-2O (Liljedahl *et al.*, 1998). These results were substantiated by H-2O over expression and anti-sense RNA mediated knockdown experiments in mouse B cell lines (Brocke *et al.*, 2003; Perraudeau *et al.*, 2000). However, more recent data has shown that following fluid-phase uptake, H-2O$^{-/-}$ B cells presented antigens equally well compared to wild type B cells (Alfonso *et al.*, 2003a; Denzin *et al.*, 2005). To study the effect of H-2O in the presentation of BCR targeted antigen, Karlsson and co-workers generated H-2O$^{-/-}$ and wild type mice expressing hapten-specific BCRs and showed that presentation was either diminished, enhanced or unaffected in the absence of H-2O (Alfonso *et al.*, 2003a; Alfonso *et al.*, 2003b). The observed differences were more epitope specific than antigen specific, as epitopes derived from the same antigen were presented differentially in the presence and absence of H-2O. This finding suggests that H-2O may control the repertoire of peptides presented by B cells depending on the interaction of the antigen with the BCR (Alfonso *et al.*, 2003a; Alfonso *et*

al., 2003b). Mass spectrometric analysis has shown that DO/H-2O influences the class II associated peptide repertoire. Although 90% of the cell surface repertoire was shown to be unaffected in the absence of DO, there was greater accumulation of peptides of unusually large sizes (> 18kDa) associated with MHC class II molecules (van Ham *et al.*, 2000). DO levels are specifically downregulated in GC B cells that increase the relative DM : DO ratio compared to naïve and memory cells (Alfonso *et al.*, 2003a; Glazier *et al.*, 2002). This might function to increase the overall antigen presentation by GC B cells. Efficient antigen presentation in GC B cells would promote GC B cell–T cell interactions that are essential for B cells to survive positive selection in the GC (Glazier *et al.*, 2002).

Alternate sites of MHCII loading:

While peptide loading on newly synthesized MHCII molecules has been extensively studied and is viewed as the primary pathway for MHCII loading, there are alternate mechanisms of peptide loading on MHCII molecules which are not well understood. One of these involves peptide loading on recycling MHCII molecules that take place in the early endosomal compartments, in both an H-2M dependent as well as an H-2M independent fashion (Griffin *et al.*, 1997; Jensen, 1995; Lindner and Unanue, 1996; Pathak *et al.*, 2001; Pinet and Long, 1998). It has been suggested that recycling MHCII molecules may present rapidly degraded antigens which may not survive transit through more acidic compartments for loading in MIICs (Pinet and Long, 1998). This has indeed been found to be the case for certain Influenza haemagglutinin derived epitopes, that are vulnerable to destruction by proteases in the more acidic late endocytic vesicles and are primarily revealed in early endocytic compartments through unfolding triggered by acidification (Sinnathamby and Eisenlohr, 2003). Additional studies using inhibitors to protein synthesis or ER-golgi transport, which

reduce availability of newly synthesized MHCII molecules, suggests that few epitopes derived from proteins such as, OVA, RNAase, tetanus toxin can preferentially load onto recycling MHCII molecules in different APCs (Griffin *et al.*, 1997; Pinet and Long, 1998; Sinnathamby and Eisenlohr, 2003).

Peptides have also been demonstrated to bind to cell surface MHCII molecules to yield complexes capable of activating CD4 T cells (Jensen, 1995; Lindner and Unanue, 1996). This binding may take place under the control of H-2M expressed on the cell surface of B cells (Arndt *et al.*, 2000). Thus, there are multiple sites where MHCII molecules can load peptides, implying that both newly synthesized as well as recycling, mature MHCII molecules are capable of being loaded with peptides.

The pMHCII complexes generated in the MIICs are then transported to the surface of the cells. In B lymphoblastoid cells, it has been observed that the limiting membrane of the MIICs can fuse directly with the plasma membrane, resulting in the release of internal MHCII containing vesicles from the cells. These secreted vesicles called exosomes, were found to be capable of antigen presentation and of inducing antigen specific T cell responses (Admyre *et al.*, 2007; Raposo *et al.*, 1996). Lysosomes in DCs have been seen to form dynamic and motile tubules after the onset of maturation, that help in the transport of pMHCII complexes to the cell surface (Chow *et al.*, 2002).

Enhanced antigen presentation in activated B cells:

Various physical and biochemical modifications take place in the MIICs following B cell activation that enhance antigen processing and peptide loading onto MHCII molecules.

Within 5 mins of BCR cross-linking, there is a marked increase in the levels of MIIC associated low molecular weight GTPases, which might facilitate enhanced trafficking of the endocytosed cargo to the late endosomes (Xu *et al.*, 1996). The phospho-protein profile of the MIICs also underwent rapid and transient changes. When BCR mediated signal transduction was blocked using inhibitors to protein kinases, there was a dose-dependent reduction in the enhancement of antigen presentation (Wagle *et al.*, 1998; Xu *et al.*, 1996). Activation leads to accumulation of newly synthesized MHCII molecules within the MIICs by protein kinase C dependant phosphorylation of Ii that augments late endosomal targeting (Barois *et al.*, 1997; Siemasko and Clark, 2001). Receptor activation increases the acidity of the MIICs, probably by activating a vacuolar proton pump (Siemasko *et al.*, 1998). It also increases the activity of different lysosomal proteases like Cat S (Lankar *et al.*, 2002) and results in the fusion of the MIIC vesicles to form large central vesicles that are > 1um in diameter (Lankar *et al.*, 2002; Siemasko *et al.*, 1998; Wagle *et al.*, 1998). The remodelling and activation of the MIIC takes several hours in DCs whereas in B cells it occurs in as little as 15 mins (Siemasko and Clark, 2001). Co-stimulatory molecules on B cells, CD80 and CD86 play an important role in engaging antigen specific T cell help (Coyle and Gutierrez-Ramos, 2001; Swallow *et al.*, 1999; Yoshinaga *et al.*, 1999). On naïve B cells, the expression of CD80 and CD86 is very negligible (Lenschow *et al.*, 1993). On activation through the BCR, the expression of CD86 is found to increase (Freeman *et al.*, 1989), which in turn induces expression of CD80 (Sahoo *et al.*, 2002).

LPS stimulation is also known to enhance expression of the co-stimulatory molecules, CD80, CD86 (Lenschow *et al.*, 1994) as well as MHCII (Barrachina *et al.*, 1999). Resting B cells are not efficient at uptake of pinocytic cargo, however, LPS mediated activation increases the pinocytic ability of B cells by about 10-fold (Krieger *et al.*, 1985). LPS

stimulation increases the expression of Rab7, which is a GTPase associated with the late endosomes, usually expressed in very low amounts by resting B cells (Bertram *et al.*, 2002). Increase in the levels of Rab7 following LPS mediated activation helps in increased transport of cargo to the late endosomal compartments and is suggested to be an important factor in modifying antigen presentation of the activated cells (Bertram *et al.*, 2002).

B cell differentiation:

B cell differentiation can give rise to plasma cells and memory B cells. Plasma cells represent the final stage of B cell differentiation and are devoted to the production of large amounts of Ig (Hibi and Dosch, 1986). Plasma cells that have been identified in the secondary lymphoid tissues that are generally short-lived in contrast to those that migrate to the bone marrow, which can be long-lived (Slifka *et al.*, 1998). Plasma cell differentiation is an irreversible process and is accompanied by significant changes in the cell. Plasma cells down-regulate surface molecules such as BCR, MHCII, B220, CD19, CD21, CD22 and CXCR5 (Calame *et al.*, 2003). This down-regulation renders the cell incapable of responding any further to the antigen. These cells up-regulate components of the immunoglobulin secretion machinery and become large; show increased levels of IgH and IgL chain transcripts along with expression of surface CD138 (Sanderson *et al.*, 1989) as well as CD93 (Chevrier *et al.*, 2009). As the plasma cells are dedicated to secretion of copious amounts of antibody, a massive increase in the biosynthetic capacity is required. The ER-stress response has been shown to provide B cells with such high metabolic rate (Wiest *et al.*, 1990). During the terminal differentiation in the B cells high rate of Ig chain synthesis disrupts the ER homeostasis, as a consequence of which misfolded or unfolded proteins accumulate in the ER. This stress signal induces an unfolded protein response

(UPR) that results in the production of ER chaperones and enzymes assisting in protein folding. UPR is activated by two different ER localized transmembrane proteins - ATF6 and IRE1, both of which sense stress through their luminal domains and transduce the signal through their cytosolic domains (Ma and Hendershot, 2003). ATF6 moves to the nucleus, where it upregulates transcription of XBP-1 (Gass *et al.*, 2002). Activated IRE-1 excises a 26-nucleotide segment from its substrate, the XBP-1 transcript; thereby activating it and rendering it stable (Calfon *et al.*, 2002; Iwakoshi *et al.*, 2003; Yoshida *et al.*, 2001). Spliced XBP-1 then activates genes involved in targeting of proteins to the ER, their folding and maturation in the ER, and trafficking between the ER, golgi, endosomes and targeting of secretory vesicles to the plasma membrane (Lee *et al.*, 2003). A third factor usually operative in mammalian cells during ER stress response is ATF4, which activates the protein CHOP as one of its downstream targets, which in turn halts protein translation and leads to cell death (Marciniak *et al.*, 2004). It has been found that XBP-1 encodes an inhibitor of the ATF4 pathway, $p58^{IPK}$ and therefore it is likely that this pathway is not activated in plasma cells during its UPR, thereby shielding the cells from death (Shaffer et al., 2004). Plasma cells have high levels of expression of CXCR4 which binds to its ligand CXCL12 expressed on splenic red pulp, lymph node, and in bone marrow, and helps in their homing to the different organs. Interactions between CXCR3 on plasma cells and the inflammatory chemokines CXCL9, CXCL10 and CXCL11 guide the movement of the plasma cells to sites of infection (Hargreaves et al., 2001; Hauser *et al.*, 2002; Wehrli *et al.*, 2001). It has been shown that shortly after immunization most antibody-forming cells occur in the peripheral lymphoid tissues, but later on, especially during secondary type responses, most antibody-forming cells are localized in the bone marrow (Benner *et al.*, 1981). The long lived plasma cells survive in the bone marrow with the help of factors such as, IL-6 (Kawano *et al.*, 1995; Minges Wols *et al.*, 2002) that bind to receptors on plasma cells, such

as very late antigen (VLA) -4 (Minges Wols *et al.*, 2002). Ligands on osteoclasts that engage the receptor BCMA on long lived plasma cells play an important role in their persistence in the bone marrow niche (O'Connor *et al.*, 2004). Studies that involved the knockdown of Blimp-1 both *in vitro* and *in vivo* (Shapiro-Shelef *et al.*, 2005) and the use of mice that do not express the complement receptor CR2 (Gatto *et al.*, 2005) have shown that these molecules act as vital factors in generation and maintenance of long lived plasma cells. Although, antigen persistence have been shown to affect humoral immune responses (Tew and Mandel, 1978), it is not essential in the maintenance of long lived plasma cells (Manz *et al.*, 1998).

The immunological memory is characterized by a rapid and robust immune response as compared to the primary response (Ahmed and Gray, 1996). This is because the threshold of activation of the memory cells is lower than that of naïve B cells with respect to the amount of antigen and T cell help required for the induction of an effector response (Yefenof *et al.*, 1986). The increased expression of the co-stimulatory molecules CD80, CD86 (Liu *et al.*, 1995; Tarlinton, 2006) and MHCII (Shimoda *et al.*, 2006) provide memory B cells with the ability to act as potent antigen presenting cells for CD4 T cells. Memory B cells show a loss of surface IgM and IgD expression and acquire expression of switched Ig isotypes (Coffman and Cohn, 1977). The cytoplasmic domain of the switched isotype, IgG contributes towards the enhancement of the magnitude of secondary responses as compared to IgM and IgD (Martin and Goodnow, 2002; Wakabayashi *et al.*, 2002). Memory cells are long-lived and can be maintained in the absence of persisting antigen (Anderson *et al.*, 2006; Maruyama *et al.*, 2000). They are similar to naïve cells with respect to cell size and the expression of the markers, BCR, B220 and CD19. In humans, memory B cells can be identified by the surface expression of CD27 (Klein *et al.*, 1998). However, in mouse no such specific marker for

memory B cells identification exists. Thus, in mice they can be scored as cells that are capable of secreting secondary isotypes in limiting dilution assays as well as cells that can mount antigen specific IgG responses in adoptive hosts (Julius *et al.*, 1972).

More recently, another subset of memory B cells has been identified that lacks the expression of B220 and most other B cell markers (Driver *et al.*, 2001; McHeyzer-Williams *et al.*, 2000; McHeyzer-Williams and McHeyzer-Williams, 2005; O'Connor *et al.*, 2002). This population termed the 'preplasma cells' do not express CD138, but adoptive transfer experiments have shown that they can produce large numbers of antigen binding B220- cells that can differentiate rapidly into plasma cells (Driver et al., 2001). It has been suggested that they form an intermediate population in a progression from post-GC B220+CD138- memory B cells to the terminally differentiated B220-CD138+ plasma cells (Shapiro-Shelef *et al.*, 2003).

The lyst mutation:

bg/bg mice occurred as a spontaneous mutation in the *lyst* gene of the C57/BL6J mouse strain and have been used subsequently as a model to study different effects of lysosomal dysfunctioning. The *lyst* gene encodes a cytosolic protein (Perou et al., 1997) that is 3801 amino acids long, has a molecular weight of 430 KD and shows about 88% homology between mouse and humans (Ward *et al.*, 2000). Despite the availability of the complete sequence of the *lyst* gene, its precise function has not yet been characterized. The structure of this protein reveals certain distinct domains like the amino terminal ARM/HEAT repeats and the carboxy terminal WD40 repeats, which are suggestive of its function in vesicular transport and protein-protein interactions (Cornillon *et al.*, 2002; Ward *et al.*, 2000). A few

models have been proposed to explain the role of LYST in the formation of the enlarged lysosomes. The first model hypothesizes that LYST normally acts as a negative regulator of homotypic and heterotypic lysosome fusion, affecting tethering/docking complexes or SNARE complexes that are necessary for membrane fusion (Oliver and Essner, 1975; Stinchcombe *et al.*, 2000). The second model proposes that LYST is a positive regulator of fission as over-expressing LYST results in a more peripheral redistribution of smaller lysosomes (Burkhardt *et al.*, 1993; Perou *et al.*, 1997). A third model suggests that LYST may play a role in vesicle trafficking and sorting of cargo to the late endosomes (Faigle *et al.*, 1998). In humans, the *lyst* mutation results in Chediak-Higashi syndrome (CHS) that is characterized by symptoms such as recurrent bacterial infections, prolonged bleeding time, ocular and cutaneous albinism, lympho-proliferative disorders and progressive neuropathy (Barbosa *et al.*, 1996; Shiflett *et al.*, 2002; Spritz, 1998). Study of cells from CHS patients and bg/bg mice reveal giant intracellular vesicles that cluster around the nucleus. These vesicles are positive for LAMP1, LAMP2, lysosomal glycoproteins, and a small subset is found to be positive for Rab7 (Burkhardt *et al.*, 1993). This indicates that the late endosomal and lysosomal compartments are affected, with lysosome and lysosome related vesicles like melanosomes (Bahadoran *et al.*, 2001), platelet dense granules (Paigen *et al.*, 1990; Salles *et al.*, 2008) and cytolytic granules in different cells (Baca *et al.*, 1989; Haliotis *et al.*, 1980) showing severe compromise in function. Early endosomes and trans-golgi network are the main sources of membrane proteins that traffic to the late endosomes. In CHS, molecules normally routed to the lysosomes from these two sources via. late endosomes, are found to accumulate in the early endosomes. This indicates a block in the vesicular transport between early and late endosomes. It is the missorting of endosomal proteins during the early to late endosomal transit that is likely to result in perturbation of lysosomal identity and its functions (Faigle *et al.*, 1998; Zhang *et al.*, 2007), which is evident in different cell types.

Melanocytes show abnormally enlarged melanosomes containing extraordinarily high amounts of melanin; and there is also a defect in the functioning of a small GTPase called Rab27a in these cells (Bahadoran *et al.*, 2001). The platelets show reduced numbers of platelet dense granules (Paigen *et al.*, 1990; Salles *et al.*, 2008). Many cells in the immune system also exhibit compromised activity, for instance, the cytotoxic T lymphocytes (CTLs) (Baca *et al.*, 1989; Stinchcombe *et al.*, 2000) and the NK cells (Haliotis *et al.*, 1980) cannot release lytic granules efficiently. The inhibitory molecule CTLA-4, that gets upregulated on T cells following activation, accumulates in the giant granules and fails to get expressed on the surface of the cells (Barrat *et al.*, 1999). This leads to development of lympho-proliferative disorders in CHS patients. Mature neutrophils in Beige mice and CHS patients lack activities of the two lysosomal proteinases, elastase and Cat G, because of the unusual presence of inhibitors for these two enzymes in the lysosomes (Takeuchi *et al.*, 1986). Antigen presentation is intimately linked to a cell's ability to process antigens in the endosomal / lysosomal pathway. In Epstein-Barr virus (EBV) transformed B cells from CHS patients, a delay in peptide loading and transport of pMHCII complexes to the surface of the cells was observed (Faigle *et al.*, 1998), although the total MHCII levels or the peptide profile exhibited on them remained largely unaffected.

Materials & Methods

Reagents:

RPMI-1640, 10 X Hank's balanced salt solution (HBSS), foetal bovine serum (FBS), glutamine and other reagents used for cell culture were purchased from Biological Industries, Life technologies, Sigma; Click's medium was from Irvine Scientific, while fine chemicals and inhibitors used in the studies were from Sigma-Aldrich, Roche, Calbiochem, Bangalore Genei, Amersham Biosciences, Merck Ltd and Pierce. Antibodies used in cell culture, flow-cytometry and microscopy were procured from BD Biosciences, eBiosciences, Southern Biotech, Bangalore Genei and Santacruz Biotechnology, Inc. Alexa Fluor conjugated secondary reagents used for microscopy were from Molecular Probes Ltd. Capture and detection antibodies used in ELISA and ELISPOT assays were from Southern Biotech. Antibodies for western blot were purchased from Cell Signaling Technologies. Detection reagents and photographic films used in western blots were from Amersham Biosciences. Reagents, columns and adaptors used for magnetic sorting of cells were all purchased from Miltenyi Biotech. Nitrophenyl substituted antigens [NP-Ficoll, NP-chicken gamma globulin (NP-CGG), NP-ovalbumin (NP-OVA)] used for *in vivo* immunizations were from Biosearch Technologies, Complete Freund's Adjuvant (CFA) from Difco and Alhydrogel (alum) from Superfos Biosector.

Mice and immunizations:

6-10 week old C57BL/6 (CD45.2), C57BL/6.SJL (CD45.1), bg/bg (harbouring the LYST mutation) and OT-II (a CD4 TCR$\alpha\beta$ transgenic strain, where the TCR recognizes OVA derived peptides in context of H-2A^b) mice were used in different studies. Mice were procured from Jackson Laboratories (Bar Harbour, USA) and bred at Small Animal Facility,

National Institute of Immunology, New Delhi. Mice were maintained and used according to the guidelines of NII Institutional Animal Ethics Committee.

For primary responses, mice were immunized with 100 μg NP-Ficoll in saline intra-peritoneally (i.p.) for TI responses and either 25 μg, 10 μg or 5 μg NP-CGG on adjuvants for TD responses. Antigen mix in alum was made by incubating the antigen (dissolved in phosphate buffered saline, PBS) with alum in a 1:1 ratio overnight at 4°C with gentle shaking. CFA emulsions were prepared by mixing antigen (dissolved in saline) and CFA in a 1:1 ratio, using a glass syringe. To study secondary immune responses, mice were challenged with 5 μg NP-CGG in saline i.p. For adoptive transfer experiments, mice were immunized with 10μg NP-CGG on alum i.p., and 15 days post immunization B cells were purified and transferred intravenously (i.v.) into irradiated OVA primed mice (immunized with 100μg OVA on CFA i.p. one month prior to cell transfer). The recipients were then challenged with 100μg NP-OVA in saline i.p. and NP-specific responses were scored on day 4 post challenge.

Cell lines used for the study:

The T cell hybridoma used for the study was 13.8, which is a CD4 restricted hybridoma, that recognizes OVA derived peptides presented on H2-A^b (Mukherjee et al., 2001). 13.8 cells have a Lac-Z reporter gene under a minimal IL-2 promoter and the nuclear factor of activated T cells (NF-AT) enhancer element of the IL-2 gene. Hence, the response of these T cells following activation can be detected using colorigenic substrates for the enzyme β-galactosidase (β-gal). Freeze-downs of 13.8 cells were thawed at 37°C, following which they were washed in complete media and plated in T-25 flasks at a density of 0.5 x 10^6 / ml,

in a total volume of 10 ml. For maintaining these cells, they were seeded at a density of 0.05 x 10^6 / ml in T75 in a total volume of 20 ml and after 48 hr approximately 12 x 10^6 cells were recovered, which were then split for subsequent culturing. For using cells in antigen presentation assays, they were seeded at a density of 0.1 x 10^6 / ml, in 20 ml media and cultured in T-75 flasks. After 24 hr approximately 1 x 10^7 cells were recovered and used for the assays.

Culture supernatant from the fibroblast cell line L929, which is known to release M-CSF as a secondary metabolite, was used as a source of M-CSF. Cells were seeded at a density of 10^5 cells / ml in a total volume of 50 ml in T175 flasks. Supernatant was collected on day 3 by which time the cells become confluent, and was then filtered to remove cell debris. A 30% L929 conditioned media was used for deriving macrophages from bone marrow cells.

Media used for cell culture:

Mouse cells were cultured in Click's medium or RPMI-1640 fortified with 2 mM L-glutamine, 1.35 g/l sodium bicarbonate, 5 mM HEPES, 50 μM β-mercaptoethanol, 100 μg/ml streptomycin, 100 IU penicillin and 10% fetal bovine serum (FBS). FBS was heat inactivated at 56°C for 45 min before use. The media hence made by adding all the above supplements has been referred to as complete media.

Cell preparations:

Spleen, peripheral lymph nodes (inguinal and popliteal) and thymus were dissected from mice euthanized by cervical dislocation and teased between a pair of frosted glass slides to

obtain single cell suspensions. Red blood cells (RBCs) in the splenocyte suspension were lysed by osmotic shock using Gey's erythrocyte lysis buffer (MacPherson et al., 2001), washed and then re-suspended in complete medium. Blood was collected from retro-orbital venous plexus into 2 U/ml heparin solution made in PBS. Heparinized blood was subjected to RBC lysis by incubating it for 5 mins with 2 ml Gey's buffer, washed with PBS and treated again with 1 ml Gey's buffer if necessary. For eliciting peritoneal exudates cells (PECs), 1 ml of 4% Brewer's thioglycollate broth (Himedia) (aged for a month in the dark at RT) was given i.p.; 72 hr later mice were euthanized and 10 ml cold saline was injected into the peritoneum and the exudate cells were harvested. For generating bone marrow derived macrophage cultures (BMDMs), femur and tibia bones were dissected from euthanized mice and the bone marrow was harvested by flushing the bone with medium through a 23G needle, dispersed into single cell suspensions, washed and the cells cultured at a density of 10^6 /ml in 6-well plates in complete medium containing 30% L929 culture supernatant. On day 3 and day 5, 60% of the culture supernatant was discarded and the cells were replenished with complete medium containing 30% L929 supernatant. Adherent cells obtained on day 7 were used in the experiment as BMDMs.

Purification of cells:

Splenocytes were labelled with magnetic anti-mouse B220 microbeads for 30 min on ice for the purification of B cells. In some cases, splenocytes were stained with biotin conjugated B220 monoclonal Ab (mAb) (for the purification of B cells) or biotinylated anti-Thy1.2 mAb (for the purification of T cells) for 45 min on ice. Cells were washed with MACS buffer (PBS containing 1% FCS) and incubated on ice for 30 min with magnetic streptavidin

beads diluted in the same buffer. Magnetically labelled cells were washed and loaded onto a positive separation column. Purification was done as per manufacturer's protocol.

Proliferation assays:

For thymidine [H^3] incorporation assay, splenocytes were cultured at a density of 5 x 10^6 / ml in 96-well flat bottom plates with titrating doses of LPS or anti-IgM in complete medium. Plates were pulsed with 0.5 μCi of [^3H] thymidine 48 h after initiation of culture and harvested 12-16 h later onto glass-fiber filters for scintillation spectroscopy. Alternatively, cells (suspended at a density of 10-15 x 10^6 / ml in PBS) were labelled with 10 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) for 20 mins at 37°C with intermittent shaking. Labelled cells were washed and labelling checked by flow-cytometry, following which the cells were stimulated with different concentrations of LPS or anti-IgM for 72 hr. CFSE dilution after indicated time was scored on gated B cells and analysed using the proliferation platform of Flow Jo software.

Estimation of plasma cell differentiation:

To score for plasma cells *in vitro*, splenocytes were cultured at a density of 10^6 / ml in 6-well plates in a total volume of 5 ml with LPS or anti-IgM F(ab')$_2$. After 3 days in culture, the cells were harvested, and subjected to live cell separation. Live cell separation was done by layering cells onto a Lympholyte-Mammal column (Cedarlane) that was spun at 700g for 20 min at 22°C. Cells at the interface were collected, washed and re-suspended in complete media following which they were stained for B220 and CD138, and plasma cells were scored flow-cytometrically.

In order to measure Ig secretion following stimulation, splenocytes were cultured with titrating doses of LPS at a density of 0.5×10^6 / 200μl in 96-well plates for 6 days following which the supernatant was estimated for total Ig using ELISA.

For ELISPOT assays, splenocytes were stimulated with different doses of LPS or anti-IgM $F(ab')_2$ for 4 days at a density of 10^6 / ml in 6 well plates in a total volume of 5 ml. On day 4, these cells were harvested and live cells separated on a Lympholyte-Mammal column were used in ELISPOT assays.

Preparation of substituted proteins:

Preparation of fluorescein isothiocyanate conjugated ovalbumin (FITC-OVA):

FITC labelling of OVA was done using a kit from Bangalore Genei. In brief, FITC was freshly dissolved in the labelling solvent and the protein to be labelled was dissolved in 0.1M Na_2CO_3-$NaHCO_3$ buffer. Protein and FITC were then mixed in a specific molar ratio of 10:1 and incubated for 2 hr at RT. The reaction was stopped by treatment with 1M NH_4Cl for 1 hr at RT. FITC- labelled protein was then separated from the free dye on a Sephadex G-25 column. Eluent fractions with an $A_{280} < 0.2$ were pooled and the fluorescein to protein ratio (F/P) was calculated as follows:

$$\text{Molar F/P} = \frac{\text{MW} \times A_{495}/195}{389 \times A_{280} - [(0.35 \times A_{495})] / E^{0.1\%}}$$

where, MW is the molecular weight of the protein, 389 is the molecular weight of FITC, absorption maxima of FITC is at 495 nm, 195 is the absorption $E^{0.1\%}$ of bound FITC at 490

nm at pH 13.0, (0.35 x A_{495}) is the correction factor due to the absorbance of FITC at 280 nm and $E^{0.1\%}$ is the absorption at 280 nm of the protein at 1.0 mg/ml.

Preparation of maleyl-OVA (Abraham et al.,1995):

OVA was dissolved in borate buffer (5mM, pH 8.5) at 20 mg/ml, followed by addition of 2.5 times (w/w) powdered maleic anhydride with constant stirring. pH was maintained between 8.5 and 9.0 with addition of 1N NaOH solution through out the process. At the end of the reaction, pH was finally adjusted to 7.5 and excess maleic anhydride was removed by dialysis against PBS at 4°C. Protein concentrations were estimated by Bi-cinchonic acid method using a commercial reagent. Various dilutions of protein and a known protein standard, bovine serum albumin (BSA), in a volume of 20 µl were taken, to which 200 µl of reagent C (obtained by mixing reagent A and reagent B in a ratio of 50:1) was added and incubated at 37°C for 20-30 min. Absorbance was measured at 570 nm on a microplate reader. Protein concentration of experimental samples was calculated by extrapolating from the standard curve obtained. The extent of maleylation was estimated using the 2,4,6 tri-nitrobenzene sulfonic acid (TNBS) assay for the loss of free ε-amino groups. Briefly, 4% NaHCO3, 0.1% TNBS and 1 mg/ml of maleylated protein were mixed in equal volumes (100 µl each) and incubated at 40°C for 2 hr, at the end of which 100 µl of 10% sodium dodecyl sulphate (SDS) was added. The reaction was terminated by adding 1N HCl (50µl), and absorbance was measured at 335 nm on microreader plate. The difference in absorbance between native and maleylated proteins indicated the extent of maleylation. Maleyl proteins that showed atleast 95% maleylation were used for the experiments.

Preparation of biotinylated OVA:

OVA was dialyzed against biotin labelling buffer (0.1M $NaHCO_3$, 0.1M NaCl, pH 7.4). 500 ml buffer was used for dialyzing 10-20 mg of the protein at 4°C with 2-3 changes over a period of 2 days following which 10 µl of 10 mg/ml biotin in dimethyl sulfoxide (DMSO) (anhydrous and freshly made) was added per mg of the dialyzed protein. The mix was incubated for 1 hr at RT and then dialyzed to remove unbound biotin, at 4°C using dialysis buffer (0.1M Tris.Cl, 0.1% NaN_3, 0.2M NaCl, pH 7.4). 500 ml of this buffer was used for dialyzing 10-20 mg of the protein with 2-3 changes over a period of 2 days. The extent of biotinylation was estimated by ELISA using a biotinylated protein of known concentration as standard.

Antigen delivery to cells:

To target OVA to the BCR, B cells were labelled on their surface with 10 µg/ml of biotinylated goat anti-mouse IgM, followed by 1 mg/ml avidin and 8 mg/ml of biotinylated OVA. The staining steps were all performed in PBS containing 1% FBS, at 4°C for 30 min. Cells were washed twice after every step and staining was done at a cell density of 10-20 x 10^6 / ml. To target OVA through the scavenger receptor, cells were pulsed at a density of 5-10 x 10^6 / ml in serum free media with 10 mg/ml of maleyl OVA for 1hr at 37°C. For pinocytic delivery of OVA, cells were pulsed at a density of 5-10 x 10^6 / ml in serum free media with 10 mg/ml of OVA at 37°C for an hour.

Antigen presentation assay:

APCs (titrating from 10^6 to 10^4 / well) were cultured in 96-well plate in a final volume of 200µl with 13.8 T cells (10^5 / well). APC – T cell interaction was maintained for various periods of time and at the end of incubation, cells were pelleted and supernatant was discarded. Cells were washed thrice with PBS and incubated with 100µl substrate buffer [0.15M chlorophenolred-β-D-galactopyranoside (CPRG) dissolved in 100mM β-mercaptoethanol, 0.125% NP-40 and 9mM $MgCl_2$] per well till colour developed (6-12 hr). Plates were read on a microplate reader at 570 nm. When cycloheximide (CHX) was used as an inhibitor of protein synthesis, cells were pre-treated with 100 µg/ml CHX for 2 hr at 37°C, following which OVA was delivered to the cells pinocytically or by receptor-mediated endocytosis for 4 hr in the continued presence of the inhibitor. Cells were fixed following treatment, washed and then plated with 13.8 T cells. No pre-treatment was done when antigen presentation was studied in presence of different lysosomal processing inhibitors. The inhibitors used were E64d (100µM), Bafilomycin A1 (100nM), NH_4Cl (100mM) and cells were plated at a density of 10^6 / ml in a volume of 5 ml during the course of inhibitor treatment.

Flow-cytometry:

Reagents used for flow-cytometry:

Reagents used to stain mouse specific markers were biotin-, fluorescein-, phycoerythrin (PE)-, PE-Cy5-, PE-Texas red-, PE-Cy7-, Pacific blue- and Allophycocyanin-coupled antibodies directed against B220, CD4, CD8, CD11b, CD11c, CD25, CD44, CD45.1, CD45.2, CD54, CD69, CD90.2, CD138, IgM, IgD, and $H\text{-}2A^{b}$. Labelled secondary detection

reagents used included $F(ab')_2$ fragments of anti-mouse IgG (Fc)-PE, anti-rat IgG (Fc)-PE, streptavidin-PE, streptavidin PE-Cy5, and streptavidin PE-Texas red. Propidium iodide (PI), Sytox red and Sytox green were used to stain the nuclei of dead cells.

Surface staining of cells:

For surface staining, 0.3 x 10^6 - 10^6 cells were incubated with 50 μl of primary staining reagent appropriately diluted in staining buffer (PBS containing 0.5% BSA and 0.1% NaN_3), on ice for 45 min in 96-well round bottom polystyrene plates. The cells were washed thrice with cold staining buffer, followed by incubation for 45 min with 50 μl of appropriate secondary reagent diluted in staining buffer at working concentration. Finally, cells were washed two to three times with ice-cold staining buffer and re-suspended in PBS.

BCR internalization assay:

To study BCR internalization, splenocytes were labelled on their surface with biotinylated anti-IgM for 45 min on ice at a cell density of 10-20 x 10^6 / ml in PBS containing 1% FBS. The labelled cells were then washed extensively with the same buffer, and incubated at 37°C for 0, 5, 30, 60 and 120 min. After each time-interval cells were washed and stained with streptavidin coupled to PE and B220-FITC. As a control, cells were labelled with biotinylated anti-$H2A^b$.

OVA-FITC internalization assay:

PECs and B cells were incubated with 10 mg/ml FITC-OVA for 20 min at 37°C, following which they were washed twice, in PBS containing 1% serum and given an acid-alkali wash

to strip off excess FITC-OVA bound to the cell surface. For the acid-alkali treatment, the cells were washed with ascorbate buffer (113 mg Ascorbic acid, 10 μl of 1M $MgCl_2$, 10 μl of $CaCl_2$ in 10 ml H_2O), followed immediately by an equal amount of PBS for neutralization. Cells were washed, re-suspended in reconstituted media, and incubated at 37°C for different time intervals, at the end of which cells were washed twice with cold 1% FBS containing PBS, resuspended in PBS and intracellular FITC signal was measured flow-cytometrically.

Staining for dead cells:

For staining dead cells, samples were first stained for different surface markers, washed and re-suspended at a density of 10^6 cells / ml. To this either PI was added at a final concentration of 1 μg/ml or sytox green at 1 μM or sytox red at a final concentration of 3 nM. For PI staining, samples were analysed immediately by flow-cytometry, whereas, for sytox green and sytox red staining, cells were incubated with the dye for 15 min at RT before they were analyzed on a flow-cytometer.

All data obtained from flow-cytometry were analyzed using Flow Jo software.

Fluorescence Microscopy:

Following stimulation with biotinylated anti-IgM, 4 x 10^6 cells were fixed (1% paraformaldehyde for 20 min at 37°C) and permeabilised (0.03% Saponin in PBS for 20 min at RT). Cells were blocked with PBS containing 1% FBS prior to incubation with antibodies in the same medium. Staining of the cells with the different antibodies was carried out at 4°C for 30 min at each step, with washes between every step. Goat anti-mouse IgM-biotin

followed by streptavidin conjugated to Alexa Fluor was used to track BCR in microscopic studies. To detect lysosomal compartments, cells were stained with mAb against LAMP1 (rat mAb) followed by Alexa Flour conjugated to donkey anti-rat IgG. Cells were washed, counted and re-suspended in PBS. 20 μl of the cell suspension was loaded onto the slides and the cover-slip was sealed with nail-polish to prevent generation of air bubbles. For image acquisition, an Olympus IX-81 inverted laser scanning confocal system was used. Pin-hole size was kept at 3.0 units, which corresponds to an axial resolution of 1μm with a 60 X 1.4 NA objective. 8 bit images were collected using the Fluoview FV10-SW software (Olympus) with a step size between 0.1μm-0.5 μm and a digital zoom of 3. Images were processed using Adobe Photoshop software.

Enzyme linked immuno-sorbent assay (ELISA):

For detection of total Ig, IgM or IgG in serum or in culture supernatants, 96-well ELISA plates were coated overnight at 4°C, with 2 μg/ml of goat anti-mouse Ig in 0.1M Na_2CO_3 - $NaHCO_3$ buffer, pH 9.5. For detection of NP-specific IgG and IgM, plates were coated with 10 μg/ml of NP-BSA (either NP_6-BSA or NP_{23}-BSA), diluted in the same buffer. The plates were blocked with 1% lactogen in PBS containing 0.05% Tween-20 (PBST) and loaded with diluted sera or culture supernatants (dilutions made in 0.125% lactogen in PBST). At each step, incubation was carried out at 37°C for an hour. Bound antibody was detected using goat anti-mouse Ig-HRP, IgM-HRP and IgG-HRP in different assays, followed by addition of 0.1M citrate-phosphate buffer pH 5.0, containing 1 μl/ml H_2O_2 and 0.5 mg/ml orthophenyldiamene (OPD). The reaction was terminated using 2N H_2SO_4 when colour started developing in the blank (where no test sample was added). Absorbance was measured at a wavelength of 490 nm using a microplate reader. NP-specific antibody titres

were estimated from a standard curve generated using serum from un-immunized mice, run in parallel.

Enzyme linked immunospot (ELISPOT) assay:

The membrane of a 96-well multiscreen filter plate was first activated by treatment with 70% ethanol at RT and then coated overnight at 4°C with either anti-mouse Ig (H+L) at 4 μg/ml or with 10 μg/ml of NP_{17}-BSA diluted in sterile, filtered PBS. Coated plates were washed with PBS and blocked with media containing 10% FBS at 37°C for an hour. Cells were suspended in complete media, plated on the membrane at titrating cell densities and incubated for 4-5 hr at 37°C. In order to detect the spots, the membrane was incubated overnight at 4°C with HRP conjugated goat anti-mouse Ig or IgG diluted in PBS, washed and 100 μl of substrate buffer [333 μl of 10 mg/ml amino ethyl carbamide solution made in dimethyl formamide was dissolved in 10 ml of 0.1M Na-acetate buffer, pH=5.0, filtered and 10 μl of 30% H_2O_2 was added] was added per well followed by incubation of the membrane at RT for 30 min. Following development of spots, the membrane was washed under tap water to terminate the reaction, air-dried and the spots were counted using an ELISPOT reader.

Limiting dilution assay (LDA):

Cells from draining lymph nodes were titrated in 96-well flat-bottom plates at cell densities ranging from 10^5 / well to 10^3 / well (36 wells for each cell input) along with 10^5 thymocytes / well as filler cells and the cultures were stimulated with 10μg/ml LPS. 12 wells of each cell input were left un-stimulated. 7 days later anti-NP IgG in the culture

supernatant was estimated by ELISA. Wells that showed an absorbance greater than three times that of un-stimulated control wells were considered as positive. Frequency of memory cells was calculated as the cell input at which 37.5% of the wells were negative for the antibody.

Calcium Flux assay:

0.5 x 10^6 B cells were suspended in 1 ml PBS containing 1% FBS, loaded with 4 μM of Fluo-3AM dye for 30 min at 37°C and was then kept for another 30 min at 37°C for de-esterification of the dye. After labelling, cells were washed, re-suspended in 100 μl of PBS in tissue culture plates and then stimulated with 100 μl of anti-IgM at a final concentration of 10μg/ml. The induction of Ca^{2+} flux was scored over time at wavelengths of 380 nm for excitation and 485 nm for emission, using a fluorimeter.

SDS-PAGE and Western blotting:

Preparation of cell lysates:

Cells were stimulated with 10 μg/ml of anti-IgM $F(ab')_2$ at a density of 5 x 10^6 / ml for different time-intervals. Following treatment, cells were washed and lysed in 200 μl of TKM lysis buffer (50mM Tris-HCl pH 7.4, 1mM EDTA, 25mM KCl, 0.02% NaN_3, 5mM $MgCl_2$), containing proteinase and phosphatase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin) along with 5% (v/v) DTT and 0.1% Triton X-100. Lysate was separated from the cell debris by centrifugation at 13,000 rpm for 20 min at 4°C, and the lysate was then boiled with 50 μl of 5X SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue). The denatured cell lysate

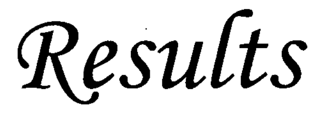
was vortexed to reduce sample viscocity and spun at 13,000 rpm for 5 min to remove any residual debris.

Western Blotting:

Proteins were separated under reducing conditions using 8%, 10% or 12% acrylamide (as described in Molecular Cloning: A laboratory Manual; Sambrook, Fritsch and Maniatis) on a minigel apparatus. 50-70 μl of the denatured cell lysate (equivalent to 1-1.5 x 10^6 cells) was loaded per well, and electrophoresis was conducted at voltage of 80V with a resultant current of 20-30 mAmps. Full range molecular weight marker was run in one lane as a reference. Separated proteins in the gel were fixed and transferred to nitrocellulose membrane by electrophoresis using Tris-Glycine buffer (25 mM Tris, 192 mM Glycine, pH 8.3) containing 20% v/v methanol at 100V for an hour in the cold. After transfer, membranes were washed and blocking was done using 5% Blotto (w/v) in PBS containing 0.1% Tween-20 at RT for 1 hr. Following blocking, membranes were incubated with the primary antibodies overnight at 4°C diluted in 5% BSA at appropriate dilutions. The primary antibodies used in the study were rabbit mAbs against total Erk1/2, phospho Erk1/2, total p38, phospho p38, phospho JNK1/2, total PLCγ2 and phospho PLCγ2. For detection, the membrane was incubated with HRP conjugated goat anti-rabbit IgG at RT for an hour and developed using enhanced chemi-luminescence. For re-probing, the membrane was stripped in stripping buffer (62.5 mM Tris pH 6.8, 1% v/v SDS, 700 μL β-mercaptoethanol) at 55°C for 15 min before addition of the next primary antibody.

Statistical analysis:

Wherever mentioned in the figure legend, data have been compared by two-tailed student's t test (with unequal variance), where $p < 0.05$ was considered significant.


Results

Normal B cell phenotype in bg/bg mice:

Beige (bg/bg) mice have a mutation in the lysosomal trafficking regulator gene (*lyst*) which affects the functions of lysosomes and lysosome-related organelles in many different cell types. Since other workers have shown that the total lymphocyte proportion in spleen of bg/bg mice is significantly higher than in the wild type (WT) control, C57Bl/6 (B6) mice (Bannai *et al.*, 2000), we wanted to ascertain if their peripheral B cell compartment is comparable to that in the B6 mice. Thus B cell subsets in the spleen were analyzed by staining splenocytes from B6 and bg/bg mice for the B cell markers B220, IgM and IgD. *Ex vivo* staining showed that the proportion of B cells in the spleen as identified by B220 staining, was significantly higher in bg/bg mice than in B6 mice (Fig. 1 A and B). The absolute numbers of B cells, calculated by multiplying the frequencies obtained by flow-cytometry to the total cell yields in spleen were also found to be higher in bg/bg mice than in B6 control mice (Fig. 1B). However, the proportions of IgM^{hi} IgD^{lo}, IgM^{hi} IgD^{hi} and IgM^{lo} IgD^{hi} cells were similar between B6 and bg/bg splenocytes as shown in Fig. 1A and B, indicating that in bg/bg mice the immature and mature B cell compartments are similar to its WT counterpart. Since one of the major factors governing the efficiency of antigen presentation is MHC class II (MHCII) expression on APCs, we analyzed MHCII levels on gated B cells in splenocytes from B6 and bg/bg mice. Results shown (Fig.1C) indicate that MHCII levels are equivalent on B cells from the two different mice strains.

LPS- mediated activation and proliferation of B cells:

LPS is a commonly used mitogen for B cells. LPS binds to TLR4-RP105 complex on the surface of B cells and this results in recruitment of various signaling intermediates and transcription factors which activates B cells and drives it towards proliferation, differentiation and death (Ogata et al., 2000; Peng, 2005). To test activation of B cells

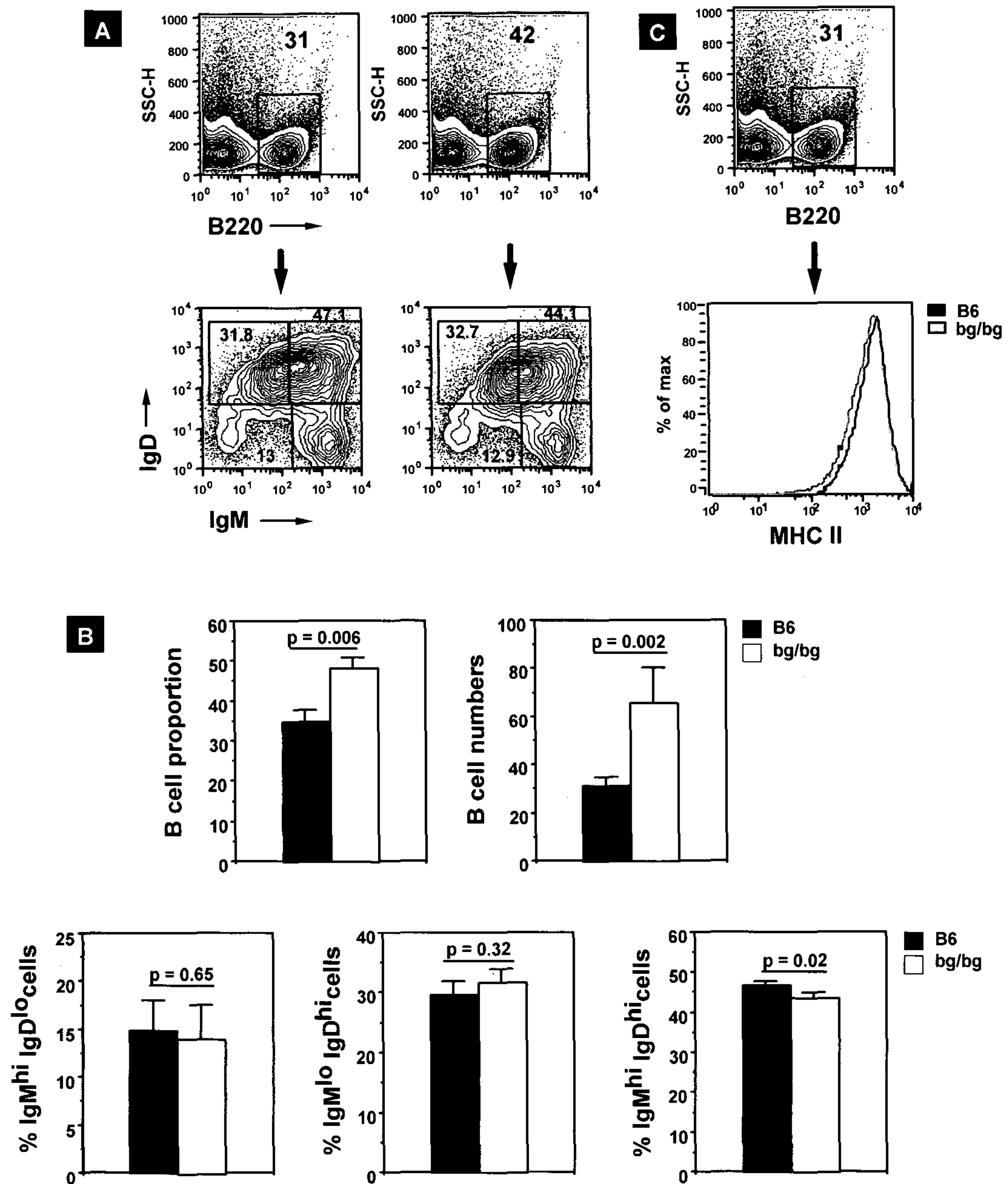


Fig. 1 B cell phenotype is similar between B6 and bg/bg mice.

A. Splenocytes from B6 and bg/bg mice were stained for surface B220, IgM and IgD, and B cell proportions and subsets in the spleen were analyzed on gated B220+ cells. **B.** Statistical representation of data for B cell proportions and B cell numbers in 10 mice (mean ± SE) over multiple experiments. Statistical representation of data for % IgM^{hi} IgD^{lo}, $IgM^{lo}IgD^{hi}$ and IgM^{hi} IgD^{hi} cells for 3 mice, mean ± SE. **C.** MHCII levels on gated B220+ cells. Representative of 3 independent experiments.

following LPS stimulation, splenocytes from B6 and bg/bg mice were treated with 10µg/ml LPS and expression of activation marker CD44 as well as up-regulation of MHCII on B cells was followed 48 hr later. Both markers showed significant upregulation as compared to unstimulated controls and the extent of upregulation was similar in B6 and bg/bg cells (Fig. 2).

Proliferation following stimulation was assessed using two different read-outs. In the first method, cells were stimulated in culture for 48 hr with titrating doses of LPS and pulsed with [^{3}H] thymidine for an additional 12 hr period. This assay quantifies incorporation of radioactive thymidine by cells that were dividing at the time the cultures were pulsed. Thymidine counts were similar for B6 and bg/bg splenocytes at almost all doses of LPS stimulation (Fig. 3A). However, as B cell numbers in the bg/bg splenocytes are greater compared to the WT splenocytes (Fig: 1B), equivalent proliferation of the two might be indicative of greater proliferation in B6 splenocytes. In the second method of estimating proliferation, splenocytes were labeled with the protein binding dye CFSE and at 72 hr after stimulation with 10µg/ml or 1µg/ml of LPS, CFSE dilution was analyzed on the live, B220 gated cells by flow-cytometry. The extent of CFSE dilution indicates number of cell divisions undergone and also the proportion of cells in each division. We found that B6 and bg/bg B cells exhibited equivalent dilution of the dye (estimated using proliferation platform of the Flowjo software) at both doses of LPS used (Fig. 3B). The above results indicated that activation and proliferation of bg/bg B cells following LPS stimulation was comparable to that of the WT control cells.

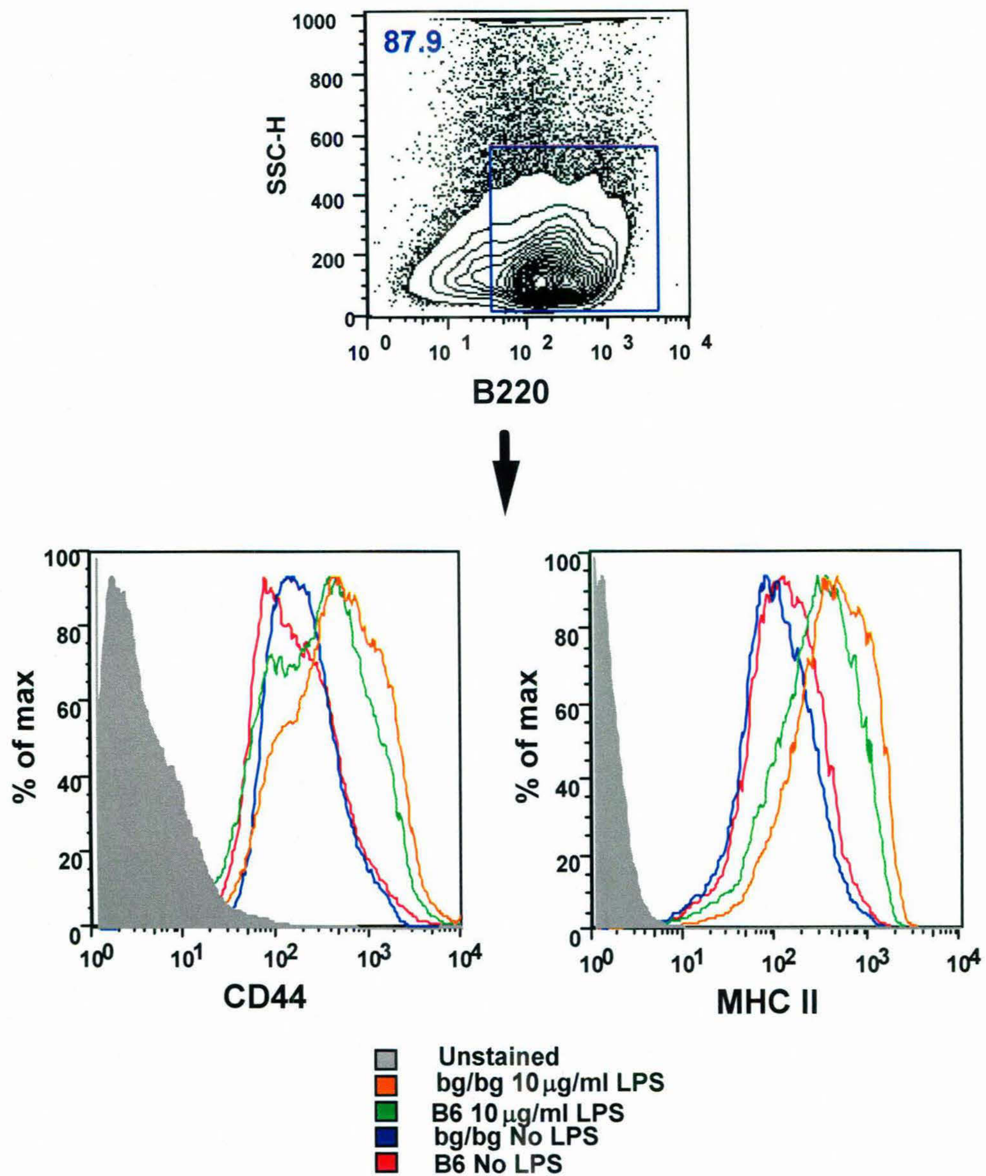


Fig. 2 Equivalent upregulation of activation markers on B6 and bg/bg B cells following LPS stimulation.

Splenocytes from B6 and bg/bg mice were stimulated with 10μg/ml LPS for 48 hr, following which expression of MHC II and CD44 on gated B220+ cells was analyzed and compared to levels on unstimulated controls. Representative of 3 independent experiments.

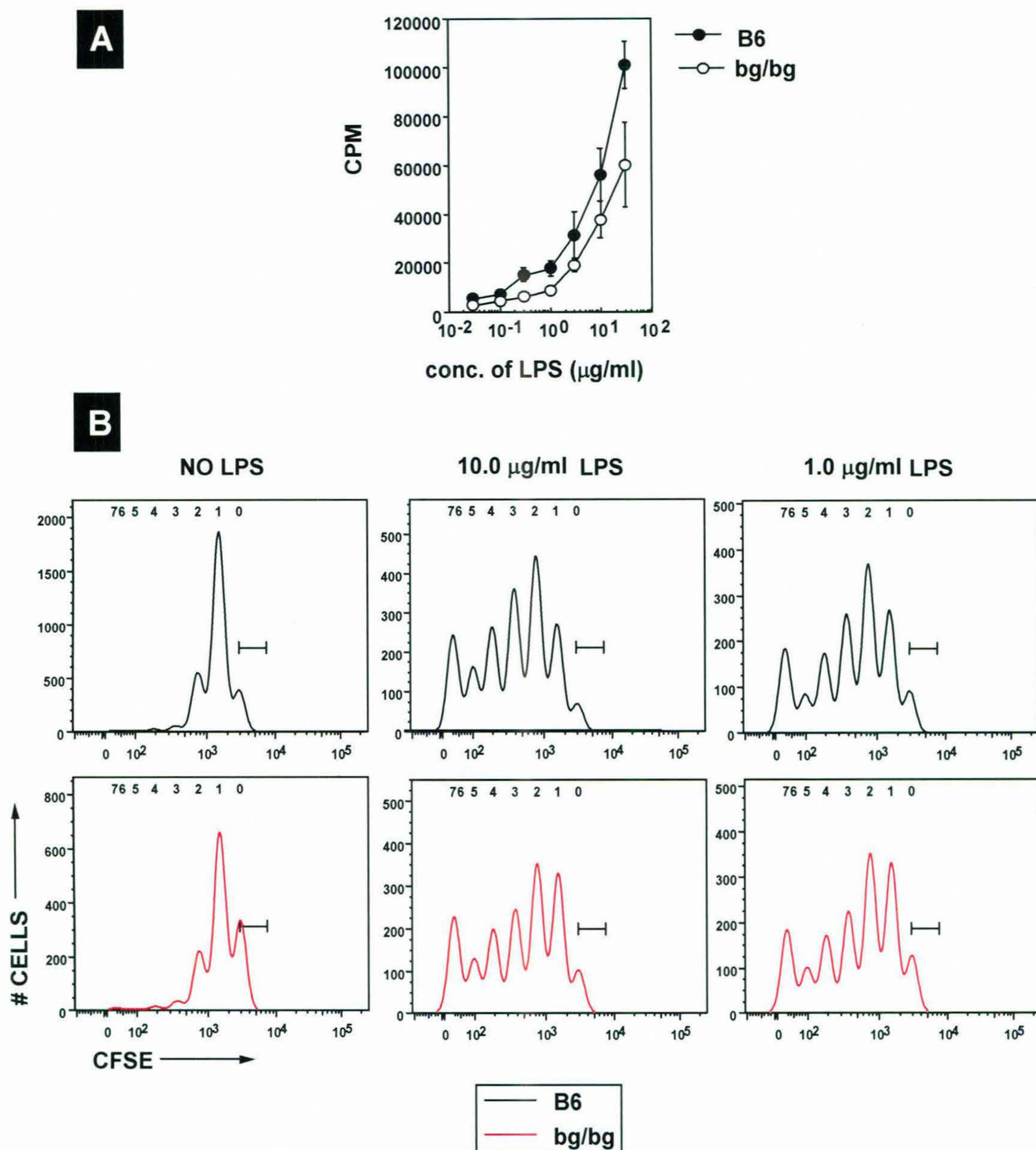


Fig. 3 B6 and bg/bg splenocytes proliferate equivalently on LPS stimulation.

Splenocytes from B6 and bg/bg mice were stimulated with titrating doses of LPS for 48 h or 72 h. **A.** Proliferation estimated by [^{3}H] thymidine incorporation at 48 h is represented as CPM of triplicate cultures ± SE. Background proliferation was < 3000 CPM. Representative of 4 independent experiments. **B.** Proliferation measured by CFSE dilution of labeled splenocytes has been depicted here for two different doses of LPS stimulation at 72 h (one experiment). CFSE profile of unstimulated cells served as control.

LPS- mediated B cell differentiation:

Three different approaches were employed to assess B cell differentiation into plasma cells following LPS mediated activation. In the first approach, CD138 or Syndecan-1 which is a transmembrane proteoglycan expressed on pre-plasmablasts and on terminally differentiated B cells was used as a marker for scoring plasma cell generation. B6 and bg/bg splenocytes were stimulated with different doses of LPS and 3 days post-stimulation the cells were harvested and loaded on a ficoll hypaque column for separation of live cells. CD138 expression on the live, B220 positive cells was studied by flow-cytometry. This study showed that the plasma cell proportions ($B220^{lo}$ and CD138+) were lower in LPS activated bg/bg splenocyte cultures as compared to the WT ones (Fig. 4A). In the second approach, splenocytes from B6 and bg/bg mice were cultured for 6 days with LPS in titrating doses. Total Ig secreted by the cells in the supernatant was estimated using ELISA and the data show that in LPS activated bg/bg splenocyte cultures, the amount of secreted Ig was less than that in B6 cultures (Fig. 4B). A third assay, the ELISPOT assay was also performed to score for the frequency of antibody secreting cells (ASCs) in splenocyte cultures that have been stimulated with LPS. Splenocytes were cultured with different doses of LPS for 4 days, following which live cells were separated using a ficoll-hypaque column. We had plated 15 million splenocytes and after 4 days recovered 6.7 and 6.2 million from B6 and bg/bg cultures respectively in the 1μg/ml LPS group, while 7.5 and 7.0 million from B6 and bg/bg cultures respectively in the 10μg/ml LPS group. These live, activated cells were then plated on ELISPOT plates in titrating cell numbers. The number of spots, which indicates the number of ASCs in the culture, were found to be lower in the bg/bg cultures as compared to the WT ones (Fig. 4C). The diameter of the spots were however, not visibly different between WT and bg/bg stimulated cells, which indicated that the secretory activity of these cells were not different.

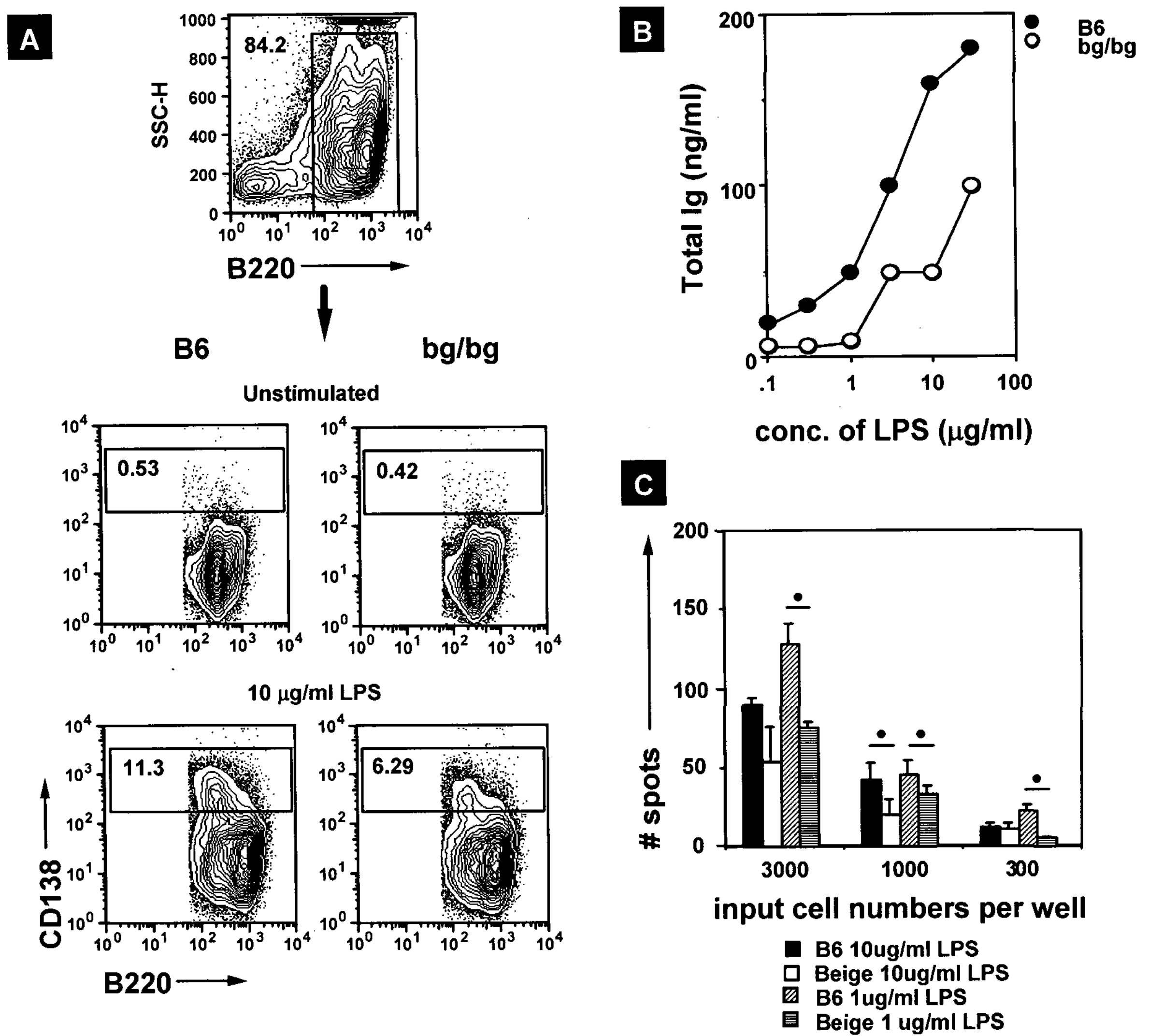


Fig. 4 bg/bg splenocytes differentiate poorly into plasma cells following LPS stimulation.

A. Splenocytes from B6 and bg/bg mice were stimulated with 10μg/ml LPS and on day 3, CD138 positive cell proportion was estimated on gated B220+ cells. CD138 staining on fresh splenocytes served as control. Representative of three independent experiments. **B.** Splenocytes from B6 and bg/bg mice were stimulated with titrating dilutions of LPS and after 6 days, total Ig in the culture supernatent was estimated using ELISA. Absorbance in the unstimulated control wells was close to that in blank. Representative of 4 independent experiments. **C.** Splenocytes from B6 and bg/bg mice were activated with different doses of LPS for 4 days. The number of ASCs (spots) at different cell densities was measured by ELISPOT assay and is presented here as mean ± SE of triplicate wells. Bars that have been marked (•) have statistically significant difference. Representative of two independent experiments.

LPS- mediated B cell death:

To study death following LPS activation in bg/bg B cells, splenocytes cultured in the presence of titrating doses of LPS and at 48 hr and 72 hr post-stimulation, B cell death was scored flow cytometrically by looking for incorporation of the dyes, propidium iodide or sytox green into nuclei of dead cells. Data shows that death was not significantly different between bg/bg and WT B cells at 48 hr or 72 hr post LPS activation (Fig. 5).

Thus, we can conclude from these results that on LPS mediated stimulation, splenocytes from WT and bg/bg mice show equivalent activation, proliferation and susceptibility to death. However, bg/bg splenocytes show compromised differentiation into plasma cells.

Responses of bg/bg B cells to anti-IgM stimulation:

Another commonly used route for achieving B cell activation is by ligating BCR complexes on the surface of B cells. We tried to follow activation of bg/bg B cells by treating them with anti-IgM (anti-μ) and scoring for proliferation and differentiation of the stimulated cells. As carried out earlier for LPS activation, B cell proliferation following BCR stimulation was assessed by two different methods; by scoring for CFSE dilution of the activated cells, as well as, by checking for thymidine incorporation by the proliferating cells. In both these studies, it was seen that bg/bg splenocytes at lower concentrations of anti-IgM stimulation, proliferated better than the B6 splenocytes (Fig. 6A and B). To assess B cell differentiation, B6 and bg/bg splenocytes were stimulated with anti-IgM $F(ab')_2$ at different doses and on day 4 post-activation, plasma cell proportions were analyzed by scoring for B220 low, CD138 positive cells flow-cytometrically. At higher doses of the stimulus, differentiation of B cells into plasma cells was poor, and the proportions were not different from that seen in unstimulated control cells (data not shown). At the lower dose of 0.1μg/ml

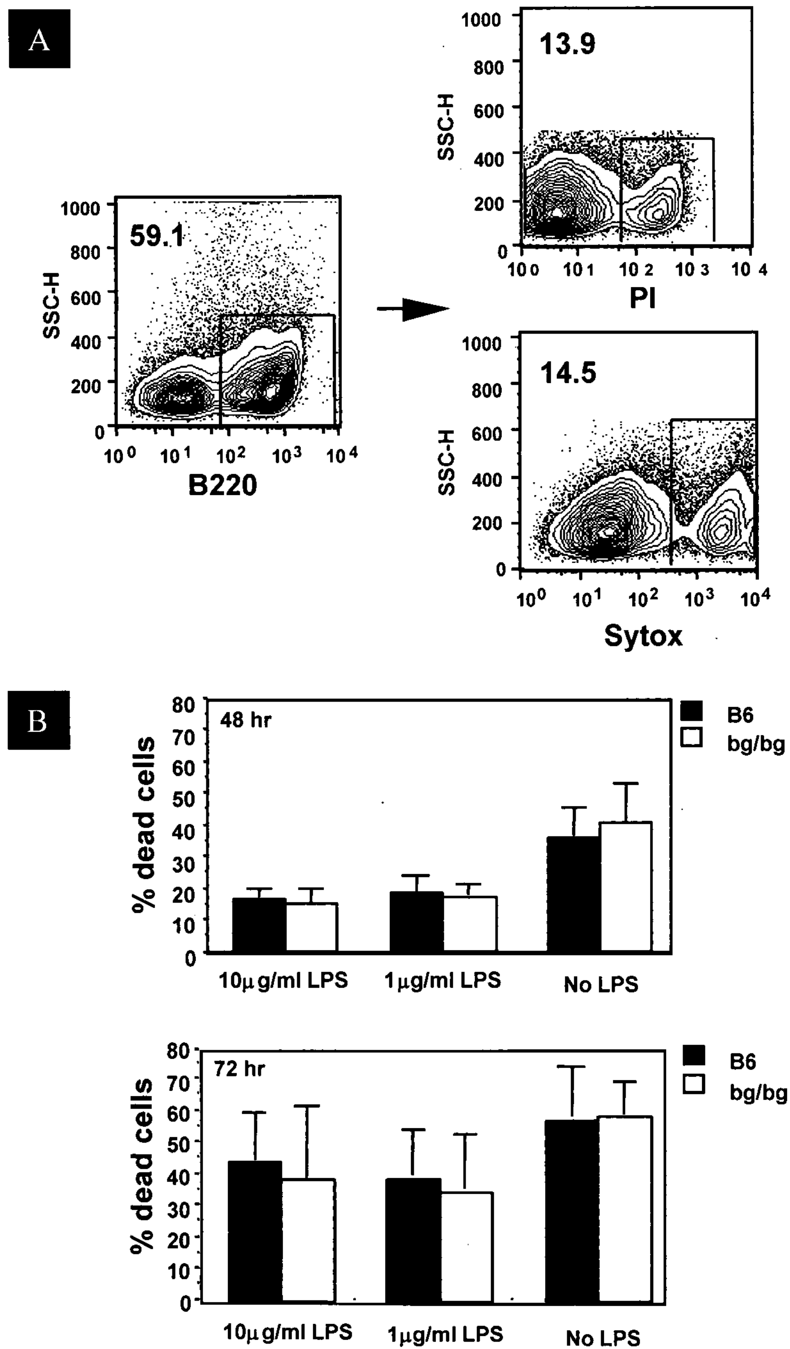


Fig. 5 Equivalent death in WT and bg/bg B cells on LPS stimulation.

Splenocytes from B6 and bg/bg mice were stimulated with LPS at different doses for 48 hr and 72 hr. At the end of each time-point, cells were harvested and dead cells identified by uptake of either PI or sytox green. **A.** Representative gating for B220, PI and sytox green have been depicted. **B.** Dead cell proportions on gated B220+ cells have been shown as mean ± SE for 3 independent experiments using either PI or Sytox green at 48 h and 72 h of stimulation.

anti-μ F(ab')$_2$, however, some differentiation into plasma cells was observed and this was found to be lower in the bg/bg cultures (Fig. 7A). Plasma cell generation was also scored as the number of ASCs in BCR stimulated cultures using ELISPOT assay. B6 and bg/bg splenocytes were cultured with anti-μ F(ab')2 at different doses for 4 days. We plated 15 million cells, and the recovery in the 0.1μg/ml group was 7.4 and 8.0 million from B6 and bg/bg cultures respectively, in the 1μg/ml group was 7.8 and 8.4 million from B6 and bg/bg cultures respectively and in the 10 μg/ml group was 7.2 and 6.5 million from B6 and bg/bg cultures respectively. After 4 days, live cells were separated and ELISPOT assay was performed to score for the ASC frequency. ASC frequencies were seen to increase with decreasing doses of the stimulus used (Fig. 7B). At all doses of stimulation, it was observed that bg/bg cultures had lower frequency of ASCs compared to that of the WT ones, although only in the 0.1μg/ml anti-μ F(ab')$_2$ group, the difference was statistically significant at all input cell densities (Fig. 7B).

Kinetics of internalization of engaged BCR in bg/bg B cells:

It can be concluded from the above data that, bg/bg B cells exhibit better proliferation at lower doses of anti-μ stimulation and a poorer differentiation into plasma cells. One possibility is that in bg/bg B cells due to the *lyst* mutation (which is known to affect the endocytic pathway) trafficking of the internalized BCR to late endosomal / lysosomal compartments (degradative compartments) is affected and in such a scenario, it is probable that the ligated BCR continues to signal for an extended period of time in bg/bg B cells, leading to better proliferation. Again, since proliferating cells need to exit cycle in order to differentiate, it is likely that greater proliferation results in a smaller pool of plasma cells. Before tracking the trafficking of internalized BCR to the endosomal compartments, as a control, we wanted to assess if the kinetics of internalization of the engaged BCR is

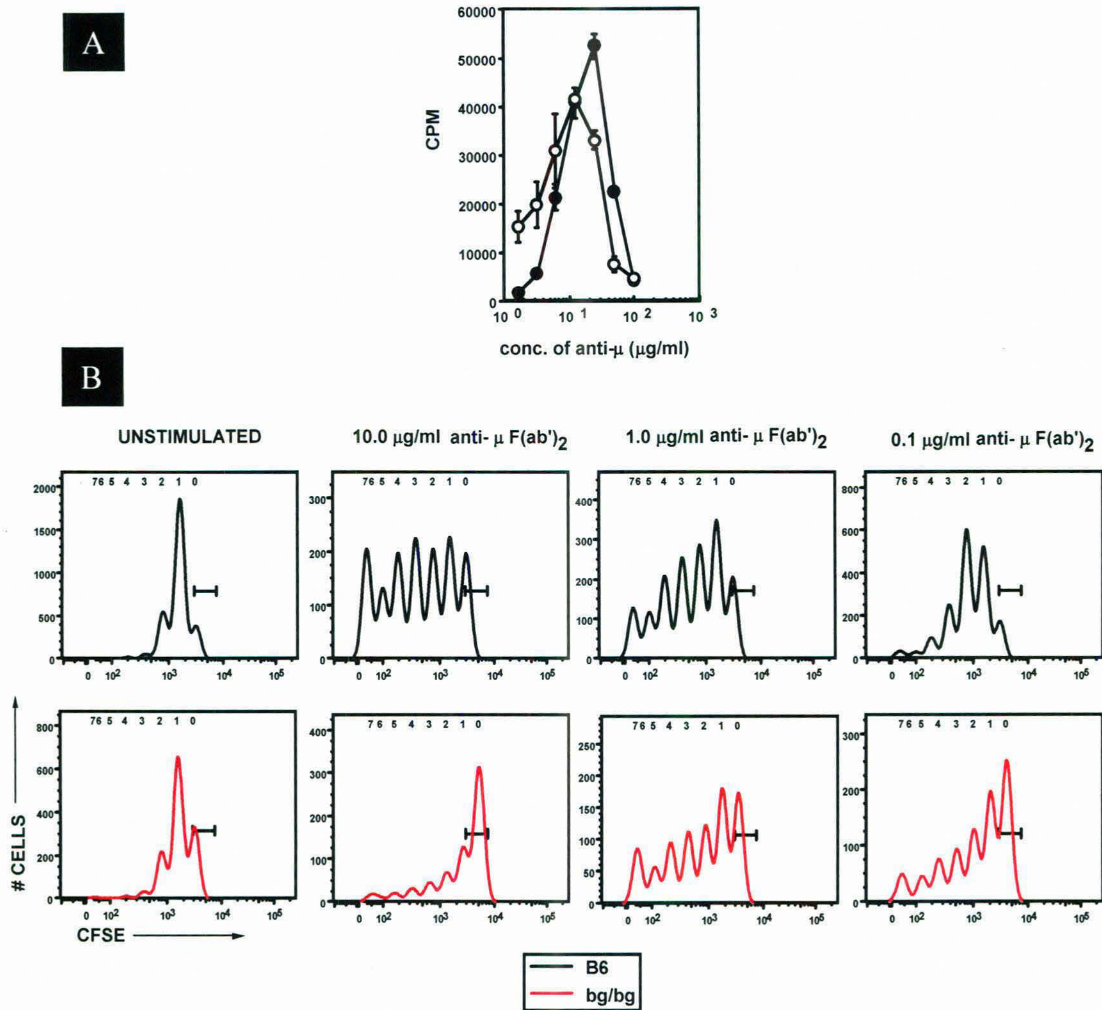


Fig. 6 bg/bg splenocytes proliferate better at lower doses of anti-IgM stimulation.

Splenocytes from B6 and bg/bg mice were stimulated with titrating doses of anti μ **(A)** or anti-μ F(ab')2 **(B)** for 48 h or 72 h respectively. **A.** Proliferation estimated by [^{3}H] thymidine incorporation at 48 h is represented as CPM of triplicate cultures ± SE. Background proliferation was < 3000 CPM. Representative of 3 independent experiments. **B.** Proliferation measured by CFSE dilution of labeled splenocytes has been depicted here for three different concentrations of anti-μ F(ab')2 stimulation at 72h (one experiment). CFSE profile of unstimulated cells served as control.

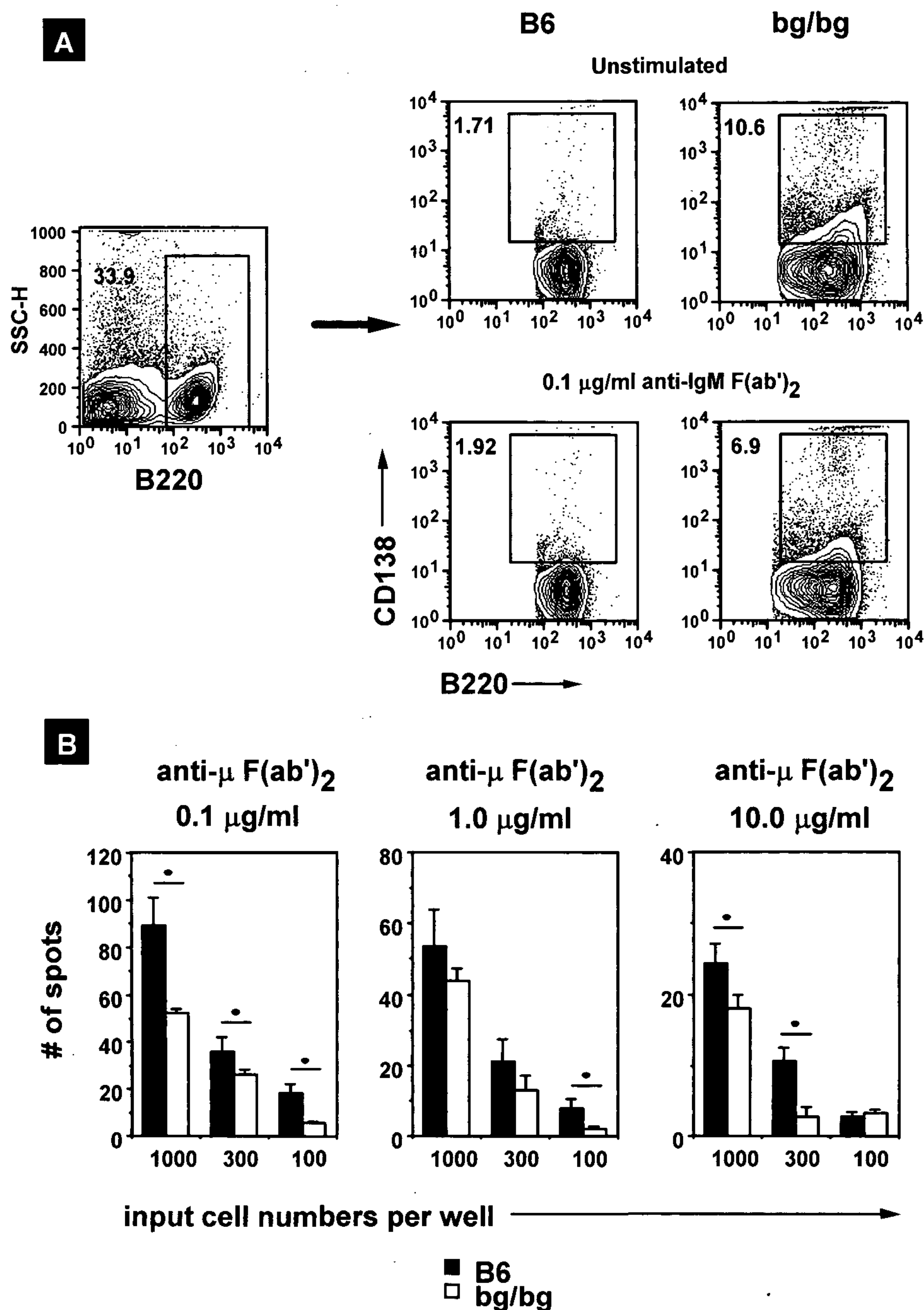


Fig. 7 anti-μ F(ab')$_2$ stimulated bg/bg splenocytes differentiate poorly into plasma cells.

A. Splenocytes from B6 and bg/bg mice were stimulated with titrating doses of anti-μ F(ab')$_2$ and on day 3, live cells were stained with B220 and CD138 to score for plasma cells. CD138 staining profile of fresh splenocytes has been shown as control. Representative of two independent experiments. **B.** Splenocytes from B6 and bg/bg mice were activated with different doses of anti-μ F(ab')$_2$ for 4 days. The frequency of ASCs at titrating cell densities was measured by ELISPOT assay and is presented here as mean ± SE of triplicate wells. Bars that have been marked (•) have statistically significant difference. Representative of two independent experiments.

comparable between bg/bg and B6 B cells. This was addressed by stimulating B6 and bg/bg splenocytes with anti-μ coupled to biotin and internalization was followed 5, 30, 60 and 120 min post-stimulation by labeling cells on their surface with streptavidin coupled to PE. Results indicated that the loss of BCR from the surface of B cells takes place in the initial 5 min of stimulation and that the rate of internalization was similar between B6 and bg/bg B cells (Fig. 8).

Trafficking of internalized BCR to lysosomal compartments in bg/bg B cells:

Since internalization of BCR was comparable between B6 and bg/bg B cells, we next followed the trafficking of the internalized BCR complex to lysosomal compartments, by using LAMP1 (Lysosome associated membrane protein 1) as a marker for lysosomes. B cells from B6 and bg/bg mice were stained on their surface with anti-μ-biotin and then incubated for different time-points at 37°C and trafficking to lysosomes over time was followed microscopically. After each time interval, cells were fixed and stained with streptavidin coupled to Alexa Fluor 488 to detect surface and internalized BCR. In order to detect the lysosomal compartments, cells were stained with a rat mAb to LAMP1 followed by an anti-rat secondary reagent that was coupled to Alexa Fluor 633. At 0 min (Fig. 9), most BCR was on the surface of the cells. Following stimulation for 5 mins, both in B6 and bg/bg B cells, BCR showed signs of capping which is an event prior to internalization of engaged BCR. In B6 cells at 30 mins following stimulation, BCR begins to co-localize with LAMP1 positive lysosomal compartments and by about 60 mins almost all of the internalized BCR is in these compartments. In contrast, if we look at the bg/bg B cells, although there are signs of co-localization of the BCR with LAMP1 positive lysosomes at 30 mins, even after 120 mins of stimulation, a significant proportion of the BCR is found to be outside the lysosomal compartments. Hence from data shown in fig. 9 it can be said that

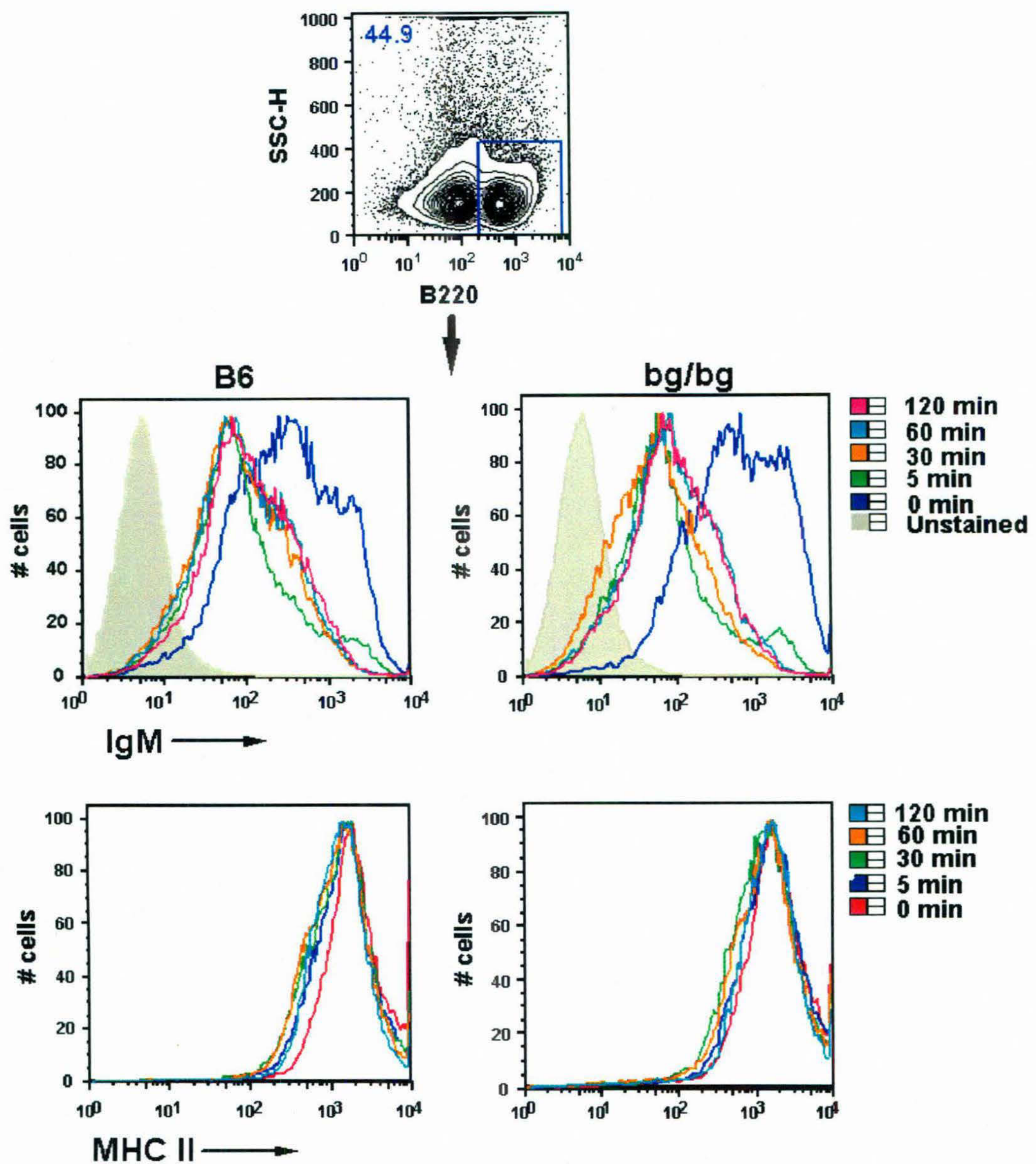


Fig. 8 Similar rates of down-modulation of engaged BCR in B6 and bg/bg B cells.

Splenocytes from B6 and bg/bg mice were stained on their surface with anti-μ-biotin and were incubated at 37°C for different time-points. At every time-point, cells were fixed and stained with B220-FITC and streptavidin-PE and surface IgM levels on gated B cells have been depicted as an overlay plot for each strain. Shaded histogram represents the unstained control. Representative of 5 independent experiments. As a control B cells were stained with MHCII-bio and the level of MHCII down-modulation, if any, was followed over time.

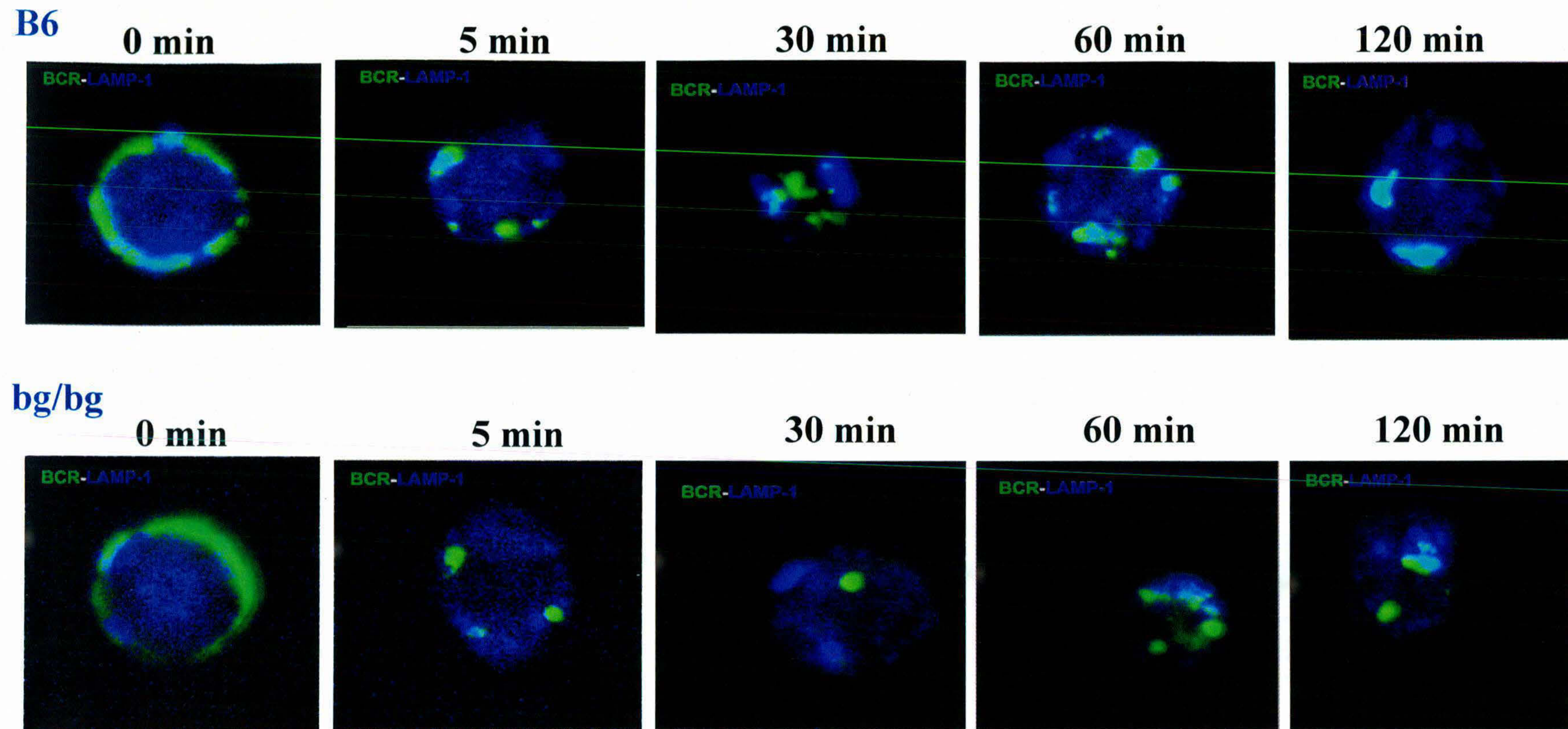


Fig 9. Delayed trafficking of internalized BCR to lysosomal compartments in bg/bg B cells.

B cells from B6 and bg/bg mice were stained on their surface with anti-μ-biotin and then incubated for different time-points at 37°C. After each time interval, cells were fixed and stained with streptavidin-alexa 488 to detect surface and internalized BCR. In order to mark the lysosomal compartments, cells were also stained with rat mAb to Lamp-1 followed by anti-rat secondary reagent that was coupled to alexa fluor 633. Representative of 3 independent experiments with approximately 7- 10 cells in each experiment.

in bg/bg B cells the internalized BCR traffics slowly to the LAMP1 positive lysosomal compartments.

Slower trafficking of internalized BCR in bg/bg B cells shown by using inhibitors to lysosomal processing:

As it was seen earlier, that ligated BCR traffics to the lysosomal compartments with delayed kinetics in bg/bg B cells, we tried to address the same point using various pharmacological inhibitors of lysosomal processing. The inhibitors used were E64d, a thiol protease inhibitor, NH_4Cl and Bafilomycin A1, agents that increase the lysosomal pH and thereby affect lysosomal processing. In fig. 10A, OVA was targeted to the BCR, by staining cells on the surface with anti-μ-biotin, avidin and OVA-biotin, and the cells were then incubated at 37°C for an hour, by which time according to microscopic data, most OVA targeted BCRs should be in lysosomal compartments in B6, whereas in bg/bg B cells there should still be a fraction that is outside the lysosomes. During the period of incubation at 37°C, cells were kept either in presence or in absence of different lysosomal inhibitors and were fixed following incubation. Antigen presentation to 13.8 T cells following inhibitor treatment was estimated (using the β-galactosidase activity of the activated T cells) and normalized to antigen presentation without inhibitor. It was found that antigen presentation was significantly higher for bg/bg B cells in the presence of all lysosomal inhibitors used. This observation was also found to be true for LPS activated B cells (Fig. 10B).

While delivery of BCR targeted antigen to lysosomes is accelerated (Cheng et al., 1999b), it is possible that pinocytic cargo may show delayed trafficking to these compartments. Hence, we tried to look for the inhibition of antigen presentation by lysosomal inhibitors when the antigen is delivered by the pinocytic route in resting B cells, activated B cells and activated

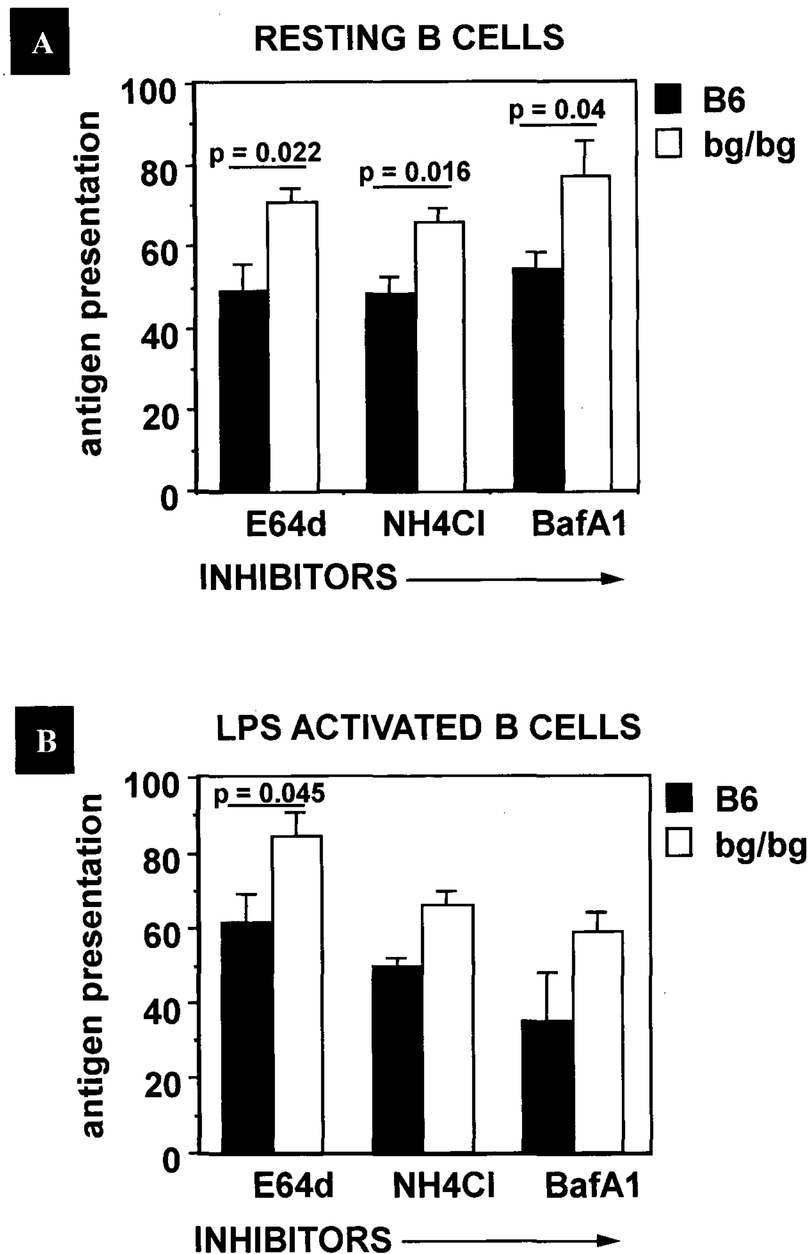


Fig.10 BCR targeted antigen traffics slowly to lysosomal compartments in resting and activated bg/bg B cells.

B cells **(A)** and LPS activated B cells (10μg/ml LPS for 48 hr; **B**) were given OVA through BCR and then were allowed to process the antigen at 37°C either in presence or in absence of lysosomal inhibitors, E64d, NH_4Cl and Bafilomycin A1. Cells were then plated with 13.8 T cells for 24 hr. Antigen presentation (%) is calculated as presentation in presence of the inhibitor normalized to presentation in the no treatment group. Mean antigen presentation for triplicate wells at a density of 0.3 million cells per well ± SE for 3 independent experiments have been depicted. p values have been indicated only for the bars where it is statistically significant.

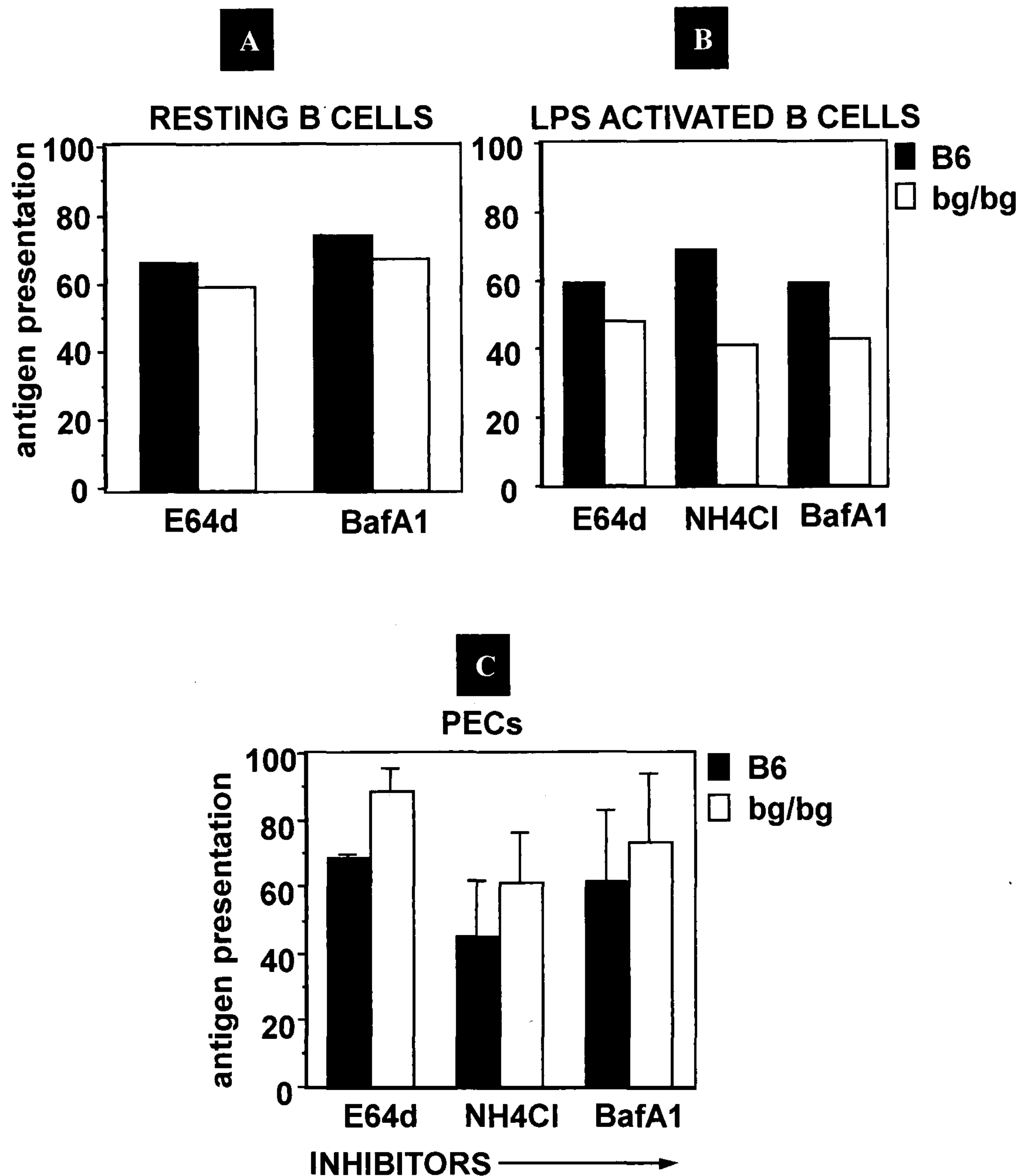


Fig. 11 Trafficking of soluble antigen to lysosomal compartments is similar between WT and bg/bg APCs.

B cells (**A**), LPS activated B cells (10μg/ml LPS for 48 hr) (**B**) and PECs (**C**) were given soluble OVA at 37°C either in presence or in absence of lysosomal inhibitors, E64d, NH_4Cl and Bafilomycin A1. APCs were then plated with 13.8 T cells for 24 hr. Mean antigen presentation for triplicate wells at a density of 0.3million cells per well has been plotted for one experiment each in **A** and **B** and with SE for three independent experiments in **C**. p value for the bars in **C** are not significant.

macrophages. WT and bg/bg APCs were incubated with soluble OVA for 30 min at 37°C in presence or absence of lysosomal inhibitors, fixed and plated with 13.8 T cells. No significant difference in inhibition between WT and mutant cells was seen in this case (Fig. 11). The data presented above indicate that LYST mutation delays trafficking of BCR targeted cargo to the lysosomal compartments and has different consequences on different pathways of antigen trafficking in a B cell.

Prolonged signaling in bg/bg B cells on BCR triggering:

The BCR and its cargo are generally destined for degradation in the lysosomal compartments. Since, the results shown earlier clearly indicate that in bg/bg B cells the internalized BCR traffics slowly to the lysosomal compartments, it may be possible that in bg/bg B cells, the BCR ligand complex remains intact for a longer period of time and continues to signal to the B cell. In B cells following BCR ligation, the kinase Btk phosphorylates PLCγ2, leading to generation of DAG and IP3. IP3 binds to internal calcium store and subsequently leads induction of calcium flux. In order to study signaling events following BCR triggering, B cells from B6 and bg/bg mice were loaded with the dye Fluo-3AM, stimulated with 10μg/ml of anti-μ $F(ab')_2$ and calcium flux was monitored using a fluorimeter (Fig. 12A). It was found that on BCR triggering, bg/bg B cells show a more sustained calcium flux than B6 B cells.

In order to check if delay in trafficking of internalized BCR to lysosomes, affects the duration of activation of signaling intermediates in bg/bg B cells, western blots were performed on lysates prepared from stimulated B6 and bg/bg B cells (stimulated for different time-points with anti-μ $F(ab')_2$ to detect induction of phospho-PLCγ2 (BCR

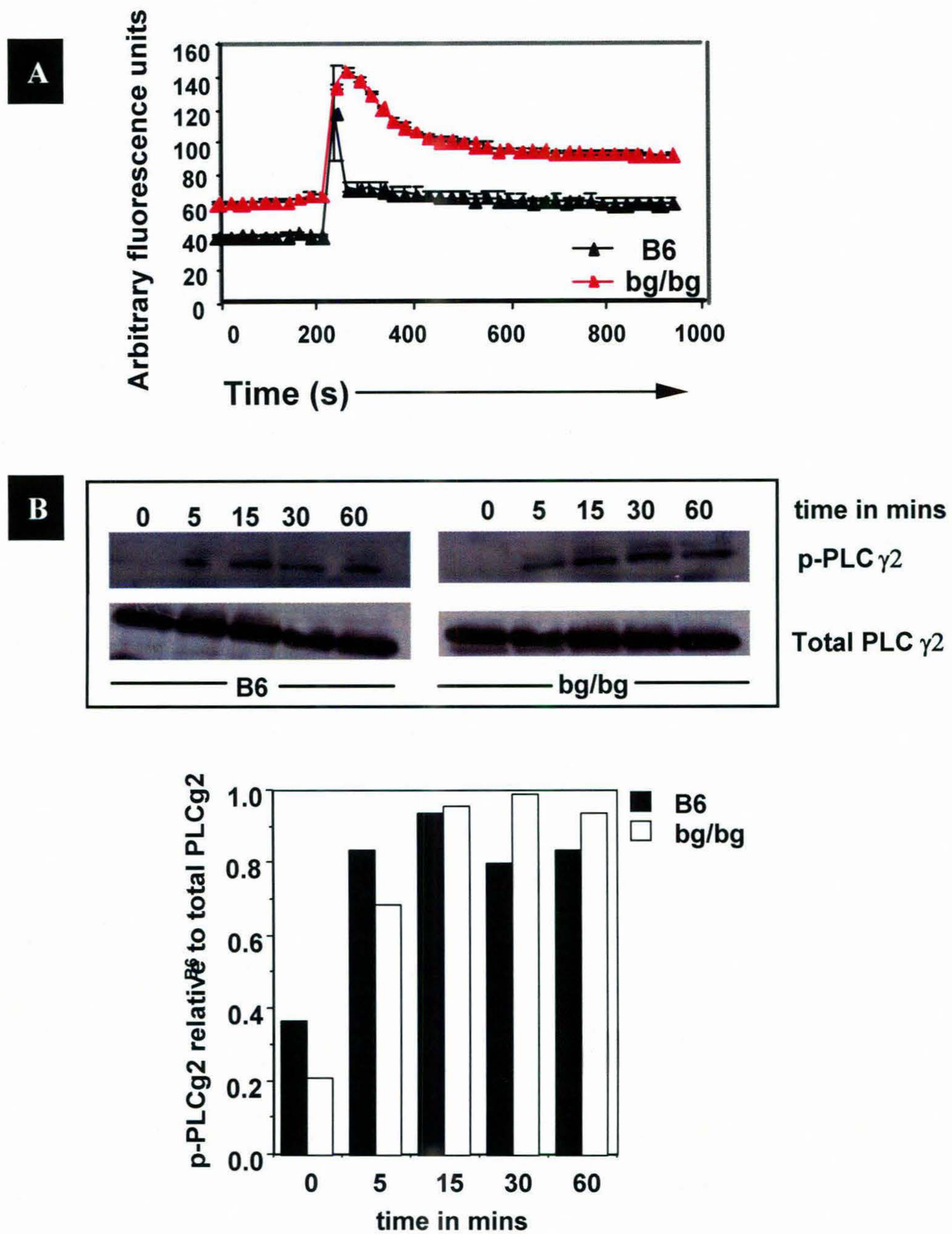
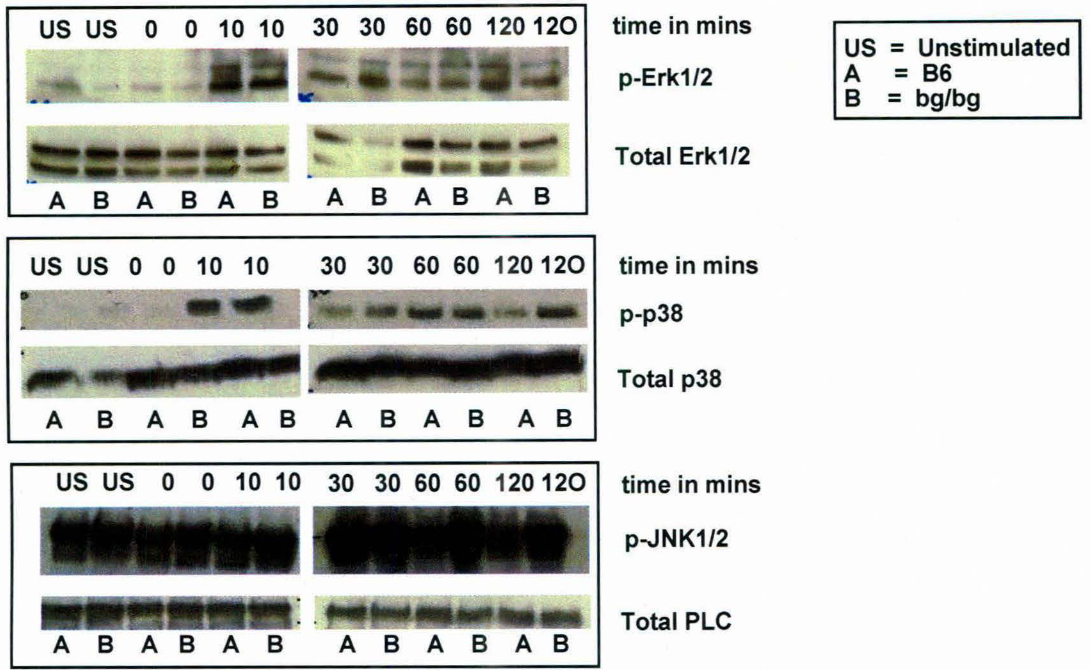


Fig. 12 Ca^{2+} flux and phospho-PLCγ2 induction in bg/bg B cells following BCR triggering.

A. B cells from B6 and bg/bg mice were loaded with the dye Fluo-3AM and stimulated with 10μg/ml of anti-μ following which Ca2+ flux was measured. Representative of two independent experiments. **B.** B cells from B6 and bg/bg mice were stimulated with anti-μ F(ab')2 and phospho-PLCγ2 levels were estimated after different time-intervals. Levels of phospho-PLCγ2 have been normalized to that of total PLCγ2 and the ratio has been depicted here for each time-point. Representative of two independent experiments

C



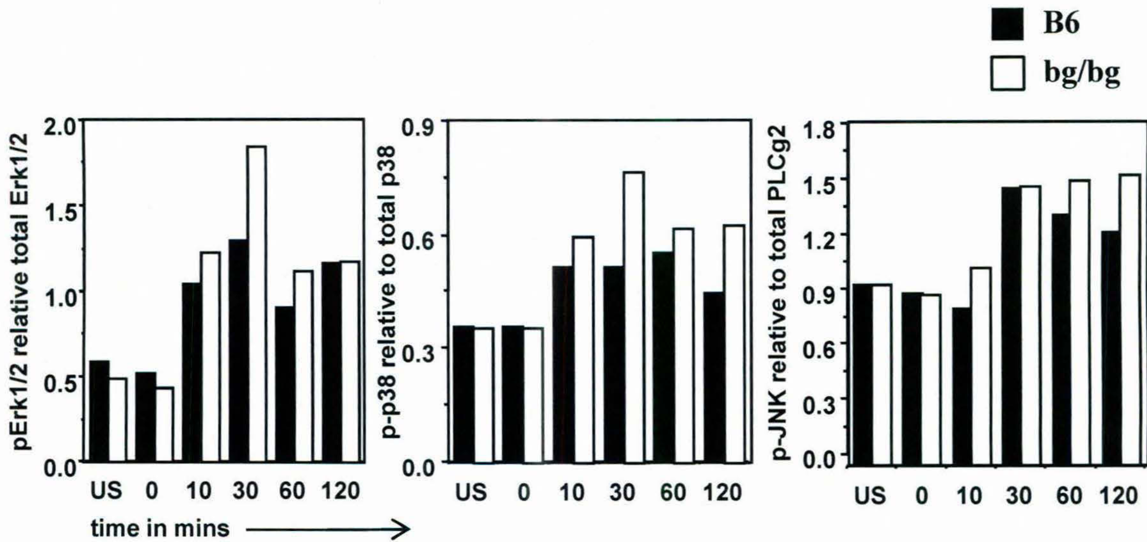


Fig. 12 contd..

C. B cells were purified from B6 and bg/bg mice and stimulated with 10μg/ml anti μ $F(ab')_2$ for different time-points. Cell lysates were probed with antibodies for phospho- and total- Erk1/2, p38, JNK1/2, PLCγ2. A ratio of phospho to total protein has been depicted here as bar graphs. Representative of three experiments each for p-Erk1/2 and p-p38. Representative of two experiments for p-JNK1/2.

proximal signaling event) and the different phospho-MAPKs (BCR distal signaling event). B cells were purified from B6 and bg/bg splenocytes and were either left unstimulated or stimulated with anti-μ F $(ab')_2$ for 0, 5, 30, 60 and 120 mins. After each time-point, cells were lysed in lysis buffer and loaded on a polyacrylamide gel. Kinetics of induction of phosphorylation of PLCγ2 (Fig. 12B) and the three MAPKs, p38, Erk1/2and JNK1/2 (Fig. 12C) was almost similar between B6 and bg/bg B cells. However, in BCR triggered bg/bg B cells the phosphorylated activated states of PLCγ2 and the MAPKs were maintained for a much extended period as compared to B6 B cells.

Slower degradation of pinocytically delivered cargo in bg/bg peritoneal exudate cells:

Engaged BCR on internalization exhibits delay in trafficking to the lysosomes in bg/bg B cells as seen in fig. 9. We also tried to deliver antigen into B cells and peritoneal exudate cells (PECs), as a source of activated macrophages, by pinocytosis, and study the fate of this pinocytic cargo inside the cells. B6 and bg/bg cells were pulsed with FITC labeled OVA, and at different time-points after pulsing, the cells were analyzed for degradation of the cargo and loss of intracellular FITC signal (Fig. 13). Macrophages take up soluble antigen very efficiently and the uptake was found to be similar between WT and bg/bg PECs. However, when we followed the loss of intracellular FITC signal over time, it was found that the loss was much slower in bg/bg PECs compared to WT, indicating that LYST mutation might result in pinocytically delivered antigen to be degraded much slowly in bg/bg mice as compared to its degradation in WT cells. We tried the same approach with B cells but the OVA-FITC signal in these cells was not robust and we were unable to detect any loss of signal over time (Fig. 13). This is probably due to the poor ability of B cells to take up antigen by pinocytosis.

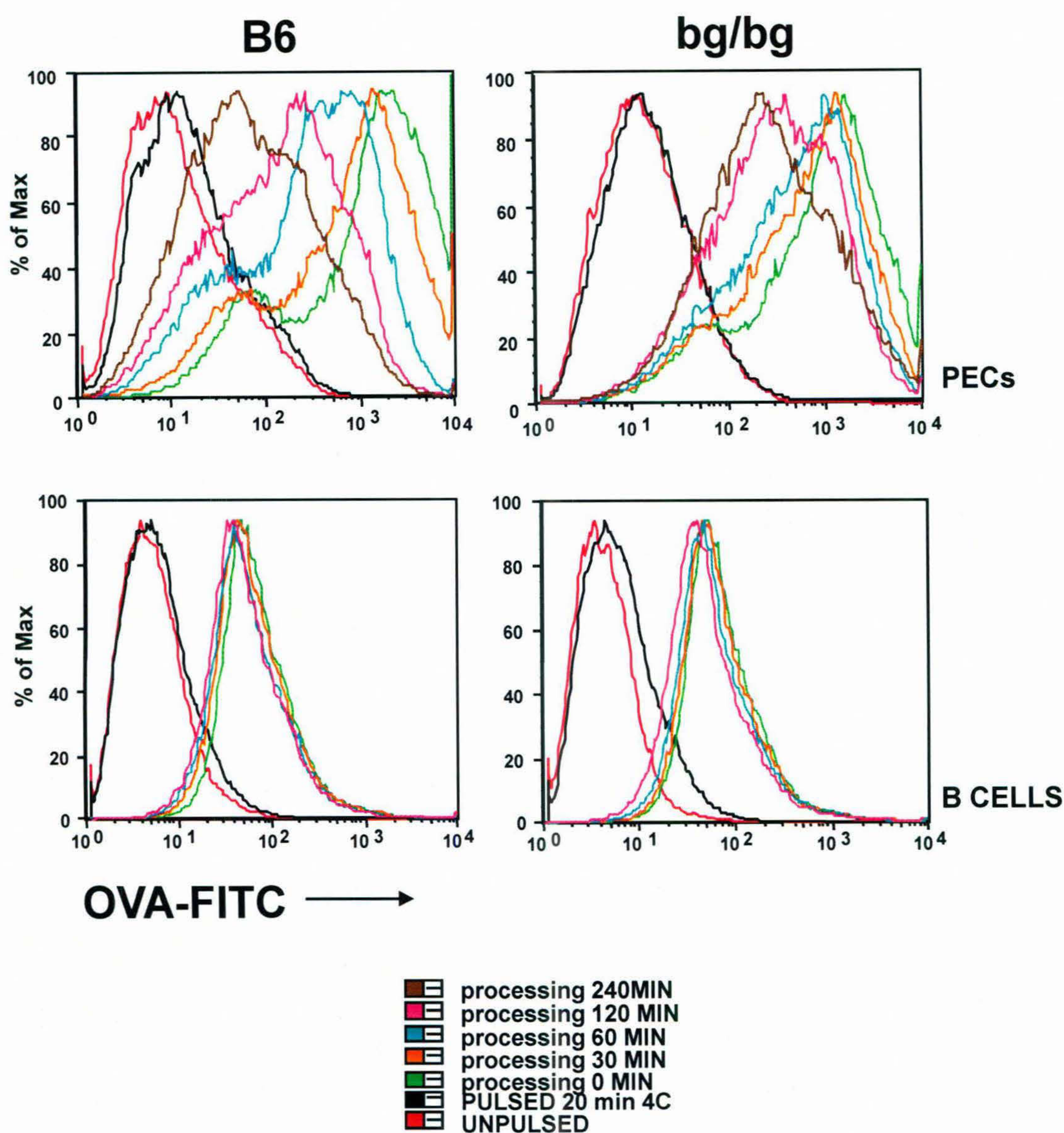


Fig. 13 Slower degradation of pinocytic cargo by bg/bg macrophages.

B6 and bg/bg PECs or B cells were allowed to take up soluble FITC- labeled OVA for 30 min at 37°C. At different time-points after uptake, the cells were analyzedfor intracellular FITC signal. Unpulsed cells and cells that were pulsed with OVA-FITC at 4°C were used as controls. Representative of 3 independent experiments.

Differential kinetics of presentation of receptor targeted and soluble antigen by resting bg/bg B cells and macrophages:

B cells can process antigens in the endosomal / lysosomal compartments and present antigen-derived peptides in context of MHCII molecules to CD4 T cells, an event that subsequently modifies B cell responses. Since, the *lyst* mutation is known to affect sorting of cargo as well as functionality of the lysosomes in different cell types, we were interested in studying the effect of this mutation on B cell antigen presentation events. B cells can take up antigen from the external milieu by different mechanisms - by endocytosis of antigen bound to the BCR or by engaging receptors other than the BCR (receptor mediated endocytosis) as well as by fluid phase uptake (pinocytosis) of soluble antigen. Our studies aimed at assessing presentation following antigen uptake through different routes in bg/bg B cells as compared to the B6 B cells. The antigen used for the studies was ovalbumin (OVA) and activation of the T cell hybridoma, 13.8 (by measuring β galactosidase activity) was used as a read-out to score for OVA presentation. For receptor mediated endocytosis OVA was delivered to the B cells either through the BCR or through scavenger receptors (SR). Targeting OVA to the BCR was achieved by labeling B cells on the surface with anti-μ-biotin, followed by avidin and OVA-biotin, while maleylation of OVA helped in targeting it to the SRs. Following antigenic stimulation, B cells were plated with 13.8 T cells for different time periods. As expected, with increasing time of T cell-APC interaction, there was greater T cell activation. In B cells, for both SR and BCR targeted antigen delivery, it was found that at early time-points of incubation with T cells (3hr, 6hr and 12 hr), B cells from bg/bg mice exhibited poorer antigen presentation compared to B6 B cells (Fig. 14A). However, by 24 hr the antigen presentation by bg/bg B cells was found to be almost equivalent to that of the B6 B cells (Fig. 14A). To study pinocytosis, B cells were incubated with soluble OVA, following which the cells were washed and plated with 13.8 T cells for

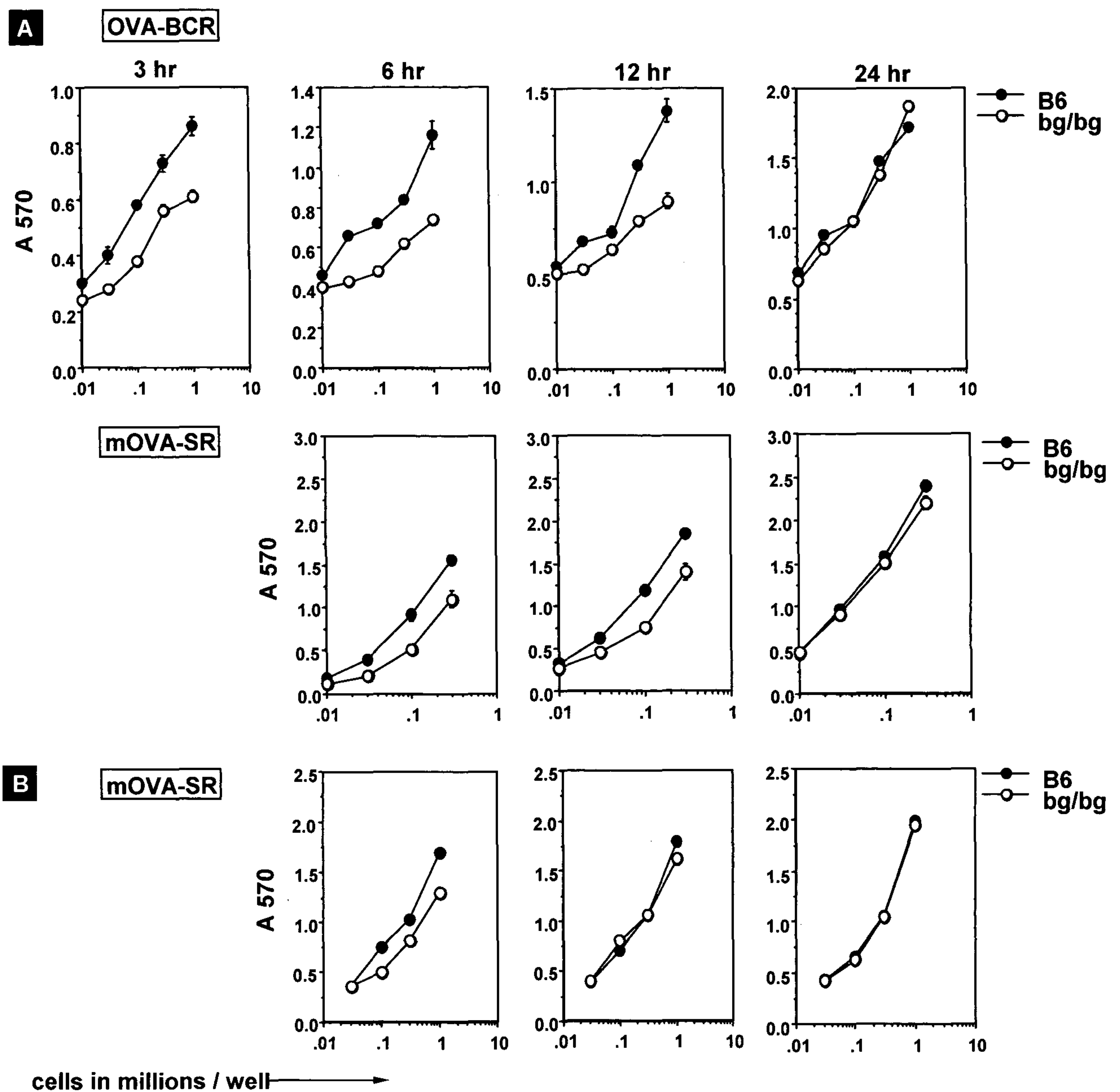


Fig. 14 bg/bg B cells and BMDMs exhibit delay in presentation of receptor targeted antigen.

B cells **(A)** and BMDMs **(B)** from B6 and bg/bg mice were used as APCs. Receptor mediated delivery of antigen was achieved by targeting OVA through the BCR (OVA-BCR) in B cells or by maleylating OVA (mOVA) and targeting it to the SR, in B cells and BMDMs. Antigen pulsed APCs were plated with 13.8 T cells for different time periods, following which T cell activation was measured colorimetrically (A570) and plotted as mean ± SE of triplicate wells. A570 of control groups < 0.4. Representative of 3 independent experiments.

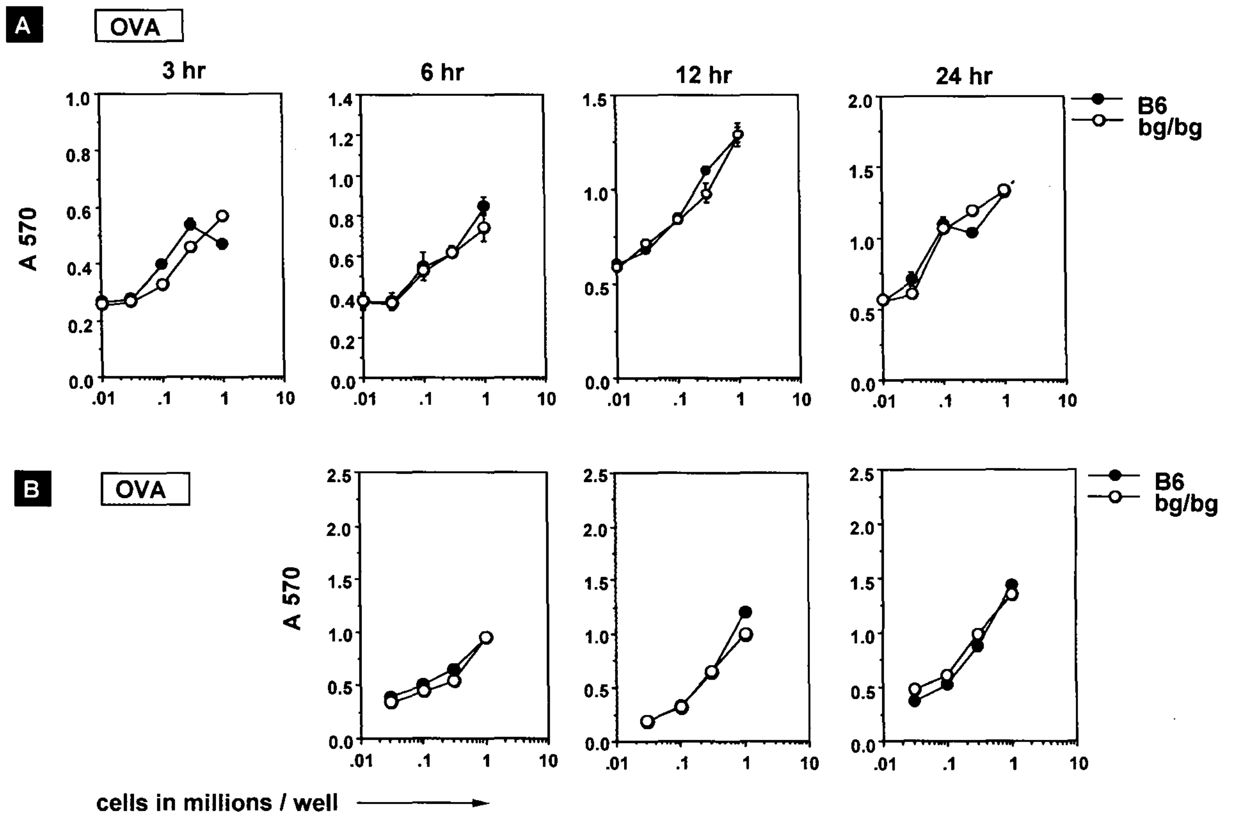


Fig. 15 B6 and bg/bg B cells and BMDMs show equivalent presentation of pinocytically delivered antigen.

B cells **(A)** and BMDMs **(B)** from B6 and bg/bg mice were given soluble OVA for pinocytic uptake. Pulsed APCs were plated with 13.8 T cells for different time periods following which T cell activation was measured colorimetrically (A570) and plotted as mean ± SE of triplicate wells. A570 of mock treated groups < 0.4. Representative of 2 independent experiments.

different time periods (Fig. 15A). Data revealed that between B6 and bg/bg B cells, presentation of soluble antigen, although poor, was similar at all time-points, unlike what was seen for receptor targeted antigen.

Bone marrow-derived macrophages (BMDMs), a source for resting macrophages, were used as controls in the B cell antigen presentation assays. Results showed that presentation of mOVA by bg/bg BMDMs was lower than B6 cells at 6hr of T cell-APC interaction, but by 12hr and 24hr the differences ceased to exist (Fig. 14B). Presentation of soluble OVA showed no difference between B6 and bg/bg BMDMs for different periods of incubation with T cells (Fig. 15B).

The above data indicate that different APCs from bg/bg mice show a lineage independent delay in presentation of receptor targeted antigen, while presentation of pinocytically delivered antigen is equivalent to that of WT cells. These data also indicate that antigens follow different endocytic pathways *en route* to lysosomes depending on their mode of uptake, and that LYST mutation affects these pathways differentially.

Poor presentation of antigen by activated bg/bg B cells and macrophages:

Activation of B cells makes them more efficient at antigen uptake and better conditioned for antigen processing and presentation by bringing about physical and biochemical modifications in the MIICs (Krieger et al., 1985; Siemasko et al., 1998). Thus, B cells were stimulated with 10μg/ml LPS for 48 hours, to achieve an activated phenotype. As control, thioglycolate elicited peritoneal macrophages were used as a source of activated macrophages. Activated B cells and macrophages were incubated with soluble OVA and put in culture with 13.8 T cells. For different time periods of T cell-APC interaction, it was

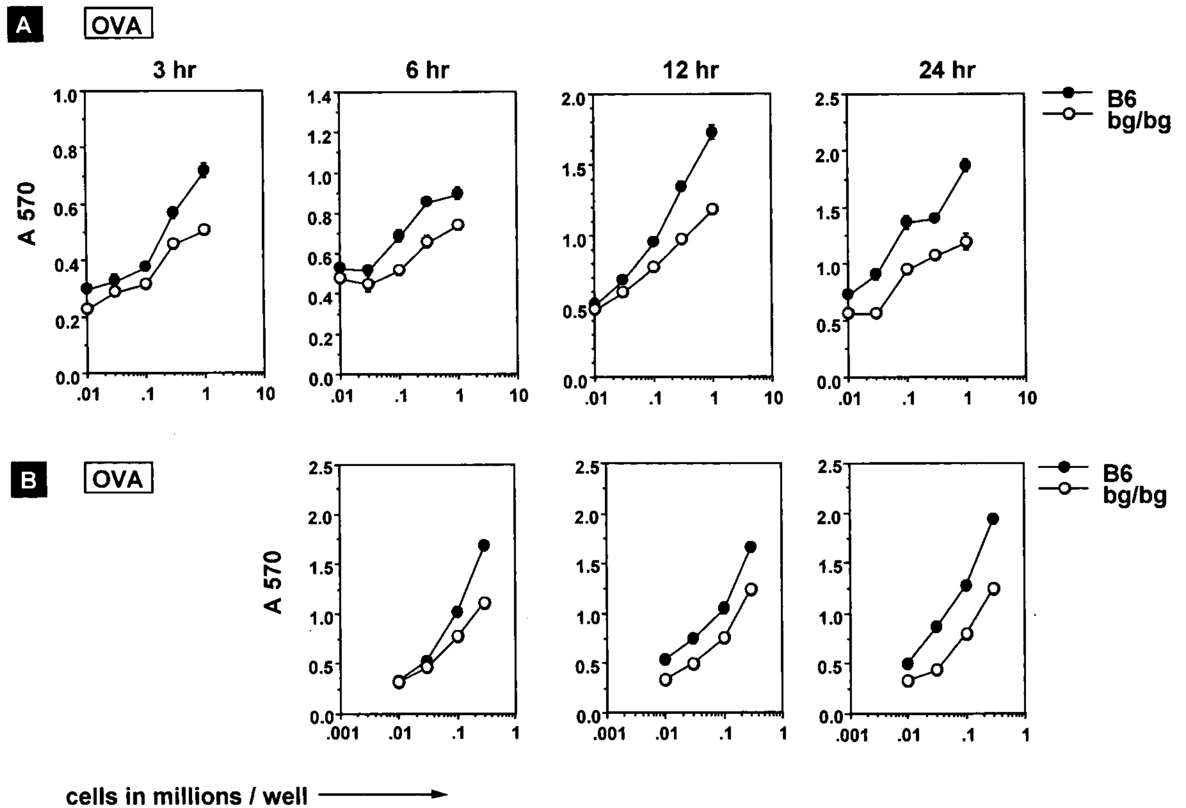


Fig. 16 Activated bg/bg B cells and macrophages show compromised presentation of pinocytically delivered antigen.

LPS activated B cells **(A)** or PECs **(B)** from B6 and bg/bg mice were given soluble OVA. Pulsed APCs were plated with 13.8 T cells for different time periods, following which T cell activation was measured colorimetrically (A570) and plotted as mean ± SE of triplicate wells. A570 of mock treated groups < 0.4. Representative of 2 independent experiments.

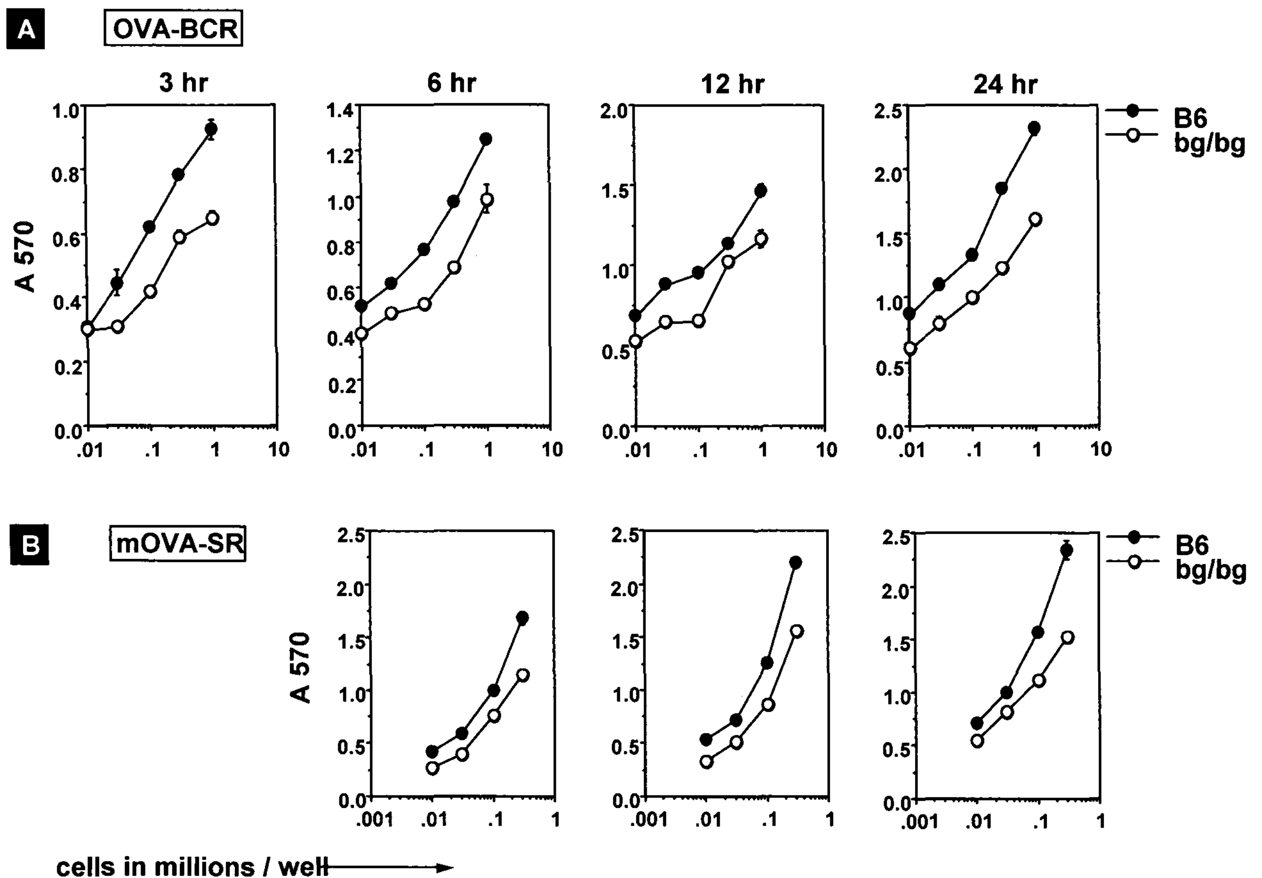


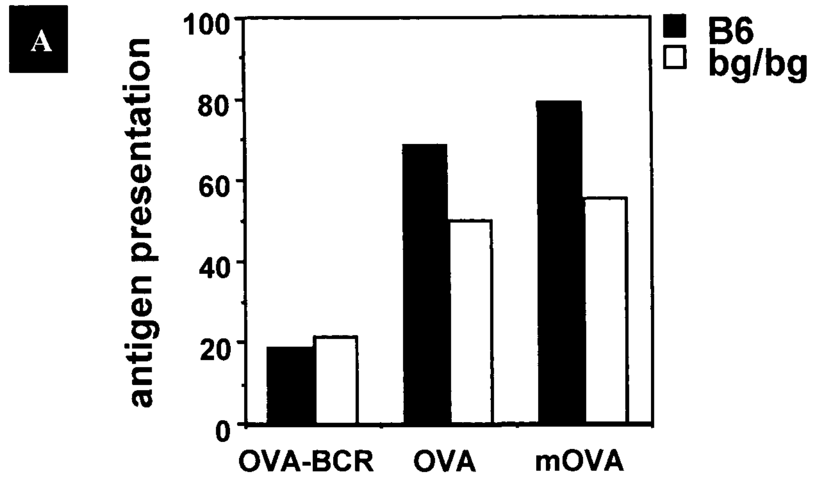
Fig. 17 Activated bg/bg B cells and macrophages show compromised presentation of receptor targeted antigen.

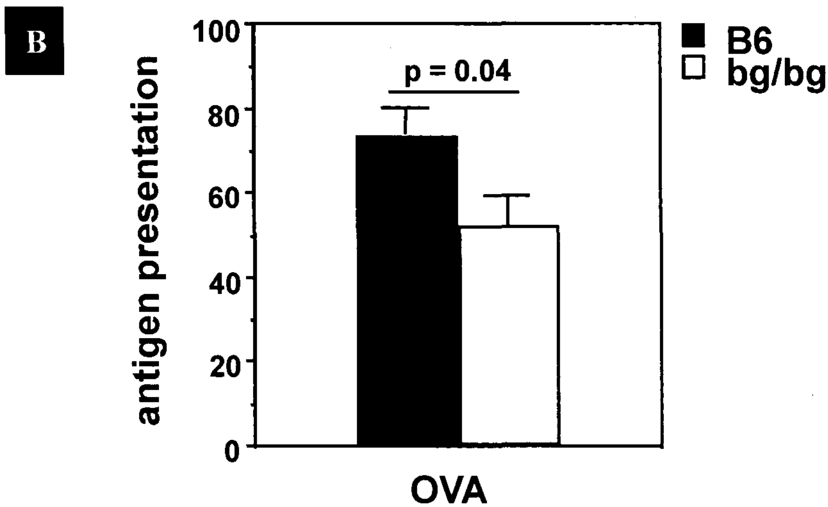
A. B cells from B6 and bg/bg mice were stimulated with 10μg/ml LPS for 48 hr and used as activated B cells. **B.** PECs were used as a source of activated macrophages. These activated cells were given OVA through their receptors, BCR (OVA-BCR) and SR (mOVA) in case of B cells and only SR for macrophages. APCs were plated with 13.8 T cells for different time periods, following which T cell activation was measure colorimetrically (A570) and plotted as mean ± SE of triplicate wells. A570 of control groups < 0.4. Representative of 3 independent experiments.

found that activated B cells and macrophages from bg/bg mice were compromised in their presentation of soluble antigen (Fig. 16A and B). OVA was either targeted through the BCR in B cells or mOVA was targeted to the SR in both B cells and PECs in order to achieve receptor mediated endocytosis. Activated B cells and macrophages from bg/bg mice exhibited compromised presentation of receptor-targeted antigen compared to B6 cells (Fig. 17A and B). This was true both for early as well as late time-points of T cell-APC interaction. Thus, on activation of APCs, the bg/bg defect becomes even more evident and now the bg/bg APCs cannot catch up with the B6 cells even after long periods of incubation with T cells.

Poor presentation of antigen by bg/bg B cells on recycling MHCII molecules:

Microscopic data (Fig. 9) showed that after an hour of ligation of the BCR, most internalized BCR in B6 B cells are present in the lysosomes, whereas, in bg/bg B cells a fraction still remained outside the lysosomes, probably in the early compartments of the endocytic pathway. In these compartments, antigenic cargo can access recycling MHCII molecules along with newly synthesized MHCII molecules. Hence, in bg/bg B cells what we observe as antigen presentation may have major contributions from recycling MHCII mediated antigen presentation. To address this possibility, we used cycloheximide, an inhibitor of protein synthesis to restrict presentation to recycling MHCII molecules. In B cells presentation of soluble antigen and SR targeted antigen on recycling MHCII molecules were also assessed. Hence, B cells from B6 and bg/bg mice were given soluble OVA, mOVA or OVA directed through the BCR, in presence or absence of cycloheximide. Following treatment, cells were washed, fixed and plated with 13.8 T cells for 24 hours. For OVA delivered through the BCR there was complete abrogation of presentation following





antigen presentation :

$$\frac{\text{Ag presentation with inhibitor treatment}}{\text{Ag presentation without treatment}} \times 100$$

Fig. 18 bg/bg B cells are compromised in antigen presentation on recycling MHCII molecules.

B cells from B6 and bg/bg mice were delivered soluble OVA, maleyl OVA (mOVA) or OVA targeted through the BCR (OVA-BCR) in presence of 100μg/ml cycloheximide for 4 hr. Prior to antigen pulsing the cells were pretreated with 100μg/ml cycloheximide for 2 hr. B cells were plated with 13.8 T cells for 24 hr. **A .** Mean antigen presentation at 0.3million cell density per well has been plotted for all three forms of OVA delivery (one experiment). **B .** Mean antigen presentation of soluble OVA at 0.3 million cell density per well has been plotted for 3 independent experiments ± SE.

inhibitor treatment in both cell types (Fig. 18A). This suggested that antigen targeted to the BCR, requires newly synthesized MHCII molecules for its presentation (Fig. 18A). Presentation of soluble OVA and mOVA was reduced following cycloheximide treatment but was not completely abrogated (Fig. 18A and B), indicating that in both cases a fraction of the antigen gets loaded onto recycling MHCII molecules.

bg/bg B cells do not show defect in activation and proliferation when in competition for T cell help:

To summarize the *in vitro* data presented above ligated BCR traffics slowly to lysosomal compartments in bg/bg B cells, which in turn results in extended signaling from the internalized BCR as well as a delay in antigen presentation to T cells. In order to study B cell responses in the light of two such forces of opposing nature operating in bg/bg B cells, B cells from B6 and bg/bg were put in competition for antigen specific T cell help *in vitro*, followed by scoring for CD69 upregulation (to assess activation) or CFSE dilution (to assess proliferation) on the activated cells.

B cells from the two congenic strains of mice, B6.SJL (CD45.1) and bg/bg (CD45.2) were given OVA through BCR. Splenocytes of OT-II mice, where the CD4 T cells have receptors that recognize OVA derived peptides in context of H2-$A^{b,}$ where cultured in presence of 100ng/ml of anti CD3 for 2 days and with 2U/ml of IL-2 for the next 2 days, to generate T cell blasts. Live, activated T cells were then put in culture with B cells that had been given OVA through BCR at titrating T cell: B cell ratios. B cells from the two different congenic strains were either cultured independently with T cells or in competition with each other for T cell help. After 12 hours of co-culture, the cells were harvested, washed and stained for the activation marker CD69 on gated CD45.1 or CD45.2 cells (Fig. 19). Data presented here

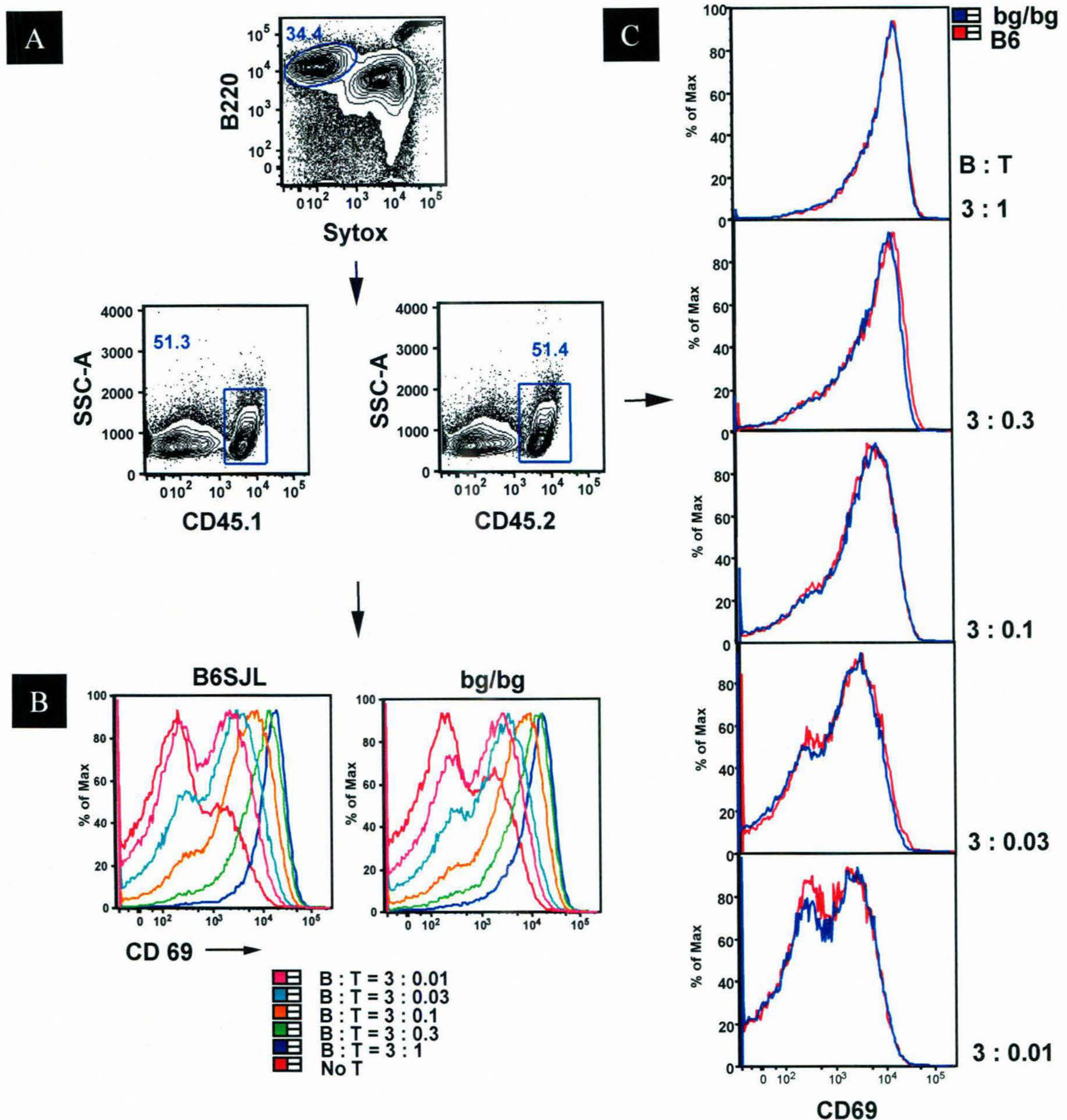


Fig. 19 CD69 upregulation on bg/bg B cells is not compromised in *in vitro* competition cultures.

B cells from B6.SJL(CD45.1) and bg/bg (CD45.2) mice were given OVA through the BCR and plated with activated OT-II T cells in titrating B cell : T cell ratios independently or in competition for T cell help. CD69 upregulation was assessed 12 hr later on live B cells gated for the congenic marker CD45. **A.** Gating for B220, sytox red, CD45.1 and CD45.2 used for the analysis has been depicted. **B.** On the gated population, titration of CD69 upregulation with titrating T cell help has been shown for independent cultures. **C.** For different B cell: T cell ratios in competition cultures, upregulation of CD69 on the gated cells have been depicted as overlay plots for the two strains of mice.

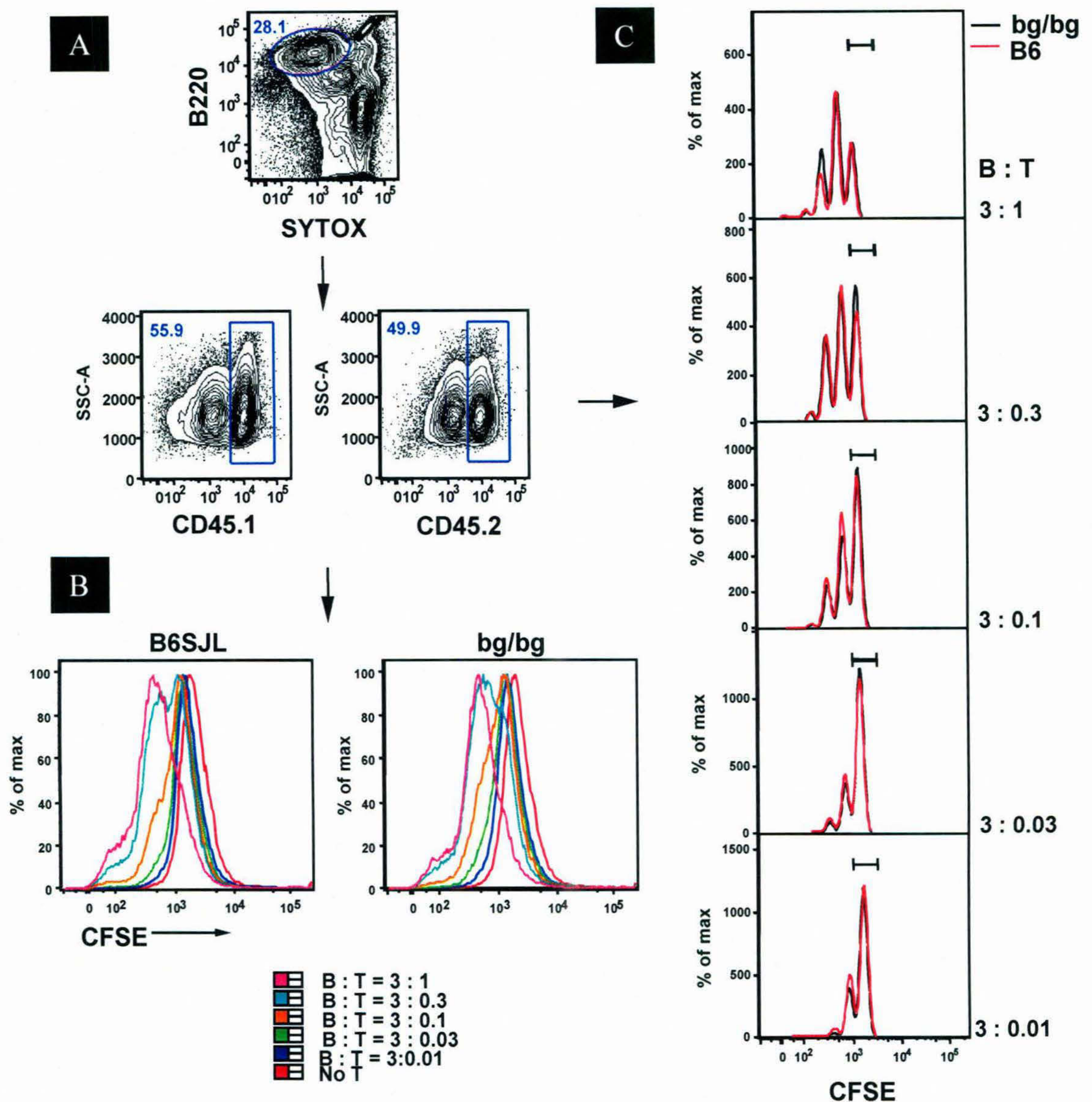


Fig. 20 CFSE dilution of bg/bg B cells is not compromised in *in vitro* competition cultures.

B cells from B6.SJL (CD45.1) and bg/bg (CD45.2) mice were labeled with CFSE, given OVA through the BCR and then plated with activated OT-II T cells in titrating B cell:T cell ratios independently or in competition for T cell help. CFSE dilution was assessed 48 hr later on live B cells gated for the congenic marker CD45. **A.** Gating for B220, sytox red, CD45.1 and CD45.2 used for the analysis has been depicted. **B.** On the gated cells, CFSE dilution with titrating T cell help has been shown for independent cultures. **C.** For different B cell: T cell ratios in competition cultures, CFSE dilution by B cells have been depicted as overlay plots for the two strains of mice.

show that CD69 was upregulated on both B6 and bg/bg B cells in independent cultures and the level of upregulation titrated with titrating T cell-B cell ratios. When the B cells were put in competition for T cell help, CD69 upregulation was still found to be equivalent on the two different B cell types. For assaying B cell proliferation, cells were labeled with CFSE, following which OVA was targeted to the BCR and the cells were then put in culture with activated T cells in varying ratios as discussed earlier. After 48 hr of T cell-B cell co-culture, CFSE dilution was analyzed on live B cells gated for the congenic CD45 marker (Fig. 20). It was found that by titrating T cell help available, there was a titration in the proportion of cells that entered division. However, B6 and bg/bg B cells showed equivalent CFSE dilution even in competition for antigen specific T cell help.

Analysis of primary T-independent responses to immunization:

Using bg/bg mice we were able to study *in vivo* priming events following immunization and responses following recall. The advantage of *in vivo* studies is that it takes into account the complexity of interactions and the contribution of other physiological parameters in the responses being studied.

B6 and bg/bg mice were immunized with 100μg of a T-independent antigen, Nitrophenyl coupled to ficoll (NP-Ficoll) and the titres of anti-NP IgM and IgG in the serum were followed over a period of 3 weeks by ELISA (Fig. 21). Antibody titres were lower in bg/bg mice compared to B6 for both NP specific IgM as well as IgG. Hence, in response to T-independent stimulation both *in vitro* and *in vivo*, bg/bg mice exhibit compromised differentiation to plasma cells.

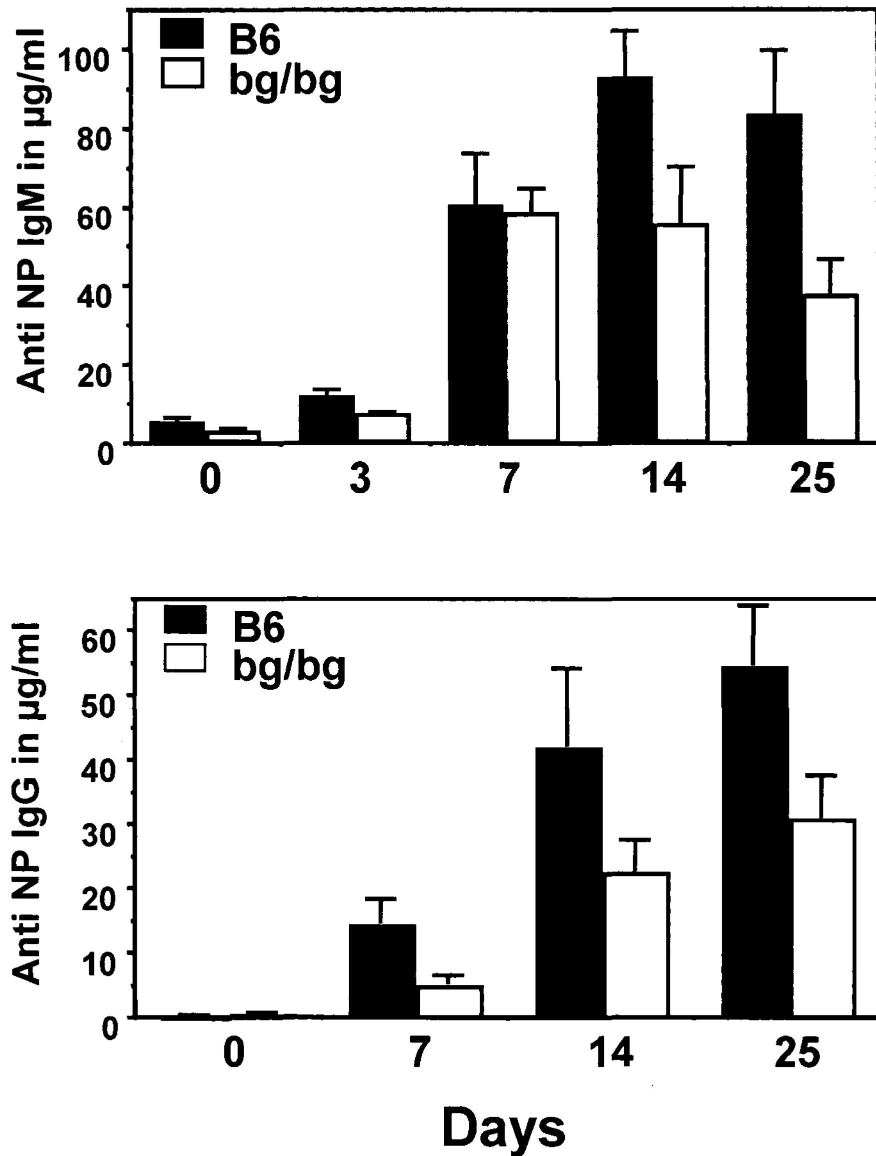


Fig. 21 Compromised IgM and IgG responses in bg/bg mice to T-independent antigen.

B6 and bg/bg mice were immunized with 100µg NP-Ficoll and on different days post immunization, NP-specific IgM and IgG titres were checked by ELISA. Data presented here are antibody titres expressed as mean ± SE for 10 mice per group in one experiment.

Analysis of primary T-dependent responses to immunization:

B6 and bg/bg mice were immunized with a T-dependent antigen, NP-CGG at two different doses, 25μg and 5μg per mouse, and NP-specific IgG titres were measured over time. We found that when immunized with a higher dose of the antigen, bg/bg mice show a better anti-NP IgG response as compared to B6 mice at all time-points (Fig. 22A). The response peaks at day 10 and then steadily declines, and by about 3 months the response comes down to pre-immunization levels. We also tried to estimate the affinity of the antibody produced, by using two different NP substituted BSA reagents to capture anti-NP IgG in the sera. NP_{6}- -substituted BSA binds anti-NP antibody that have high affinity, whereas, NP_{23}-substituted BSA binds both high as well as low affinity antibodies. A ratio of the two gives an idea of affinity maturation of NP specific antibody over time. It was found that the affinity of the antibodies increased over time but was not different between the two strains of mice (Fig. 22C). However, in some cases, the antibody titers detected by the two reagents showed almost equivalent absorbances, and hence the affinity maturation calculated for those time-points may not be accurate. When B6 and bg/bg mice were immunized with a lower dose of the T-dependent antigen NP-CGG (5μg per mouse), the anti-NP IgG levels were not found to be significantly different in B6 and bg/bg mice over a period of 5 months (Fig. 22B). In this case the affinity maturation of anti-NP antibody in bg/bg mice was found to be faster than that in the B6 mice (Fig. 22D).

From data discussed earlier, we had seen that bg/bg mice show variation in their primary antibody response depending on the dose of the antigen. At a lower dose of the antigen (10μg NP-CGG) we also tried to score for the frequency of ASCs in the draining lymph nodes of immunized mice. B6 and bg/bg mice were immunized with 10μg NP-CGG per mouse on CFA. On days 7, 14 and 21 post-immunization, the draining lymph nodes were

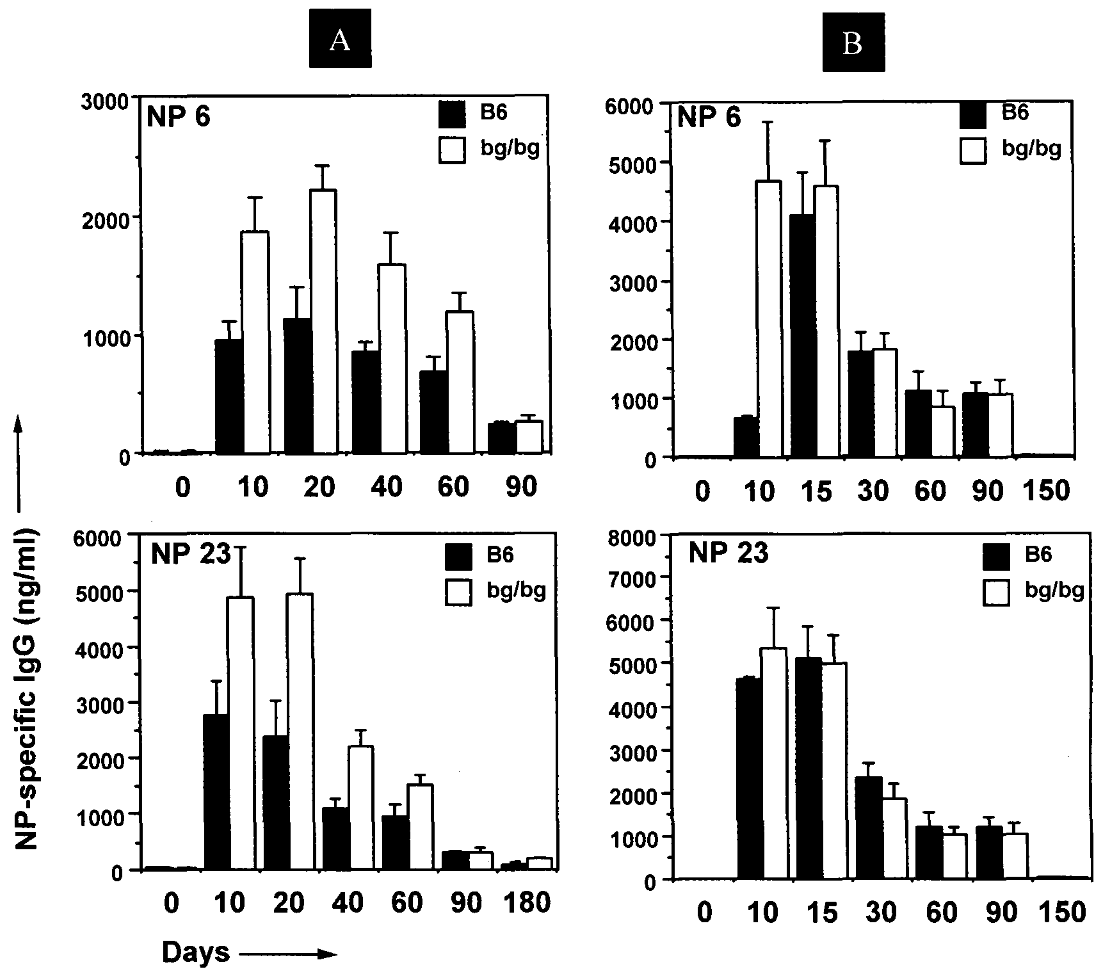
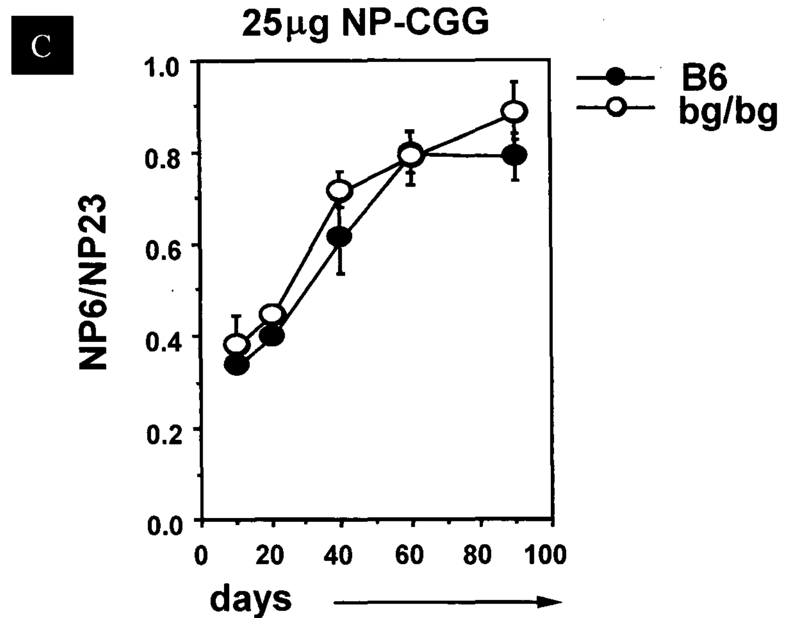


Fig. 22 Antibody responses following primary immunization with high or low doses of TD antigen.

B6 and bg/bg mice were immunized with either 25μg **(A)** or 5μg **(B)** of NP-CGG on alum i.p. Anti-NP IgG titres were followed over time by ELISA using either NP6-BSA or NP23-BSA to detect high affinity and total antibodies respectively. Data presented above are mean ± SE for 7-10 mice per group in one experiment.



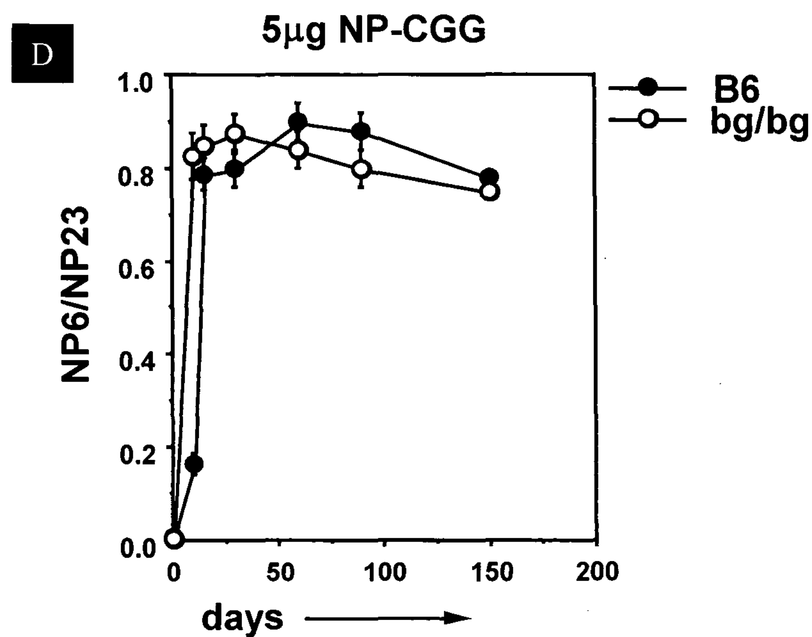


Fig. 22 contd.....

The affinity of the antibodies generated over time following primary immunization with either 25μg **(C)** or 5μg **(D)** NP-CGG was estimated as the ratio of high affinity anti-NP IgG (captured using NP6) to total anti-NP IgG (captured using NP23) and has been depicted here as a ratio (NP6/NP23). Data presented above are mean ± SE for 7-10 mice per group in one experiment.

harvested and cells were titrated on ELISPOT plates to score for the number of NP-specific antibody secreting cells (ASCs). Differentiation into antibody secreting cells was found to peak at day 14, and by day 21 the proportion of antigen specific plasma cells in the draining organ showed significant reduction (Fig. 23). The ASC frequency in the draining lymph nodes on day 7 was higher in bg/bg mice, but at the subsequent time points, however, no difference in the ASC frequency was observed between the two strains of mice.

Analysis of secondary T-dependent antibody responses to immunization:

i. Limiting dilution assay (LDA):

We tried to assess frequency of memory B cells in the draining lymph nodes of NP-CGG immunized B6 and bg/bg mice upto 3 weeks in a limiting dilution assay, using the approach of polyclonal activation of re-stimulable memory cells, followed by antigen-specific ELISA. Thus, B6 and bg/bg mice were immunized with 10μg NP-CGG on CFA s.c., and at different time-points post immunization the draining lymph node cells were harvested and given a polyclonal stimulus in the form of LPS. After 6 days of culture, the supernatant was assessed for anti-NP IgG using ELISA. Wells that showed an absorbance greater than three times that of unstimulated control were considered positive for the antibody. The cell input at which 37.5% of the wells were negative was considered to be the frequency of re-stimulable memory cells. The frequency of memory B cells in the lymph nodes at different time-points following immunization as estimated by LDA have been tabulated below:

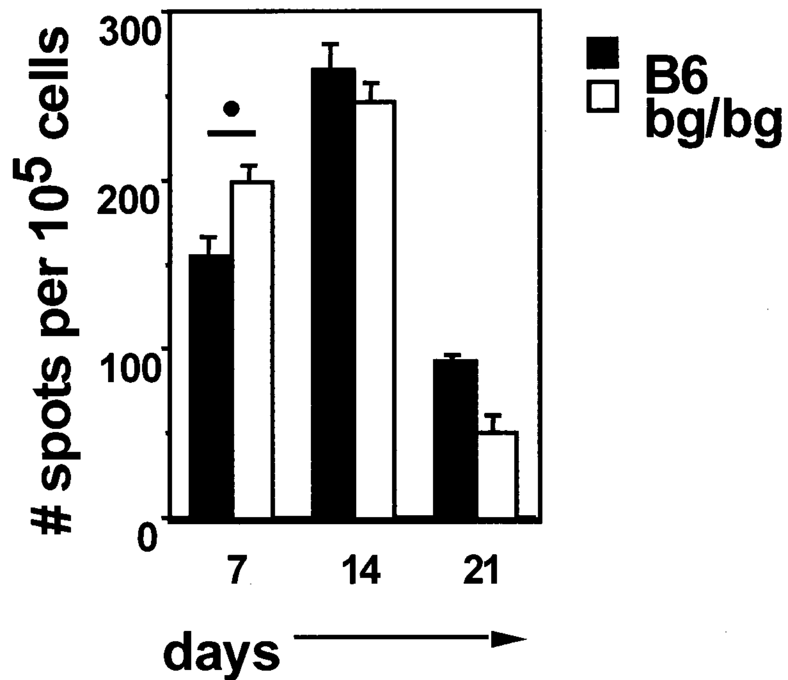


Fig. 23 Equivalent plasma cell frequency in WT and bg/bg draining lymph nodes following primary immunization.

B6 and bg/bg mice were immunized s.c. with 10μg NP-CGG and after days 7, 14 and 21, NP specific plasma cells in the draining lymph nodes was assessed using ELISPOT assay. The values depicted here as antibody secreting cells (ASCs) are mean ± SE of triplicate wells and have been normalized to frequencies in unimmunized controls. Data presented above are for cells pooled from 2 mice per time-point in one experiment. Bars that have been marked (●) have statistically significant difference.

DAYS	B6	bg/bg
7	1 in 2 X 10^4	1 in 8 X 10^5
14	1 in 8 X 10^3	1 in 2 X 10^4
21	1 in 1 X 10^4	1 in 3 X 10^4
42	1 in 1 X 10^5	1 in 3 X 10^4

Frequency of memory cells in bg/bg was lower on days 7 and 14 (Fig. 24) but after about a month following immunization, the frequencies were almost equivalent between B6 and bg/bg mice. However, it should be noted here that the proportion of B cells in draining lymph nodes of B6 mice and bg/bg mice may be different, and since the input cells were not normalized for B cell numbers, the frequency of memory B cells tabulated above is not accurate.

ii. Adoptive transfer experiment:

Scoring for memory B cells by LDA had some limitations as discussed earlier. In order to get a more accurate estimate of memory response in bg/bg mice, the following adoptive transfer experiment was performed. Carrier primed mice were generated by immunizing B6 mice with 100μg OVA on CFA intraperitoneally. On the other hand, B6 and bg/bg mice were immunized with 10μg NP-CGG on alum i.p., and 2 weeks following immunization B cells were purified from the splenocytes of the primed mice. These B cells were transferred intravenously into one month old, irradiated, carrier primed mice. One day post B cell transfer the recipients were immunized with 100μg NP-OVA in saline i.p. 4 days after challenge, NP specific antibody titres in the recipients were recorded by ELISA (Fig. 25).

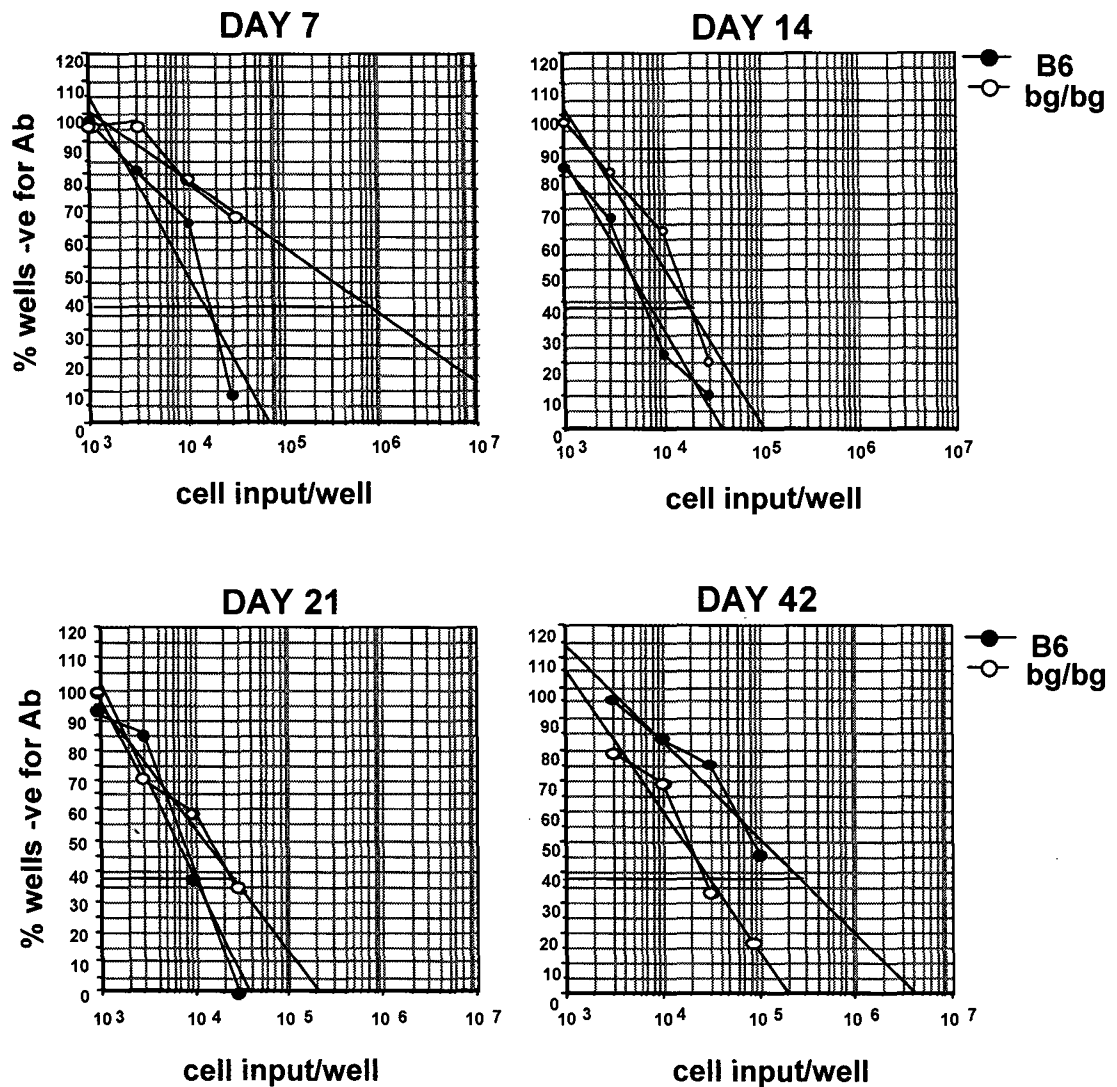


Fig. 24 Limiting dilution assay (LDA) to assess memory cell frequency in WT and bg/bg mice following primary immunization.

B6 and bg/bg mice were immunized with 10μg NP-CGG on CFA s.c. and cells from the draining lymph nodes were harvested on days 7, 14, 21 and 42 following immunization. Limiting dilution assay was performed on these cells and the % of wells with their absorbances below the cut off have been plotted against the cell input density per well. Memory B cell frequency was calculated from the plot as the cell input number per well for which 37.5% wells were negative for the antibody. Data presented above are for cells pooled from 2 mice per time- point in one experiment.

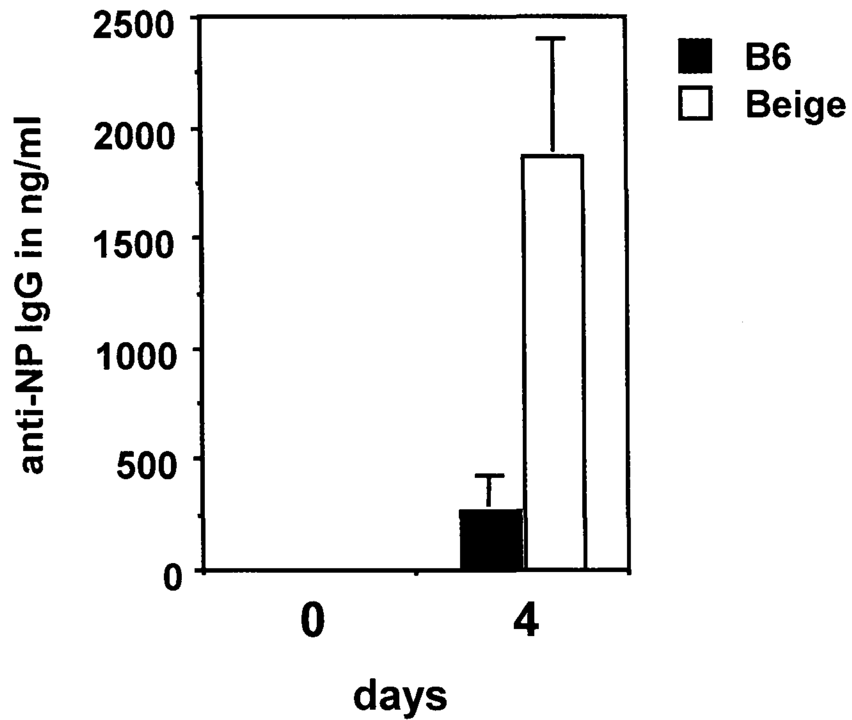


Fig 25. bg/bg B cells show better memory response following challenge.

B cells were purified from B6 and bg/bg mice, 15 days post immunization with 10μg NP-CGG and transferred into irradiated OVA primed recipients. The recipients were then immunized with 100μg NP-OVA and ELISA was done to estimate NP specific IgG responses, 4 days after challenge. Data presented here is mean ± SE for 3 mice per group in one experiment. Representative of two independent experiments.

Irradiated recipients that had received bg/bg B cells, showed a significantly higher antibody response following challenge compared to the ones that had received B6 B cells.

Greater accumulation of plasma cells in spleen and bone marrow of bg/bg mice following challenge:

It has been reported that plasma cells from the spleen migrate to the bone marrow within the first few days of a secondary challenge and accumulate there over time, thereby establishing bone marrow as the major source of antibody production in the body (Benner et al., 1981). We were interested in assessing if the *lyst* mutation affected this phenomenon of long lived plasma cell accumulation in the bone marrow. Thus, we immunized B6 and bg/bg mice with 10μg NP-CGG on CFA s.c. NP-specific IgG titres in sera of these mice were assessed over time to ascertain when the primary response dies down (Fig. 26A). On day 60 following primary immunization, when the antibody titres reached pre-immunization levels, these mice were challenged with 5μg NP-CGG in saline. An assessment of plasma cell proportions in the bone marrow on day 5 following challenge revealed an equivalent proportion between bg/bg and B6 mice. However, after about two months (day 65) when we scored for the plasma cell frequency in the bone marrow of bg/bg mice, the proportions were significantly higher than that in WT bone marrow cells (Fig. 26 B and C). Alongside long-lived plasma cell frequencies, we also scored for generation of ASC in the draining organ following challenge. At two different time-points following secondary immunization, day 5 (early) and day 65 (late), splenocytes were harvested from these mice and subjected to NP-specific ELISPOT assay (Fig. 26B). On day 5 following challenge, there was a greater proportion of NP-specific ASCs in bg/bg splenocytes than in B6. By day 65, the proportion of antigen specific plasma cells in spleen decreased both in B6 mice as well as in bg/bg mice, but the difference persisted (Fig. 26C). These data suggest that generation of plasma

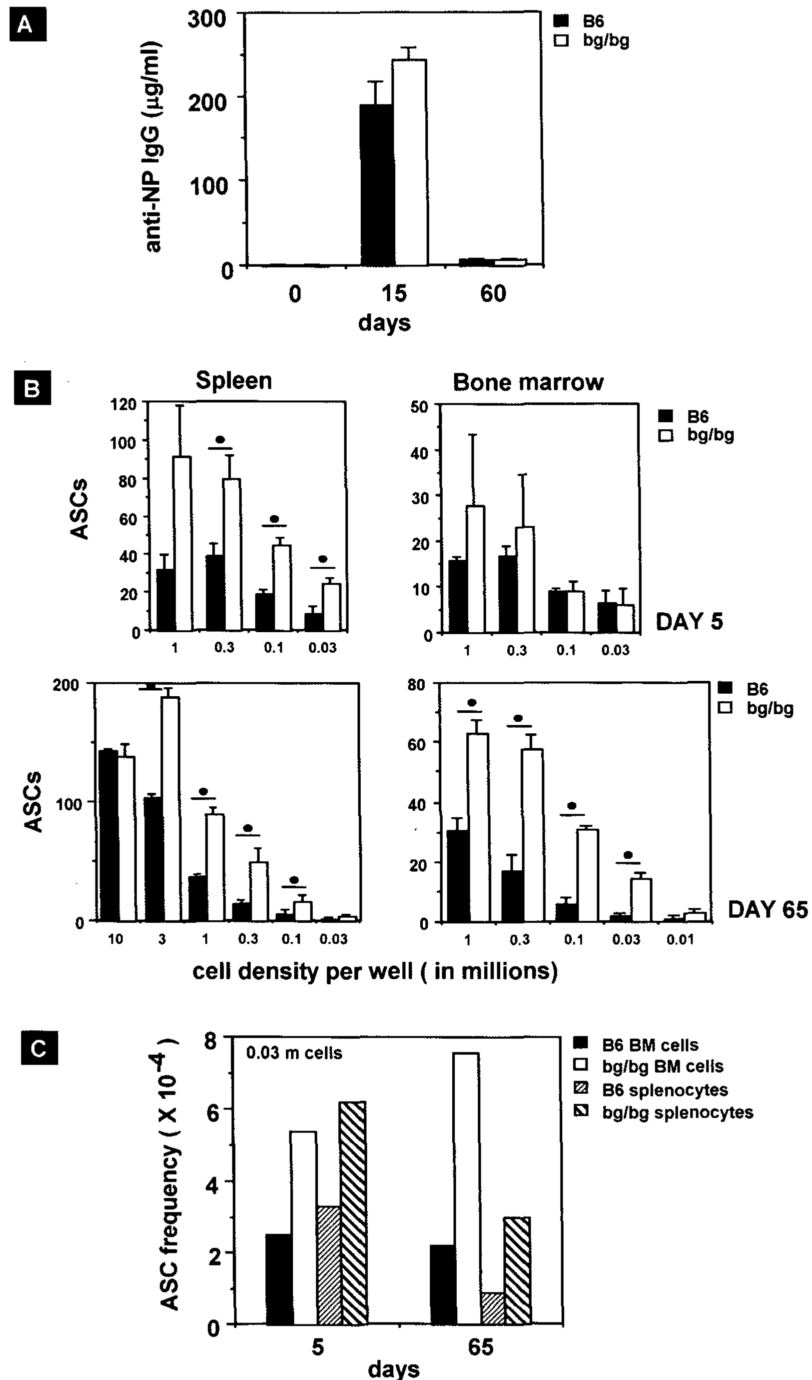


Fig. 26 Greater accumulation of long-lived plasma cells in bone marrow of bg/bg mice following challenge.

A.Primary antibody response of B6 and bg/bg mice immunized with 10µg NP-CGG on CFA s.c. Once the primary response subsided mice were challenged with 5µg NP-CGG i.p. **B.** ASC frequencies following challenge, in spleen and bone marrow of immunized mice and unimmunized control mice have been plotted against titrating cell numbers as mean ± SE of triplicate wells. Bars marked as (•) have statistically significant difference. **C.** ASC frequency at 0.03 million cell density per well have been plotted for the different organs on day 5 and day 65.

cells in the draining organ (spleen) following challenge is better in bg/bg mice compared to B6 mice, and also there is greater accumulation of long-lived plasma cells in the bone marrow of bg/bg mice over time.

Discussion

The endosomal pathway is a complex intracellular membrane system that has evolved in eukaryotic cells to efficiently take up material from the external environment or internalize cell surface molecules and deliver them to degradative machinery so as to accomplish various functions, such as, nourishment, transmission and termination of signals from different receptors and turnover of different cell membrane proteins (Mellman, 1996; Mukherjee *et al.*, 1997). Some cells of the immune system, called the antigen presenting cells like DCs, macrophages and B cells use this pathway for efficient uptake and degradation of antigens present in the extracellular milieu and present these antigen derived fragments to T cells in order to evoke a successful immune response. These cells have also modified certain components in the endocytic pathway for more effective antigen presenting functions, an example being the presence of specialized late endosomal / lysosomal compartments known as the MIICs (Tulp *et al.*, 1994). The ability of B cells to present antigenic peptides to T cells and to procure help either in the form of cognate cell to cell interactions or as cytokines is essential for the development of an effective humoral response to most protein antigens. MHCII molecules accumulate in the MIICs, where the conditions support degradation of antigens to generate peptides that can be effectively loaded onto these molecules (Neefjes, 1999; Stern *et al.*, 2006; Tulp *et al.*, 1994). In B cells, antigens can reach this pathway either following pinocytosis or after binding to specific receptors on the B cell surface. B cells are highly efficient at internalizing antigens that bind to their surface BCR, and it has been reported that several signaling intermediates that are recruited to an engaged BCR aid in the sorting of the BCR cargo containing vesicles to the endocytic compartments (Lankar *et al.*, 1998; Siemasko *et al.*, 2002). Hence, it can be hypothesized that the BCR signals from the endocytic compartments following internalization before it gets degraded in the lysosomes as is the case with many hormonal and growth factor receptors (Bevan *et al.*, 1996; Burke *et al.*, 2001). Thus, in such a

scenario degradation of the BCR in the lysosomes would be an important parameter controlling the duration of BCR signaling. The relative efficiency of these different processes, BCR degradation and termination of BCR signaling as well as B cell antigen presentation, will contribute towards shaping B cell response to antigens. We used bg/bg mouse in our studies where mutation in the *lyst* gene results in accumulation of giant intracellular vesicles of late endosomal / lysosomal characteristics and affects functioning of lysosomes and lysosome related vesicles in different cell types, such as, melanocytes, cytotoxic T cells, platelets and NK cells (Baca *et al.*, 1989; Bahadoran *et al.*, 2001; Burkhardt *et al.*, 1993; Paigen *et al.*, 1990). Apart from a study that reports defects in B cell antigen presentation in CHS patients (Faigle *et al.*, 1998), the human homologue for bg/bg mouse, the role of *lyst* mutation in controlling B cell responses has not been explored. Thus, in this study we tried to assess B cell signaling and antigen presentation events and their effects on different B cell responses in the bg/bg mice.

Initial studies were aimed at analyzing whether the B cell phenotype in bg/bg mice is comparable to its WT counterpart, the C57BL/6 mice. We assessed B cell proportions and absolute B cell numbers in splenocytes from bg/bg mice and found that they were significantly higher compared to those in WT mice. However, the proportions of the different mature and immature B cell subsets scored by staining for IgM and IgD on B220+ cells in the splenocytes were found to be comparable between the two groups (Figure: 1A and B). Having observed that the proportion of cells in the B cell compartments were unaffected in bg/bg mice, we then studied B cell responses following activation, using either BCR independent (LPS) or BCR dependent (anti-IgM) mechanisms of B cell stimulation. B cells respond to a commonly used mitogen, LPS (a component of the cell wall in gram negative bacteria) by undergoing activation, followed by which they enter

multiple rounds of proliferation, differentiation into plasma cells and death. LPS recognition in B cells is mediated by a complex that contains TLR4-MD2 along with RP105-MD1 receptors (Akashi *et al.*, 2000; Ogata *et al.*, 2000; Shimazu *et al.*, 1999) which signals downstream using adaptors such as MyD88 or TRAM/TRIF and induces B cell activation. Our studies attempted to compare bg/bg B cell responses to WT cells on stimulation with LPS. Following LPS stimulation, upregulation of the activation marker CD44 and MHCII (Figure: 2), proliferation (Figure: 3) and susceptibility to death (Figure: 5) were found to be comparable between WT and bg/bg B cells. Next we checked for differentiation into plasma cells, either by scoring for the frequency of the ASCs or by measuring the amounts of Ig secreted in the culture supernatent. It was found that bg/bg B cells differentiate poorly into plasma cells as compared to WT B cells (Figure: 4). It is known that ligation of TLR4 by LPS, results in internalization of this complex with subsequent degradation in the lysosomes. It has also been suggested that the pathway downstream of TLR4 that involves the MyD88 adaptor protein, is initiated at the plasma membrane while the MyD88 independent pathway involving adaptors such as TRAM and TRIF is initiated from the early endosomes following TLR4 internalization (Kagan *et al.*, 2008). Since the *lyst* mutation is known to affect late endosomal sorting of cargo (Faigle *et al.*, 1998), it might be interesting to study receptor internalization following ligation, trafficking through the endocytic compartments and the effect of endosomal sorting on regulation of receptor signaling. Since it is not feasible to track an LPS ligated TLR4 in the endosomes, we tried stimulating B cells through their surface BCR, one of the common means to activate B cells. In different experiments we used anti-IgM, anti-IgM-biotin and anti-IgM $F(ab')_2$, ligands that would polyclonally stimulate the B cells through their BCR.

BCR ligation results in the clustering of the BCRs into clathrin-coated pits and in the presence of lipid rafts and actin this ligated BCR is subsequently internalized (Stoddart *et al.*, 2005). BCR internalization following ligation was found to be equivalent in WT and bg/bg B cells (Figure: 8). Following internalization, the BCR along with its ligand, gets sorted into the different compartments of the endocytic pathway and finally reaches the MIICs. In antigen presenting cells, MIICs are specialized compartments that accumulate large amounts of MHCII and have conditions highly conducive for antigen processing and presentation (Neefjes, 1999; Stern *et al.*, 2006; Tulp *et al.*, 1994). MIICs have characteristics overlapping with late endosomal and lysosomal compartments, and one of the lysosomal features that they share is the presence of LAMP1 on their membranes (Peters *et al.*, 1991). Since there are reports of the LYST protein being associated with microtubules as well as being involved in the sorting of cargo from early endosomes to late endosomes (Faigle *et al.*, 1998), we tried to assess if the trafficking of internalized BCR complexes to LAMP1 positive compartments is affected in bg/bg B cells (Figure: 9). Our results indicated that the ligated BCR in case of the bg/bg B cells show slower kinetics of trafficking to the LAMP1 positive compartments as compared to WT B cells. For instance, within 60 min of BCR triggering, almost all the internalized BCR in WT B cells was inside the LAMP1 compartments, while in bg/bg B cells there was still a fraction that remained outside these compartments.

In an experiment performed to confirm this observation, we studied antigen presentation in the presence of inhibitors of lysosomal processing. Given the microscopy data that after an hour of BCR ligation in WT B cells most of the internalized BCR-ligand complexes are in the lysosomal compartments, inhibitors to lysosomal processing should inhibit antigen presentation much more effectively in these cells than in bg/bg B cells, where a fraction of

the BCR-ligand complex is still outside the lysosomal compartments. Three different lysosomal processing inhibitors were used for the study, E64d (a thiol protease inhibitor), Bafilomycin A1 (that specifically inhibits the action of vacuolar proteases, thereby preventing the acidification of the endocytic compartments) and NH4Cl (that increases the pH of the acidic compartments). It was observed that antigen presentation was inhibited in both WT and bg/bg B cells following inhibitor treatment, but when normalized to their respective untreated controls, the inhibition of antigen presentation was significantly higher in WT B cells (Figure: 10A). Similar results were obtained using activated B cells from WT and bg/bg mice (Figure: 10B). This reinforces the point that the internalized BCR ligand complex in bg/bg B cells traffics at a slower rate to the lysosomal compartments compared to WT B cells.

In addition to the cargo that is delivered to cells by receptor mediated endocytosis, we also tried to study the fate of antigens that are endocytosed through the pinocytic route. PECs from bg/bg mice that were given soluble fluorescein labeled antigen exhibited a delay in the degradation of the cargo as compared to PECs from WT mice (Figure: 13). We tried the same approach using B cells from the two strains of mice, but since B cells are inefficient at pinocytosis, we failed to generate interpretable data. We also used lysosomal inhibitors to study the kinetics of trafficking of pinocytic cargo by a similar mechanism as discussed earlier. In PECs, the inhibition of antigen presentation by lysosomal processing inhibitors was less in bg/bg cells as compared to WT cells (Fig. 11C), but the difference was not statistically significant. These experiments need to be repeated using both PECs and B cells in order to obtain data of statistical relevance.

As discussed earlier due to slower trafficking in the bg/bg B cells even after an hour of BCR ligation, there is a substantial fraction of the internalized BCR that has not reached the late endosomal / lysosomal compartments. It is known that loading of antigens can take place at alternative sites other than the MIICs, either on the cell surface (Arndt *et al.*, 2000; Lindner and Unanue, 1996) or in the early endosomal compartments (Pinet and Long, 1998), where the antigen can access mature recycling MHCII molecules (Pathak *et al.*, 2001). Since, antigen presentation in bg/bg B cells could possibly take place from early endosomal compartments following one hour of BCR triggering we were interested in assessing the efficiency of antigen presentation by these B cells on the recycling MHCII molecules. In order to restrict presentation to the recycling MHCII molecules, we used a protein synthesis inhibitor, cycloheximide that would block synthesis of new MHCII molecules. It was seen that in the presence of cycloheximide, there was complete abrogation of antigen presentation when the antigen was delivered through the receptor in B cells from both strains of mice (Figure: 18A). This indicates that receptor targeted antigen can be presented only on newly synthesized MHCII molecules and possibly require late endocytic compartments for processing. When we assessed presentation of pinocytically delivered antigen on recycling MHCII molecules, we found that WT B cells presented antigen on recycling MHCII molecules with a greater efficiency as compared to bg/bg B cells (Fig: 18A and B). The same was observed with presentation of maleylated antigen (Fig: 18A). Although maleylated antigen is targeted through the scavenger receptor, the conditions necessary for its presentation appears to be different from that required for presentation of BCR targeted antigen. Till date nothing much is known about any defect in the early endosomal sorting of cargo and early endosomal functioning in bg/bg B cells. Thus, in order to dissect the cause of poor presentation of maleylated antigen on recycling MHCII molecules in bg/bg B cells, it would be necessary to track the

trafficking of a maleylated antigen, such as, maleyl OVA (using an antibody directed against OVA) to the recycling endosomes (marked by Rab11) in bg/bg B cells by confocal microscopy and compare that to trafficking in WT B cells.

One possible outcome of slower trafficking of the receptor targeted antigen to lysosomal compartments in bg/bg B cells could be a delay in degradation and as a consequence a prolonged association between the BCR and its ligand. Not much has been reported about the signaling of internalized BCR from the endocytic compartments, while many other receptor-ligand complexes are known to signal from the endosomes (Bevan *et al.*, 1996; Burke *et al.*, 2001), before they get degraded in the lysosomes. However, different BCR signaling intermediates like the Syk kinase (Lankar *et al.*, 1998), as well as the phosphorylated ITAMs in the CD79a molecule are important in the sorting of BCR containing cargo to the early endosomes, while the adaptor protein BLNK (Siemasko *et al.*, 2002) and Vav (Siemasko and Clark, 2001) are important for sorting and entry of the cargo into the MIICs. It has also been reported that the presence of CD79a and CD79b are both essential for the effective sorting of the internalized cargo to the processing compartments in B cells (Siemasko *et al.*, 1999). In the light of such observations, it is plausible to argue that the internalized BCR possibly remains signaling competent even after internalization. On the basis of this hypothesis we wanted to analyze if a slower targeting of BCR to the MIICs has any consequence on the duration of signaling from the BCR in bg/bg B cells. Thus, we assessed activation of different signaling intermediates that are known to be downstream of BCR ligation. PLCγ2 is a molecule which is recruited to the signalosome complex that assembles on BLNK following BCR ligation, and subsequently undergoes activation (Kurosaki *et al.*, 2000). Activated PLCγ2 is known to act on PIP_3 leading to the generation of two secondary messengers, DAG and IP3. IP3 induces calcium flux from the

intracellular calcium stores in the cell. This is an event that happens early in the signal transduction pathway downstream of the BCR. We found that on BCR triggering, bg/bg B cells showed a prolonged activation of PLCγ2 (estimated by measuring the phosphorylated state of the protein), compared to the WT B cells (Figure: 12B). Using a fluorophore, we also measured the Ca^{2+} flux in BCR activated cells, and found that calcium flux in the bg/bg B cells was sustained for a longer period as compared to WT cells (Figure: 12A). A primary integration point of BCR signaling is the MAPK family, consisting of three members, ERK, JNK/SAPK and p38 MAPK (Kurosaki, 1999). Phosphorylation of these kinases at distinct sites leads to their activation, following which they can induce various transcription factors. When we studied the activation profile of these kinases in BCR ligated cells, we found a similar profile in WT and bg/bg B cells. However, in bg/bg B cells, the activated state of different kinases was maintained for a longer period of time compared to WT B cells (Figure: 12C). Thus, slower trafficking of the internalized antigen BCR complex to the degradative compartments in bg/bg B cells possibly results in longer association of the BCR with its ligand leading to prolonged activation of different BCR associated signaling intermediates.

Downstream signals from an engaged BCR are known to result in B cell activation which leads the cells through multiple rounds of proliferation, accompanied by differentiation into plasma cells or death. When we checked proliferation of B cells following BCR ligation (by measuring thymidine incorporation as well as by estimating CFSE dilution), we found that bg/bg B cells proliferated better than the WT B cells at lower doses of the BCR trigger (Figure: 6). Prolonged signaling from the internalized BCR in bg/bg B cells is a likely reason for greater proliferation observed in these cells. Differentiation of B cells was estimated either by staining for the plasma cell marker, CD138, or by assessing the number

of ASCs after BCR stimulation. In both assays, we found that differentiation into plasma cells was compromised in bg/bg B cells compared to WT cells (Figure: 7). Differentiation into plasma cells is brought about by modulation in the levels of several transcription factors, such as, upregulation of Blimp-1 (Shapiro-Shelef and Calame, 2005; Turner *et al.*, 1994) and IRF-4 (Shaffer *et al.*, 2002)and downregulation of Bach-2 (Ochiai *et al.*, 2006). Study of modulation of these factors following BCR stimulation might give an insight into the cause of poor differentiation observed in bg/bg B cells.

Degradation of the BCR ligand complex is also important for the generation of antigenic peptides that are subsequently loaded onto MHCII molecules and presented to T cells. Our earlier data showed that trafficking of internalized BCR to the LAMP1 positive MIIC compartments is slower in bg/bg B cells. Also, degradation of the pinocytic cargo is slower in bg/bg PECs compared to WT cells. Antigen presentation is intimately linked to the cell's ability to traffic internalized cargo successfully to sites of degradation and it has been reported that in B cells from CHS patients, peptide loading onto MHCII molecules as well as the transport of the pMHCII complexes to the cell surface is delayed (Faigle *et al.*, 1998). In order to study antigen presentation in bg/bg B cells, we initially estimated the levels of MHCII molecules on the surface of B cells from both strains of mice since it is an important factor that determines the efficiency of antigen presentation. We found that the MHCII levels were comparable between WT and bg/bg B cells (Figure: 1C). In order to study antigen presentation, B cells were given antigen through two different routes, by receptor-mediated endocytosis, involving the BCR and the SR or by pinocytosis. It was found that antigen delivered as a pinocytic cargo was presented equally well by B cells from both strains of mice (Figure: 15A). On the other hand, antigen targeted through the BCR showed slower kinetics of presentation in bg/bg B cells (Figure: 14A) for the initial

12 hr of T cell – B cell interaction, while after 24 hr the differences were no longer existent. In B cells, BCR crosslinking helps in an accelerated transport of the internalized BCR ligand complex to the MIICs, thereby accelerating the rate of degradation of this complex (Cheng *et al.*, 1999). Also, signaling from the BCR has been suggested to result in physical and biochemical modifications of the MIICs that improve the processing capability of these compartments (Siemasko *et al.*, 1998; Xu *et al.*, 1996). Under conditions of efficient antigen processing in BCR ligated cells, the defect in antigen trafficking to the degradative compartments in bg/bg B cells become evident when we check for their ability to activate T cells. But as there is only a delay in trafficking and no permanent defect, antigen presentation in bg/bg B cells catch up with the WT cells by about 24 hour of interaction with T cells. Uptake of pinocytic cargo by WT B cells, on the other hand, is not very efficient and so it takes a longer time in this case to achieve T cell activation as compared to receptor targeted antigen. This may be a reason why no difference in presentation of pinocytically delivered antigen was evident between WT and bg/bg B cells even at the early time-points of B cell – T cell interaction. When we analyzed the presentation of both pinocytically delivered antigen and receptor-targeted antigen in activated B cells, the results obtained were surprisingly different from those obtained with resting cells. LPS activated B cells from bg/bg mice showed poorer presentation irrespective of the route of antigen delivery (Figure: 16A and 17A). LPS activation of B cells is known to increase its pinocytic ability by about 10 fold as compared to resting B cells (Krieger *et al.*, 1985). LPS activation also results in increase in the levels of MHCII (Barrachina *et al.*, 1999) and different molecules involved in the process of endocytic trafficking, such as Rab7 (Bertram *et al.*, 2002). Such changes would enhance antigen presenting functions of B cells. Thus, while antigen processing and presentation would increase following LPS activation in both WT and bg/bg B cells, the defects in endosomal /

lysosomal pathway in bg/bg would make differences in antigen presentation between B cells from the two strains all the more pronounced.

In the B cell antigen presentation assays described so far we used macrophages as controls, cells which are highly efficient at antigen presentation. Also, the use of APCs other than B cells helped us derive a more general idea about antigen presentation in bg/bg mice, independent of the cell lineage. Analysis of antigen presentation by resting macrophages (BMDMs), revealed that, as in case of B cells, macrophages also show a delay in presentation of antigen targeted through the receptor (in this case scavenger receptors alone) (Figure: 14B) as opposed to pinocytically delivered antigen that is presented with equivalent efficacy by both WT as well as bg/bg cells (Figure: 15B). The results obtained using activated macrophages were similar to those observed for activated B cells, with presentation being poor in bg/bg macrophages for both receptor targeted as well as pinocytic routes of antigen delivery (Figure: 16B and 17B). These data indicate that irrespective of their lineage, the antigen presenting functions of both B cells and macrophages are affected by the *lyst* mutation.

On the basis of the two observations, first, that the internalized BCR ligand complexes signal for an extended period of time in bg/bg B cells, and second, that the receptor targeted antigen is presented with slower kinetics by bg/bg B cells, we wanted to investigate the efficiency of activation of bg/bg B cell when put in competition with WT B cells for antigen specific T cell help *in vitro*. Assessment of upregulation of CD69 on B cells at the end of 12 hr of co-culture (Figure: 19) and CFSE dilution by B cells at the end of 48 hr of co-culture (Figure: 20) with antigen specific T cells revealed that there was no difference in activation or proliferation of antigen primed B cells between the two strains of

mice. It may be possible that the effects of prolonged BCR signaling and delayed T cell help recruitment on B cell activation and proliferation, nullify one another *in vitro* and the resultant B cell responses are therefore similar between the two strains of mice. One drawback of using *in vitro* systems for studying B cell responses is that it does not take into account the complexity of different other cellular interactions that exist in a physiological scenario, such as the ability of DCs to activate T cells following immunization. In order to track B cell responses of WT and bg/bg B cells under conditions of *in vivo* competition for antigen specific T cell help, we attempted generating mixed bone marrow chimeric mice using WT and bg/bg cells. In the control group, where we had transferred only bg/bg bone marrow cells into irradiated WT recipients, the endogenous cells also repopulated along with the transferred cells giving rise to chimerism, and hence the experiment had to be abandoned. But these experiments need to be repeated to study bg/bg B cell responses under conditions of competition *in vivo*.

The lysosomal functioning defect in bg/bg B cells was found to have effects on B cell signaling, proliferation, differentiation and antigen presentation as compared to WT B cells, in the *in vitro* studies. We wanted to study if such effects observed *in vitro* translate into differences in B cell responses between the two strains of mice *in vivo* following immunization. We had seen earlier that when stimulated through the BCR, bg/bg B cells exhibit compromised differentiation *in vitro*. We confirmed this finding *in vivo* by using NP-Ficoll, a TI antigen (Maizels *et al.*, 1988), where B cells with BCRs specific for the hapten (NP) undergo differentiation into plasma cells, without involvement of T cell help. Following immunization, the levels of NP-specific IgM and IgG titers were estimated at different time intervals (Figure: 21) and the results showed that NP specific antibody levels of both isotypes were lower in bg/bg mice compared to WT mice. Thus, it appears that on

being triggered through their BCR alone, bg/bg B cells exhibit compromised plasma cell differentiation.

Next, we tried to follow B cell responses following immunization with a TD antigen, NP-CGG. When a dose of 25μg NP-CGG was used for immunization, serum antibody levels were greater in the bg/bg mice compared to WT mice. However, at a lower doses of NP-CGG, the serum antibody titers (Figure: 22A and B) were not different between WT and bg/bg mice over a period of time. We also scored the frequency of ASCs in the draining lymph nodes (Figure: 23) following immunization with a lower dose of NP-CGG and found that excepting at an early time-point (day 7), the frequencies at the subsequent time-points were not different between WT and bg/bg mice. Earlier *in vitro* as well as *in vivo* data have shown that plasma cell differentiation following BCR stimulation was compromised in bg/bg B cells compared to WT B cells (Figure: 7). Contradictory results observed using TI and TD antigens may be because, in case of TD antigens functions of different other cells, like the ability of DCs to stimulate T cell and the efficiency of activated T cells to interact with antigen primed B cells, would also influence B cell differentiation. We also assessed affinity maturation of the antibody response and found that the affinity of the NP-specific antibody increased over time as was expected. However, in the group immunized with a higher dose, affinity maturation was not different between the two strains of mice, but at the lower dose, the affinity maturation was faster in bg/bg mice compared to WT mice (Figure: 22C and D).

Another outcome of antigenic stimulation of B cells is the generation of memory, a hallmark of the adaptive immune response that helps in defence against subsequent challenges with the same antigen (Arpin *et al.*, 1995). The features commonly used for

distinguishing memory B cells are the presence of isotype switched BCRs, mutated BCRs and the ability of these cells to rapidly differentiate into plasma cells following stimulation. We used LDA to score for the frequency of re-stimulable memory B cells in the draining lymph nodes of immunized mice. At early time points the frequency of memory B cells was found to be lower in bg/bg cells than in WT cells, although by about a month the frequency of memory B cells were almost comparable in the draining lymph nodes of both mouse strains (Fig: 24). In LDA, we did not account for the B cell frequencies in the draining lymph node cells, and hence the estimate of memory B cell frequency was inaccurate. To assess memory responses, we next performed an adoptive transfer experiment, in which antigen primed B cells from WT and bg/bg mice were transferred into irradiated carrier primed WT recipients. Following transfer the recipients were challenged and antibody response was scored on day 4. As compared to LDA, in this case a single time-point data was sufficient to give us an estimate of the memory response. It was found that the response of bg/bg B cells to antigenic challenge was much better than that of WT B cells in two independent experiments (Fig: 25). We also scored for the frequency of antigen specific ASCs in the spleen (draining organ) following challenge and found that the frequency of ASCs were higher in the spleen of bg/bg mice compared to that in WT mice 5 days following challenge (Fig. 26B and C). Thus, these data taken together indicate that memory responses in bg/bg mice following antigenic challenge is better than that of WT mice.

T cell help also induces B cell differentiation into long-lived plasma cells that accumulate in the bone marrow and are maintained for long periods of time in that niche with the help of signals received from the stromal cells (Cassese *et al.*, 2003; Radbruch *et al.*, 2006). It has been shown that the proportion of the long lived plasma cells increase in the bone

marrow upon antigen re-encounter (Benner *et al.*, 1981). The proportion of plasma cells peak in the draining organ after challenge between days 3 and 5 and then gradually declines. In the bone marrow on the other hand, the proportions peak at day 5 following challenge and remain constant over an extended period of time. So, we tried to score for the accumulation of antigen specific long lived plasma cells in the bone marrow at an early time point and a late time point upon secondary immunization. After 5 days of challenge, the frequency of ASCs were equivalent in WT and bg/bg bone marrow cells, however, after two months following challenge there was a significantly greater proportion of plasma cells in the bone marrow of bg/bg mice as compared to WT mice (Fig: 26B and C), which indicated that there is greater accumulation of long lived plasma cells in the bone marrow of bg/bg mice over time.

Thus, following immunization with low doses of TD antigen in bg/bg mice, we find that plasma cell proportions are equivalent to the WT mice except at an early time-point, affinity maturation is faster, memory cell generation is greater and the accumulation of long-lived plasma cells are significantly higher than that in WT mice. The two vital factors that are important in modulating the final outcome of B cell responses are the strength and duration of the BCR signal and the ability of B cells to recruit T cell help. Since the formation of T cell – B cell conjugates under physiological conditions can take few days (Garside *et al.*, 1998) and the delay in the ability of bg/bg B cells to recruit T cell help was seen only during the initial few hours *in vitro*, it may be possible that, the *in vivo* B cell responses that we observe are largely due to the effects of prolonged signaling from the BCR in bg/bg B cells. Therefore, in order to assess contributions of both the factors, T cell help and BCR signaling, in regulating B cell responses *in vivo*, future studies require generation of mixed bone marrow chimeric mice. Under such conditions where B6 and

bg/bg cells would be in competition for antigen as well as for T cell help, the relative contributions of BCR signaling and T cell help recruitment in modulating different B cell responses *in vivo* would possibly become more evident.

This study provides an insight into B cell responses in the bg/bg mice, a system where the endosomal trafficking and lysosomal functionality are compromised. Initially, we assessed the ability of bg/bg B cells to respond to a commonly used B cell mitogen LPS, and found that activation, proliferation and death were similar to that of WT B cells. However, differentiation into plasma cells was compromised. Next we ligated BCR on the surface of B cells, in order to track the fate of a receptor targeted antigen in bg/bg B cells following internalization and found that they traffic slowly to the LAMP1 positive compartments. Slower trafficking was found to have two consequences, first, the internalized BCR ligand complex continued to signal for a longer duration of time in bg/bg B cells resulting in better proliferation as seen at lower doses of stimulation and also a compromise in plasma cell differentiation, and second, a delay in presentation of BCR targeted antigen. This defect in presentation of receptor targeted antigen was found to be lineage independent as it was also observed in bg/bg macrophages. Presentation of pinocytic antigen was, however, similar in B cells and macrophages from the two strains of mice indicating that the trafficking of pinosomes, and of endosomes following receptor-mediated uptake, have differential susceptibility to the bg/bg defect.

At a low dose of primary immunization, plasma cell generation was unaffected except at an early time-point and affinity maturation was found to be more rapid. Accumulation of long lived plasma cells and memory response following challenge were better in bg/bg mice compared to WT mice. Since the delay in T cell help recruitment observed *in vitro* was only during the initial hours of T cell – B cell contact, one needs to study *in vivo* B cell responses in a competition scenario using mixed bone marrow chimeric mice to successfully analyze the relative contributions of BCR signaling and T cell help recruitment in modulating different B cell responses.

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