Regulatory roles of PI3Ks and PH domain-containing adaptor protein Bam32 in humoral immune responses

By

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Thesis Abstract

PI3Ks (phosphoinositide 3-kinases), a family of enzymes expressed in immune cells, are activated in response to a wide variety of stimuli by generating second lipid messengers. A subset of singnaling molecules containing lipid-binding pleckstrin homology (PH) domains are downstream molecules of PI3K signaling pathway, essential to mediate the functional outcomes of PI3Ks. Bam32 / DAPP1 is a PH domain-containing adaptor protein, which was discovered from human tonsil germinal centers (GCs); however, its biological function related to GCs, where efficient T-cell-dependent (TD) antibody responses are generated, is unknown. This thesis is focused on the effect of genetic or pharmacological blockade of PI3K p110δ activity on T and B cells, and the role of Bam32 in GC responses.

Type 2 cytokine responses are significantly decreased in p110δ–inactivated mice, whereas Type 1 cytokine responses are increased or comparable after primary and secondary immunization. Hallmarks of asthma, airway inflammation and respiratory hyper-responsiveness are dramatically reduced in those mice. Adoptive transfer of OVA-primed splenocytes from normal, but not p110δ-inactivated mice could induce airway eosinophilia in naïve, airway-challenged recipient mice. These data demonstrate a novel functional role for p110δ signaling in induction of Type 2 responses in vivo and may offer a new therapeutic target for Th2-mediated airway disease.

Paradoxically, serum IgE levels are markedly increased in OVA-immunized p110δ-inactivated mice despite lower level of swich factor IL-4. In vitro studies showed that p110δ is required to restrain IgE class switch recombination in a B-cell intrinsic manner. Blockade of PI3K activity using broad-spectrum PI3K inhibitors PIK-90 and PI-103 generates similar results. In vivo administration of p110δ–selective inhibitor IC87114 into OVA-immunized mice results in selective elevation of antigen-specific IgE production. Disruption of p110δ signaling leads to increased germline transcription at the epsilon locus (εGLT) and increased induction of activation induced cytidine deaminase (AID) enzyme, suggesting deregulation at the level of the isotype switch process. Moreover, p110δ signaling selectively regulates the expression level of transcription factor Bcl6 and IRF4, which may be responsible for the regulation of AID and εGLT.

PI3K signaling regulates multiple steps of GC development, and Bam32 may be involved. GCs dissipate prematurely in Bam32-deficient mice after immunization with OVA/alum. In vitro, Bam32-deficient B cells are functional competent in proliferation, chemotaxis, isotype switching and plasma cell differentiation in response to signals present in GCs. In vivo, Bam32-deficient GC B cells proliferate normally; however, they are more apoptotic. Adoptive transfer studies indicated that intrinsic defect of $Bam32^{-/-}$ B cells leads to premature GC dissolution. Additionally, GCs formed by $Bam32^{-/-}$ B cells contain fewer T cells, implying that Bam32 is required for B cell-dependant T cell accumulation within established GCs. Treatment of $Bam32^{-/-}$ mice with agonistic anti-CD40 fully restored GC persistence and IgG1 isotype switching, demonstrating that

Bam32-deficient GC B cells are functionally competent when access to cognate signals is not limiting. Collectively, those data demonstrate that Bam32 is not required for GC initiation, but rather functions in a late checkpoint of GC progression associated with T cell recruitment and GC B cell survival.

In general, by focusing on PI3K p110δ and its downstream adaptor protein Bam32, my studies clearly indicate that p110δ is a potential therapeutic target for the treatment of Th2-induced airway inflammation. The unexpected immunomodulatory acitivity on IgE switching associated with multiple PI3K inhibitor compounds is first discovered in this thesis, suggesting that more need to be investigated in this aspect before those inhibitor compounds are widely used in the clinic. Furthermore, the specific regulatory role of Bam32 in GCs represents a unique model for us to study the late GC checkpoint in regarding to in vivo GC B cell and T cell interaction, which is an important issue need to be clarified in order to fully understand GC responses.

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Abbreviations

ADCC	Antibody dependent cell-mediated cytotoxicity
AID	Activation induced cytidine deaminase
APCs	Antigen presentation cells
AP-1	Activator protein 1
Bam32	B-lymphocyte adaptor molecule of 32 kDa
BCAP	B-cell PI3K adaptor protein
BCL-6	B cell lymphoma 6
B-CLL	B-cell-derived chronic lymphocytic leukemia
BCRs	B cell receptors
BHR	Bronchial hyper-responsiveness
BLIMP1	B-lymphocyte-induced maturation protein 1
BLNK	B cell linker
BrdU	Bromodeoxyuridine
Btk	Bruton tyrosine kinase
C/EBO	CCAAT/enhancer binding protein
CFSE	Carboxyfluorescein diacetate succinimidyl ester
C_H	Heavy chain constant
CSR	Class switch recombination
DAG	Diacylglycreol
DAPP1	Dual-adaptor for phosphotyrosine and 3-phosphoinositides 1
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FDCs	Follicular dendritic cells
FO	Follicular
FOXO	Forkhead Box O
Gabs	Grb2-associated binder adaptor/scaffolding proteins
GC	Germinal center

GEFs	Guanine nucleotide exchange factors
GPCR	G-protein-coupled receptors
GLTs	Germline transcripts
GTPase	Guanosine triphosphatase
HLH	Helix-loop-helix
H_2O_2	Hydrogen peroxide
ICs	Immune complexes
ICOS	Inducible T cell co-stimulator
Ig	Immunoglobulin
IKK	IκB kinase
IP3	Inositol(1,4,5)-trisphosphate
IP3Rs	IP3 receptors
IPEX	Immune dysregulation, polyendocrine enteropathy, X linked syndrome
IRF4	Interferon regulatory factor 4
IRS	Insulin receptor substrate
LPS	Lipopolysaccharide
MALTs	Mucosa-associated lymphoid tissues
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic peptide-1
mLN	Mesenteric lymph nodes
MHC	Major histocompatibility complex
mTORC2	Mammalian target of rapamycin complex-2
MZ	Marginal zone
NFAT	Nuclear factor of activated T cells
NK	Natural killer
PAE	Porcine aortic endothelial
Pax5	Paired-box protein 5
PD1	Programmed cell death 1
PDGF	Platelet-derived growth factor

PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI	Phosphorylate phosphatidylinositol
PI3Ks	Phosphoinositide 3-kinases
PI(3)P	Phosphatidylinositol-3-phosphate
PI(3,4)P2	Phosphatidylinositol-3,4-biphosphate
PI(3,4,5)P3	Phosphatidylinositol-3,4,5-tris-phosphate
PI(4,5)P2)	Phosphatidylinositol 4,5-bisphophate
PIP5Ks	Phosphatidylinositol-4-phosphate 5-kinases
PKA/B/C	Protein kinase A/B/C
PNA	Peanut agglutinin
PRAS40	Proline-rich Akt substrate of 40 kDa
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
PX	Phox homology
RAG	Recombination-activating gene
RIAM1	Rap1-GTP interacting adaptor molecule
SKAP	Src kinase- associated phosphoprotein
SH2	Src homology 2
STAT6	Signal transducer and activator of transcription 6
TAPP	Tandem pleckstrin homology domain protein
TD	T cell-dependent antigens
T_{FH}	T follicular helper cells
TI-1 antigens	T cell-independent antigens type 1
TI-2 antigens	T cell-independent antigens type 2
TLR4	Toll-like receptor 4
TSC2	Tuberous sclerosis complex 2
VASP	Vasodilator-stimulated phosphoprotein
WASP	Wiskott-Aldrich syndrome protein

Chapter 1 General Introduction

1-1 Humoral immunity

The immune system has evolved to specifically recognize and fight against non-self antigens through T and B lymphocytes. T and B lymphocytes are the major players involved in cellular immunity and humoral immunity respectively. Here, we will focus on the humoral immunity, which is mediated by the antibodies produced from B cells. Antibodies have several important functions in vivo related to their capabilities of specifically binding to antigens, interacting with Fc receptors expressed on various cell types, and activating the complement system. Thus, antibodies can neutralize toxins; they can coat and opsonize invading pathogens to facilitate subsequent phagocytosis; they can bind to Fc receptors expressed on macrophages, neutrophils or natural killer (NK) cells to induce antibody dependent cell-mediated cytotoxicity (ADCC); antibodies of IgM and IgG isotypes can potently activate complement via a classical pathway, leading to an osmotic death of antibody-coated pathogens; antibodies of IgE isotype can activate eosinophils via FcεR, resulting in expulsion of parasites [1]. Therefore, possessing all the capacities mentioned above, pathogen-specific antibodies can rapidly fight against and clear invading pathogens.

An antibody molecule, also known as immunoglobulin, is composed of two identical heavy chains and two identical light chains, which are termed based on their molecular weights. They form a roughly Y-shape molecule, containing variable or V regions located at the two arms of the Y end, and constant or C region located at the stem of the

Y. V region was so named because it varies between different antibody molecules, and it determines antigen-binding specificity; whereas only a few variations are observed in C region, and based on those differences five classes of immunoglobulins, IgM, IgD, IgG, IgA and IgE, were categorized as isotypes [2]. Before secretion, antibodies are expressed on B cell surface as B cell receptors (BCRs) with a carboxy terminal hydrophobic membrane-anchoring sequence. On mature naïve B cells, antigen-specific mIgM and mIgD are expressed as BCRs, associated with transmembrane antigen-nonspecific Igα and Igβ to transmit signals, and BCRs of each B cell bear the same antigen specificity [2]. An individual possesses heterogeneous B cells expressing a vast diversity of BCRs, ready to recognize almost any invading foreign antigen. When the individual encounters a foreign antigen, only a small population of B cells specifically recognizing the antigen is activated, and then they will proliferate rapidly, undergoing a process termed "clonal expansion". As a result, those B cells will differentiate into antibody-producing plasma cells or they will become memory cells which react immediately upon antigen recall [3].

1.1.1 T cell-independent antibody responses

Some antigens can induce B cells to produce antibodies in the absence of T cells. Those antigens are termed as T cell-independent antigens, and they are further subdivided into type 1 and type 2. T cell-independent antigens type 1 (TI-1 antigens) can activate polyclonal B cells expressing BCRs with different antigen specificity, as exemplified by lipopolysaccharide (LPS) from Gram-negative bacteria, and highly organized and repetitive viral protein coats. However, in vivo only high dose of LPS can activate polyclonal B cells via pattern recognition receptor (PRR) Toll-like receptor 4 (TLR4),

and the antibodies generated are not antigen-specific [4]. At low dose, both BCR and TLR4 need to be activated to give rise to antigen-specific antibody responses, which is typically modeled experimentally using BCR ligands coupled to LPS [5].

In contrast to TI-1 antigens, T cell-independent antigens type 2 (TI-2 antigens) contain multiple epitopes, which are parts of antigens recognized by BCRs. Upon antigen recognition, extensive crosslinking of BCRs triggers activation of B cells. Different from TI-1 antigens, the full activation of B cells by TI-2 antigens needs secondary signals, which can be provided through nonspecific interaction with T cells, NK cells and possible other innate immune cells [6]. The typical TI-2 antigens are bacterial polysaccharide, Dextran, and hapten (small non-immunogenic molecule) -conjugated Ficoll.

Specialized mature B cell subtypes, peritoneal B1 cells (CD5+ B cells) and marginal zone B cells (located at the border of the splenic white pulp), are the predominant responders of TI antigens [7, 8]. TI-1–reponding cells produce IgM mainly, whereas TI-2–reponding cells produce IgM and IgG3. Cytokines, such as IFN-γ and BAFF, secreted by dendritic cells or NK cells, may promote the IgG3 isotype switching from IgM [9].

1.1.2 T cell-dependent antibody responses

Antibody responses against non-self protein antigens need the help provided from T cells that recognizing the same antigen. In T cell-deficient mice, protein antigens cannot induce B cells to produce antigen-specific antibodies [10]. Therefore, protein antigens

are known as T cell-dependent antigens (TD antigens). Similar to TI-2 antigens, full activation of naïve B cells needs accessory signals in addition to TD antigen-triggered BCR crosslinking, but those accessory signals are provided by antigen-specific T cells instead of bystander cells or through massive crosslinking. In the absence of accessory signals, the antigen-specific B cells will become anergic, unable to be activated.

As illustrated in figure 1.1, in order to obtain T cell help, B cells act as antigen-specific antigen presentation cells (APCs) first. After binding to specific antigens, BCRs trigger a signaling cascade, leading to internalization of BCR-antigen complexes. Consequently, empty BCRs will be re-expressed on the cell surface, whereas antigens will be degraded into peptides and loaded onto major histocompatibility complex (MHC) II molecules via endosomal / lysosomal pathway [11, 12]. T cells can recognize the peptide:MHC II complexes expressed on B cells via TCRs in an antigen-specific and MHC-restricted manner, which is termed cognate recognition. Interaction between antigen-specific T and B cells is further stabilized by pairs of co-receptors expressed on T and B cells, such as ligation between CD28 (expressed on T cells) and CD80/CD86 (expressed on B cells) [13, 14]. Signals provided by TCRs and co-receptors trigger T cells to produce membrane-bound or secreted molecules, acting as the accessory signals to stimulate recognized B cells. CD40 ligand, one critical membrane-bound molecule of T cells, binds to CD40 on B cells; facilitates B cells entering cell cycle; upregulates co-receptor expression; and promotes B cell survival [15]. Secreted cytokines determine which type of antibody isotype to be produced. For example, in the presence of IL-4, IgM+ B cells preferentially switch to IgG1 and IgE-producing cells [16].

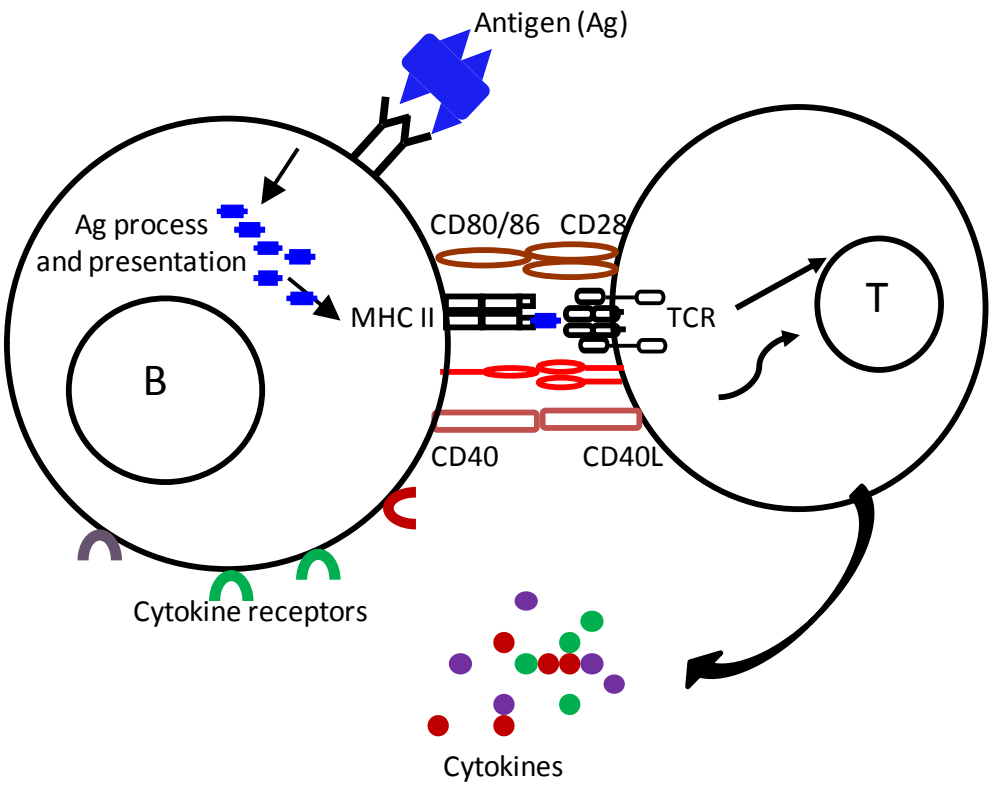

Figure 1. 1 Diagram of cognate T and B cell interaction

With the help provided by cognate T cells, TD antibody responses, distinct from TI antibody responses, are able to generate antibodies of high affinity via somatic hypermutation, a process that point mutations are randomly introduced into the V-regions of immunoglobulin genes at very high rate, and affinity maturation, which allows the survival of B cells with high affinity BCRs; Also, accessory signals enable B cells to express antibodies possessing the same antigen-specific V-regions associated with C-regions of IgG, IgA or IgE, a process known as class switch recombination (CSR) [2]. In addition, TD antibody responses lead to the generation of memory B cells, which are responsible for the rapid re-call responses against re-entered antigens. All those characteristics of TD antibody responses mentioned above will be explained further in the following paragraphs.

1.1.3 Germinal centers

Germinal center (GC) is a specialized anatomical structure observed in the secondary lymphoid organs, including spleen, lymph nodes, and mucosa-associated lymphoid tissues (MALTs) [17]. The GC is known to be the place where efficient T-dependent antibody responses happen: BCRs expressed on B cells undergo somatic hypermutation, affinity maturation and isotype switch recombination, and selected B cells finally differentiate into memory B cells and long-lived plasma cells. Because of the important role of GCs in humoral immunity, new technology has recently been developed to investigate how GCs are regulated in vivo, and our concepts on the GC response have been improved.

1.1.3.1 Basic model of GC development

The basic model of GC development is illustrated in figure 1.2 [18]. Once non-self protein antigen is introduced in vivo, CD4 T cells in the T cell zones of peripheral lymphoid organ get activated via interaction with antigen presenting cells (APCs). Meanwhile, B cells encounter the antigen in the follicle, and then they will migrate to the border of T and B cell zones, where B cells interact with cognate T cells to receive help. After fully activation, the B cells can quickly become either IgM-producing plasmablasts, or seeders of GCs. Those B cells destined to be the precursors of GCs migrate back to a follicle, and reside close to follicular dendritic cells (FDCs), bearing immune complexes (ICs) containing the specific antigen [18-20]. The B cells acquire unique GC B cell phenotype by losing surface IgD expression, and becoming peanut agglutinin (PNA) and activation antigen GL7 positive. During the GC initiation stage, GC B cells proliferate

quickly within the FDC network. Then, GCs mature and become segregated by two compartments, light and dark zones, which are named based on the histological observation. The light zone is mainly composed of FDCs, GC B cells and GC T cells. The dark zone has been proposed to extend out of the dense FDC network, where GC B cells undergo rapid proliferation [21]. Recent studies showed that GC B cells in both light and dark zones are proliferative, and they can travel between two zones by upregulating and/or downregulating cell surface chemokine receptors [21-23].

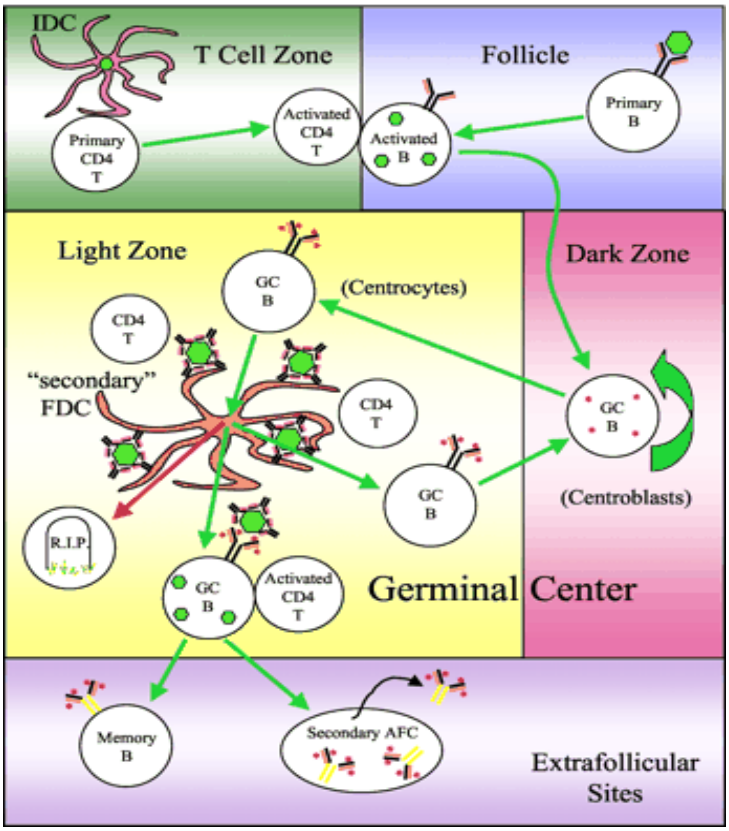

Figure 1. 2 Basic model of GC responses [18]
Green arrows indicate cell migration routes, and red arrows indicate negative selection-induced cell death by apoptosis (R.I.P). IDC is abbreviation of interdigitating DC, AFC is for antibody-forming cells.

1.1.3.2 Functional outcomes of GCs

GC response is a necessary step for the establishment of productive humoral immunity, which means that high affinity antigen-specific antibodies of switched isotypes are generated at significant levels, and the antibody responses can be induced more quickly and more abundantly upon the secondary challenge of the same antigen. Enzymes essential for immunoglobulin hypermutation and class switch recombination are activated in GC B cells [24, 25]; therefore, GC B cells can vary their BCR isotypes and binding affinities to the antigen. Antibody affinity maturation happens within GCs since GC B cells need to compete for the limited antigens trapped on FDCs, and also compete for the subsequent interaction with cognate GC T cells to obtain survival signals, especially CD40 ligands [26, 27]. GC B cells expressing BCRs with low affinity have disadvantages to compete for antigen binding will undergo apoptosis, and then the dead cells are phagocytosed by nearby macrophages [23]. GC is a specialized organization also required for the differentiation of class-switched plasmablasts and memory B cells, and they will migrate out of GCs after becoming plasmablasts and memory B cells [28]. Plasmablasts migrate to the bone marrow to become long-living antibody-producing plasma cells [29], whereas blood-born memory B cells have the strong propensity to migrate back to the follicles of secondary lymphoid organs, ready for the rapid responses of the antigen re-visit [28, 30].

1.1.3.3 Kinetics of GC responses

A typical GC response can be readily divided into 4 stages. The first stage is the initiation stage: GC seeding cells migrate into FDC network. The second stage is the

early GC stage: GC B cells proliferate within FDC zone and expand to non-FDC zone. The third stage is the mature GC stage: distinct GC dark and light zones are segregated. And the fourth stage is the GC dissolution stage: the dark zone disappears. However, the kinetics of GC responses varies depending on the type of antigen received. For example, the initiation of GCs takes about 4 to 6 days in mice after immunization of hapten-coupled TD antigens. Early GCs appear from day 7 to 9, and GCs mature and reach peaks by days 10 to 14. Later on, the GCs slowly dissipate [31]. In contrast, when mice are immunized with sheep red blood cells (SRBCs), which can induce robust polyclonal GC responses in the absence of adjuvant [32], GC responses enter to the early GC stage at day 4 post-immunization and reach the peak response by day 6 to 8, and then they become to decay, which lasts for almost 40 days until only residual GC B cells are able to be detected [33]. Based on a mathematical model, antigen doses have negligible effect on GC kinetics; however, the antigen dose and the half life of antigen-antibody complexes, also called immune complexes, deposited on FDCs are correlated with the GC size [34]. It is still debatable whether the availability of antigens or the help provided by GC T cells determines the duration of GC responses [34, 35], and my study in this thesis emphasizes the important role of GC T cells in GC progression.

1.1.3.4 The role of FDCs in GC responses

The relationship between the FDC network and initiation and maturation of GCs suggests the important role of FDCs in GC development. In support of this idea, lymphotoxin α-deficient mice, absent of FDC organization, showed no GC responses [36]. However, there are contradictory findings implying that FDC network is not pivotal for the GC

reaction. For example, although FDC organization is also absent in lymphotoxin β-deficient mice, GCs can still be observed in the mesenteric lymph nodes (mLN) [37]. However, GC responses in those mice terminate immaturely [38], indicating the requirement of FDCs for a normal GC response.

Up to now, it is still not clear about the molecular basis of the interaction between FDCs and B cells. This interaction may rely on the capabilities of FDCs trapping immune complexes and secreting chemokines. FDCs express abundant Fc receptors and complement receptors, which efficiently capture antigens in the form of immune complex. A widely held paradigm is that GC B cells compete for tight interaction with FDCs based on BCR affinity; however, recent findings have challenged this idea. In mice unable to form immune complex or deficient in complement receptors antigen is undetectable on FDCs; however, GC formation and antibody affinity maturation can still occur [39]. The direct evidence comes from in vivo imaging studies, which demonstrated that GC B cells migrate within FDC network rapidly without prolonged cell-cell contact, suggesting GC B cells can capture antigen in a rapid way [23]. Furthermore, FDCs secrete chemokine CXCL12 (SDF-1) and CXCL13 (BLC), ligands of CXCR4 and CXCR5 respectively [40]. GC B cells express CXCR5 and different levels of CXCR4, CXCL13+ FDCs have been proposed to attract CXCR5+ $CXCR4^{low}$ GC B cells into the light zone [22].

1.1.3.5 The role of GC T cells in GC responses

It is well known that CD4 T cells play important role in the processes of GC responses. CD4 T cells are required for antibody somatic hypermutation and the generation of

memory B cells [41, 42]. TI-2 antigens can induce follicular GCs in the T cell –sufficient or –deficient mice [43, 44]. However, those GCs formed in the absence of T cells are short lived and fail to give rise to affinity matured antibody. Signals provided by CD4 T cells via cell surface molecule, such as CD40L, CD28, OX40 and ICOS (inducible T cell co-stimulator), or secreted cytokines, including IL-4 and IL-21, have been demonstrated to be very important [27]. Recently, GC T cells have been described as a specified CD4 T cell subset, termed T follicular helper cells (T_{FH} cells). T_{FH} cells differentiation is driven by the transcription factor BCL6 (B cell lymphoma 6), and are characterized to express high levels of ICOS, PD1 (programmed cell death 1), and CXCR5 [45].

Recent advances in real-time imaging of GCs have shed light on the understanding of in vivo B:T interaction within GCs [23, 46]. All together, those studies suggest that GC B cells compete to form functional conjugates with antigen-specific GC T cells, which are less abundant in GCs. In GCs, T and B cells migrate rapidly and randomly, however T cells migrate more rapidly. Cognate interactions between GC T and B cells are able to reduce the T cell velocity to match that of B cells [23]. A recent study suggested that T:B interactions are required to recruit and /or retain CD4 T cells within GCs [46].

1.1.4 Antibody class switch recombination (CSR) and somatic hypermutation

CSR and somatic hypermutation of antibodies occur in the GCs. CSR, also known as isotype switching, is a region-specific DNA recombination mechanism by replacing the currently expressed immunoglobulin (Ig) heavy chain constant (C_H) region gene with one

downstream C_H gene. Thus, after CSR the Igs maintain the same antigen specificity; however, they acquire different effector properties. The process of CSR can be readily divided into three steps: targeting, cleavage, and DNA repair [47]. Switch (S) regions, highly repetitive sequences upstream of each C_H gene except Cδ, need to be targeted first to initiate CSR. The accessibility of S region is regulated by the sterile germline transcripts (GLTs), which are induced by cytokines [48, 49]. Then, DNA lesions are introduced into the targeted S regions leading to DNA cleavage. Lastly, the cleaved ends are rejoined by DNA repair machinery, looping out a circular DNA containing segment between Sμ and the targeted S region.

Somatic hypermutation is a transcriptionally regulated event, characterized by introducing point mutations into Ig variable region genes. The random nature of somatic hypermutation means most mutations will not increase affinity, thus selection process is critically important for allowing the generation of dominating clones with high affinity BCRs in each GC [50]. GC B cells are intrinsically hyper-apoptotic because of DNA mutations, and they express various apoptosis-inducing genes [51]. Some mutations are deleterious, leading to abrogation of cell surface BCR expression, and subsequently the B cells will undergo immediate apoptosis [52]. Most mutations result in expression of BCRs with low or intermediate affinity for antigens, leading to disadvantages of B cells to obtain limited antigens trapped on FDCs and to interact with cognate T cells. Therefore, without sufficient BCR signaling and survival signals provided by T cells, those GC B cells will also be eliminated by apoptosis. Very few mutations generate high affinity BCRs, and only B cells with high affinity BCRs can successfully capture antigens,

and present antigens to cognate T cells to receive survival signals that rescue them from the fate of apoptosis. It has been demonstrated that activation induced cytidine deaminase (AID) enzyme, which deaminates cytosines in particular DNA regions, is required in both CSR and somatic hypermutation [24, 25, 53]. AID is specifically upregulated in GC B cells [24], and its deletion leads to blockage of CSR and somatic hypermutation.

1.1.5 Humoral memory

One hallmark of the GC responses is to generate humoral memory, which is persistent antibody production after T cell-dependent antigen exposure. Humoral memory is accomplished by generating the pools of memory B cells and plasma cells. Memory B cells possess different cell surface markers from naïve B cells, and they mount faster, larger and qualitatively different secondary antibody responses [54]. CD27 has been used as a memory marker for human memory B cells [55, 56]. However, there are no commonly used specific markers for murine memory B cells, which are usually detected based on antigen specificity and switched isotype without GC-specific markers [57, 58]. Furthermore, recent studies suggested that murine memory B cells can be divided into mutated and unmutated subsets by the expression of CD80 and CD35 [59].

Plasma cells are responsible for long persistent antibody titers after infection. It was proposed that plasma cells are short-lived, and the pool of plasma cells is maintained by continuously differentiation of memory B cells into plasma cells [60, 61]. However, bromodeoxyuridine (BrdU) labeling experiments demonstrated that BM plasma cells are

long-lived [62]. Also, recent studies showed that the plasma cell pool is independent of memory B cells by using αCD20 antibody or irradiation to delete memory B cells [63, 64]. The decision made for GC B cells to differentiate into plasma cells is orchestrated by a set of transcription factors. And interestingly, transcription factors required for the maintenance of GC B cells and plasma cells are usually cross-inhibitory. For example, BLIMP1 (B-lymphocyte-induced maturation protein 1) is a master regulator for plasma cell differentiation; however it inhibits the induction of genes that are critical for GC B cell development, such as BCL6 (B-cell lymphoma 6), PAX5 and AID. Therefore, the differentiation from GC B cells to plasma cells initiated by BLIMP1 is irreversible [65]. In contrast, BCL6, which is required for GC formation and is expressed at high level by GC B cells [66, 67], directly represses the expression of BLIMP1 [68], preventing the premature plasmacytic differentiation.

1-2 IgE and its relationship with allergic diseases and asthma

IgE isotype is tightly regulated for its production because IgE plays not only beneficial roles in immunity against parasitic infection, but also notorious roles in the pathogenesis of allergic diseases and asthma. In normal individuals, serum IgE is maintained at a low level compared to other isotypes. IgE levels are increased in individuals with parasitic diseases, such as helminth infection [69]; atopic conditions, such as asthma, allergic rhinitis and atopic dermatitis [70]; and rare genetic disorders, such as Wiskott-Aldrich syndrome, hyper-IgE syndrome and IPEX (immune dysregulation, polyendocrine enteropathy, X linked syndrome) [71-73]. The incidence and health concern of allergic diseases and asthma are ever-growing in industrialized countries, and allergen-specific

IgE has been considered as a hallmark of allergic diseases, and the following paragraphs will be focused on the aspects of regulation of IgE production, and the role of IgE in allergic diseases and asthma.

1.2.1 IgE CSR

T cell-derived signals, such as CD40 and IL-4Rα signaling, drive the germline transcription of immunoglobulin gene Cε encoding heavy chain of IgE isoform, leading to the production of "sterile" Cε germline transcripts (εGLTs), which are essential for the induction of IgE CSR. They also induce the expression of AID enzyme, which is required for the general CSR, and of cause here for IgE CSR.

Production of εGLTs is controlled by the promoter of Iε exon (Figure 1.3), which contains binding sites of many transcription factors, such as STAT6 (signal transducer and activator of transcription 6), Bcl6, NF-κB, PU.1, PAX5 (paired-box protein 5), C/EBO (CCAAT/enhancer binding protein), AP1 (activator protein 1), and E-box binding sites [74]. All those transcription factors promote the transcription of εGLTs except BCL6, which is a negative regulator competing with STAT6 for the same binding site [75]. T_H2 cytokines IL-4 and IL-13 induce the transcription of εGLTs via the activation of STAT6 [76]; whereas CD40L expressed on T cells facilitates εGLTs transcription in B cells through inducing NFκB nuclear translocation, which can synergize with STAT6 to promote εGLT induction [77]. In the 5' upstream region of AID gene, there are binding sites for transcription factors, including STAT6, NFκB, PAX5 and E47 [78-80]. Similarly, synergy between STAT6 and NFκB also applies to the induction of AID [78]. Collectively, IgE-switching in B cells is favored in the immune responses of T_H2 type.

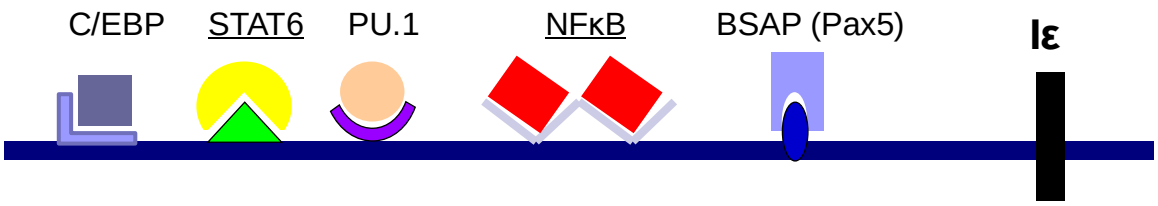

Figure 1. 3 Transcription factors that bind to the promoter regions of epsilon germline transcript

IgE-switched cells can be derived directly from IgM+ cells or indirectly from IgG1+ cells by sequentially CSR [81]. Both are accomplished at DNA level by DNA recombination to form a hybrid between Sε and Sμ or Sμ/γ first. Then, the DNA sequence spanning from Sμ or Sμ/γ to Sε is excised out, leaving VDJ adjacent to the Cε sequence. Finally, the ligation between the rest Sμ and Sε gives rise to the full length and functional IgE heavy chain [82].

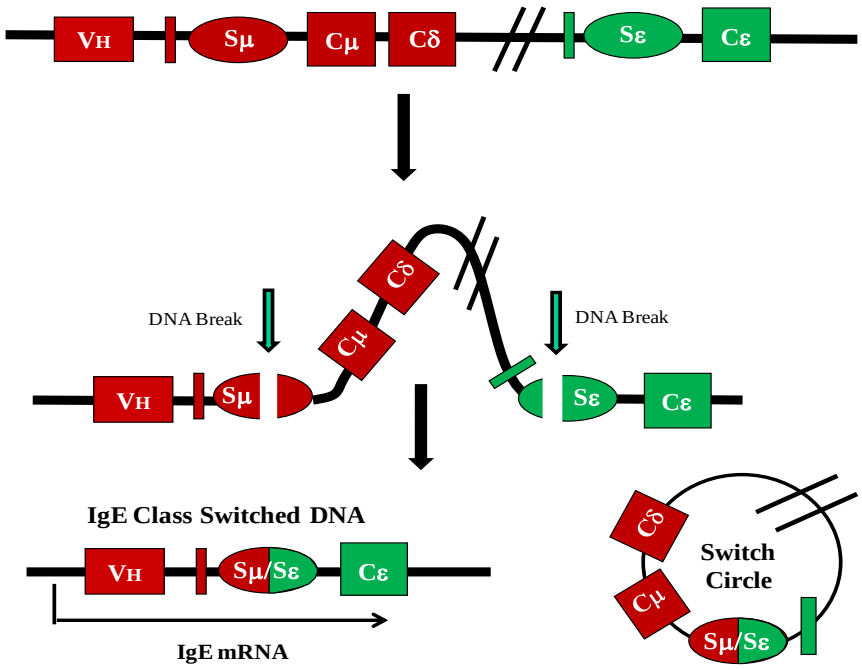

Figure 1. 4 Diagram of IgE switch recombination

1.2.2 Central role of IgE in type I hypersensitivity

IgE is critical for type I hypersensitivity. Serum IgE is bound mostly by high affinity receptor FcεRI, which is expressed on mast cells, basophils and activated eosinophils [83]. Mast cell is the main cell type orchestrating allergic reactions. These cells reside at significant numbers in mucosal, epithelial and subendothelial connective tissues [83], and IgE bound on mast cells persists for longer time compared to the short half life of serum IgE [84]. Re-exposure of allergen causes crosslinking of mast cell-bound allergen-specific IgE, leading to mast cell activation and degranulation, a process to release granules. Inflammatory mediators, lipid mediators, cytokines and chemokines are released locally to induce the rapid clinical responses [85]. In addition, released cytokines and chemokines attract eosinophils and lymphocytes, resulting in late phase allergic responses [86]. If these mediators are released in the airway, they will give rise to clinical onsets of allergic asthma, including bronchial mucosa edema, mucus production and smooth muscle constriction, followed by inflammatory cell infiltration [74]. If the large amounts of preformed mediators are released into blood stream, anaphylactic shock will be induced, characterized by systemic vasodilaltion and extreme airway constriction [87]. Up to now, anti-IgE therapy has been shown to be beneficial to treat patient with moderate or severe allergic asthma [88], implying the critical role of IgE in the pathogenesis of allergic asthma.

1.2.3 Regulation of IgE production

IgE production is regulated at cellular and molecular levels, resulting in 10,000-50,000 fold less serum free IgE levels than that of IgG in non-allergic individuals. Even in

allergic individuals, the increased level of IgE is still far below that of IgG by at least 1000 fold. Some known mechanisms of IgE regulation are outlined below, and we have identified a further regulatory mechanism in this thesis.

1.2.3.1 Linkage with cell division

It has been demonstrated that both IgG1 and IgE-switching are cell division-dependent. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling experiments showed that cells seldom switch to other isotypes in the early cell divisions (0-3) during in vitro culturing. Moreover, IgE-switching needs even more cell divisions than IgG1-switching under the same culture condition [89, 90], and the frequency of IgE-switched cells is almost 10 fold less than that of IgG1-swtiched cells. It is still not clear why isotype switch is cell division-linked. But GLTs are abundant only when cells reach the boundary of G1/S phase of cell cycle [91]. The more stringent cell division requirement for the switching to IgE may simply due to the long distance between Sμ and Sε or requires sequential switch.

1.2.3.2 Regulation by cytokines

As mentioned above, IL-4 and IL-13 secreted by T cells are required for IgE isotype switch. Several other cytokines can affect IgE production through modulating the production of IL-4 and IL-13, or regulating the process of CSR in the presence of IL-4 and IL-13. In human B cells, IL-10 appears to have dual roles in IgE production induced by IL-4 [92, 93]. IL-10 inhibits the switching of IgM+ cells to IgE+ cells by inhibiting εGLTs; however, IL-10 also potentiates IgE synthesis and secretion in IgE-switched cells through promoting plasma cell differentiation. IL-18 is another cytokine, which

promotes IgE production in IL-4-dependent manner [94, 95]. IL-18 has no direct effect on IgE CSR. Instead, IL-18 administration or over-expression in vivo leads to hyper-activation of T cells with a bias to a T_H2 type response, which is responsible to the increased levels of serum IgE and IgG1.

Meanwhile, some cytokines inhibit IgE production. IFN-γ is a typical one, not only inhibiting the production of IL-4 and IL-13 from T_H2 cells, but also directly repressing the transcription of εGLTs [96]. Isotype switching to IgG1 and IgE are equally inhibited by IFN-γ. By contrast, IL-21 shows a selective inhibitory effect on IgE switching, but not on IgG1 switching. Immunization of IL-21R–deficient mice leads to higher production of IgE, but lower IgG1 generation, than that of control mice [97]. Similarly, in vivo administration of IL-21 specifically inhibits antigen-specific IgE production after immunization [98]. In vitro culture studies showed that IL-21 inhibits the transcription of εGLTs by inducing negative regulator of IgE CSR, the inhibitor of differentiation 2 (Id2) [99, 100]. In addition, IL-21 has been found to suppress sequential switching from IgG1 to IgE [101]. Collectively, cytokines described above can positively or negatively regulate the IgE production, either by regulating the generation of T_H2 cytokines or by directly affecting the transcription of εGLTs.

1.2.3.3 Regulation by low-affinity IgE receptor CD23

CD23, unlike other immunoglobulin receptors, belongs to the C-type (calcium dependent) lectin superfamily, and it is constitutively expressed on B cells and FDCs, and can be induced on many immune cells upon activation by antigens or cytokines [102].

Membrane bound CD23 without IgE ligation is susceptible to proteolysis, leading to the release of soluble CD23 [103]. Because CD23 is expressed on the surface of large amount of cells, CD23 may work as a buffer system to prevent the sudden increase of free serum IgE and its subsequent deadly threat. In addition, CD23 seems to possess both positive and negative effects on IgE production under different circumstances. When the IgE level is low, membrane bound CD23 is cleaved to be soluble, which can bind to B cell membrane IgE and CD21 simultaneously, promoting IgE synthesis [104, 105]. When the IgE level is high, membrane bound CD23 is protected from cleavage and the interaction between IgE and membrane CD23 negatively regulates IgE production. Immunization of CD23-deficient mice leads to increased total and antigen-specific IgE compared to control mice [106, 107]. On the contrary, over-expression of CD23 results in decreased IgE production after immunization [107].

1.2.3.4 Negative regulation by transcription factors

Two main transcription factors, BCL6 and ID2, have been demonstrated to negatively regulate IgE switching. BCL6 is constitutively expressed in B cells at low level. In GC B cells, BCL6 protein expression is greatly elevated due to posttranscriptional regulation [66]. BCL6-deficient mice show impaired GC responses, together with enhanced serum IgE level and Th2 cytokine production [67]. Further studies indicate that BCL6 competes with STAT6 to bind to the STAT6-binding sites located in the Iε promoter, thus inhibiting IL-4-stimulated εGLT transcription [75]. In addition, no IgE is induced in BCL6 and STAT6 dual deficient mice, indicating that increased IgE generation in BCL6-deficient mice is dependent on the activities of STAT6 [75].

ID2 is a helix-loop-helix (HLH) protein, lacking a DNA-binding motif; therefore, it acts as a negative regulator by interacting with other HLH transcription factors. TGFβ and IL-21, both suppressing IL-4-induced IgE CSR, have been reported to induce ID2 expression in B cells [100]. Immunization of ID2-deficient mice gives rise to increased serum level of antigen-specific IgE compared to that of the control mice [108]. The mechanism is that ID2 interacts with HLH transcription factor E2A, preventing E2A binding to the Iε promoter, and thus leading to reduced induction of εGLTs [109]. In addition, ID2 negatively regulates AID expression by modulating the activity of Pax5 binding to the promoter region of AID [79].

1.2.4 Role of IgE in asthma pathogenesis

Asthma is characterized by airway inflammation, bronchial hyper-responsiveness (BHR), and reversible airway obstruction triggered by allergens or non-allergens, and the clinical symptoms are dyspnea, chest tightness, wheezing and cough. The pathogenesis of asthma is a complicated process, which has not been fully understood. However, there is strong association between the elevated IgE levels and allergic asthma, indicating that IgE is a key component in the pathogenesis of allergic asthma [110, 111]. Even in nonallergic asthma, high IgE level has been identified as a risk factor [112].

Asthma mouse models have been widely used for better understanding the role of IgE in the pathogenesis of asthma. Passive transferring of antigen-specific IgE into mice followed by intranasal challenge with the same antigen is able to induce evidence of asthma, such as airway eosinophilia and BHR in the mice [113], suggesting IgE alone is

sufficient for asthma induction. However, further studies imply that T cells are absolutely required for the IgE-induced asthmatic responses. For example, in T-cell deficient mice, passive transfer of IgE and subsequent allergen challenge fail to elicit airway inflammation and BHR [114]. Moreover, both eosinophilic airway inflammation and allergen-induced BHR can be observed in IgE-deficient mice and mast cell-deficient mice, arguing against the central role of IgE in the pathogenesis of asthma [115, 116]. In addition, using IgE-transgenic mice, Maezawa Y et al. showed that IgE crosslinking alone did not induce airway eosinophilia and hyperresponsiveness; instead, IgE crosslinking in the airway resulted in CD4 T cell recruitment [117]. They also found that if Th2 cells already exist, IgE crosslinking leads to recruitment of Th2 cells into airway and enhances Th2-mediated eosinophil airway infiltration. All together, it is safe to draw conclusion that under many conditions, IgE-FcεR-mediated mast cell activation and inflammatory mediator release contribute to the airway inflammation and BHR. As a consequence, anti-IgE monoclonal antibody, Omalizumab, which prevents serum free IgE from binding to FcεRI and CD23, has been successfully used in the clinic to treat allergic asthma.

1-3 B cell signaling pathways

The decision that B cells make in the stages of B cell development (such as from pro-B to pre-B, from pre-B to immature B, and from immature B cells to mature B cells), and in the stages of B cell activation and differentiation (such as from naive B cells into GC B cells, plasma cells, and cells secreting different antibody isoforms) is directed by intracellular signaling molecules. Those molecules are activated upon ligation of various

cell surface receptors, including BCRs, TLRs, cytokine and chemokine receptors, complement receptors, etc [2]. They are trasduced intracellularly in a stepwise manner to form multiple signaling pathways, leading to the generation of second messengers and/or DNA-binding transcription factors, which will subsequently determine the fate of B cells. Here, I will briefly review PLCγ2 and ERK pathways, which are partially related to my studies, before moving on to PI3K signaling pathway, which is the main focus of this thesis.

1.3.1 PLCγ2 pathways

Upon antigen recognition, B cell surface BCRs are aggregated, leading to activation of protein tyrosine kinase (PTK) Syk, adaptor molecule B cell linker (BLNK), and Bruton tyrosine kinase (Btk) [118]. Syk and Btk phosphorylate and activate PLCγ2, which can hydrolyse the plasma membrane lipid phosphatidylinositol 4,5-bisphophate [PI(4,5)P2)] to generate inositol(1,4,5)-trisphosphate (IP3) and diacylglycreol (DAG) [119]. The water-soluble IP3 directly binds to IP3 receptors (IP3Rs) in the endoplasmic reticulum (ER), leading to the immediate release of Ca2+ into the cytoplasm [120]. And the depletion of stored Ca2+ in the ER results in sustained Ca2+ influx through channels in the plasma membrane [121]. Increased intracellular Ca2+ facilitates binding of Ca2+ - calmodulin to protein phosphatase calcineurin, leading to activation of its phosphatase activity [122]. Calcineurin dephosphorylates the cytoplasmatic subunits of nuclear factor of activated T cells (NFAT) tracription complexes, resulting in its transportation into nucleus [123]. NFAT, which cooprates with other transcription factors, regulates the expression of multiple target genes [124-126]. In B cells, interferon regulatory factor 4

(IRF4) appears to be one of them [127], which has been found to regulate GC B cell and plasma cell differentiation [128, 129].

PLCγ2 not only transduces this calcium/NFAT pathway, but also mediates the activation of nuclear factor (NF)-κB. NF-κB transcription factors are restained in the cytoplasm via interaction with the inhibitor IκB. Phosphorylation of IκB by the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and IKKγ, results in NF-κB activation [130]. PKCβ, activated by PLCγ downstream second messengers, DAG and calcium, has been demonstrated to selectively regulate IKKα phosphorylation indirectly, which subsequently activates NF-κB [131]. NF-kB regulates the expression of various genes, including cytokines, chemokines, regulators of cell survival and apoptosis (such as Bcl-xL), and cell cycle regulators [132], and thus NF-kB is critical for B cell survival, proliferation and differentiation.

1.3.2 Ras/Raf/MEK/ERK pathway

Extracellular signal-regulated kinase 1/2 (ERK1/2), also called mitogen-activated protein kinases (MAPKs), are protein serine/threonine kinases that are activated in B cells in response to a variety of extracellular stimuli, including antigens, TLR ligands, CD40 ligand [118, 133-135]. Here, I will briefly introduce the pathway that leads to BCR-induced ERK activation, since the signaling adaptor molecule I am studying was shown to be involved in this pathway [136, 137].

ERK is readily activated by the cascade of Ras/Raf (the MAP3K in the pathway)/MEK (the MAP2K in the pathway); however, there are multiple ways coupling BCR to the active form of Ras, GTP-bound Ras [118]. The classical pathway suggested for BCR-mediated Ras activation is that Syk phosphorylates adaptor protein Shc [138], which will bind to another adaptor protein Grb2. Since Grb2 is contitutively associated with SOS, a guanine nucleotide exchange factor for Ras, they form a Shc/Grb2/SOS signaling complex [139]. SOS activates membrane-anchored Ras by exchanging GDP for GTP [140]. Another model is that Syk activates adaptor protein BLNK, which functions as Shc to mediate SOS recruitment and subsequent Ras activation [141]. In addition, another guanine nucleotide exchange factor, RasGRP3, also activates Ras [142]. Evidence suggests that PKCβ, downstream molecule of PLCγ2, phosphorylates and activates RasGRPs [143]. Upon activation, GTP-bound Ras binds to and activates Raf-1, which in turn phosphorylates MEK1/MEK2 in corporation with B-Raf. Finally, activated MEK1/MEK2 phosphorylate ERK1/ERK2, which will form dimmers and translocate to the nucleus, leading to the induction of transcription factors, such as Ets, TCF, ELK-1, and SAP-1, which control cell proliferation and differentiation [144, 145].

1.3.3 PI3K pathway

PI3Ks (phosphoinositide 3-kinases) are a family of enzyme which can phosphorylate phosphatidylinositol lipids at the D3 position of the inositol ring to generate second messengers, critical for a wide range of cellular functions, including cell survival, proliferation, migration, and the trafficking of intracellular organelles [146]. In B cells, ligation of cell surface receptors, such as BCRs, cytokine receptors, and CD40, leads to

PI3K activation, which is required for subsequent activation of many functional important signaling molecules, including PLCγ2. Genetic blockade or attenuation of PI3K activity in mice showed that PI3K signaling pathway is essential for B cell development and activation [147]. Additionally, PI3K signaling has also been suggested to be involved in GC responses and plasma cell differentiation. In the following paragraphes, I will introduce some basic concept of PI3Ks and their functional outcomes in B cells.

1.3.3.1 Expression isoforms of PI3Ks

Based on structural similarities and lipid substrate specificity, PI3Ks are divided into three classes: I, II and III. Of those, the most well-characterized enzymes are class I PI3Ks. Class I PI3Ks are further sub-divided into class IA and B, consisting of one catalytic subunit and one regulatory subunit. Three class IA catalytic subunits, termed p110α, p110β, and p110δ, can bind one of the 50-, 55-, or 85-kDa regulatory subunit to form a heterodimeric enzyme. Regulatory subunits of class IA contain Src homology 2 (SH2) domains, which can direct the catalytic subunit to the plasma membrane upon receptor tyrosine phosphorylation [148]. There is only one mammalian isoform of class IB PI3K (PI3Kγ), consisting of a catalytic subunit p110γ and a regulatory subunit p101 or p84/p87 [149]. PI3Kγ is activated by G-protein-coupled receptors (GPCR), such as chemokine receptors. Three mammalian isoforms of class II PI3Ks are identified, with a characteristic carboxy-terminal C2 domain [150], termed PI3K-C2α, PI3K-C2β and PI3K-C2γ. *In vivo* expression of both PI3K-C2α and PI3K-C2β are ubiquitous, whereas PI3K-C2γ is preferentially expressed in liver. There is only one mammalian isoform of

class III PI3K, consisting of a catalytic subunit Vsp34 and a regulatory subunit p150, known as Vps34 (PIK3C3).

1.3.3.2 Generation of lipid second messengers by PI3Ks

Class IA PI3Ks (PI3Kα, β, and δ) and class IB PI3K (PI3Kγ) are able to phosphorylate phosphatidylinositol (PI), phosphatidylinositol-4-phosphate [PI(4)P] and phosphatidylinositol-4,5-biphosphate [PI(4,5)P2], which locate in cell plasma membrane, to generate phosphatidylinositol-3-phosphate [PI(3)P], phosphatidylinositol-3,4-biphosphate [PI(3,4)P2], and phosphatidylinositol-3,4,5-tris-phosphate [PI(3,4,5)P3]. Of those lipid products, PI(3,4,5)P3 is critical for class IA PI3K signaling by acting as second messenger to recruit PI3K downstream signaling molecules. *In vitro* studies showed that Class II PI3Ks preferentially convert PI and PI(4)P to PI(3)P and PI(3,4)P2. They can also utilize PI(4,5)P2 as their substrate, but at very rare situation and is very inefficient [151]. How these enzymes are activated *In vivo* is still unclear. There is evidence suggesting that PI3K-C2α lies downstream of the monocyte chemotactic peptide-1 (MCP-1) receptor [152] and the insulin receptor [153]. Class III PI3K Vps34 phosphorylates PI to generate PI(3)P in the internal membrane compartments of the endosomal/lysosomal system [154], which is fundamental for vesicular and protein trafficking.

1.3.3.3 Protein domains interacting with PI3K lipid products

Lipid products generated by PI3K are functionally important, acting as second messengers by recruiting lipid-binding signaling molecules to the plasma membrane where those lipids are present. Signaling molecules regulated by PI3Ks can selectively

bind the phosphorylated lipids generated by active PI3Ks, not the correspondent enzyme substrates. Specific binding between molecules and 3-phosphoinositides mostly is mediated by the following protein domains: pleckstrin homology (PH) domains, Phox homology (PX) domains, and FYVE (after the name of proteins Fab1, YOTB, Vac1, and EEA1) domains [155]. PH domain, about 120 amino acids in length, shows characteristic 3D structure consisting of two perpendicular anti-parallel β sheets, followed by a C-terminal amphipathic helix [156]. A subset of PH domains preferentially bind to PI(3,4)P2 and PI(3,4,5)P3 generated by class I PI3Ks. However, individual PH domain of different proteins binds to PI(3,4)P2 and/or PI(3,4,5)P3 with different affinity. Both PX and FYVE domains are able to bind to PI(3)P, product of class II/III PI3Ks [157]; however, FYVE domains show a restricted specificity towards PI(3)P [158]. In contrast, PX domains have a broader PI binding specificity. For example, some PX domains bind to PI(3,4)P2 or PI(4,5)P2 selectively [159].

1.3.3.4 Expression and activation of PI3Ks in B cells

Class I PI3K signaling is extensively studied in the context of lymphocyte signaling, whereas little is known about class II/III PI3Ks in lymphocytes. Thus, I will focus on class I PI3Ks in the following paragraphs. Of class I PI3Ks, catalytic subunits p110δ and p110γ are predomiantly expressed in hematopoietic cells, whereas p110α and p110β are ubiquitously expressed. In B cells, all those isoforms of class I PI3Ks are expressed, however, the expression level of p110δ is relatively higher than that of other isoforms, contributing to almost 50% of class IA PI3K activities, and p110δ has been found to be more critical for B cell functions [160-164].

In recognition of antigens, cytokines, CD40L, or LPS (TLR4 ligand), PI3Ks are immediately activated in B cells. BCR-triggered PI3K activation has been extensively studied, even though it is still unclear about the details of the coupling between PI3Ks and BCRs [165, 166]. BCR co-receptor CD19 appears to be involved in PI3K activation by providing binding sites, Y-X-X-M motifs, for the SH2 domains of p85 regulatory subunit of PI3Ks [167, 168]. Re-location of the regulatory subunit of PI3Ks brings catalytic subunit to the plasma membrane, where the catalytic subunit undergoes conformational change to expose its enzymatic site. However, CD19 is not the only molecule that tansduces BCR-induced PI3K activation [169, 170]. Adaptor molecules, such as BCAP (B-cell PI3K adaptor protein) and Gabs (Grb2-associated binder adaptor/scaffolding proteins), which also contain Y-X-X-M motifs, may be involved in PI3K activation [171, 172]. IL-4-induced PI3K activation has been suggested to require the adaptor protein, insulin receptor substrate (IRS)-2, which binds to the phosphorylated insulin/IL-4 receptor motif [173-175]. It is even less clear about how CD40 and TLR4 signaling leads to PI3K activation, and PI3K signaling pathway seems to be not critical for CD40 and TLR4-induced B cell proliferation [161, 176].

1.3.3.5 Major enzymes downstream of class I PI3K signaling pathway

A variety of enzymes containing PH domains selective for 3-phosphoinositides diversify the class I PI3K signaling outcome. Here, we will introduce two major enzymes, which are functionally important and closely correlate with class I PI3K activity.

(1) Serine–threonine kinase Akt

Akt, the human homologue of the viral oncogene v-akt, is also named as protein kinase B (PKB), related to protein kinase A (PKA) and C (PKC) [177]. Three isoforms of Akt (Akt1, Akt2 and Akt3) have been discovered. Each isoform seems to have redundant, yet distinct, roles, as indicated by the studies of individual isoform knock-out mouse [178, 179]. Knockout of both Akt1 and Akt2 leads to impaired thymocyte development and survival [180]. The PH domain in the N-terminal region of Akt binds to both PI(3, 4)P2 and PIP3, contributing to the Akt recruitment to the plasma membrane. Full activation of Akt needs phosphorylation of two crucial amino acids by phosphoinositide-dependent kinase 1 (PDK1) and PDK2 respectively [181]. Mammalian target of rapamycin complex-2 (mTORC2) appears to be one of the PDK2s, acting upstream of Akt [182]. Many diverse biological functions induced by PI3K signaling come from active Akt, thus Akt phosphorylation has been conveniently used as an indicator of PI3K activity.

Akt plays critical roles in cell survival and apoptosis by regulating several pro-apoptotic and anti-apoptotic proteins. For example, Akt signaling inactivates pro-apoptotic proteins, such as BAD, MDM2, caspase-9 and Forkhead Box O (FOXO) transcription factor [183-186]. Akt can also up-regulate prosurvival genes, such as Bcl-2 family member MCL-1, through phosphorylating GSK3 [187]. One of the major targets of Akt is mTORC1, which is triggered by both nutrients and growth factor signaling to promote cell growth [188]. Akt activation of mTORC1 appears to occur through an indirect effect by inhibiting the tumor suppressor tuberous sclerosis complex 2 (TSC2) [189, 190] and the proline-rich Akt substrate of 40 kDa (PRAS40) [191, 192]. Both TSC2 and PRAS40

negatively regulate mTORC1, but this negative regulation is removed after they are phosphorylated by Akt. In terms of lymphocyte activation and development, the FOXO family of transcription factors (FOXO1, FOXO3a, FOXO4) has recently emerged as another key target of Akt [193]. Akt phosphorylates FOXOs, resulting in the translocation of those transcription factors from nucleus to cytoplasm; thus, target genes repressed or promoted by FOXOs are activated or de-regulated. FOXOs regulate the expression of a variety of genes, which are involved in cell-cycle progression (e.g. cyclin D1/2, $p27^{Kip1}$, and BCL6), apoptosis (e.g. Bcl-XL, BNIP3, Bim), and cell differentiation (e.g. Id) [194-199].

(2) Tyrosine kinase Btk

Btk is a Tec-family kinase expressed by B cells, connecting PI3K signaling to calcium flux and other downstream signaling events [200]. The PH domain of Btk selectively binds to PIP3 with high affinity, promoting membrane recruitment of Btk [201]. Point mutations of the PH domain of Btk lead to reduced calcium influx and immunodeficiency in mice and humans [202]. It has been believed that membrane recruitment of Btk is crucial for its activation by adjacent Src kinases, such as Lyn [203]. Then the active Btk further phosphorylates and fully activates PLCγ2, which can hydrolyzes PI(4,5)P2 to yield IP3 and diacylglycerol (DAG), contributing to early calcium spike and sustained calcium influx respectively [204]. Btk also appears to regulate calcium mobilization through the association with phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks), which re-generate PI(4,5)P2, the substrate for Btk upstream and downstream regulators, PI3Ks

and PLCγ2 [205]. Similar to Btk, Itk is the related Tec kinase expressed in T cells, linking PI3K activity to PLCγ1 phosphorylation, and to calcium influx [206].

1.3.3.6 Roles of PI3Ks in B cell development and activation

Using genetic approach, various knockout mice targeting PI3K catalytic or regulatory subunit are established. Of these, mice with single deletion of p110δ, p110γ or p85α are viable and extensively studied. Most recently, pharmacological inhibitors possessing high selectivity to individual PI3K family member or catalytic subunit have been developed, providing another useful tool for functional studies. Thus, all those tools enable us to understand the roles of PI3Ks in B cell development and activation. In this thesis, we have used some of theses tools to uncover new regulatory roles for p110δ.

(1) B cell development

In the bone marrow, the earliest B cell progenitors are pro-B cells, at which stage the immunoglobulin (Ig) heavy chain undergoes gene rearrangement. Only cells with successfully rearranged Ig heavy chain on the surface will express functional "pre-B cell receptors (pre-BCRs)", which allow the transition of pro-B cells into pre-B cells. At the stage of pre-B cells, Ig light chain gene rearrangement occurs to form BCRs. Cells that express functional BCRs associated with signaling components Igα and Igβ become immature B cells, and antigen recognized by this BCR complex induces cell death instead of proliferation [2]. Immature B cells migrate out of the bone marrow and become transitional B cells temporarily before they emerge as mature B cells in the periphery. Mature B cells are divided into two subsets: B1 cells, small population mainly located in

body cavities, and B2 cells, the majority of mature B cells found in circulation and second lymphoid organs. B2 cells can be further subdivided into follicular (FO) and marginal zone (MZ) B cells based on their locations and functions [2].

Studies using genetic approaches showed that PI3Ks regulate B cell development at each stage as mentioned above. Deletion of either catalytic subunit p110δ or regulatory subunit p85α leads to a partial block at pro-B to pre-B transition, almost complete loss of B1 cells, reduced total number of splenic FO B cells [161, 207]. In addition, p110δ-deficient mice have dramatic reduction of MZ cells [161]. On the contrary, mice lacking the inositol phosphatase PTEN, which antagonizes PI3K activity, generate expanded population of B1 and MZ B cells [208]. At the immature B cell stage, basal level of PI3K signaling in cells carrying nonautoreactive BCR appears to be essential to turn off recombination-activating gene (RAG) expression and to promote positive selection [209, 210]. PI3K downstream transcription factor FOXO1 is able to directly bind to RAG genes, facilitating the transcription of both RAG1 and RAG2; thus, PI3K signaling may negatively regulate RAG expression by inactivating FOXO1 to allow positive selection in immature B cells [211]. However, FOXO1 is critical for the progress from pro-B cells to pre-B cells, since FOXO1 deficiency in pro-B cells leads to impaired expression of IL-7Rα and RAG [212].

(2) B cell activation

B cells are activated when cell surface receptors or ligands, such as BCRs, Toll-like receptors (TLRs), CD40 and IL-4R, are engaged. As we mentioned in the previous

paragraph, downstream signals derived from those cell surface receptors activate PI3Ks. Using genetic approaches to block PI3K activity, studies show that anti-IgM-stimulated B cell proliferation is substantially reduced [161, 207, 213]. Pharmacological inhibitor studies indicate that persistent PI3K activation triggered by BCR aggregation is required for B cells to survive and to undergo cell cycling [214]. The lack of PI3K activity also results in reduction of B cell proliferation upon stimulation of other mitogens, such as CD40L, IL-4 and LPS. On the contrary, Pten-deficient B cells, exhibiting elevated basal PI3K activity, are hyperproliferative in response to various stimuli [215]. In addition to B cell proliferation and survival, PI3K isoform p110δ signaling is required for chemokine-mediated B cell homing and migration [164]. In line with the important roles of PI3Ks in B cell activation, humoral immune responses are markerly impaired in p85α-deficient, p110δ-deficient, and CD19-deficient mice [161, 207, 216]. Constitutive elevation of PI3K signaling leads to neoplastic transformation, which has been found to be a general theme in cancer [217].

1-4 PH domain-containing adaptor proteins

The discovery and characterization of lipid-binding PH domains have led to substantial advances in understanding of how PI3Ks activate cellular responses [218, 219]. The human genome contains approximately 350 PH domain proteins; however, only a fraction of these are likely to have high affinity lipid binding properties [220]. A subset of PH domains showing selective PI(3, 4)P2 and／or PI(3,4, 5)P3 binding have been found to present in various signaling molecules, including not only enzymatic proteins, such as protein kinases (Btk, Akt, PDK1), guanine nucleotide exchange factors (GEFs) (Vav,

Tiam1, GRP1, Sos, and P-Rex1), and guanosine triphosphatase (GTPase)-activating proteins (Arap3, Gap1m, centaurin-a), but also scaffold／adapter molecules, such as Grb-associated binder 1／2／3 (Gab1／2／3), Src kinase- associated phosphoprotein (SKAP)／adhesion and degranulation-promoting adapter protein (ADAP), B-lymphocyte adaptor molecule of 32 kDa (Bam32) / dual-adaptor for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), and Tandem pleckstrin homology domain protein (TAPP) 1／2 [201, 221-224]. Even though protein kinases are the most extensively studied ones to dissect PI3K downstream signaling, adaptor proteins emerged as functional important molecules which finely tune PI3K signaling.

1.4.1 Bam32 / DAPP1

Bam32, also named DAPP1 or 3' phosphoinositide-interacting SH2 domain-containing protein, was identified by three groups based on its 3-phosphoinositide-binding ability [224-226]. My supervisor Dr. Aaron Marshall concurrently identified Bam32 as a germinal center-associated gene using subtractive hybridization polymerase chain reaction to clone genes expressed in follicular dendritic cells isolated from human tonsil [227]. Bam32／DAPP1 contains both a C-terminal PH domain and an N-terminal SH2 domain with a single tyrosine phosphorylation site located centrally (Figure 1.5). Bam32 genes from other species are also present in sequence databases, and its amino acid sequence is evolutionary highly conserved among human, mouse, rat, and chicken with 80–90% homology [228]. In human, Bam32 expression appears to be restricted to hematopoietic cells, with highest levels in B cells. Further experiments indicated that Bam32 expression levels can be modulated when human B cells are activated in vitro

[227]. In mouse, Bam32 mRNA can be readily detected in B cells, and also expressed at lower levels in T cells, dendritic cells, and macrophages [136]. As discussed in detail below, functional studies have clearly implicated Bam32 in B-cell activation and further suggest potential roles in T-cell and mast cell function.

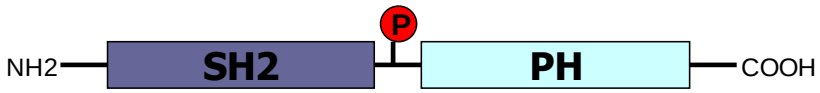

Figure 1. 5 Basic structure of Bam32 / DAPP1

1.4.1.1 Structural studies of Bam32/DAPP1 PH domain

Structural analyses of PH domains indicate that, although they possess low amino acid sequence homology, they form a conserved backbone structure [229]. This conserved β-sheet structural fold has three variable loops in one open end, of which the β1-β2 loop is thought to be critical in determining affinity and specificity for 3-phosphoinositide binding [229, 230]. The PH domain of Bam32/DAPP1 has been well characterized and was shown to bind to both PIP3 and PI(3, 4)P2 with high affinity and specificity in vitro [224]. Sequence and structural analyses confirmed that conserved residues required for 3-phosphoinositide binding are present in the β1-β2 loop of Bam32/DAPP1 PH domain and are positioned correctly to contact 3-phosphoinositide [219, 231]. When compared with the structure of Btk or Grp1 PH domains, both known to bind to PIP3 only, the Bam32/DAPP1 PH domain forms more hydrogen bonding interactions with 4-phosphate of the lipid head group and no hydrogen bonds with 5-phosphate [231]. This mode of phosphoinositide binding is very similar to that of TAPP1 (see below), and remarkably, a single glycine to alanine substitution could convert the Bam32 PH domain to a selective binder of PI(3, 4)P2, like the TAPPs [232]. A more recent study using surface plasmon

resonance (SPR) analysis indicated that Bam32／DAPP1 PH domain shows some degree of preference for PI(3, 4)P2 over PIP3 [233].

1.4.1.2 Plasma membrane recruitment of Bam32／DAPP1

In line with the in vitro lipid-binding and crystal structure studies, Bam32／DAPP1 PH domain has been identified to be essential for plasma membrane recruitment of this molecule upon cell activation. The first evidence came from enhanced green fluorescent protein (EGFP)-tagged Bam32／DAPP1 fusion protein studies in BJAB B cells, showing that Bam32 ／ DAPP1-EGFP was recruited to the plasma membrane after BCR crosslinking, and this membrane translocation was dependent on both PI3K activity and PH domain integrity [227]. Similar results were also observed in GFP-Bam32／DAPP1 overexpressing porcine aortic endothelial (PAE) cells or DT40 chicken B cells stimulated with platelet-derived growth factor (PDGF) or mouse anti-chicken IgM, respectively [225]. In the BCR-activated human B-cell model, membrane recruitment of Bam32／DAPP1 is delayed and sustained for longer time after receptor ligation, compared with the pattern of Btk membrane translocation, which is rapid and transient [234]. The different kinetics of PH domain-mediated membrane recruitment correlated well with the kinetics of in vivo-generated PIP3 and PI(3, 4)P2 [234]. After BCR cross-linking, PIP3 levels reach their maximum within 30 s, then rapidly return to the basal level; on the contrary, PI(3, 4)P2 levels peak several minutes after stimulation, and then remain at plateau levels for 30 min or more in BJAB cells [234].

One important pathway for producing PI(3, 4)P2 in B cells and mast cells is removing the 5-phosphate from PIP3 via the action SHIP phosphatase [235, 236]. Thus, SHIP can dampen PIP3-dependent downstream signaling events but enhance PI(3, 4)P2-dependent downstream signaling events. In other words, the level of SHIP activity can determine which arm of PI3K signal will be favored, by balancing the generation of PIP3 and PI(3, 4)P2 signalosomes. In B cells, ligation of the inhibitory Fc receptor FcγRII recruits and activates SHIP [237]. Studies carried out in our lab showed that coligation of FcγRII with BCR inhibited membrane recruitment of Btk PH domain, whereas Bam32／DAPP1 membrane translocation is not inhibited but in fact occurs earlier [238]. This same differential effect of SHIP was also seen in cells overexpressing membrane-targeted SHIP [238]. Based on these findings, and others discussed below, it is reasonable to propose that 'inhibitory' signaling molecule SHIP may be more accurately referred to as a modulator of PI3K signaling, because it can also exert positive effects on a subset of PI3K effector molecules, such as Bam32／DAPP1 and TAPPs.

1.4.1.3 Bam32／DAPP1 tyrosine phosphorylation

Bam32／DAPP1 contains 10 tyrosine residues in total, of which only one (Tyr-139) is phosphorylated. Y139 is located centrally between the SH2 and PH domains in a IYESV motif, a predicted target sequence for Src family kinases [239]. This tyrosine and the sequence context are completely conserved among a variety of species [228]. In B cells, Dr. Marshall et al. found that Bam32／DAPP1 is phosphorylated after BCR ligation or pervanadate stimulation [227]. Several studies have provided clear biochemical and genetic evidence that Src kinases, but not Tec or Syk kinases, can efficiently

phosphorylate Bam32 / DAPP1 at Y139 in vitro and in vivo [225, 240, 241]. Despite clear evidence that Bam32 is mono-phosphoylated on Y139, all studies find that phosphorylation leads to a bandshift of 3–4 kDa in its mobility on SDS-PAGE gel, which may be due to an SDS-stable conformational change or additional modifications present on the tyrosine-phosphorylated molecule [227]. Consistent with the latter possibility, a mass spectrometry survey of phosphopeptides generated in activated mast cells identified both a peptide corresponding to Y139-P Bam32 and a doubly phosphorylated peptide corresponding to Y139-P / S141-P Bam32 [242]. Previous studies [225, 240, 241] showed that Bam32 phosphorylation is abrogated when cells are stimulated in the presence of PI3K inhibitors or when the phosphoinositide-binding pocket of its PH domain is mutated. This finding suggests that PI3K-dependent membrane recruitment may be required to bring Bam32 into proximity of Src kinases present in lipid rafts. Another possibility is that 3-phosphoinositide binding to the PH domain may be required to release the molecule from an 'auto-inhibited' conformation analogous to that recently reported for SKAP-hom [243]. In vitro Bam32 / DAPP1 phosphorylation by Src kinases is not appreciably altered in the presence or absence of lipid vesicles containing PIP3, suggesting that PIP3 binding to the PH domain does not directly affect the accessibility of Y139 to kinases [240]. Interestingly, in vivo tyrosine phosphorylation of Bam32 / DAPP1 was blocked by mutation of the phosphotyrosine-binding pocket in the SH2 domain [225]; however, this same SH2 domain mutant can still be directly phosphorylated by Src in vitro [241]. Thus, both PH domain-mediated membrane recruitment and SH2 domain-mediated protein interactions seem to be required for Bam32 to gain access to Src kinases in vivo.

1.4.1.4 Bam32/DAPP1-mediated signaling pathways

Published data indicate that Bam32 is functions as PI3K effector molecule in immune cells, regulating distinct branches of signaling pathways related to cell survival, proliferation, or cytoskeletal rearrangement and receptor internalization. In accordance with the predominant expression of Bam32 in B cells, the most significant impact of Bam32 in signal transduction is described in this cell type.

Initial data suggested Bam32 may be important for regulation of PLCγ2 activity and calcium responses. Dr. Marshall et al. found that tyrosine-phosphorylated Bam32/DAPP1 co-immunoprecipitated with PLCγ2 in B cells after pervanadate stimulation. Further in vitro binding studies showed direct interaction could occur between Bam32/DAPP1 SH2 domain and tyrosine-phosphorylated PLCγ2 [227]. Consistently, Bam32-deficient DT40 B cells were reported to have reduced tyrosine phosphorylation of PLCγ2 upon BCR crosslinking as well as reduced calcium responses and NFAT transcriptional activity [244]. Those data implied that full activation of PLCγ2 in DT40 B cells requires involvement of Bam32/DAPP1. However, Bam32-deficient B cells were found to have normal PLCγ2 phosphorylation and calcium responses [136]. Thus, the Bam32-PLCγ2 interaction may not be functionally critical in mammalian cells.

It has been found that Bam32/DAPP1 overexpression inhibits BCR-induced NFAT activation in BJAB cells, suggesting that this adapter may have some inhibitory signaling functions. This effect required an intact PH domain but not an intact SH2 domain [227],

but it does not seem to be associated with altered calcium responses (A. Allam and A. J. Marshall, unpublished data). Given the high binding affinity of the Bam32 PH domain to 3-phosphoinositides, it is tempting to speculate that overexpression of such molecules may deregulate membrane recruitment of other PI3K effector molecules, by competing for binding to the same 3-phosphoinositides. However, unpublished data in our lab have demonstrated that membrane recruitment of Btk-PH or TAPP-PH domain EGFP fusion proteins is not detectably inhibited in BJAB cells stably overexpressing untagged Bam32 (S. Hou and A. J. Marshall, unpublished data). This finding suggests, at least in this cell line model (PTEN-deficient), competition for 3-phosphoinositides is not a major factor.

Major intracellular signaling pathways initiated by BCR crosslinking can converge on activation of mitogen-activated protein kinases (MAPKs), which are essential for B-cell proliferation, differentiation, and survival. Studies in human, mouse, and chicken cells all consistently indicate that Bam32 can regulate BCR-induced activation of ERK and JNK but not p38 MAPK [136, 244-246]. MAPK activation is dependent on activation of Ras and Rac GTPases and is relayed by sequential phosphorylation of upstream MAP4Ks, MAP3Ks, and MAP2Ks in a process coordinated by scaffold proteins [247]. Han et al. [136] found that Bam32-deficient B cells had defects in the ERK pathway that spanned from the MAP2K to MAPK4 levels, suggesting Bam32 modulates this pathway at a receptor-proximal step. They found evidence that Bam32 may directly interact with and promote activation of HPK1 (a MAP4K); however, this result has not been reproduced by other laboratories. In contrast, phosphorylation of Raf, a key component of the canonical Ras-ERK pathway, was not affected [136]. How Bam32/DAPP1 interacts with HPK1,

and the significance of Bam32 ／ DAPP1-dependent HPK1 activation in the MAPK pathways, are still open questions to be answered.

Dr. Atef Allam, previous Ph.D student in our lab, found evidence indicating that Bam32 promotes BCR-induced activation of Rho GTPases Rac1 and cdc42, which is likely to both feed into MAPK activation pathways and impact on cytoskeletal rearrangement. Bam32-deficient DT-40 B cells showed reduced Rac activation, while overexpression of Bam32 led to enhanced Rac activation [246]. Overexpression of Y139F-mutant Bam32 strongly attenuated Rac activity, suggesting phosphorylation is critical for this function. In Bam32／DAPP1-dual deficient DT40 cells, co-capping of Rac1 with the BCR appears to be reduced, suggesting that Bam32 may directly or indirectly regulate Rac targeting [246]. In addition, Bam32-deficient mouse B cells have been found to have clear defects in activation of Rac1 and cdc42 but not Rac2 or Rap1 [248]. Rac1 is known to be an important upstream activator of the JNK pathway [249, 250], thus it is possible the requirement for Bam32 in MAPK activation may reflect its role in activation of Rac. The mechanism by which Bam32 modulates Rac activity and localization is currently unknown.

1.4.1.5 Roles of Bam32／DAPP1 in B-cell immune responses

In consistent with those findings described above showing that Bam32/DAPP1 is involved in many aspects of BCR-triggered signaling pathways, previous studies indicate that this adaptor plays important roles in BCR-induced functional responses. Firstly, Bam32/DAPP1 may be functionally active in the process of BCR-mediated antigen

presentation, including BCR capping, internalization, endosomal / lysosomal degradation, and cell surface presentation [251]. It was found that Bam32／DAPP1 co-localizes with BCR throughout the processes of BCR patching, capping, and internalization [252]. And throughout the BCR-Bam32 ／ DAPP1 internalization processes, the complexes co-localize with molecules critical for endosomal trafficking, such as clathrin, transferrin, and F-actin [252]. In addition, Bam32-deficient DT40 cells showed impaired BCR internalization [252]. My colleague, Dr. Monther Al-Alwan, took a further step to determine whether this molecule functions in antigen processing and presentation to T cells using Bam32-deficient primary mouse B cells, which will be mentioned in the chapter IV discussion section of this thesis. Secondly, Bam32/DAPP1 may be required for BCR-induced cell survival. BCR cross-linking was reported to induce significantly increased frequency of apoptosis in Bam32-deficient DT40 cells [244]; however, no difference in apoptosis was observed in Bam32-deficient primary mouse B cells [136], indicating that involvement of this adaptor in this signaling pathway depends on the cellular context. Thirdly, two independent Bam32-deficient mouse lines concur that BCR-induced proliferation is markedly impaired [136, 245]. Consistent with this defective BCR-triggered proliferation and markedly reduced B1 cells, both Bam32-deficient mice showed remarkably reduced TI-2 antibody responses, as assessed by either immunization with hapten-conjugated Ficoll or vaccination with the natural TI-2 antigen, bacterial capsular polysaccharide [136, 245]. Fournier et al. [245] further demonstrated that this defect led to defective protection from infection with an encapsulated bacterium Streptococcus pneumoniae. In this thesis, we will describe further evidence for the roles

of Bam32/DAPP1 in TD antibody responses by carefully examining GC responses in Bam32-deficient mice.

1.4.2 Other PH domain-containing adaptors related to Bam32

TAPP1 and TAPP2 are adaptor proteins containing PH domains, which share sequence homology to Bam32 [234]. TAPP genes are evolutionary conserved, and in human both TAPP1 and TAPP2 are expressed in a wide range of cell types including the hematopoietic cells [228]. Both biochemical and surface plasmon resonance (SPR) assays demonstrate that PH domains of TAPPs enable them to interact with phosphoinositides, showing preference of PI(3,4)P2 over PIP3 [233, 240]. Consistent with this unique binding capability, membrane recruitment of TAPPs upon BCR aggregation is slow and persistent, mirroring the kinetics of PI(3,4)P2 production in vivo [238]. In addition, It was found that hydrogen peroxide (H_2O_2) treatment, which induces the generation of membrane PI(3,4)P2 [253], leads to TAPP2 membrane recruitment [254]. Up to now, it is still unclear about which signaling molecules interact with TAPPs and wheather TAPPs are functionally important in immune cells. One most recent study carried out by our lab members were designed to answer those questions, and they observed that the cytoskeletal proteins, utrophin and syntrophin, are TAPP2-associated proteins in B lymphocytes. Interestingly, in B-cell-derived chronic lymphocytic leukemia (B-CLL) cells, TAPP2 and syntrophin expression levels correlate pretty well with ZAP-70, a known prognostic marker of the more aggressive B-CLL subtype. Furthermore, TAPP2 overexpression leads to increased adhesion to ECM proteins, while the knockdown of either TAPP2 or utrophin results in decreased adhesion responses in B

cells [255]. All together, this study suggests that PI(3,4)P2 branch of PI3K activity is conducted by TAPPs to recruit syntrophin/utrophin, which is required for activation-induced B cell adhesion.

Grb2-associated binder (Gab) adaptor / scaffolding proteins, including Gab1, Gab2, and Grb3, contain an N-terminal PH domain which binds to PIP3, a C-terminal several conserved tyrosine phosphorylation motifs, and proline-rich motifs interacting with SH2 and SH3 domains [256]. Gabs are recruited to the cell membrane in response to cytokines, growth factors and antigen receptors in PH-dependent and independent manners [257, 258]. Gabs not only act as PI3K downstream targets, but also can activate PI3Ks via binding to the p85 subunit [258]. Another major signaling pathway Gabs are involved in is to activate ERK through interaction with the protein tyrosine phosphatase SHP-2 [259, 260]. Similar as Bam32, Gab2 also exerts inhibitory function in some contexts, such as TCR signaling. And this inhibitory effect seems to be mediated by the Gab2-associated SHP-2 and LAT [261, 262]. Because of the pivotal roles of Gabs in the activation of PI3K/Akt and ERK pathways, Gabs have been shown to be required for cell proliferation, survival and differentiation upon ligation of cell surface receptors in immune cells. For example, Gab2 phosphorylation is required for immature B cell survival after BCR aggregation [263]. In mast cells, Gab2 appears to have both positive and negative effects on FcεRI-induced signaling and activation [264, 265]. Studies of Gab1 and Gab2 knockout mice also implicate that Gabs play important roles in immune system. For example, Chimeras reconstituted with Gab1-deficient fetal liver cells exhibit decreased pre-B cells and increased myeloid cells [266]. In addition, T cell-independent

type 2 antigen (TI-2) responses were enhanced in the chimeras [266]. In Gab2-deficient mice, mast cells are significantly reduced in various tissues, accompanied with impaired activation and degranulation [267].

Src kinase-associated phosphoprotein (SKAP) adaptors, including SKAP55 and SKAP-hom (SKAP-55 homolog), are recently discovered PI3K downstream adaptors. They contain a PH domain, a C-terminal SH3 domain, an N-terminal dimerization domain (DM), and multiple tyrosine phosphorylation sites [268]. SKAP55 is mainly expressed in T cells, but not in B cells, whereas SKAP-hom is widely expressed in hemotopoietic cells [268]. In vitro lipid binding assay showed that the PH domain of SKAP-hom is able to bind to PIP3 with high affinity, and also to PI(3,4)P2 at significant level [243]. Interestingly, the PH domain alone actually inhibits the membrane recruitment of SKAP-hom, but the 3-phosphinositide binding can reverse this auto-inhibition state [243]. SKAP adaptors are found to be required for receptor-mediated cytoskeleton rearrangement, integrin clustering, and adhesion through interaction with ADAP (adhesion and degranulation promoting adaptor protein) [269]. ADAP has been found to regulate cytoskeleton machinery by binding to the main molecules in this pathway, such as VASP (vasodilator-stimulated phosphoprotein) and WASP (Wiskott-Aldrich syndrome protein) [270, 271]. Furthermore, SKAP55/ADAP complex is able to interact with RIAM1 (Rap1-GTP interacting adaptor molecule), which can activate integrin [272]. SKAP55-deficient T cells as well as ADAP-deficient T cells show defect in TCR-induced integrin adhesion [273]. Similarly, B cells deficient in SKAP-hom show reduced adhesion in response to BCR stimulation [274].

In conclusion, those adaptor molecules, including Bam32 / DAPP1, need PH domain for their membrane recruitment upon ligation of cell surface receptors, which is in PI3Ks dependent manner and is required for their subsequent functional outcomes. Generally, those adaptor molecules, unlike PI3Ks or PI3K downstream kinases, do not have major effects on immune responses; however, they are required to diversify and refine many branches of immune responses. It is likely that additional PH-domain containing adaptors will be discovered to better understand the complexity and intertwinement of multiple intracellular signaling transduction pathways and their functional outcomes. This thesis particularly focuses on Bam32 / DAPP1 to uncover its roles, which fine tune one aspect of humoral immune responses, GC responses.

1-5 Summary of thesis rationale, hypothesis and results

PI3Ks are activated in a variety of immune cells, such as B cells, T cells, NK cells, eosinophils, neutrophils, and mast cells, upon receptor ligation. PIP3 generated by activated PI3Ks is located in the membrane, leading to membrane recruitment and subsequent activation of PH domain-containing enzymes and adaptors, which further transduce signals. PI3K downstream enzymes, including Akt, Btk/Itk, and PDK-1/2, regulate cell survival, proliferation, differentiation, and cytoskeleton rearrangement. PI3K downstream adaptors, such as Bam32, TAPP1/2, Gabs, and SKAPs, link to branches of signaling pathways regulating immune responses. In this thesis, my general hypotheses are: 1) **Genetic inactivation of PI3K p110δ attenuates allergic airway inflammation**; and 2) **Bam32 regulates the initiation, maintenance or convalescence of the germinal center response to ensure the humoral immune response effective.**

Overrall, studies carried out in chapter 2 and 3 expand our knowledge of p110δ signaling in the regulation of Th2-dominated inflammatory disease, allergic asthma. Chaper 3 describes a novel finding of PI3K activity in controlling IgE switching in B cells, a key component of allergic inflammation, which could have a big impact on the pharmaceutical development of PI3K inhibitor compounds. Lastly, Chapter 4 pushes forward our understanding of the molecular regulation of GC responses by identifying the role of Bam32 in a later stage of GC progression.

Chapter 2 Roles of PI3Ks in Controlling Allergic Airway Inflammation and Hyperresponsiveness

2-1 Specific introduction

Class IA PI3Ks, activated by stimulation through a variety of receptor types, including antigen receptors, co-stimulatory receptors and certain cytokine receptors, are key signaling enzymes regulating B and T cell functions [146, 275]. Studies on mice bearing genetic deletions of the Class IA regulatory subunits have revealed critical roles of these molecules in B lymphocyte development and activation [207, 213]. Effects of regulatory subunit deficiencies on T cells are less clear: deficiency of the p85α regulatory subunit for class 1A PI3Ks was reported to have no effect on T cell activation in vitro, while deficiency in the p85β regulatory subunit increased T cell proliferation in vitro [276]. One group found that p85-deficient mice have enhanced responses to Leishmania infection [277], but reduced immunity to nematode infection [278], suggesting that impaired Class IA PI3K signaling leads to an immune dysregulation rather than a general immunodeficiency. However, the interpretation of these studies is complicated because regulatory subunits each affect the stability and activity of multiple catalytic subunits and may have adaptor functions in signaling independent of PI3K catalytic subunits.

Recent work has begun to explore the specific roles of different PI3K catalytic subunit isoforms in T cell functions. Analysis of p110α and p110β deficiency has been hindered

by the lethality of these mutations [279, 280]. We have used a targeted mutagenesis approach to generate mice expressing normal levels of p110δ bearing an inactivating point mutation in the kinase domain [161]. It was shown that T cells from p110δ-D910A mice had reduced antigen-induced proliferation in vitro, but this appears in some cases be overcome by strong signaling through co-stimulatory receptors [161]. Our lab also found that after Ovalbumin (OVA) immunization, the primary immune response of p110δ-D910A mice is type 1 dominant, showing enhanced production of type 1 cytokines, but reduced production of type 2 cytokines. In addition, our lab showed that dysregulated IL-10 production in p110δ-D910A mice is responsible for the finding of increased level of IFN-γ by analyzing IL-10 production and by using anti−IL-10 neutralizing antibodies.

The rationale of this chapter is based on the previous finding in the lab, showing that genetic inactivation of p110δ leads to Th1-skewed primary immune responses after OVA immunization. Since Th2 type responses have been demonstrated to play important role in the pathogenesis of asthma, I hypothesized here that altered Th1/Th2 balance caused by genetic mutation of p110δ catalytic isoform may be protective against asthma pathogenesis.

2-2 Material and Methods

2.2.1 Mice

P110$\delta^{D910A/D910A}$ mice have been described previously, and the P110$\delta^{D910A/D910A}$ mutation completely inactivates catalytic activity of p110δ subunit [161]. They were backcrossed

on a C57BL/6 or Balb/c background for 9 generations. Age and sex matched control wild-type (WT) C57BL/6 or Balb/c mice were purchased locally or from Charles River Canada. Control and P110$\delta^{D910A/D910A}$ mice were used between 7 and 12 weeks of age. All mice were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg) in compliance with the guidelines established by the Canadian Council on Animal Care.

2.2.2 OVA sensitization and airway challenge

Mice were immunized by intraperitoneal (i.p.) injection of 2 μg ovalbumin (OVA) protein (Sigma) adsorbed onto 2.0 mg $Al(OH)_3$ (alum) adjuvant (Imgect Alum; Pierce Chemical Co, Cheshire, United Kingdom) and boosted 14 days later with a second identical immunization. On days 28 and 31 after the initial immunization, the mice were deeply anesthetized by using isoflurane, and then the mice were challenged intranasally with 50 μg OVA in 40 μl of sterile saline. On day 35 after the initial immunization, the mice were sacrificed to collect blood, bronchoalveolar lavage fluid (BALF), lung, and spleen. The establishment of asthma model and experimental design are illustrated in figure 2.2.1.

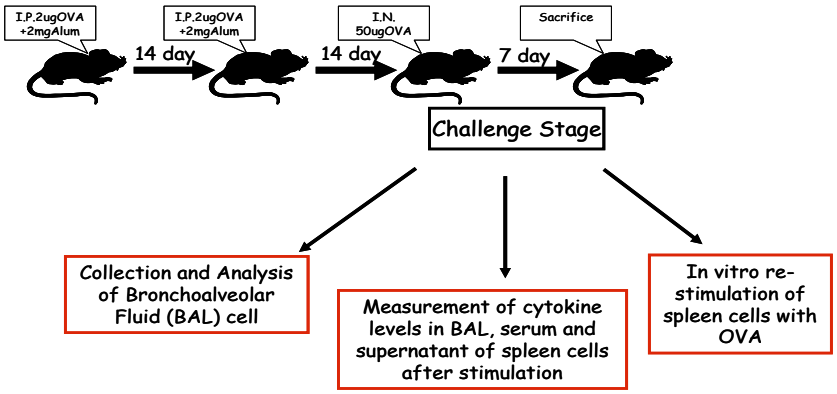

Figure 2.2. 1 OVA sensitization and airway challenge mouse model

2.2.3 Cardiac puncture, BALF, and lung collection

The mouse was deeply anesthetized by using NO or Halothane, and then the abdomen and the chest was surgically opened to expose the heart. Directly insert needle into the heart, and slowly draw the blood out. After euthanasia, the trachea of the mouse was cannulated, and the lung was slowly washed twice with 1ml cold phosphate buffered saline (PBS). After BALF collection, the lung tissue was cut and fixed directly in the 10% buffered formalin.

2.2.4 BALF cell cytospin and differential cell counting

After counting of BALF cells under microscopy, cells were resuspended in PBS to make concentration 1×10^6/ml. 50μl of this suspension was loaded to the cuvette attached to a superfrost microscope glass slide (Fisher Scientific, Ontario, Canada). Cells were then centrifuged at 1000 rpm for 5 min to prepare slides. The slides were air-dried, fixed with ethanol, and then stained with Fisher Leukostat Stain Kit (Fisher Scientific, Ontario, Canada). The number of monocytes, lymphocytes and eosinophils were determined based on their morphology and staining characteristics by two independent observers.

2.2.5 Histopathological analysis of lung tissue

Formalin-fixed lung tissues were dehydrated and embedded in paraffin by using tissue processor (Sakura FineTek Inc.). Paraffin-embedded tissues were sectioned to be 5μm thick by using a Leica RM2245 microtome (Leica Microsystems Inc, Ontario, Canada) and then attached to a microscope superfrost plus glass slide (Fisher Scientific, Ontario, Canada). Tissues sections were de-waxed and then stained with hematoxylin and eosin

(H & E) as following. The slides were stained in hematoxylin (Fisher Scientific, Ontario, Canada) for 15min, and then rinsed in tap water. The slides were then de-stained in 0.5% acid alcohol (5ml concentrated HCL in 1L 70% Alcohol) for seconds and rinsed in tap water. Finally, the slides are counterstained in Eosin (Fisher Scientific, Ontario, Canada) for 5min and rinsed in tap water. After H&E staining, the slides were examined for pathological changes by Olympus CK2 light microscopy. Bronchial mucus and mucus-containing goblet cells within airway bronchial epithelium were stained by a periodic-acid Schiff (PAS) staining kit (Sigma, St. Louis, MO).

2.2.6 In vitro re-stimulation of splenocytes and supernatant collection

Aseptically removed spleens were homogenized using a ground-glass tissue grinder to prepare the single-cell suspensions. Red blood cells were removed by adding ice-cold ACK (Ammonium-Chloride-Potassium) lysing buffer (0.83% NH_4Cl, 0.1% $KHCO_3$). Cell suspensions were then centrifuged and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 20μM 2-mercaptoethanol. Triplicates of splenocytes from each mice were cultured in round bottom 96-well plate at 7.5×10^6 cells/ml (280 μl/well) alone or with 300 μg/ml OVA stimulation in complete RPMI-1640 culture medium at 37°C in a 5% CO_2 atmosphere. Culture supernatants were harvested at 48 and 72 hours for the measurement of cytokine levels (IL-4, IL-13, CXCL10 and IFN-γ).

2.2.7 ELISA analysis of cytokines and chemokines

Levels of cytokines and chemokines in culture supernatants and BALF were detected by using sandwich ELISA. ELISA antibody pairs for IL-4, IFNγ, CXCL10 were purchased from Peprotech, and the antibody pair for IL-13 from R&D systems, Minneapolis, MN. All cytokine and chemokine standards are purchased from Peprotech. The following table shows the concentrations of those antibodies and the starting concentrations of standards used for ELISA.

	Capture antibodies	**Detection antibodies**	**Standards**
IL-4	1 μg/ml	0.2 μg/ml	2000 pg/ml
IL-13	1 μg/ml	1 μg/ml	1000 pg/ml
IFN- γ	2 μg/ml	0.3 μg/ml	5000 pg/ml
CXCL10	0.5 μg/ml	0.1 μg/ml	4000 pg/ml

Briefly, 96-well ELISA plates were coated with 50 μl/well capture monoclonal antibodies in coating buffer (0.1 M $NaHCO_3$, pH 8.2). After overnight incubation at 4°C, the plates were blocked with 100 μl/well blocking buffer (0.05% Tween-20, 1% bovine serum albumin (BSA) in 1×PBS, pH 7.4) for 2 hours at room temperature (RT) and then washed with washing buffer (0.05% Tween-20 in 1×PBS, pH 7.4). Serial diluted BAL fluids (from 1:2 to 1:16) or supernatants (from 1:2 to 1:16) and standards were added to the wells at 50 μl/per well, and then incubated overnight at 4°C. After extensive wash, biotinylated detecting mAbs were added to the well and incubated at 37°C for 2 hours. The plate was then washed and incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, INC) at 37°C for 45min. Finally,

the plates were washed and then added with 50 µl /well of alkaline phosphatase substrate (Sigma-Aldrich, Canada) in substrate buffer. The plates were read at 405 nm and 630 nm at ELISA reader by using SoftMax Pro software.

2.2.8 Sensitized splenocyte adoptive transfer experiments

Wild-type and p110$\delta^{D910A/D910A}$ mice at C57BL/6 background were immunized with 2 µg OVA/2 mg alum by i.p. route. 14 days later, spleens were aseptically removed to obtain single cell suspension as mentioned previously. Splenocytes were re-stimulated in vitro with 600 µg/mL OVA at the concentration of 7.5×10^6 cells/ml for 3 days. Re-stimulated splenocytes were harvested and washed in PBS twice. Then, the splenocytes were re-suspended in PBS and transferred to non-irradiated naïve recipient C57BL/6 mice by i.p. route (5×10^6 cells per recipient). Three days after adoptive transfer, the mice were challenged intranasally with 50 µg OVA and 5 days later the resulting airway eosinophilia was quantified as above.

2.2.9 Analyses of airway methacholine responsiveness

As a functional correlate to the degree of airway inflammation, respiratory responsiveness was measured in unrestrained animals using whole body plethsymography (Buxco, Troy, NY). This work was done in collaboration with Dr. Ganesh Srinivasan and Dr. Andrew Halayko at the Lung Function lab in Manitoba Institute of Child Health (MICH). After system equilibration for 30-60 minutes, a model 5500D De-Vilbiss Electronic Nebulizer was used to expose mice to aerosolized physiological saline for 3 min and then increasing concentrations of nebulized

methacholine. The settings for dilution flows and aerosol time period were selected according to the manufacturer's recommendations. Mice were allowed to stabilize for at least 5 minutes to allow breathing patterns return to baseline levels between each aerosol exposure. Recordings were taken for 3 min after each nebulization. During plethysmography, pressure differences measured between a mouse chamber and a reference chamber during expiration and inspiration are used to calculate enhanced paused (Penh), a parameter that is a function of total pulmonary airflow during the respiratory cycle. Penh is dependent on breathing pattern and can correlate well with airway resistance measured in ventilated mice [281, 282]. Respiratory responsiveness was calculated from the correlation between change in Penh and concentration of inhaled methacholine from 0-25 mg/ml.

2-3 Results

2.3.1 After allergen sensitization and airway challenge, Th2 cytokines generated by p110δ$^{D910A/D910A}$ splenocytes are significantly reduced

Dr. Baher Nashed in our lab found that p110δ-D910A mice, bearing a kinase-inactive p110δ, not only exhibit selective deficits in lymphocyte proliferative responses to specific stimuli [160, 161], they also demonstrate skewed type 1 cytokine production after primary immunization. These results showed that p110δ-D910A mutants developed weak type 2 cytokine production as indicated by two- to five-fold reductions in the OVA-induced production of IL-4, IL-5 or IL-13. In contrast, p110δ-mutant cells exhibited robust secretion of the type 1 cytokine IFNγ and the IFN-γ inducible chemokine CXCL10.

Examination of serum CXCL10 levels revealed increases in OVA-immunized p110δ-mutant mice, consistent with a systemic skewing towards type 1 responses.

To determine whether the deficit in type 2 cytokine production can be overcome by repeated immunization, OVA-primed wild-type or p110δ–D910A mice were given an intraperitoneal booster immunization, followed by respiratory OVA challenge, in a protocol known to evoke strong type 2 responses and type 2-mediated airway inflammation [283]. Splenocytes obtained from OVA sensitized and challenged mice were re-stimulated in vitro with 300 μg/ml OVA, showing that induction oof IL-4 and IL-13 was greatly reduced in p110δ mutants (Figure 2.3.1A and B), consistent with a persistent inability to develop strong type 2 responses. IFN-γ production by p110δ mutants was similar to wild-type controls under these conditions (Figure 2.3.1C); however, significantly enhanced CXCL10 secretion by 2-fold increase from p110δ–mutant cells was still observable (Figure 2.3.1D).

Figure 2.3.1

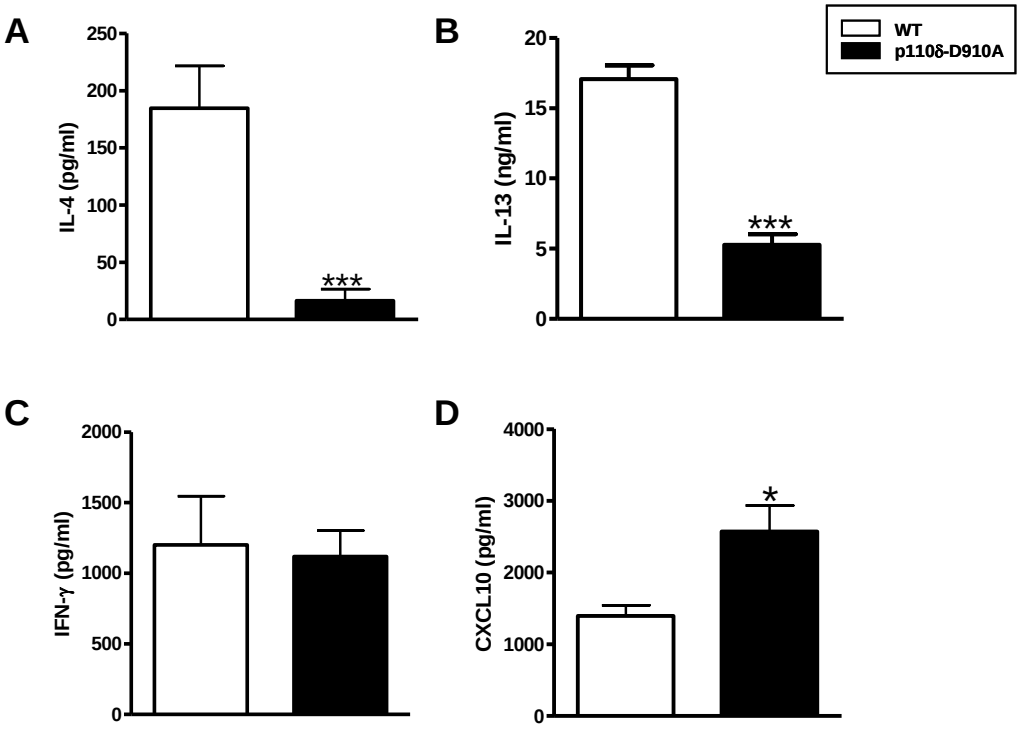

Figure 2.3.1 The secondary immune response reveals persistently reduced type 2 cytokine production in p110δ mutant mice

Spleen cells were isolated from wild type C57BL/6 and p110δ-D910A mice on C57BL/6 background after a second OVA immunization and intra-nasal challenge. This immunization protocol has been shown to give strong type 2 responses leading to airway inflammation in both C57BL/6 and Balb/c mice. Spleen cells were cultured with 300 μg/ml OVA and supernatants were analyzed for IL-4 (A), IL-13 (B), IFN-γ (C) and CXCL10 (D) by ELISA. In this and subsequent figures, open bars indicate controls (WT) and solid bars indicate p110δ mutant mice. Results represent the average and standard error of 6-12 mice per group over three experiments. *, $p<0.05$ as compared to the wild type; **, $p<0.01$ as compared to the wild type; ***, $p<0.001$ as compared to the wild type.

2.3.2 Reduced level of Th2 cytokines is also observed in BALF

To examine the local inflammatory milieu in the airway, the cytokine production profile was detected in the bronchoalveolar lavage fluid (BALF) of mice after intranasal OVA challenge. Consistent with the splenocyte recall results, BALF collected from p110δ mutants contained substantially reduced levels of IL-4, in line with similar levels of IFNγ and significantly increased levels of CXCL10 (Figure 2.3.2). Thus, local cytokine production in the lung, presumably secreted by recruited immune cells, also showed Th1 skewed phenotype.

Figure 2.3.2

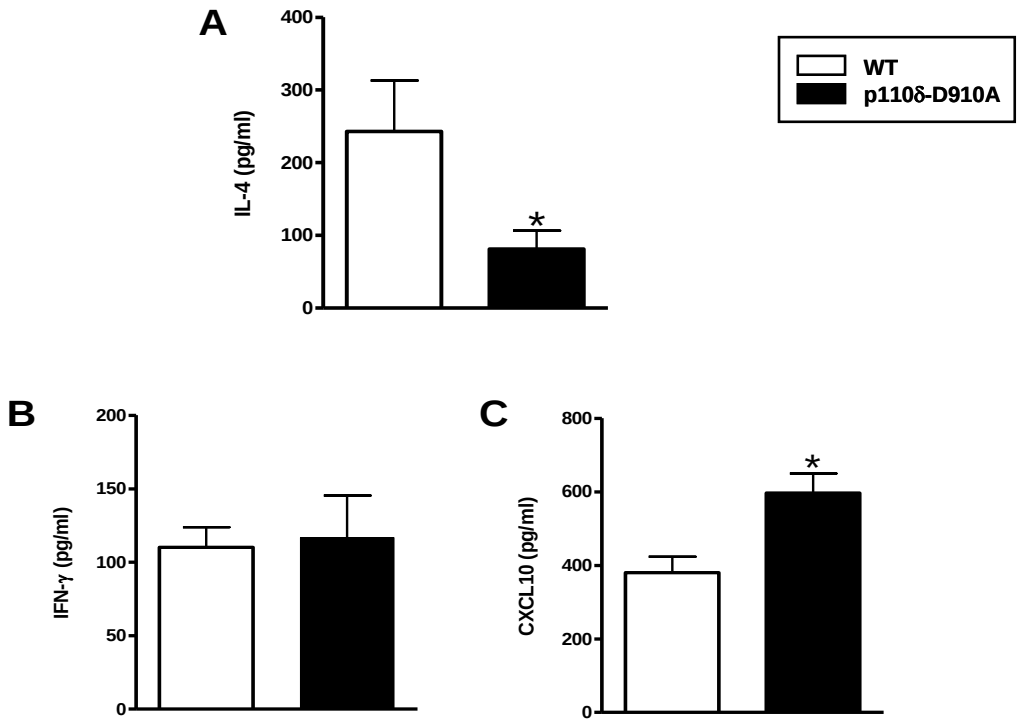

Figure 2.3.2 Imbalanced Th1/Th2 cytokine production in the BALF of p110δ mutant mice

1 ml Bronchoalveolar lavage fluid (BALF) was collected 6 days after intranasal challenge with OVA. Levels of IL-4 (A), IFN-γ (B) and CXCL10 (C) in the BALF were detected by ELISA. Results represent the average and standard error of 6-12 mice per group over three experiments. *, $p<0.05$ as compared to the wild type.

2.3.3 Eosinophilic airway inflammation is prevented in p110δ$^{D910A/D910A}$ mice

To assess whether the persistent reduction in type 2 cytokine production is associated with reduced type 2-mediated functional responses, we examined airway inflammation induced after intranasal challenge with OVA. We found that after intranasal challenge, total numbers of inflammatory cells infiltrated into the lung of p110δ mutants was dramatically reduced compared with wild-type mice (Figure 2.3.3A). Of those infiltrated cells, the percentage of eosinophils was further reduced by more than half in p110δ mutants (Figure 2.3.3B). Lung sections harvested from wild-type or mutant mice were stained with H&E and periodic acid-Schiff (PAS) to reveal local inflammation and mucous-producing goblet cells. OVA-exposed WT mice showed numerous inflammatory cell infiltrations in the lung interstitium around the airways and pulmonary blood vessels (Figure 2.3.3C and E). In contrast, inflammation is dramatically absent in the lung tissue of OVA-challenged p110δ mutants (Figure 2.3.3D and F). In addition, PAS staining clearly showed goblet cell hyperplasia and mucus production in the airways of OVA-challenged WT control mice (Figure 2.3.3G), but mucous production was visibly reduced in p110δ mutants (Figure 2.3.3H).

Figure 2.3.3

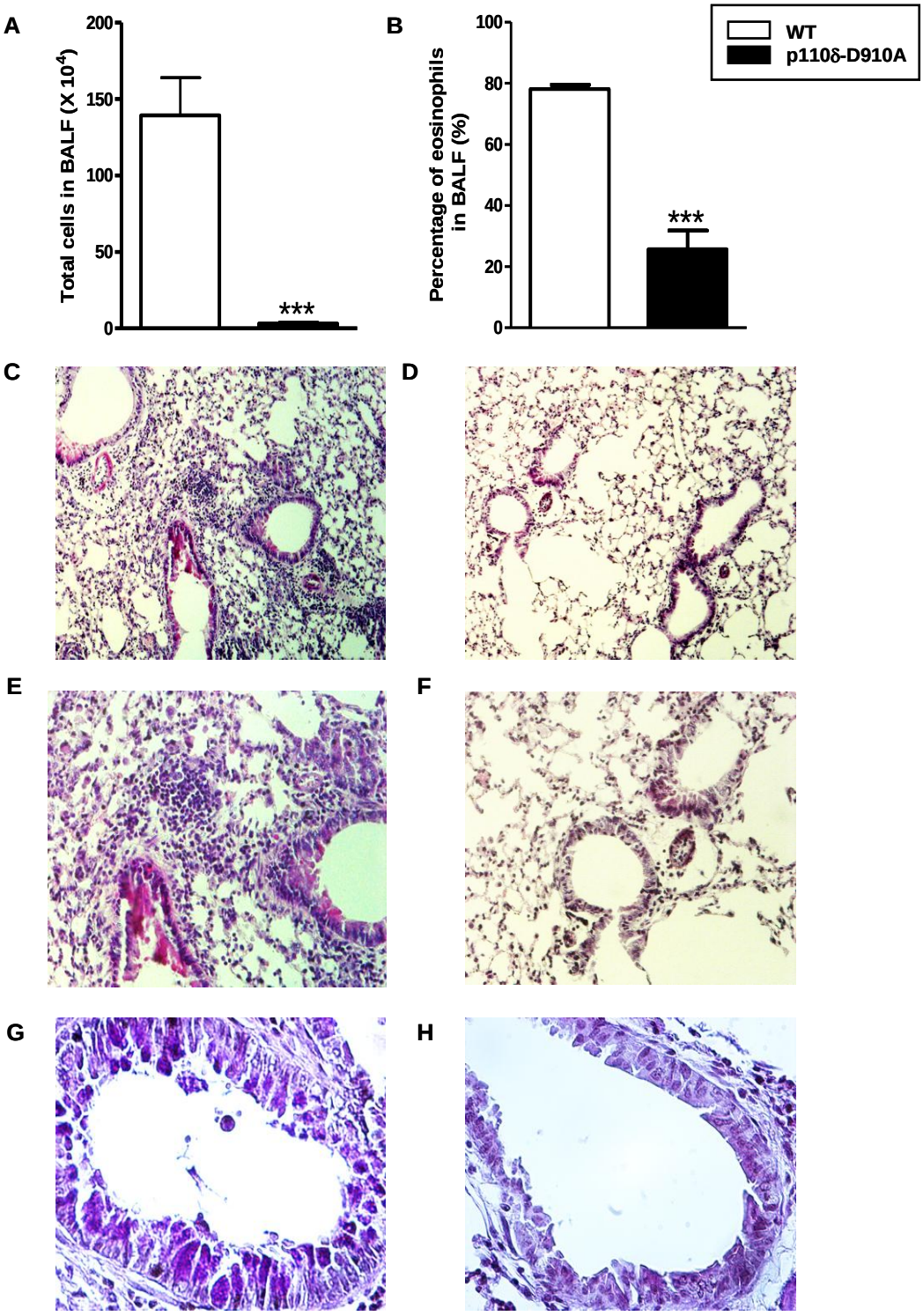
A
Total cells in BALF (X 10⁴)
200
150
100
50
0
***
B
Percentage of eosinophils in BALF (%)
100
80
60
40
20
0
***
WT
p110δ-D910A
C
D
E
F
G
H

Figure 2.3.3 Reduced induction of airway inflammation in p110δ mutant mice

After OVA sensitization and intranasal challenge, BALF and lung tissues were collected. A) The total cell numbers in the BALF were determined using a haemocytometer. Results represent the average and standard error of 6-12 mice per group over three experiments. ***, $p<0.001$ as compared to the wild type. B) BAL cell cytospin slides were stained with Fisher Leukostat Stain kit, and the percentage of eosinophils was determined by randomly counting 100 cells under light microscopy. Results represent the average and standard error of 6-12 mice per group over three experiments. ***, $p<0.001$ as compared to the wild type. The lung sections were stained by haematoxylin and eotaxin (H&E) for inflammation (C and D, 100×; E and F, 200×) and by periodic acid–Schiff (PAS) staining kit (Sigma) for mucus and mucus-containing goblet cells in the bronchial epithelium (G and H, 400×). (C) and (E) are representative H&E staining of lung sections from WT mice; (D) and (F) are representative H&E staining of lung sections from p110δ-D910A mice. G) Representative PAS staining of lung sections from WT mice; H) Representative PAS staining of lung sections from p110δ-D910A mice.

2.3.4 Attenuated airway hyperresponsiveness in p110δ$^{D910A/D910A}$ mice

To determine whether the reduced airway inflammation impacted significantly on lung function, respiratory responsiveness was assessed using whole body plethsymography. In naïve mice, no differences in respiratory responsiveness between wild-type and p110δ mutants were observed following administration of graded doses of the bronchoconstrictor methacholine (Figure 2.3.4A). This indicates that p110δ mutation has no intrinsic impact on lung function in the absence of airway inflammation. After priming and induction of airway inflammation, wild-type animals showed substantially increased respiratory responsiveness, while p110δ mutants were resistant to induction of hyper-responsiveness compared to naïve wild-type mice (Figure 2.3.4B). Hence, inactivation of p110δ has a dramatic impact on development of respiratory hyper-responsiveness to inhaled methacholine, which are consistent with reduced Th2 cytokine production and attenuated airway inflammation.

Figure 2.3.4

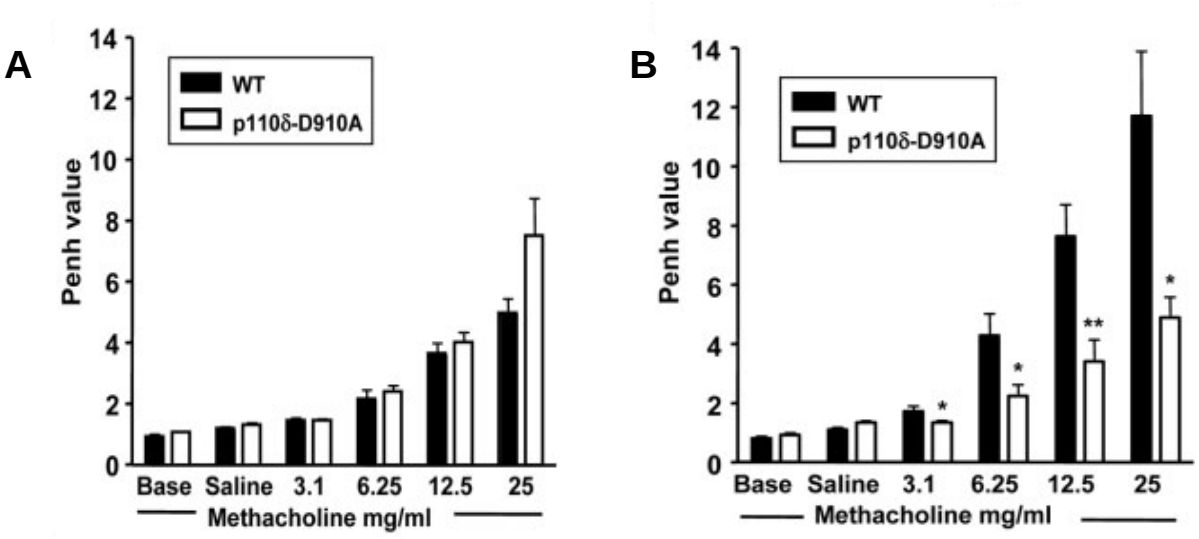

Figure 2.3.4 Reduced induction of respiratory hyper-responsiveness in p110δ-D910A mice

Measurement of respiratory responsiveness to increasing concentrations of methacholine were performed on naïve and airway-challenged Balb/c and p110δ-mutant BALB/c mice. Results are expressed as Penh values and represent the average and standard error of 8 mice per group over two experiments. A) Naïve p110δ-mutant mice have similar respiratory responsiveness to controls. B) p110δ-mutant mice have significantly reduced respiratory hyper-responsiveness compared to controls. Responses were measured five days after initial intranasal challenge. *, $p<0.05$ as compared to the wild type. **$p<0.01$ as compared to the wild type.

2.3.5 Splenocytes, not structural cells, of p110δ$^{D910A/D910A}$ mice are responsible for dramatically reduced airway inflammation

To determine whether the difference in airway eosinophilic infiltration is due to a lack of p110δ activity in hematopoietic cells or airway structural cells, we transferred spleen cells from OVA-immunized p110δ mutants or wild-type mice to naïve mice of either genotype and then intranasally-challenged with OVA (Figure 2.3.5 A). The results showed that primed spleen cells from wild-type mice induced similar levels of eosinophil migration when adoptively transferred to naïve wild-type or p110δ mutant recipients (Figure 2.3.5 B). This demonstrates that p110δ-D910A mice can support inflammatory responses within their airways given a source of appropriately primed immune cells. In contrast, primed spleen cells from p110δ mutants only induce one-fifth the level of eosinophil migration, regardless of whether they are transferred into wild-type or mutant mice (Figure 2.3.5 B). These results demonstrate that the resistance to airway inflammation is due to the requirement for p110δ signaling in hematopoietic cells.

Figure 2.3.5

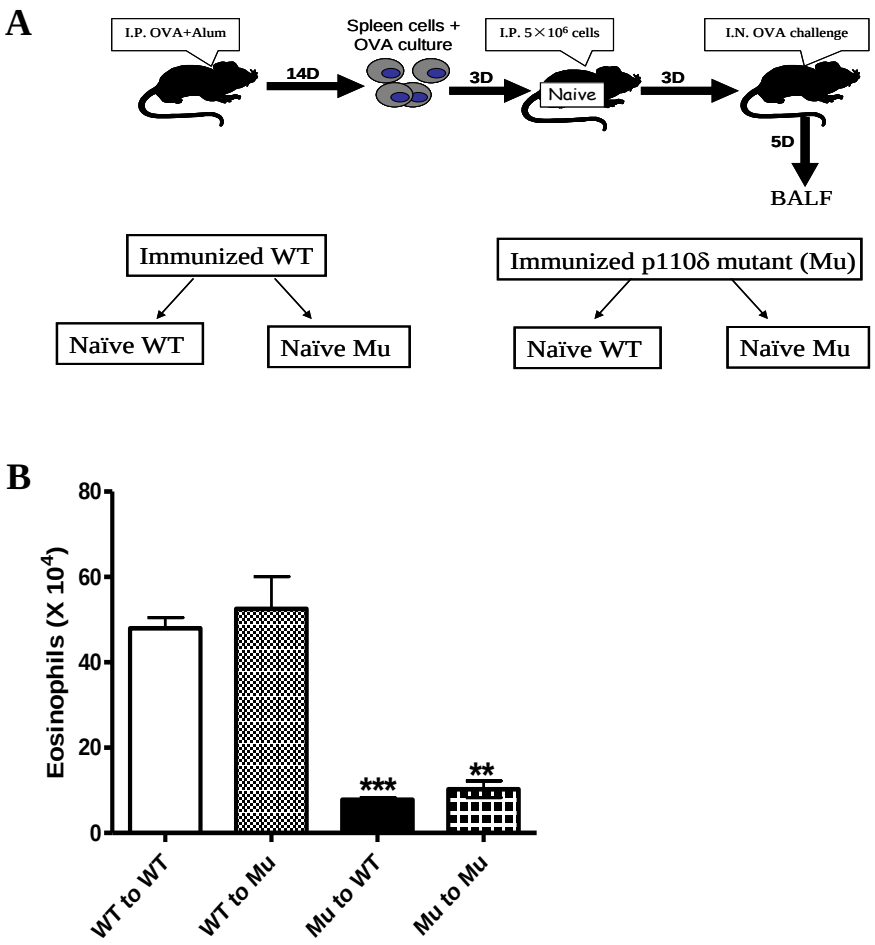

Figure 2.3.5 Adoptive transfer of primed p110δ mutant splenocytes induces much less airway eosinophila than primed wild-type cells

A) Schematic illustrating the adoptive transfer system. Splenocytes from OVA-primed mice are restimulated with OVA for 3 days and then transferred to naïve mice. Three days after transfer, recipient mice are challenged with OVA intranasally. Five days after challenge, bronchoalveolar lavage is harvested and eosinophils are counted. B) Eosinophil counts in BAL from all genotype combinations of primed cell donors and naïve recipient mice, showing that p110δ mutant donor cells are unable to induce strong airway eosinophilia. **$p<0.01$ as compared to the WT to WT adoptive transfer group.

2.3.6 Serum IgE levels are surprisingly increased in p110δ$^{D910A/D910A}$

We next examined whether reduced Th2 responses in p110δ mutant could affect systemic IgE Ab levels, which are essential for mast cell degranulation and asthma pathogenesis. However, to our surprise, after OVA sensitization and challenge, levels of serum total IgE are increased by 5-fold compared with that of WT control (Figure 2.3.6). OVA-specific IgE was subsequently detected by using biotinylated-OVA, revealing that there is more than 10-fold of increase of OVA-specific IgE in p110δ mutant (Figure 2.3.6). The discrepancy between IL-4 secreted by splenocytes and systemic IgE production in p110δ mutant drove us to do further studies to understand the reason, which will be elaborated in the next chapter.

Figure 2.3.6

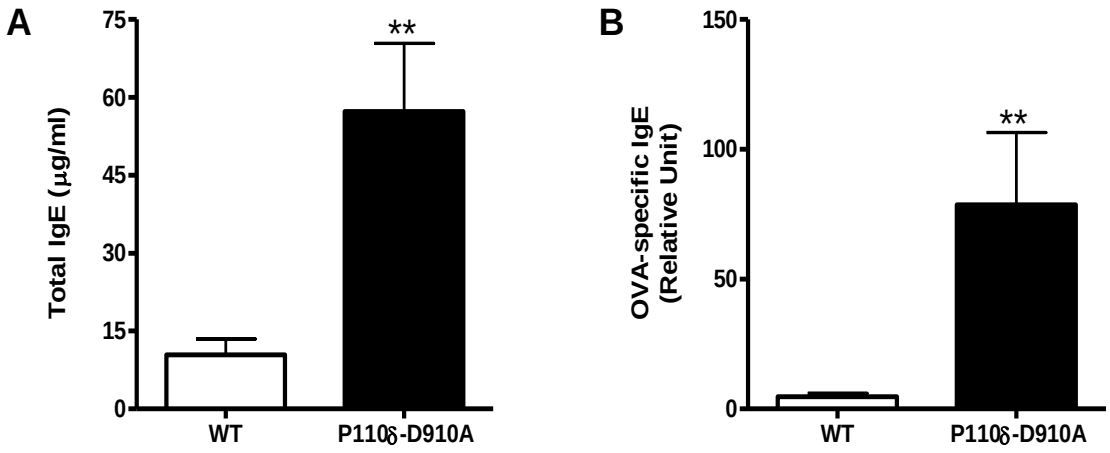

Figure 2.3.6 Increased levels of serum IgE in OVA-challenged p110δ mutant mice

Sera were collected at day 6 following OVA intranasal challenge and examined for total and OVA-specific –IgE using ELISA (A and B). B) To give relative unit of OVA-specific IgE, sera from 6 immunized p110δ mutant mice were pooled and the pooled serum was assigned as intra-standard: 1:100 dilution equals to 100 relative unit. Results represent the average and standard error of 5-7 mice per group over two experiments. **, $p<0.01$ as compared to the wild type.

2-4 Discussion

2.4.1 Regulatory roles of p110δ signaling in type 1 and type 2 immune responses

Consistent with the findings observed by my colleagues showing that p110δ mutant mice generated weak type 2, but rather potentiated type 1 response after a single OVA immunization, here I found that the observed 75-95% reduction in type 2 response is maintained even after multiple immunizations with strongly type 2-promoting conditions, and can be observed both locally (in the lung) and systemically (in the spleen). Collectivel, my data suggest a critical requirement for p110δ signaling in generation of type 2 responses. After OVA immunization, IFN-γ production in p110δ mutants is similar or even enhanced compared with that of WT mice, which is unexpected given the fact that T cell proliferation and IL-2 production upon antigen stimulation are impaired in the absence of p110δ signaling [161].

Leishmania major infection model has been used to investigate the role of PI3K signaling in Th1 and Th2 development because Th1 responses are required for disease control and Th2 responses are associated with disease progression. Using p85α (regulatory subunit of class IA PI3Ks) –deficient mice, Fukao and colleagues found that PI3K signaling negatively regulates IL-12 production in dendritic cells stimulated by toll like receptor ligands, leading to enhanced IFN-γ and TNF-α production by p85α-deficient T cells upon *leishmania major* infection [277]. We collaborated with Dr. Jude Uzonna's lab in studies showing that p110δ mutant mice are resistant to *leishmania major* infection,

despite diminished Th1 and Th2 cytokine production [284]. Further experiments demonstrated that the resistance is conferred by the dramatically reduced effector functions of IL-10–producing regulatory T cells [284].

Here, in the OVA/alum model, potentiated type 1 response was generated after primary immunization, which could be explained by the intrinsic pre-disposition of p110δ-deficent T cells towards Th1 differentiation. However there is no evidence for such a mechanism using an in vitro differentiation model with purified TCR transgenic cells [285]. Rather, we find here that p110δ-D910A mice mount a weak regulatory IL-10 response, which in turn contributes to high "de-regulated" IFNγ expression. Thus, in the presence of the physiological regulatory circuits, reduced IL-10 allows p110δ deficient T cells to develop a predominant, unopposed type 1 response. One possible reason for enhanced IFNγ production in OVA/alum immunization is that this is a strong "Th2-polarizing" stimulus, where IFNγ is strongly suppressed in normal mice. In contrast, *Leishmania major* infection model in C57BL/6 background strongly promotes Th1 differentiation, where the suppression of IFNγ by IL-10 is negligible in normal C57BL/6 mice. This interpretation is consistent with the previously described sporadic inflammatory bowel disease phenotype occurring in older p110δ–inactivated mice [161], since IBD is known to involved de-regulated Th1 responses and is opposed by IL-10 [286].

The persistent inability of p110δ–inactivated mice to generate type 2 cytokines and effector responses suggests that PI3-kinase signaling plays a critical role in generation of

these responses that cannot be compensated by other signaling pathways. PI3K downstream signaling molecules ITK and PDK1 have also been reported to be required for Th2 differentiation in vivo [287-289]. Excessive addition of IL-4 *in vitro* in the presence of other physical costimulus can not efficiently drive p110δ–inactivated T cells into IL-4–producing cells. Thus, it is tempting to speculate that IL-4 receptor signaling is deficient in p110δ–inactivated T cells since signaling through the IL-4 receptor is known to activate PI3-kinase signaling [290, 291]. The phenotype I describe here is similar to that seen in STAT6-deficient mice, which are defective in IL-4 signaling [292, 293]. Since IL-4 signaling is critical in feed-forward amplification of type 2 responses, defective IL-4 signaling in T cells could at least partially explain reduced type 2 priming in vivo.

2.4.2 Regulatory roles of p110δ signaling in Th2–associated disease: asthma

Consistent with defective induction of type 2 responses in p110δ mutant mice, I found a striking reduction in expression of Th2-dependent airway inflammatory responses. The reduced airway inflammation in p110δ-inactivated mice is most likely to result from defective induction of type 2 responses, since type 2 cytokines such as IL-4, IL-5 and IL-13 are known to play critical roles in triggering airway eosinophila [294-297]. Notably, reduced Th2 airway inflammation had significant effects on lung function, as p110δ-inactivated mice were resistant to the development of methacholine induced respiratory hyperresponsiveness after allergen challenge. In addition, I found that primed splenocytes from p110δ-inactivated mice have greatly reduced ability to transfer airway

inflammatory responses to naïve wild-type mice confirming that they are functionally impaired in this regard.

My results provide clear evidence that p110δ signaling is required for Th2-mediated airway inflammation. Previous studies found that transient blockade of all PI3K isoforms at the airway challenge stage led to substantial reductions in airway inflammation [298, 299]. Additionally, a recent report found that administering a p110δ-specific inhibitor compound at the airway challenge stage led to significant reductions in local type 2 cytokine production and airway inflammation [300]. This evidence is consistent with roles for PI3K signaling at the stage of Th2-mediated effector function, in addition to the role in priming that I demonstrate here. Given the known critical role for type 2 cytokines in initiation and maintenance of airway inflammation, it is likely that the block in type 2 cytokine production I have identified contributes significantly to inhibited airway responses in these mice. However, our data do not rule out the possibility that p110δ inactivation may additionally impact upon other functions relevant to airway inflammation, such as cell migration and homing. One study found that p110γ, but not p110δ, was required for neutrophil migration into the lungs after intranasal chemokine administration [301]; however no analogous studies have been reported for eosinophils. My data show that adoptive transfer of wild-type splenocytes (which contain very few eosinophils) can induce similar levels of airway eosinophilia in p110δ-D910A and wild-type hosts, arguing against a defect in eosinophil mobilization in these mutants.

PI3K signaling is also known to be important in regulation of airway-resident smooth muscle cells [302], which play critical roles in airway inflammation and airway hyper-responsiveness [303]. For example, one paper showed that IL-13-induced tracheal smooth muscle hyper-responsiveness in vitro is dependent on the activities of p110δ isoform [304]. However, the p110δ isoform is unlikely to be the major PI3K isoform expressed in airway smooth muscle, and my data show that p110δ-mutants exhibit airway inflammation equivalent to that of wild type controls if provided with primed wild-type immune cells. Thus, the major defect arising from p110δ deficiency lies at the level of qualitatively altered immune capacity rather than structural cells.

My results demonstrating the profound impact of p110δ deficiency on the normal development of type 1 vs type 2 biased immunity raises the possibility that therapies targeting PI 3-kinase signaling may be beneficial in the context of pre-established Th2-mediated diseases such as asthma or allergic rhinitis. Such a strategy has the potential to target the pathogenic process at several levels, including generation of type 2 cytokine responses. Indeed, development of isoform-specific inhibitors of PI 3-kinases with varied pharmacologic properties is being actively pursued [305, 306], which should allow the assessment of such strategies in the near future.

2.4.3 Paradoxically increased IgE levels in OVA/alum–immunized p110δ mutant mice

Even though dramatically reduced IL-4 production and attenuated airway inflammation and hyperresponsiveness were observed in OVA sensitized and challenged p110δ mutant

mice, I found enhanced antigen-specific IgE responses in those mice instead. It seems that increased IgE production was not sufficient to induce asthma pathogenesis of p110δ mutant mice in the asthma model I tested. However, we need to interpret the results carefully because p110δ signaling is essential for mast cell differentiation, activation and survival [278, 307]. In p110δ mutant mice, the numbers of mast cells distributed in various tissues are more or less reduced. Moreover, crosslinking of IgE–triggered mast cell degranulation is also impaired with p110δ inactivation. Thus, elevated IgE level in p110δ mutant mice does not lead to enhanced mast cell degranulation, and accordingly impact on local inflammation.

It is intriguing to understand why IgE production is increased when IL-4 is less available in p110δ mutant mice, as we know that IL-4 is definitely required for B cells to become IgE-producing cells [76, 308]. Both B cell intrinsic and extrinsic factors may account for this phenomenon. I suspected that reduced number of mast cells expressing high affinity IgE receptor FcεRI may lead to elevated serum free IgE due to less capture of IgE in tissues. A second possibility is that PI3K signaling may negatively regulate IgE-switching in B cells, which will be tested in the next chapter.

2.4.4 Summary of main conclusions and overall significance

In summary, p110δ signaling is required for Th2 cytokine production, whereas Th1 cytokine secretion is not altered or even enhanced in p110δ-inactivated mice, indicating that genetic inactivation of p110δ favors Th1-skewed immune responses. In OVA sensitized and nasal challenged p110δ-inactivated mice, Th2-mediated eosinophilic

airway infiltration and respiratory hyper-responsiveness are markedly attenuated. Adoptive transfer experiments suggest that p110δ signaling in hematopoietic cells, but not structure cells, is responsible for the induction of airway eosinophilia. The significance of this chapter is for the first time using p110δ–inactivated mice to demonstrate that p110δ signaling is one key player in OVA-induced airway inflammation and respiratory hyper-responsiveness. And these data suggest that p110δ is a possible therapeutic target for Th2-mediated airway disease.

Chapter 3 Roles of PI3Ks in regulating B cell antibody responses

3-1 Specific introduction

PI3K signaling has been shown to be critical for B cell development and activation. In vivo inactivation of catalytic subunit p110δ leads to partial block from pro-B to pre-B cells in BM, dramatic decrease of mature follicular B cells in peripheral lymphoid organs, and almost absence of MZ B cells and B1 cells [309]. P110δ-deficient B cells have defective proliferation and survival upon BCR crosslinking [161]. Furthermore, T cell-dependent antibody responses are impaired in p110δ-D910A mice associated with lack of GCs [161]. Our previous studies clearly showed that Th2 cytokine production is almost absent in p110δ-D910A mice [310], which may be another regulatory layer for reduced T cell-dependent antibody production.

Since Th2-mediated airway inflammation and hyperresponsiveness are dramatically attenuated in p110δ-D910A mice [310] and in mice treated with dominant-negative form of the PI3K regulatory subunit p85α [299], it is tempting to target PI3Ks for the treatment of human allergy and asthma. Targeting these enzymes pharmacologically also holds promise to treat inflammatory diseases and hematological cancers [311, 312]. As

such, the development of PI3K isoform-specific inhibitors is currently an area of intense activity [306, 313].

The rationale of this chapter is the previous unexpected findings that after nasal OVA challenge, serum total and OVA-specific IgE levels were significantly higher in p110δ-D910A mice than that of control mice despite lower switch factor IL-4. This unexpected result made us wonder whether PI3Ks intrinsically regulate B cell antibody production, especially IgE generation.

3-2 Material and Methods

3.2.1 Mice and immunization protocol

p110δ$^{D910A/D910A}$ mice[161] (C57BL6 background) were examined between 8 and 14 wk of age along with age- and sex-matched control mice (bred locally or purchased from Charles River Canada). WT or p110δ$^{D910A/D910A}$ mice were immunized i.p. by injection of 2 μg OVA/2 mg alum, and boosted 2 weeks later with a second identical immunization. 7 days after OVA challenge, the mice were sacrificed to collect serum for detection of antibody responses.

3.2.2 ELISA analysis of total and OVA-specific antibodies

Concentrations of capturing and detecting antibody pairs for measuring total IgM, IgG1 and IgE are listed in the following table. Purified IgM, IgG1 and IgE standards are purchased from BD Bioscience, and the starting concentration for IgM and IgG1 standards is 20 ng/ml, for IgE standard is 100 ng/ml. Serial dilution of serum samples

begins at $1:10^6$ for WT, at $1:10^5$ for $p110\delta^{D910A/D910A}$ mice to detect IgM and IgG1. For IgE detection, serial dilution of serum samples begins at 1:50.

	Capture antibodies	**Detection antibodies**
Total IgM	1 μg/ml	0.2 μg/ml
Total	1 μg/ml	1 μg/ml
IgG1	2 μg/ml	0.3 μg/ml
Total IgE		

For determination of OVA-specific IgM and IgG1, ELISA plates were coated with 20 μg/ml OVA. Serum samples were added to the wells by serial dilution from $1:10^5$ (WT mice) or $1:10^4$ ($p110\delta^{D910A/D910A}$ mice). Same biotinylated mAbs for measuring total Igs are used for detection. For determination of OVA-specific IgE, ELISA plates were coated with the capturing mAb, rat anti-mouse IgE. Serum samples added to the wells were diluted from 1:10. Bound IgE was detected with biotinylated OVA. OVA was biotinylated using biotin-succinimide (Sigma) in borate buffer at pH 8.8 according to the vendor's protocol. OVA-specific Abs were semi-quantified according to the standard curve obtained from serial dilutions of a hyper-immune mouse serum sample.

3.2.3 Splenic primary B cell purification

Splenic B cells were purified by negative selection using the αCD43 MicroBeads and MACS columns (Miltenyi Biotech). In brief, 1×10^8 splenocytes were resuspended in 900 μl MACS buffer (0.5% BSA in 1×PBS) in 15ml tube, and then 100 μl αCD43

MicroBeads were added to the tube. After 15 min incubation on ice, the cells were washed with 10 ml MACS buffer. After centrifuge, the cell pellet was resuspended in 1 ml MACS buffer, and then added on the top of pre-washed MACS column. About 30 ml buffer running through the column was collected for B cells. B cell purity was around 95-98%, checked on flow cytometry by staining with fluorescence-labeled αCD19 or αB220.

3.2.4 B cell culture and PI3K activity inhibition in vitro

Purified B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, $2x10^{-5}$ M 2-ME) at final concentration 1×10^{6}/ml with 2 μg/ml LPS (Sigma) or 2 μg/ml αCD40 (BD Bioscience) together with 20 ng/ml IL-4 (R&D Systems). For further flow cytometric analysis, cells were stimulated in vitro for 3 days; for further ELISA analysis of secreted Igs in supernatant, cells were stimulated in vitro for 7 days. Pharmacological inhibitors were added at the indicated concentrations at the beginning of the culture period. Pan-PI3K inhibitor PI-103, PIK-90 and p110β-selective inhibitor TGX-115 compounds were synthesized and characterized as previously described.[306] The p110δ-selective inhibitor IC87114[314] was provided by Calistoga Pharmaceuticals (Seattle, WA) and p110β-selective inhibitor TGX-221[315] was a gift of Simone Schoenwaelder and Shaun Jackson (Monash University). mTOR inhibitor Rapamycin, Akt inhibitor IV and X was purchased from Calbiochem. Btk inhibitor LFM-A13 was purchased from Cayman Chemical.

3.2.5 IC87114 treatment of mice in vivo

For in vivo pharmacological PI3Kδ inhibition, WT mice were orally dosed using gavage with 50 μl 12.5 mg/ml IC87114 (dissolved in PEG400) to give 25 mg/kg dosage or equal amount of vehicle (PEG400) as control twice a day for 12 days. This dosing regimen resulted in peak serum levels of IC87114 in the 2-10 μM range, which are over 10 times higher than the IC50 (half maximal inhibitory concentration) without obvious side-effects. 1h after the first dosing, the mice were immunized with 10 μg OVA / 2 mg alum by i.p. route. At day 12, the mice were sacrificed to collect blood for antibody detection.

3.2.6 Flow cytometric analysis of intracellular antibodies in B cells

Antibodies used for flow cytometry staining were purchased from BD Bioscience, except for those specified. Before intracellular staining for anti-IgE, cells were treated for 1 min with 200 μl ice-cold acid buffer (0.085 M NaCl, 0.005 M KCl, 0.01 M EDTA, and 0.05 NaAcetate pH4) to remove passively-absorbed IgE bound to CD23.[316] The samples were then neutralized with 20 ml imcomplete RPMI1640 medium and washed twice before staining. Cells were incubated with Fc blocking antibody (mAb 2.4G2) first on ice for 10 min, and then the cells were washed and fixed in 500 μl 2% paraformaldehyde (PFA) at RT for 30 min. After fixation, cells were washed and then permeabilized with 500 μl permeabilizing buffer (0.1% Saponin, 2% FBS, 1×PBS pH7.4) on ice for 10 min. The cells were intracellularly stained with FITC-labeled anti-IgM, FITC-labeled anti-IgG1, FITC-labeled anti-IgE or PE-labeled anti-IgE (eBioscience) in permeabilizing buffer on ice for 20 min. Stained cells were analyzed using a FACSCalibur flow cytometer and data were plotted using FlowJo software (TreeStar, Portland, OR).

3.2.7 Flow cytometric analysis of cell division

Cells were labeled 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) to track cell division because CFSE dye is equally diluted to the descendants after cell divides. To label the cells, firstly, splenocytes were suspended in warm complete culture medium at concentration 1×10^7/ml. CFSE was diluted to 2.5 μM using warm PBS. Equal volumes of cells and CFSE were mixed in the 50 ml tube at RT in the dark with continuous gentle rocking. After 5 min, staining was quenched by adding warm equal volume of FBS. Then the cells were washed twice, counted, and resuspended in complete culture medium for in vitro culture. Non-stimulated CFSE-labeled cells were used for compensation when cells were acquired by using flow cytometry.

3.2.8 Real-time PCR

Cells harvested from the culture were pelleted in 1.5 ml eppendorf tubes, and then vigorously mixed with 1 ml Trizol reagent (Invitrogen). According to the Trizol protocol provided by the manufacturer, RNA was isolated and concentrations and purity were determined by UV absorbance at 260 nm and 280 nm. cDNA was synthesized from 2 μg of RNA using SuperScript II Reverse Transcriptase (Invitrogen), in the present of random primers, 10 mM dNTP, dithiothreitol (DTT) and RNasin. 1 μl cDNA product was used for the following quantitative real-time PCR (RT-PCR). RT-PCR was performed using SyberGreen amplification mix on the LightCycler System (Roche). The following LightCycler run protocol was used: denaturation program (95°C for 10 min),

amplification and quantification program repeated 40 times (95°C for 15 s, 59°C for 15 s, 72°C for 45 s with a single fluorescence measurement), melting curve program (60–95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement) and finally a cooling step to 40°C. Primer sequences used for detection of germline transcripts (μ, γ_1, and ε GLT), postswitch transcripts, AID, Blimp1, BCL6, IRF4, Pax5, ID2, E2A and β-actin were listed in the following table. β-actin was used as relative expression control to normalize sample variation.

	Forward primers	**Reverse primers**
μ GLT	CTCTGGCCCTGCTTATTGTTG	GAAGACATTTGGGAAGGACTGACT
γ1 GLT	GGCCCTTCCAGATCTTTGAG	GGATCCAGAGTTCCAGGTCAC T
ε GLT	TGGGATCAGACGATGGAGAATAG	CCAGGGTCATGGAAGCAGTG
γ1 postswitch transcript	CTCTGGCCCTGCTTATTGTTG	GGATCCAGAGTTCCAGGTCAC T
ε postswitch transcript	CTCTGGCCCTGCTTATTGTTG	CCAGGGTCATGGAAGCAGTG
AID	GGAGACCGATATGGACAGCCTTCTG	TCAAAATCCCAACATACGAAATGC
Blimp1	ACAGAGGCCGAGTTTGAAGAGA	AAGGATGCCTCGGCTTGAA
BCL6	TCAGAGTATTCGGATTCTAGCTGTGA	TGCAGCGTGTGCCTCTTG
IRF4	GGCTTCACAATCTTCAAGGTGGAC	CACACTTTCCTGTCGGGCTTAGAC
Pax5	CCGCCAAAGGATAGTGGAACTTG	CACAGTGTCATTGTCACAGACTCGC
ID2	AGCATCCCCCAGAACAAGAAGGTG	ATCGTCTTGCCCAGGTGTCGTTCT
E2A	CTAGCCCCTCAACGCCTGTG	CGGTGCCAACAGCGTGGCT
β–actin	TGGAGAAGAGCTATGAGCTGCCTG	GTGCCACCAGACAGCACTGTGTTG

3-3 Results

3.3.1 Serum IgE antibody is selectively increased in p110δ$^{D910A/D910A}$ mice

Mice bearing an inactivating mutation in the p110δ isoform of PI3K were previously shown to have markedly reduced IgM and IgG antibody responses after immunization with T-dependent or T-independent antigens [161]. Consistent with previous findings, unimmunized mice had substantially lower basal levels of IgM and IgG1 (Figure 3.3.1A). In contrast, basal levels of IgE were within the normal range (Figure 3.3.1A). Upon immunization of p110δ D910A/D910A mice with OVA, total IgE levels were markedly higher than normal, whereas IgM and IgG1 responses were markedly attenuated (Figure 3.3.1B). Strong IgE responses are also observed using TNP-KLH immunization (data provided by Dr. Okkenhaug). Levels of OVA-binding IgE were also increased in p110δ D910A/D910A mice (Figure 3.3.1C), consistent with increased antigen-specific induction of this antibody isotype. These results demonstrate that disruption of p110δ signaling leads to increased IgE levels in vivo. This is paradoxical, given that Type 2 cytokine responses induced by OVA immunization, including production of major IgE switch factor IL-4, were markedly inhibited in these mice [310].

Figure 3.3.1

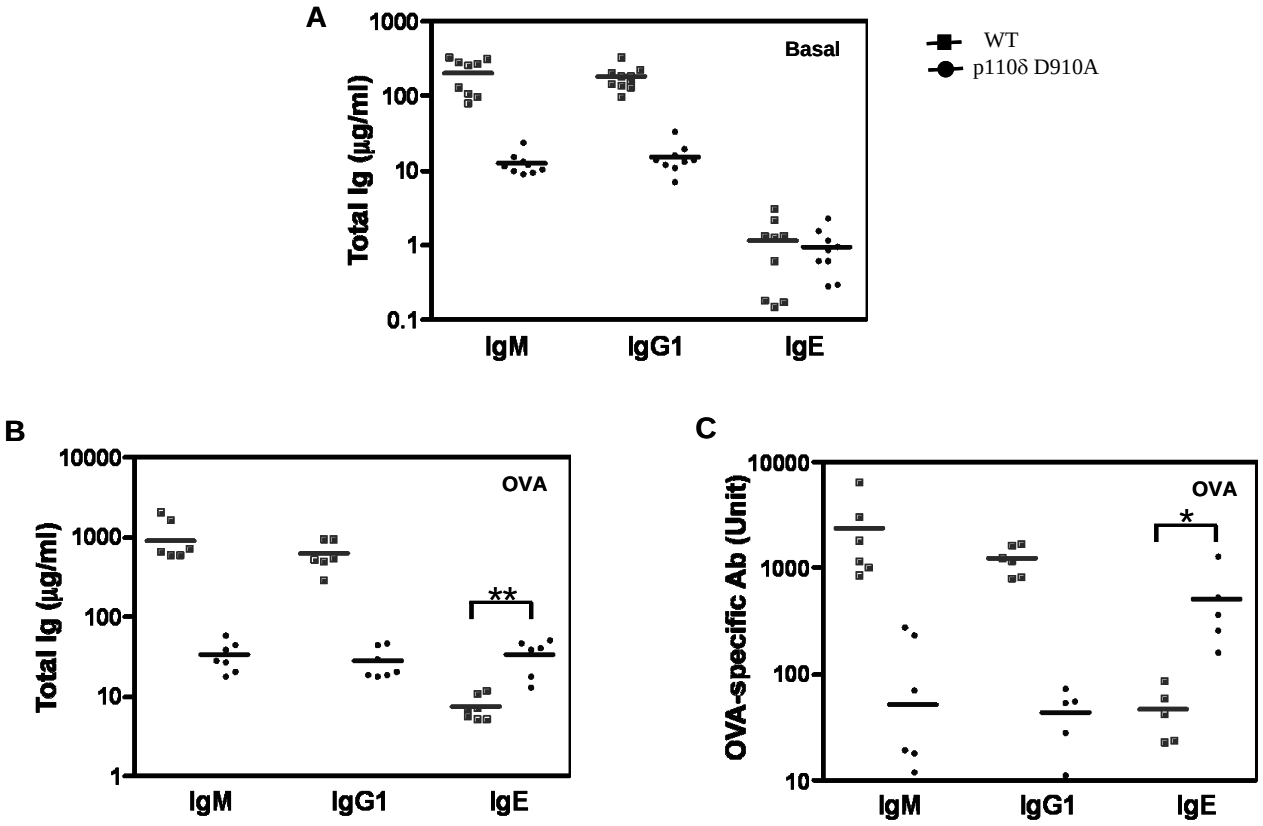

Figure 3.3.1 p110δ-D910A mice have selectively elevated IgE levels after OVA immunization

Mice were immunized with OVA/alum and boosted at day 14. Serum collected pre-immunization (A) or 7 days after the second immunization (B) were assessed for the indicated antibody isotypes by ELISA. (C) Levels of antigen-binding IgM, IgG1 or IgE antibodies were assessed after immunization. Solid square represented wild type, and open circle represented p110-D910A; * $p<0.05$; ** $p<0.005$ (Students T-test) compared to wild-type.

3.3.2 Increased frequency of switched cells, especially IgE-producing cells, are generated in p110δ$^{D910A/D910A}$ B cell cultures

To test whether p110δ$^{D910A/D910A}$ B cells are intrinsically predisposed towards IgE isotype switching, we assessed isotype switching of purified B cells *in vitro*. Splenic B cells purified from WT or p110δ$^{D910A/D910A}$ mice were stimulated with anti-CD40 and IL-4, known to facilitate isotype switch to IgG1 and IgE [82], and then IgG1- or IgE-expressing cells were detected by flow cytometry (Figure 3.3.2A). Strikingly, p110δ$^{D910A/D910A}$ B cells showed an approximately 5-fold higher frequency of IgE-positive cells in anti-CD40+IL4 cultures. IgG1-switched cells were also present at a higher frequency in p110δ$^{D910A/D910A}$ cultures; however, the increase is only 2.5-fold for this isotype (Figure 3.3.2A). Proportionally similar increases were observed in cultures containing LPS+IL4 (Figure 3.3.2B). Increased IgG1 and IgE expression was consistently observed in p110δ$^{D910A/D910A}$ cultures and was highly significant (Figure 3.3.2C and D).

To examine whether this inhibitory effect on isotype switching conducted by p110δ signaling is IL4–specific, anti-CD40/LPS+IFNγ stimulations are used to test IgG2a switching. A small but consistent twofold increase of the frequency of IgG2a+ cells was also observed in p110δ$^{D910A/D910A}$ cultures (Figure 3.3.2E and F).

Figure 3.3.2

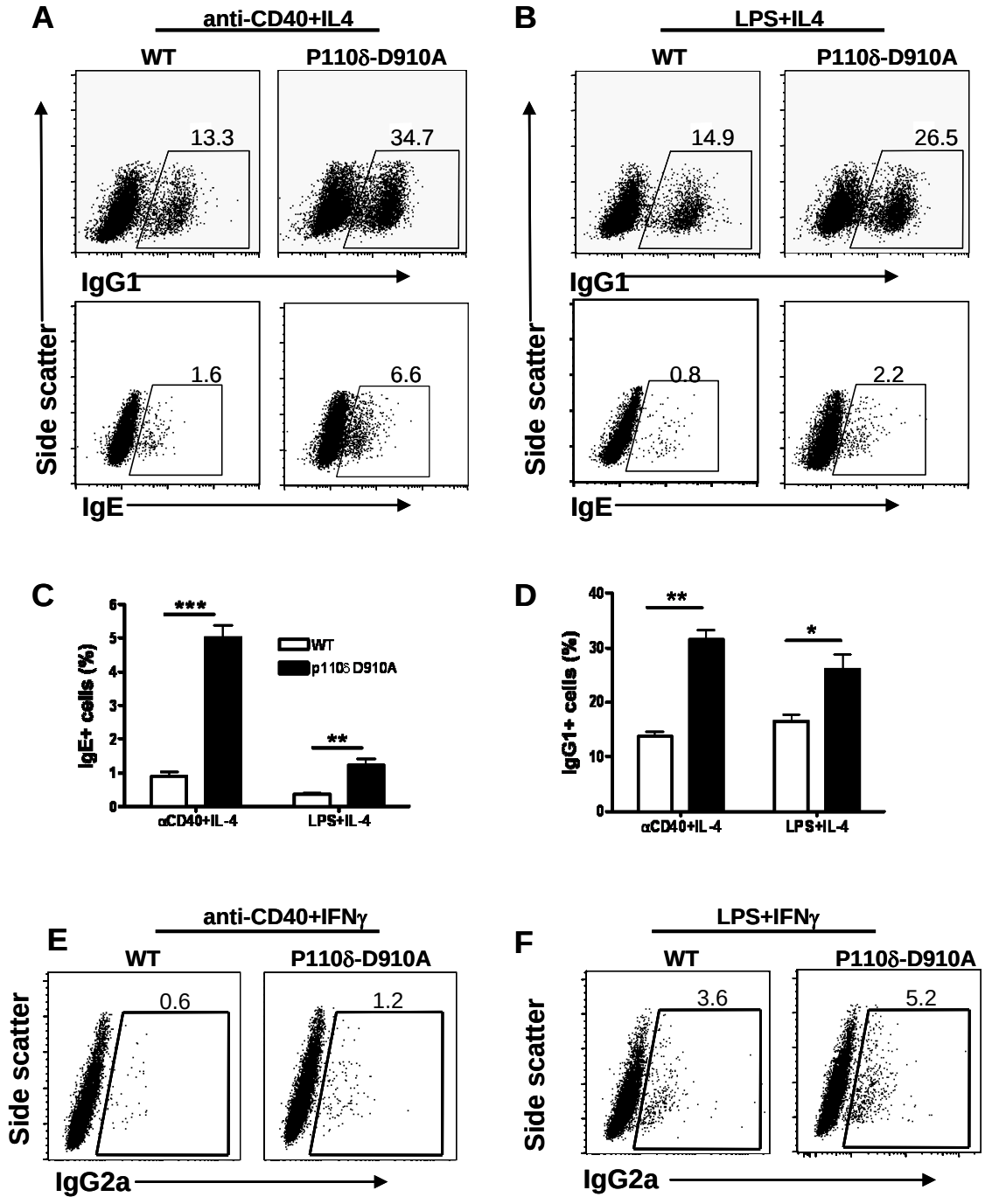
A
anti-CD40+IL4
WT
P110δ-D910A
13.3
34.7
IgG1
Side scatter
1.6
6.6
IgE
B
LPS+IL4
WT
P110δ-D910A
14.9
26.5
IgG1
Side scatter
0.8
2.2
IgE
C
IgE+ cells (%)
***
**
WT
p110δ D910A
αCD40+IL-4
LPS+IL-4
D
IgG1+ cells (%)
**
*
αCD40+IL-4
LPS+IL-4
E
anti-CD40+IFNγ
WT
P110δ-D910A
0.6
1.2
Side scatter
IgG2a
F
LPS+IFNγ
WT
P110δ-D910A
3.6
5.2
Side scatter
IgG2a

Figure 3.3.2 p110δ-D910A B cells show elevated class switch to different isotypes in vitro

WT or p110δ-D910A B cells were stimulated with anti-CD40+IL-4 (A), or with LPS+IL-4 (B), or with anti-CD40 (2 μg/ml)+IFN-γ (50 ng/ml) (E), or with LPS (2 μg/ml)+IFN-γ (50 ng/ml) (F). After 3 days of culture, cells were collected and treated with ice-cold acid buffer to remove surface binding immunoglobulins. Then cells were stained for intracellular IgG1 or IgE by using FITC-labeled anti-IgG1 or PE-labeled anti-IgE (A and B), or stained for intracellular IgG2a by using FITC-labeled anti-IgG2a (E and F). Each set of experiments has been done 3-7 times, and the representative FACS data plotted with side scatter was shown (A, B, E and F). Average percent IgE^+ (C) or $IgG1^+$ (D) cells and SEM from four independent experiments. * $p<0.05$; ** $p<0.005$; *** $p<0.001$ compared to wild-type.

3.3.3 Selective pharmacologic inhibition of p110δ in vitro greatly enhanced antibody class switching in B cells

To examine the effect of acutely disrupting p110δ signaling in wild-type B cells, we titrated the IC87114 inhibitor into B cell cultures stimulated with anti-CD40+IL-4 and examined generation of IgE-switched cells by using PE-labeled anti-IgE from eBioscience (Figure 3.3.3A and B). Significant effects were observed at doses as low as 0.25 μM IC87114, and increases in IgE-switched cells of up to 10-fold were observed at 10 μM (Figure 3.3.3B). To corroborate the IgE staining, we also used FITC-labeled anti-IgE from BD Bioscience to stain intracellular IgE, and similar results were observed (Figure 3.3.3D). IC87114 also potentiated switching to IgG1, with observed increases up to 4-fold (Figure 3.3.3C). Similar increases were seen when IC87114 was added to LPS+IL4 cultures (Figure 3.3.3E). Therefore, data obtained above are consistent with the results using B cells with genetic inactivation of p110δ, indicating p110δ signals negatively regulates IgE switch.

Figure 3.3.3

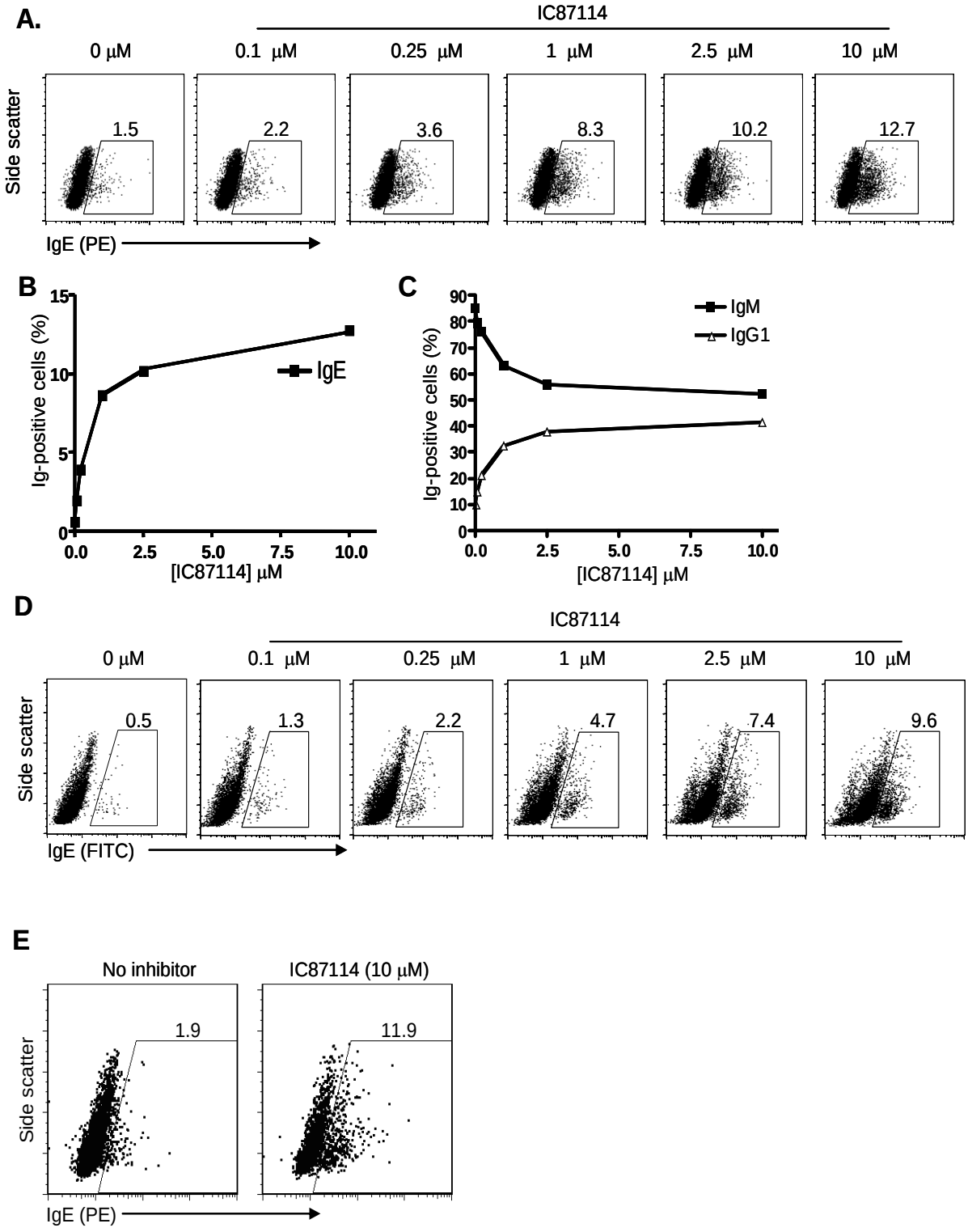
A.
IC87114
0 μM
0.1 μM
0.25 μM
1 μM
2.5 μM
10 μM
Side scatter
1.5
2.2
3.6
8.3
10.2
12.7
IgE (PE)
B
Ig-positive cells (%)
IgE
[IC87114] μM
C
Ig-positive cells (%)
IgM
IgG1
[IC87114] μM
D
IC87114
0 μM
0.1 μM
0.25 μM
1 μM
2.5 μM
10 μM
Side scatter
0.5
1.3
2.2
4.7
7.4
9.6
IgE (FITC)
E
No inhibitor
IC87114 (10 μM)
Side scatter
1.9
11.9
IgE (PE)

Figure 3.3.3 p110δ inhibitor potently enhances antibody class switching in B cells

(A) WT B cells were stimulated with anti-CD40+IL4 in the presence of the indicated doses of p110δ selective inhibitor IC87114. At day 3 cells were stained for IgM, IgG1 or IgE. IgE+ cells were detected using PE-labeled anti-IgE mAb from eBioscience. And the representative FACS data of IgE+ cells from three independent experiments were shown. Graph showing the percent IgE+ cells (B) or IgG1 and IgM+ cells (C) over a range of IC87114 doses of the representative experiment as shown in A. (D) WT B cells were cultured for 3 days with anti-CD40+IL4 in the presence of indicated doses of IC87114, and then stained for IgE using an independent FITC-labeled anti-IgE mAb from BD Bioscience. (E) WT B cells were cultured for 3 days with LPS+IL4 in the presence or absence of 10 μM IC87114, and then stained for IgE using PE-labeled anti-IgE mAb.

3.3.4 Inhibition of class IA PI3Ks activities potentiates the generation of IgE-switched cells

To determine the effect of inhibiting a broader spectrum of class IA PI3Ks, we tested a number of additional PI3K inhibitors to determine their effect on isotype switch. Several chemically-distinct inhibitor compounds with a range of target selectivities [306, 315] were able to potentiate switching to IgE to varying degrees (Figure 3.3.4A). Cultures containing IC87114 or broad-spectrum PI3K inhibitors PIK-90 or PI-103 also produced increased levels of secreted IgE (Figure 3.3.4B). At doses above 2 μM, PIK-90 and PI-103 inhibited IgE production; however this is likely due to the known ability of these compounds to inhibit the mammalian target of rapamycin (mTOR) at these high doses [306]. Consistent with this interpretation, the mTOR inhibitor rapamycin strongly suppressed IgE switching, even in the presence of IC87114 (Figure 3.3.4A). TGX115 and TGX221, selectively inhibit PI3K p110β activity at low dose, while they can also inactivate p110δ and other isoforms at high doses. Figure 3.3.4A showed that inactivation of p110β alone (low dosage of TGX115 and TGX221) had no effect on IgE-switching; however, high doses of TGX115 and TGX221 also potentiated IgE-switching. Together, these pharmacological data concur with the results using genetic-inactivation of p110δ, providing strong evidence that signaling via class IA PI3Ks functions in normal control of IgE isotype switch.

Figure 3.3.4

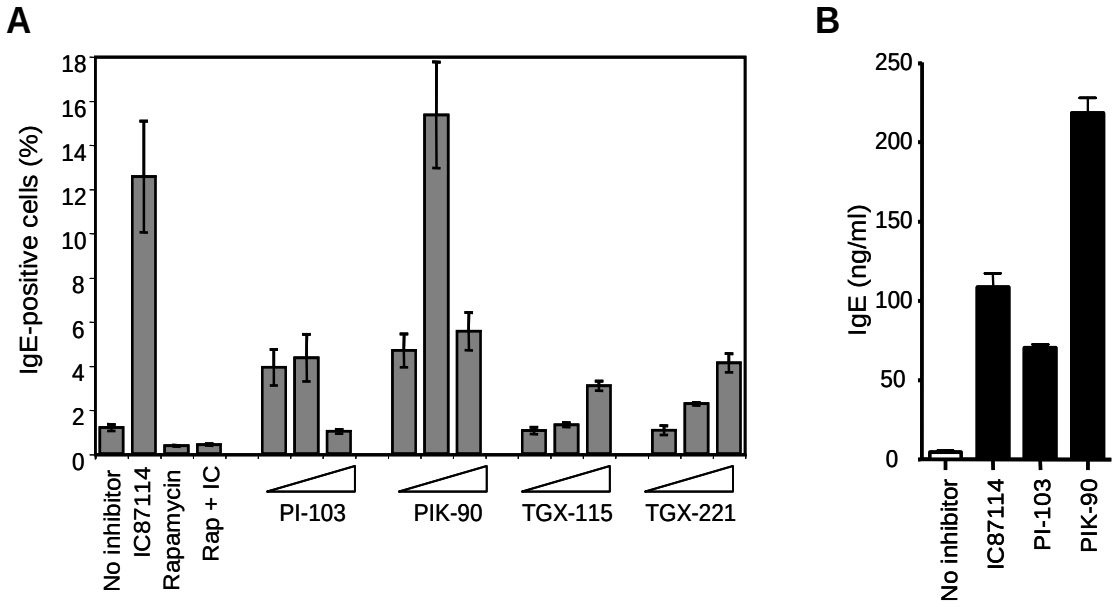

Figure 3.3.4 Inhibition of class IA PI3Ks activities potentiates the generation of IgE-switched cells

(A) Various PI3K inhibitor compounds were added to culture and the frequency of IgE+ cells was assessed. PI3K inhibitors PI-103, PIK-90, TGX-115 and TGX-221 were added at 0.1, 1 or 10 μM. IC87114 was used at 10 μM and rapamycin was used at 0.01 μM. Results represent the mean and SEM of 3 experiments. (B) Effect of PI3K inhibitors on secreted IgE levels. Cells were cultured with anti-CD40+IL4 in the presence of the indicated inhibitors and supernatants were harvested after 6 days. IC87114 was used at 10 μM, PI-103 and PIK-90 were used at 1 μM. IgE levels in the supernatants were determined by ELISA.

3.3.5 Blockage of PI3K activity potentiates the generation of IgE-switched cells associated with cell division

It has been well documented that the appearance of class-switched cells is closely related to cell divisions. This is especially true for IgE-switched cells, where substantial numbers only appear after the third or fourth division. To examine the relationship between IgE switch and cell division, B cells were labeled with CFSE prior to the culture in the presence of anti-CD40+IL-4 and stained with PE anti-IgE at day 4 (Figure 3.3.5A). This analysis revealed that PI3K-inactivated B cells divided normally as WT B cells in the culture; however, there was a striking deregulation of division-linked IgE switch under conditions when PI3K signaling is disrupted, with significant populations of IgE+ cells appearing by the second cell division and increasingly disproportionate levels of switch at each subsequent division (Figure 3.3.5B).

Figure 3.3.5

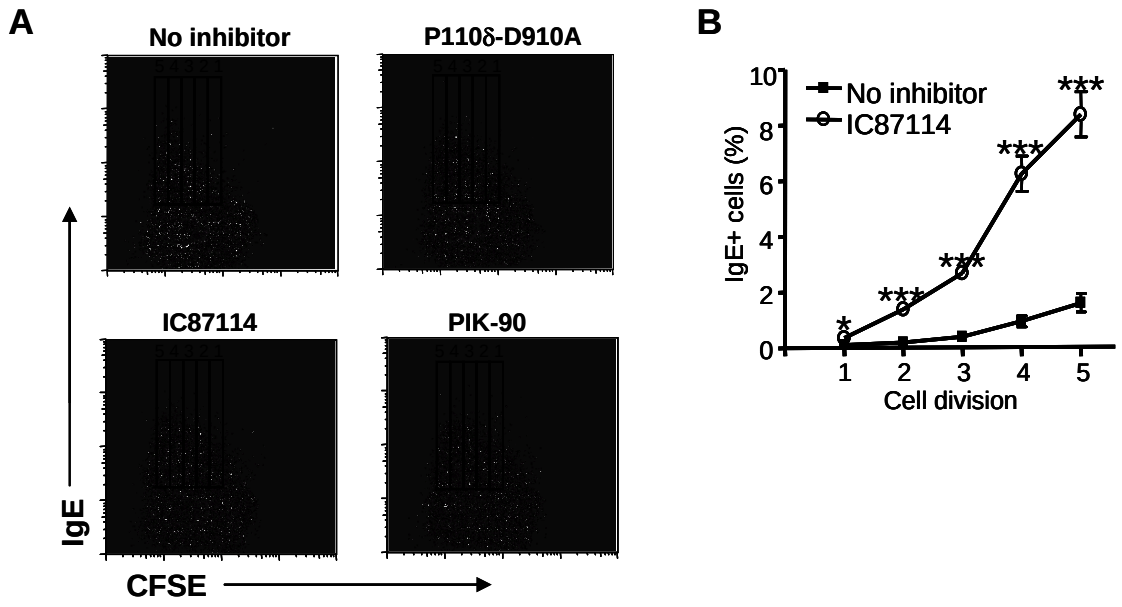

Figure 3.3.5 Blockage of PI3K activity potentiates the generation of IgE-switched cells associated with cell division

(B) WT or p110δ-D910A B cells were labeled with CFSE prior to culture with anti-CD40+IL-4. At day 4, cells were harvested and stained with PE-labeled anti-IgE. Bottom panels show data for WT B cells cultured in the presence of the IC87114 (10 μM) or PIK-90 (1 μM). Vertical lines mark cell divisions as assessed by twofold dilutions of CFSE. Each division is indicated by the number. (B) Graphical showing average percent ± SEM IgE+ cells in WT cell cultures in the absence or presence of 10 μM IC87114 at divisions 1 to 5 from three independent experiments. * $p<0.05$; *** $p<0.001$ compared to wild-type at the same division.

3.3.6 PI3K inhibition leads to deregulated sequential switching from IgG1 to IgE

A substantial proportion of the IgE response is generated through sequential switch recombination from IgG1 to IgE [317, 318]. This process normally occurs after the IgG1+ cells have undergone several additional cell divisions [90] and generates small but detectable IgG1+IgE+ intermediate populations [309]. We thus performed double-staining experiments to determine frequencies of IgE+IgG1+ cells. In $p110\delta^{D910A/D910A}$ or WT B cell cultures in the presence of PI3K inhibitors, the percentage of IgE+IgG1+ cells was greatly increased up to 10 fold compared to that of WT B cell cultures (Figure 3.3.6). In other words, in WT B cell cultures, about 2% of IgG1+ cells co-expressed IgE; while in PI3K inhibitor-treated cultures or in $p110\delta^{D910A/D910A}$ cultures, the percentage of IgG1+ cells co-expressing IgE increased to 5-15% (Figure 3.3.6). Thus, PI3K signaling negatively regulate the sequential switching from IgG1 to IgE.

Figure 3.3.6

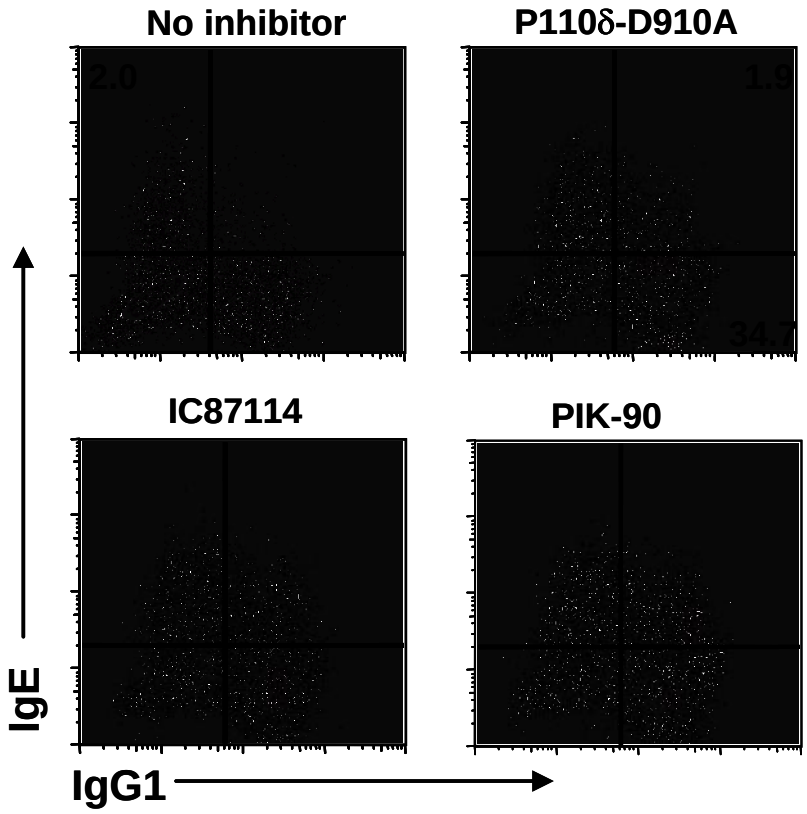

Figure 3.3.6 PI3K inhibition leads to deregulated sequential switching from IgG1 to IgE

WT or p110δ-D910A B cells cultured for 3 days with anti-CD40+IL-4 were double-stained for intracellular IgE and IgG1 using PE-labeled anti-IgE and FITC-labeled anti-IgG1. Bottom panels show data for WT B cells cultured in the presence of the p110δ–selective inhibitor IC87114 (10 μM) or pan-PI3K inhibitor PIK-90 (1 μM).

3.3.7 Pharmacological inhibition of p110δ has no significant effect on plasma cell differentiation

Blimp1 is the master regulator for plasma cell differentiation, and there is evidence indicating Blimp1 may repress AID expression and subsequent CSR [310, 311]. It was also suggested that PI3K signals are needed for plasma cell differentiation [311]. Thus one possibility is that increased CSR is due to a differentiation block in GC B cells to prevent them becoming plasma cells. Therefore, we further examined whether p110δ inhibition affected plasma cell differentiation by detecting the percentage of CD138+ plasmablasts and Blimp1 expression at RNA and protein level in cultures in the absence or presence of IC87114. Here, we do not present the results of p110δ-D910A B cell cultures, showing that CD138+ cell generation was impaired and Blimp1 expression was dramatically reduced in both anti-CD40+IL-4 and LPS+IL-4 cultures (data not shown). The reason is that marginal zone B cells, about 5% of normal splenic B cell population, is absent in p110δ-D910A splenic B cells, and marginal zone B cells are known to rapidly become plasma cells upon TLR ligand stimulation [319]. Thus, the absence of marginal zone B cells in p110δ-D910A cultures may confound the results. When WT B cells were stimulated with anti-CD40+IL-4 or LPS+IL-4 in the absence or presence of IC87114, p110δ inhibition did not lead to significantly reduced percentages of CD138+ cells in day 3 cultures (Figure 3.3.7A, B and C). Compared to normal WT B cells, p110δ–inactivated cells showed no difference of Blimp1 expression at both RNA and protein levels in the day 3 cultures (Figure 3.3.7D and E). Collectively, those data suggest that PI3K p110δ signaling has no significant or dramatic effect on plasma cell differentiation, and Blimp1 is not a downstream molecule of PI3K signaling required for CSR suppression.

Figure 3.3.7

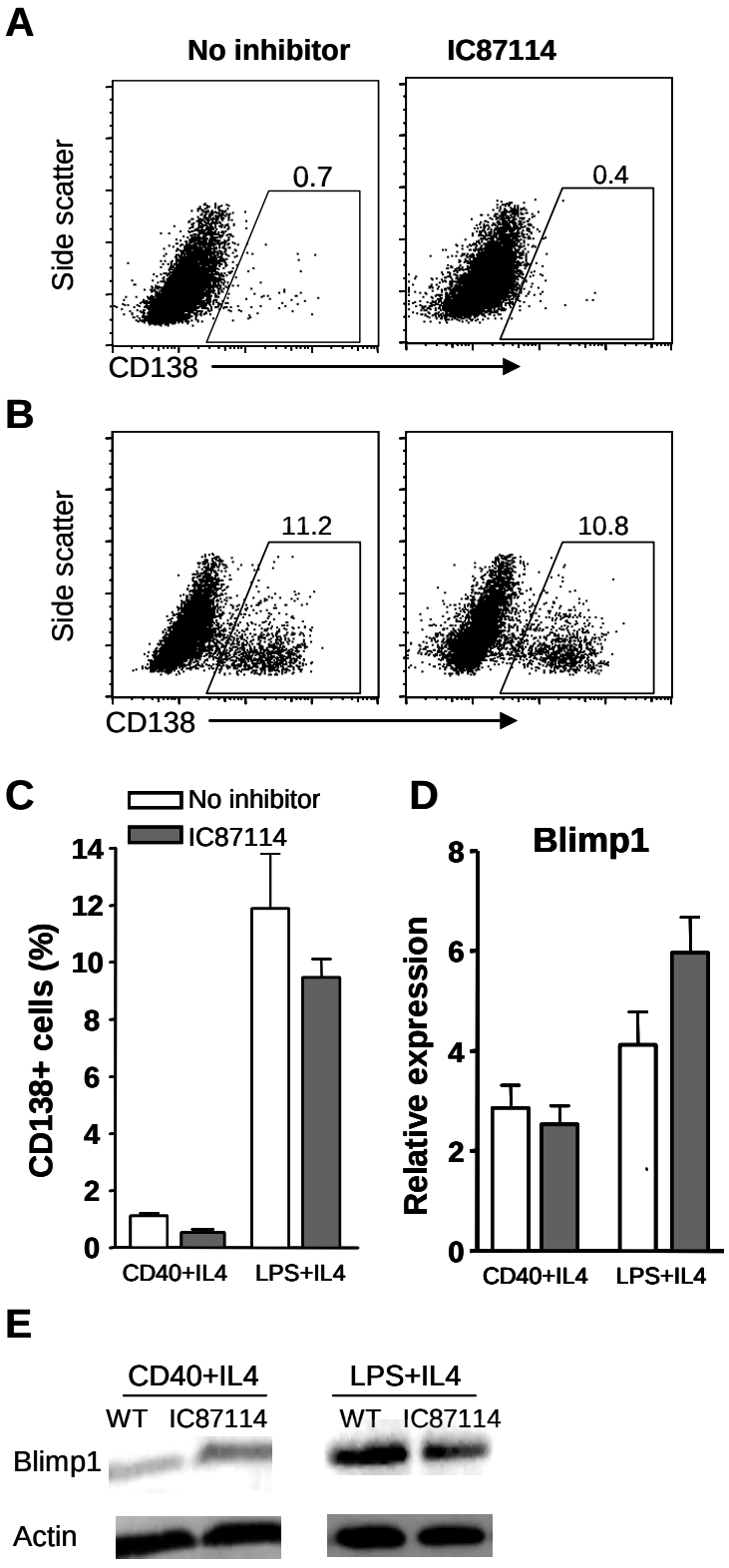
A
No inhibitor
IC87114
Side scatter
0.7
0.4
CD138
B
Side scatter
11.2
10.8
CD138
C
No inhibitor
IC87114
CD138+ cells (%)
14
12
10
8
6
4
2
0
CD40+IL4
LPS+IL4
D
Blimp1
Relative expression
8
6
4
2
0
CD40+IL4
LPS+IL4
E
CD40+IL4
LPS+IL4
WT
IC87114
WT
IC87114
Blimp1
Actin

Figure 3.3.7 Pharmacological inhibition of p110δ has no significant effect on plasma cell differentiation

WT B cells were stimulated with anti-CD40+IL-4 (A), or with LPS+IL-4 (B) in the absence or presence of IC87114. After 3 days of culture, cells were collected and stained for the marker of plasmablast CD138 (A and B). Each set of experiments has been done 4 times, and the representative FACS data plotted with side scatter was shown (A and B). (C) Average percent CD138+ cells and SEM from four independent experiments. (D) At day 3, cells were harvested to extract RNA from the indicated cultures. Then, RNA was transcribed to cDNA, followed by LightCycler RT-PCR to determine Blimp1 expression. Data are expressed as normalized expression relative to the corresponding β–actin control, and represent the average and SEM of four independent cultures. (E) Protein lysates of those day 3 cultures were blotted with anti-Blimp1 antibody.

3.3.8 The effects of inhibiting PI3K downstream kinases on CSR and plasma cell differentiation

It has been well demonstrated that Akt and Btk are two major downstream kinases of PI3Ks, so next we want to investigate whether PI3Ks regulate CSR via Akt-dependent pathway or Btk-dependent pathway by using small compounds to inhibit the activities of Akt or Btk. Addition of Btk-selective inhibitor LFM-A13 in the anti-CD40+IL-4–stimulated B cell cultures does not potentiate IgG1 and IgE switching in vitro (Figure 3.3.8A), indicating that PI3K signaling suppresses CSR in a Btk-independent manner. In addition, Btk seems not involved in plasma differentiation, as indicated by CD138 expression (Figure 3.3.8B). In order to block Akt activities in vitro, two different types of inhibitors were added in the B cell cultures. The presence of Akt inhibitor X has no effect on CSR and plasma cell differentiation; however, addition of Akt inhibitor IV slightly enhanced IgE-swiched cells and IgG1+IgE+ sequential switched cells (Figure 3.3.8A). Interestingly, increased MFI of IgG1 staining is observed in activated B cells treated with Akt inhibitor IV, indicating those cells containing abundant IgG1 are plasmablasts or quisi-plasmablasts (Figure 3.3.8A). In line with this, plasma cell marker CD138 expression is also significantly increased in Akt inhibitor IV–treated B cells (Figure 3.3.8B). Based on the above data, we can not draw a conclusion whether Akt downstream signals regulate CSR and plasma cell differentiation. One possible reason for the inconsistent results obtained by using different Akt inhibitors is that they may inhibit different panel of Akt downstream targets and they have different off-targets. In the future, other Akt inhibitors will be chosen to find out which result is more believable, and a panel of signaling molecules will be detected to compare their inhibitory activities.

Figure 3.3.8

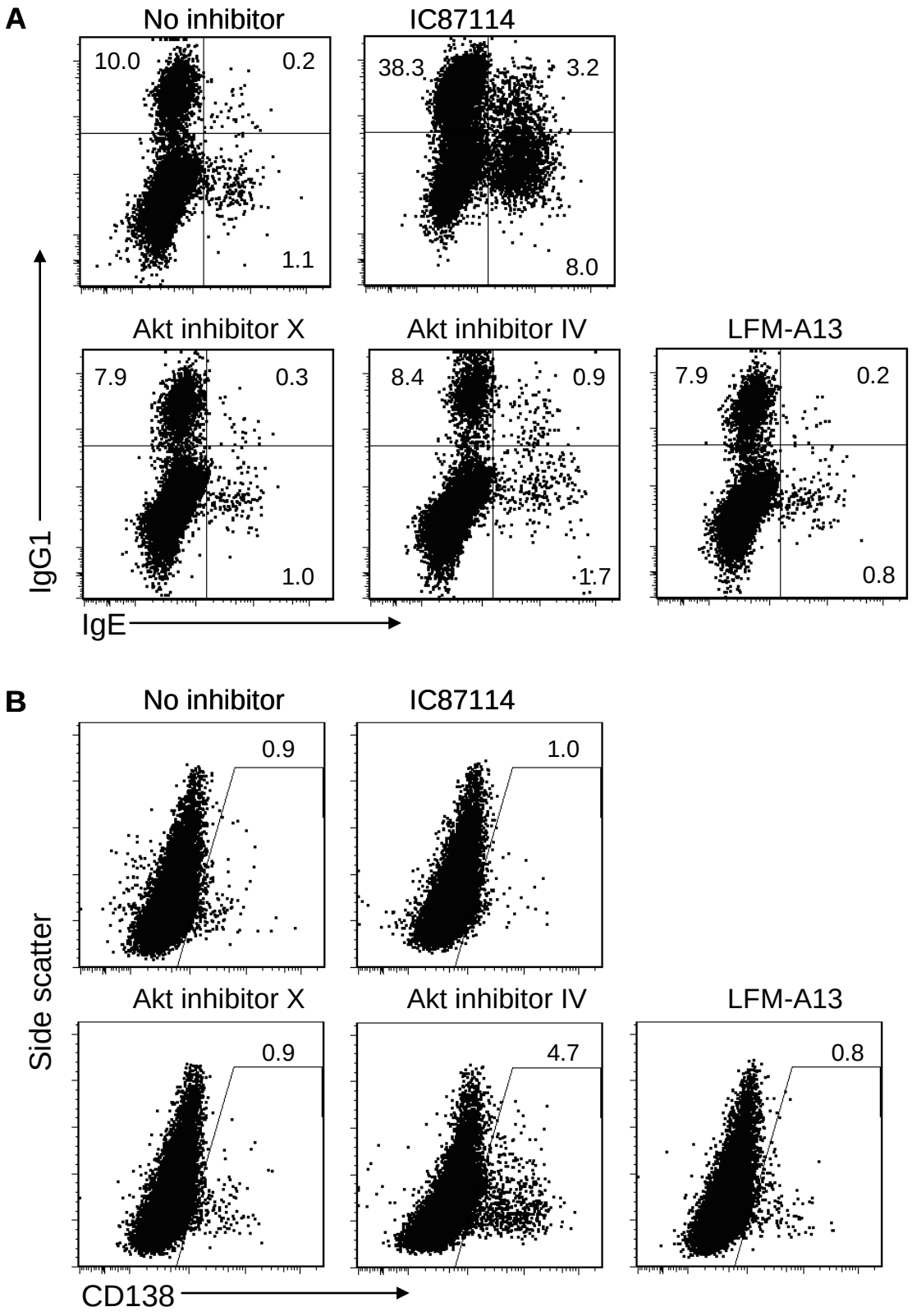
A
No inhibitor
IC87114
10.0
0.2
1.1
38.3
3.2
8.0
Akt inhibitor X
Akt inhibitor IV
LFM-A13
7.9
0.3
1.0
8.4
0.9
1.7
7.9
0.2
0.8
IgG1
IgE
B
No inhibitor
IC87114
0.9
1.0
Akt inhibitor X
Akt inhibitor IV
LFM-A13
0.9
4.7
0.8
Side scatter
CD138

Figure 3.3.8 The effects of inhibiting PI3K downstream kinases on CSR and plasma cell differentiation

WT B cells were cultured for 3 days with anti-CD40+IL-4 in the absence or presence of different inhibitor: 10 μM IC87114, 0.05 μM Akt inhibitor IV, 1 μM Akt inhibitor X, or 10 μM Btk inhibitor LFM-A13. After 3 days of culture, cells were collected, and then surface stained for CD138 and intracelluarlly stained for IgG1 and IgE using biotinylated-anti-CD138 (2^{nd} antibody using PE-Cy5.5-labeled avidin), APC-labeled anti-IgG1, and PE-labeled anti-IgE (A and B). Btk inhibition experiments have been repeated for at least 3 times, showing similar results. Akt inhibition experiment has only done once, however different doses of Akt inhibitor IV or X were used in the cultures, giving similar results as presented in figure A and B. A and B are representative FACS plot data showing IgG1+, IgE+ and CD138+ cells gated on live cells.

3.3.9 p110δ suppresses ε germline transcript and AID expression

CSR is associated with germline transcription of constant regions that is thought to be important in determining accessibility of loci to switch recombination machinery [320]. We examined levels of ε germline transcripts (εGLTs) using quantitative RT-PCR. It was found that εGLTs are significantly increased in p110δ$^{D910A/D910A}$ B cell cultures, and this increase is apparent as early as 24 h after culture initiation (Figure 3.3.9A). A similar increase in εGLTs was seen in cultures of WT B cells after addition of IC87114 and was observable in both anti-CD40+IL4 cultures and LPS+IL4 cultures (Figure 3.3.9A). Disruption of p110δ signaling also increased γ1 GLTs within the first day of culture, but this increase is no longer apparent by day 3 of p110δ$^{D910A/D910A}$ culture (Figure 3.3.9B), presumably due in part to deletion of the γ1 switch region in cells undergoing sequential switch. Significant reduced γ1 GLTs were even observed in IC87114-treated cultures by day 3 (Figure 3.3.9B), one possible reason is the less availability of γ1 switch region, for frequencies of both total and sequential switched IgE+ cells are higher in IC87114-treated cultures than those in p110δ$^{D910A/D910A}$ cultures (Figure 3.3.6). Post-switch transcripts were also measured in cells of day 3 cultures. Consistent with the IgE and IgG1 staining data, both ε and γ1 post-switch transcripts were increased when p110δ activity were inhibited, with more significant fold-increase of ε post-switch transcripts (Figure 3.3.9C).

We also examined expression of AID, an enzyme critical for CSR [25]. In WT B cell cultures, AID transcripts were present at low levels at the 24 hour time point, and then increased by over 100-fold by day 3 of culture. However, in p110δ$^{D910A/D910A}$ B cells or

WT B cells treated with IC87114, AID expression was initiated earlier, with significant expression at 24h (Figure 3.3.9D). At day 3, cultures with impaired p110δ activity expressed about twofold more AID transcripts than WT B cells. Together, these data support the model that class switching to IgE is enhanced from the outset of B cell activation, due to initial deregulation at the level of εGLT and AID expression.

Figure 3.3.9

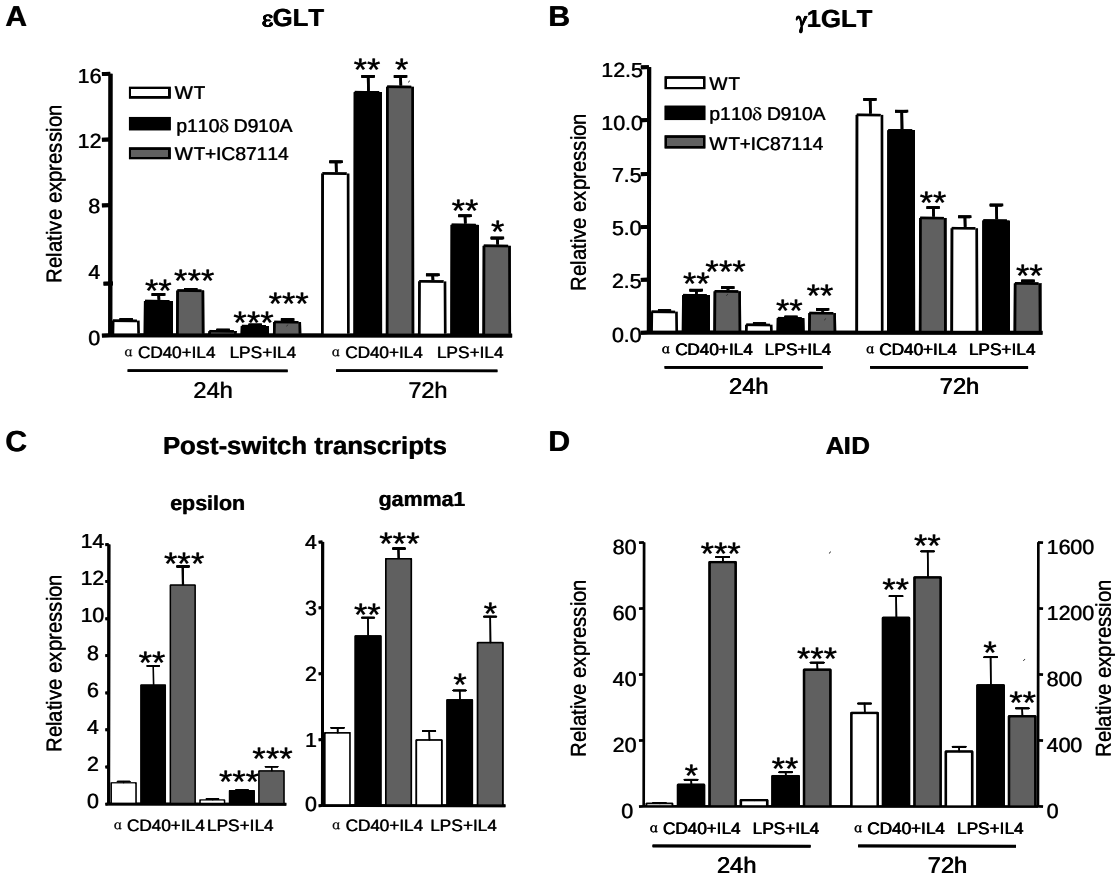

Figure 3.3. 9 p110δ regulates ε germline transcription and AID expression

RNA was extracted from B cells stimulated under the indicated conditions and levels of the indicated transcripts were determined by LightCycler RT-PCR. (A) ε germline transcripts measured at 24 or 72 h of culture (B) γ1 germline transcripts measured at 24 or 72 h of culture (C) ε and γ1 post-switch transcripts measured at 72 h of culture (D) AID transcripts measured at 24 and 72 h of culture. Data are expressed as normalized expression relative to the corresponding β actin control, and represent the average and SEM of at least 3 independent cultures. NS (no significance); * $p<0.05$; ** $p<0.005$ (Students T-test) was compared to wild-type under the same stimulation condition at the same time point.

3.3.10 p110δ is involved in both CD40 and IL-4 receptor signaling pathways to regulate ε germline transcription and AID expression

Mice with Y500F mutation in the insulin/IL-4 receptor (I4R) motif of IL-4Rα, which uncouples the receptor from PI3Ks, showed a similar phenotype of potentiated antigen-specific IgE production with no difference of IgG1 production [321]. Thus, it is reasonable to speculate that p110δ inactivation specifically interferes with IL-4R rather than CD40 signaling to up-regulate IgE production. Here, we examined εGLT and AID transcription in B cell cultures separately stimulated with either anti-CD40 or IL-4. As expected, WT B cells stimulated with either anti-CD40 or IL-4 alone, failed to generate abundant εGLT and AID transcripts (Figure 3.3.10). Upon anti-CD40 stimulation, pharmacological inhibition of p110δ activity was able to facilitate AID expression at very early time point, but had very limited effect on the transcription of εGLT (Figure 3.3.10A and B). For unknown reasons, p110δ-D910A B cells did not show this effect on AID (Figure 3.3.9A and B). In IL-4 cultures with p110δ disruption, εGLT was dramatically increased by day 1 and 3 (Figure 3.3.10A and B), and AID transcription was also slightly increased in those cultures. Results obtained from anti-CD40+IL4 cultures are consistent with the previous data (Figure 3.3.9A and D), showing synergistic effect of CD40 and IL-4R signaling on AID, but not on εGLT transcription (Figure 3.3.10). In summary, those data suggest that disruption of PI3K activity clearly affects IL-4R induction of εGLT transcription. The effect of PI3K inhibition on AID expression may involve direct modulation of CD40 signaling, but the current data are inconclusive.

Figure 3.3.10

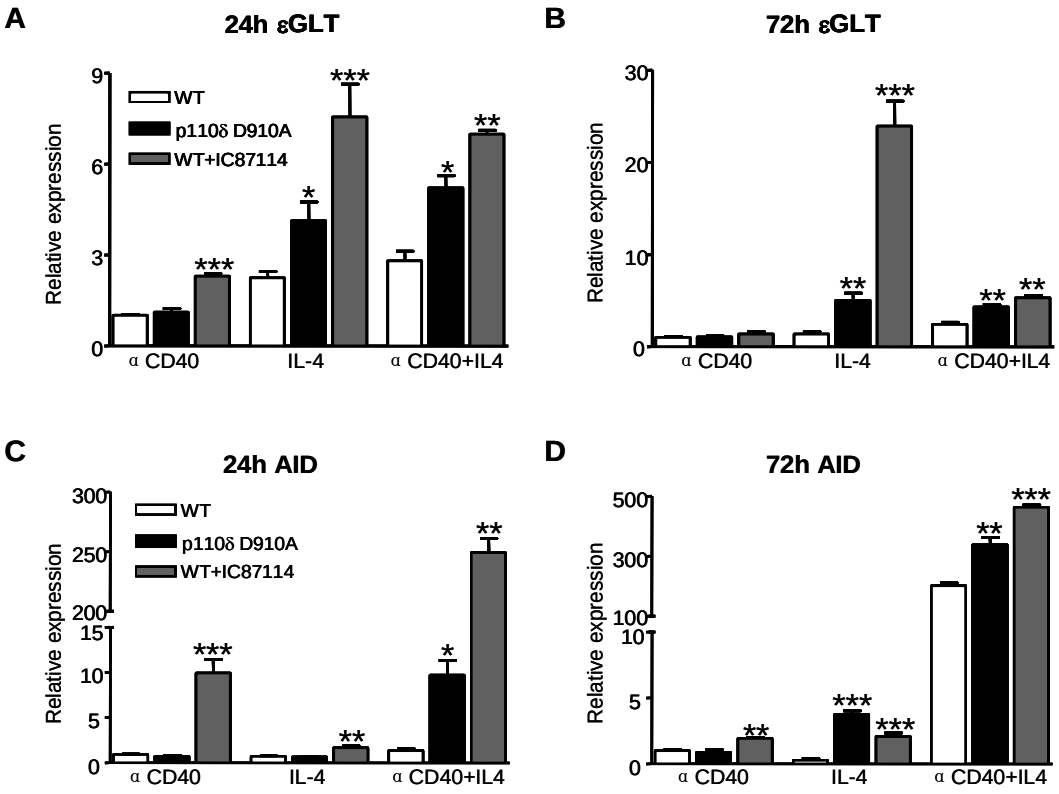

Figure 3.3.10 p110δ is involved in both CD40 and IL-4R signaling pathways to regulate ε germline transcription and AID expression

RNA was extracted from B cells stimulated with anti-CD40 and IL-4 alone or together, and then was transcribed to cDNA, followed by LightCycler RT-PCR to determine the indicated transcripts. (A) and (B) ε germline transcripts measured at 24 or 72 h of culture (C) and (D) γ1 germline transcripts measured at 24 or 72 h of culture. Data are expressed as normalized expression relative to the corresponding β actin control, and represent the average and SEM of at least 3 independent cultures. NS (no significance); * $p<0.05$; ** $p<0.005$; *** $p<0.001$ (Students T-test) was compared to wild-type under the same stimulation condition.

3.3.11 p110δ regulates the expression of transcription factors Bcl6 and IRF4

We further set out to determine whether p110δ regulates CSR by altering the expression level of a set of transcription factors known to regulate the transcription levels of AID and/or εGLT. Pax5 and E-proteins (translated from E2A locus) are able to bind to the promoter regions of AID and εGLT, enhancing AID and εGLT expression [79]. Inhibitor of differentiation (Id2) represses the transcription of AID and εGLT by interfering with Pax5 and E2A [79]. Bcl6, transcriptional repressor, also modulates CSR through competitive inhibition of NF-κB binding to the promoter regions of AID and IgE locus [75]. Interferon regulatory factor 4 (IRF4) has been demonstrated to be required for CSR through indirectly regulating AID expression [128], and interestingly IRF4 is also necessary for plasma cell differentiation, which may be accomplished by interacting with Bcl6 and Blimp1 [129]. Quantitative RT-PCR results indicated that p110δ inhibition did not affect Pax5 and Id2 expression in B cells stimulated with either anti-CD40+IL4 or LPS+IL4 (Figure 3.3.11A and B). However, slight but significant increase of E2A transcripts was observable in IC87114-treated B cell cultures, compared to wild-type B cell cultures (Figure 3.3.11C). Meanwhile, expression level of IRF4 was increased by twofold in all cultures except LPS+IL-4 stimulated p110δ B cell culture, which was influenced by the absence of marginal zone B cells (Figure 3.3.11D). Finally, we found that Bcl6 expression was significantly reduced under all conditions of p110δ inhibition (Figure 3.3.11E). Taken together, Bcl6 and IRF4 may be downstream molecules of PI3K signaling, and the downregulation of Bcl6 and upregulation of IRF4 following PI3K inactivation may be responsible for the potentiated IgE production we observed.

Figure 3.3.11

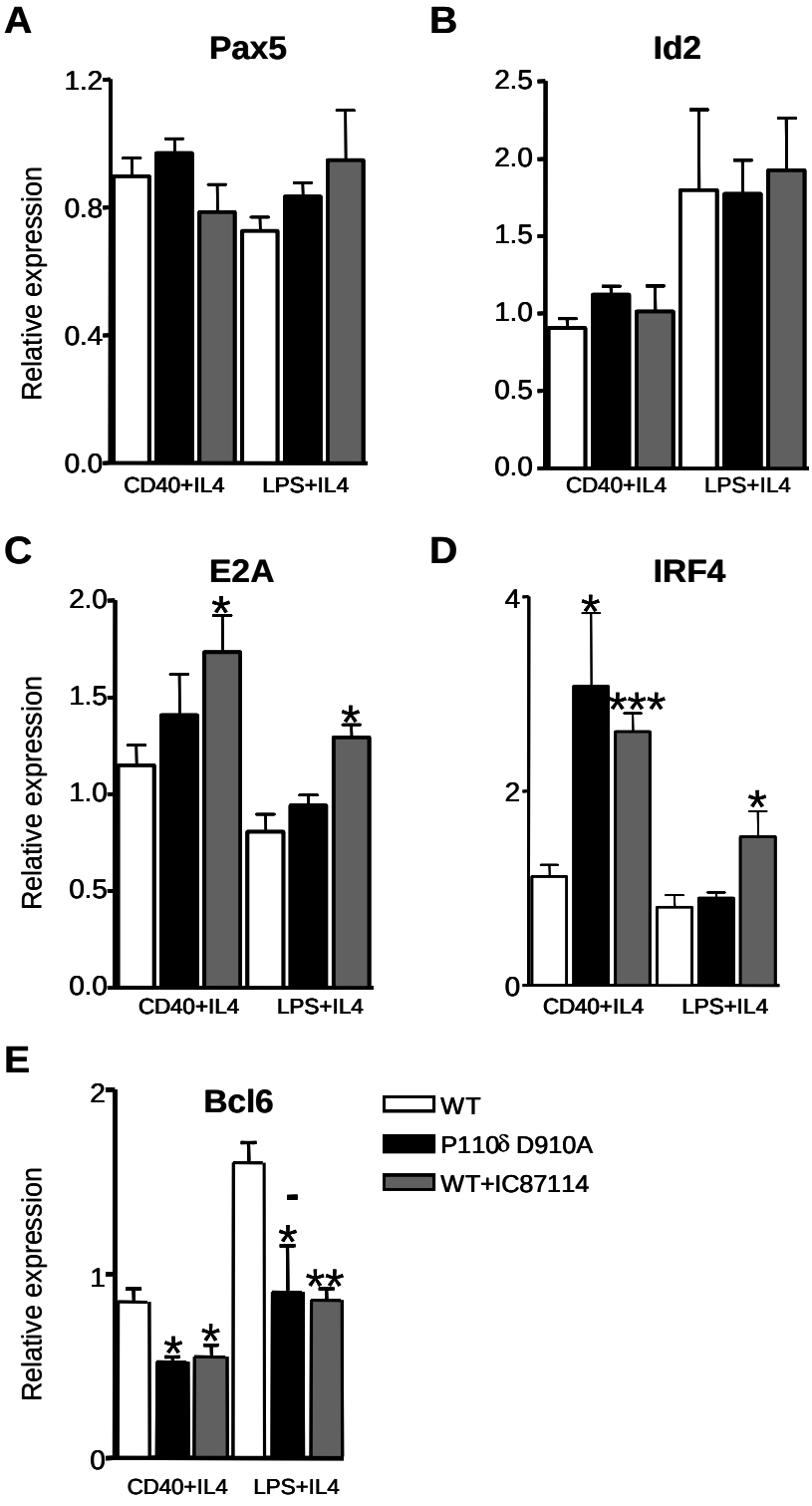
A
Pax5
Relative expression
1.2
0.8
0.4
0.0
CD40+IL4
LPS+IL4
B
Id2
2.5
2.0
1.5
1.0
0.5
0.0
CD40+IL4
LPS+IL4
C
E2A
Relative expression
2.0
1.5
1.0
0.5
0.0
*
*
CD40+IL4
LPS+IL4
D
IRF4
4
2
0
*
***
*
CD40+IL4
LPS+IL4
E
Bcl6
Relative expression
2
1
0
*
*
*
**
CD40+IL4
LPS+IL4
WT
P110δ D910A
WT+IC87114

Figure 3.3.11 p110δ regulates the expression of transcription factors Bcl6 and IRF4

WT or p110δ–D910A B cells were stimulated with anti-CD40+IL-4 or with LPS+IL-4 (B) in the absence or presence of IC87114. RNA was extracted from the 24 h of culture, and then RNA was transcribed to cDNA, followed by LightCycler RT-PCR to determine Pax5 (A), Id2 (B), E2A (C), IRF4 (D) and Bcl6 (E) expression. Data are expressed as normalized expression relative to the corresponding β actin control, and represent the average and SEM of at least 3 independent cultures. * $p<0.05$; ** $p<0.005$ (Students T-test) was compared to wild-type under the same stimulation condition.

3.3.12 Higher IgE production, accompanied with lower IL-4 recall response, appears in mice treated with selective p110δ inhibitor

To evaluate whether in vivo administration of p110δ inhibitor compound can also cause enhanced IgE production, mice were orally treated with IC87114 prior to and during OVA immunization. At day 12 after immunization, significantly lower percentage of splenic B220+Fas+GL7+ GC cells were present in IC87114-treated mice (Figure 3.3.12A), indicating that this oral treatment was effective in reducing the germinal center response, as seen in $p110\delta^{D910A/D910A}$ mice [161]. Sera were also collected for measurement of the total and OVA-specific antibodies. In contrast to $p110\delta^{D910A/D910A}$ mice, IC87114-treated mice did not have reduced levels of total and OVA-specific IgM or IgG1 (Figure 3.3.12B and C). However, both total and OVA-specific IgE were significantly increased in IC87114-treated mice, by 10- and 7-fold respectively (Figure 3.3.12B and C). Thus, in the absence of lymphopenia or other developmental abnormalities known to be present in the genetic inactivation model [161], acute inhibition of p110δ has a marked selective effect on IgE responses in vivo. To determine whether oral IC87114 treatment may affect production of cytokines regulating CSR, we assessed levels of IFN-γ, IL-4, IL-12 and IL-13 produced after OVA re-stimulation in vitro. The results indicate that IC87114-treatment leads to reduced IL-4 and IL-13 production, whereas production of IFN-γ and IL-12 are similar or increased compared to vehicle-treated groups (Figure 3.3.12D). Together, these results indicate that acute pharmacological inhibition of p110δ leads to selective increase in IgE responses in vivo despite reduced production of the critical IgE switch factor IL-4.

Figure 3.3.12

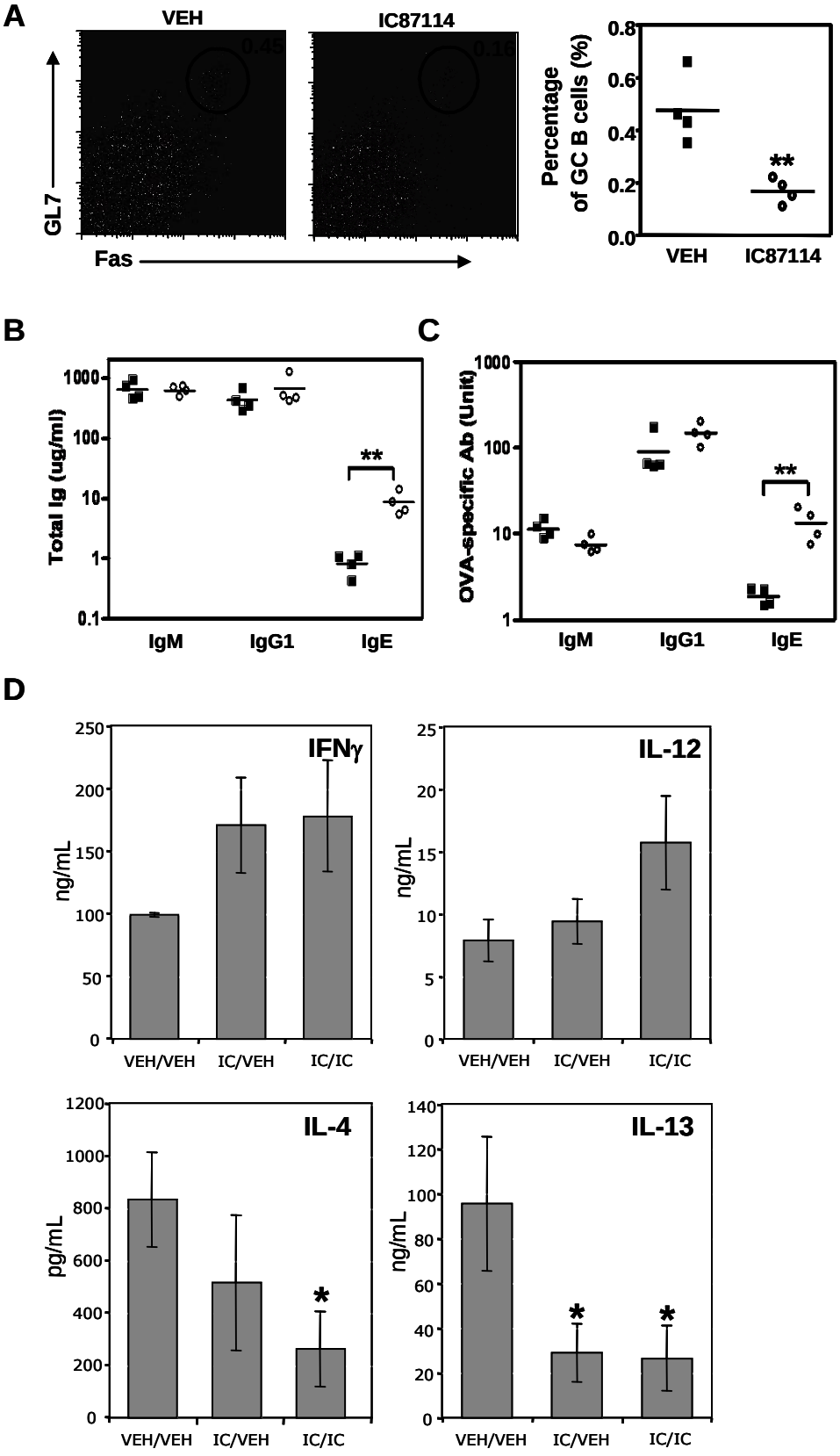
A
VEH
IC87114
0.45
0.18
GL7
Fas
Percentage of GC B cells (%)
0.8
0.6
0.4
0.2
0.0
**
VEH
IC87114
B
Total Ig (ug/ml)
1000
100
10
1
0.1
**
IgM
IgG1
IgE
C
OVA-specific Ab (Unit)
1000
100
10
1
**
IgM
IgG1
IgE
D
IFNγ
ng/mL
250
200
150
100
50
0
VEH/VEH
IC/VEH
IC/IC
IL-12
ng/mL
25
20
15
10
5
0
VEH/VEH
IC/VEH
IC/IC
IL-4
pg/mL
1200
1000
800
600
400
200
0
*
VEH/VEH
IC/VEH
IC/IC
IL-13
ng/mL
140
120
100
80
60
40
20
0
*
*
VEH/VEH
IC/VEH
IC/IC

Figure 3.3.12 Pharmacological p110δ inhibition in vivo selectively generates higher IgE production after OVA immunization

Mice were orally treated with 25 mg/kg p110δ inhibitor IC87114 or vehicle (PEG400) 1h before OVA immunization. After immunization, IC87114 or vehicle was delivered twice per day for 12 days. At day 12, serum and splenocytes were collected. For A-C, solid squares represent vehicle-treated mice, and open circle represented IC87114-treated mice. *(A)* The frequency of B220+Fas+GL7+ GC cells in spleen were analyzed by FACS. The right panel shows the percentages of GC B cells for individual mice, illustrating a significant reduction in the IC87114-treated group. *(B)* Sera were analyzed by ELISA to determine total IgM, IgG1 and IgE *(C)* Sera were analyzed by ELISA to determine OVA-binding IgM, IgG1 and IgE. *(D)* Spleen cells were re-stimulated in vitro with 300 μg/ml of OVA with or without IC87114. Culture supernatants were analyzed for IFN-γ, IL-12p40, IL-4 and IL-13 by ELISA. VEH/VEH = cells from vehicle-treated mice cultured with no inhibitor; IC/VEH = cells from IC87114-treated mice cultured with no inhibitor; IC/IC = cells from IC87114-treated mice cultured in the presence of 10 μM IC87114. * $p<0.05$; ** $p<0.005$ (Students T-test) was compared to vehicle group.

3-4 Discussion

3.4.1 The possible molecular mechanisms underlying negative regulation of PI3K signaling in IgE production

p110δ has well established functions in antigen receptor signaling in both B and T lymphocytes; however the importance of PI3K signaling in T-dependant B cell differentiation is not yet clear. p110δ inactivation does result in severely impaired IgM and IgG antibody responses; however, this is likely due at least in part to impaired helper T cell function [285, 322]. Here we show that the ability of p110δ-deficient B cells to mount antigen-specific IgE responses *in vivo* is not impaired. At face value, this result suggest that, at least for cells switching to this antibody isotype, B cell activation and differentiation to antibody secretion can proceed relatively efficiently in the absence of p110δ signaling. It is particularly striking that elevated IgE responses can be generated despite the weak Th2 cytokine responses generated in these mice [310].

Our results indicate that PI3K inhibition greatly increases IgE-switching in a B cell autonomous manner, probably due to up-regulation of AID and ε germline transcripts. In normal B cells, AID expression is induced when T-dependant activation signals are received, and expression correlates spatially and temporally with CSR and somatic hypermutation. With p110δ inhibition, B cells still express AID in an activation-dependent manner, but dramatic increases of AID mRNA were observed early after activation. AID transcription in normal B cells is initiated by synergistic signaling via NF-κB (originating from CD40 or TLR ligation) and STAT6 (originating from IL-4R)

[323]. Evidence generated to date does not support any role of p110δ in CD40/IL4 activation of STAT6 or NF-κB [160]; thus the precise signaling mechanisms linking p110δ to transcription of AID and εGLT remains to be determined. When we were pursuing this study, an elegant study conducted by Dr. Robert C. Rickert's group corroborated our data by showing that LPS+IL4–induced switching to IgG1 is inhibited by PI3K signaling due in part to regulation of AID [324]. This study provides evidence that the effect of PI3K on AID expression is mediated by Akt inactivation of forkhead transcription factor Foxo1. Forced nuclear expression of Foxo1 in both wild-type and $Pten^{-/-}$ (elevated PIP3 levels) B cells resulted in potentiated IgG1-switching cells associated with enhanced AID expression [311] in response to LPS+IL4 stimulation. On the contrary, Foxo1-deficient B cells failed to upregulate AID expression and thus to undergo IgG1 CSR upon LPS+IL4 stimulation, whereas the expression levels of γ1 GLTs were unchanged [317]. However it is presently unclear whether the AID gene is a direct transcriptional target of Foxo1 or whether Akt/forkhead may indirectly regulate AID via other factors.

The RT-PCR results suggest that transcriptional suppressor Bcl6 may be another downstream molecule of PI3Ks, responsible for increased AID and εGLTs when PI3K activity is inhibited. Since Bcl6 can competitively inhibits STAT6 binding to the STAT6-binding sites located at the promoter region of AID gene and Iε promoter region [75], it is possible that reduced Bcl6 expression in p110δ-inactivated B cells causes upregulation of AID and εGLTs. It has been demonstrated that Bcl6 negatively regulates εGLT expression [75]; however, we need further experimental evidences to ascertain the

relationship between Bcl6 and AID expression. Besides, it is necessary to investigate the protein level of Bcl6 based on the fact that protein does not always correlate to Bcl6 transcripts [318]. For example, the level of protein Bcl6 is greatly elevated in GC B cells; however, there is no difference of the amount of Bcl6 transcripts between GC B cells and naïve B cells [66]. Moreover, phosphorylated Bcl6 upon BCR crosslinking is easy to be degraded by the ubiquitin proteasome pathway [319].

Interestingly, IRF4 appeared to be another possible target regulated by PI3K signaling. IRF4 is required for AID expression by directly or indirectly binding to its promoter region [129]. In addition, IRF4 has been shown to bind to the promoter region of Bcl6 to repress its transcription [320]. Here, we found that p110δ inhibition leads to increased IRF4 transcription and decreased transcription of Bcl6 in anti-CD40+IL4 and LPS+IL-4 cultures. Thus, it is possible that the downregulated Bcl6 is caused by increased IRF4 only if we have evidences to link the PI3K activation with IRF4 suppression. Since Bcl6 is associated with GC B cell transcriptional program while IRF4 is associated with pre-plasmablast program, it is tempting to speculate that p110δ blockade interferes with establishment of full GC B cell transcriptional program, altering balance between GC B cells and plasma cells

3.4.2 Possible reasons regarding to the disconnect between IgG1 and IgE responses observed *in vivo* when p110δ activity is inhibited

Our in vivo results show a disconnect between IgG1 and IgE responses in both p110δ-mutant mice and IC87114-treated mice, with marked elevation in IgE, but not IgG1. In

contrast, short-term in vitro studies show that p110δ blockade leads to elevation in both IgG1- and IgE-switching. One possible reason for this disconnect is that p110δ inactivation leads to uncontrolled sequential switch from IgG1 to IgE, resulting in lower IgG1 secretion responses that are not reflective of the high proportion of cells that initially switched to IgG1. Sequential switch from IgG1 to IgG4 to IgE has been shown to contribute to IgE responses in allergic individuals [325], thus regulation of sequential switch is likely relevant to human allergic disease. It was recently demonstrated that IL-21 can suppress sequential switching from IgG1 to IgE [101], and IL-21 deficiency led to elevated IgE, but impaired IgG1 production [97], implicating this cytokine as a selective regulator of IgE versus IgG1 that can act at the level of sequential switch. It will be of interest to investigate whether inhibition of sequential switching directed by IL-21R or other receptors is PI3K pathway-dependent.

A variety of evidence suggests that the germinal center (GC) program of B cell activation promotes AID-mediated somatic hyper-mutation and isotype switch to IgG, but tends to disfavor IgE responses [326]. A recent study provided direct evidence that IgE+ cells are located outside of GCs and tend to express plasma cell characteristics soon after their generation, suggesting that IgE production is associated with a unique quasi-plasmablast genetic program that is opposed by the transcriptional repressor Bcl6 [101]. Bcl6 is a key regulator for the GC B cell transcriptional program, and Bcl6-deficient mice, like p110δ-inactivated mice, lack robust GC responses and generate high IgE responses [75, 327], supporting the idea that IgE switch is actively suppressed as an integral part of the GC B cell program. Since IL-21 can be produced by GC T cells, local production of IL-21 may

represent an additional mechanism for exogenous repression of sequential switch to IgE within GC. Thus it is possible that partial inhibition of the GC program under conditions of PI3K blockade may disrupt normal mechanisms that keep IgE responses in check, further contributing to enhanced IgE, but suppressed IgG levels.

3.4.3 Implications derived from PI3K pharmacological inhibitor studies

Our pharmacological inhibitor data largely concur with the results using genetic-inactivation of p110δ, providing strong evidence that signaling via this PI3K isoform is essential for B cell-intrinsic control mechanisms limiting IgE isotype switch. Our results show that a variety of structurally diverse inhibitor compounds with a range of target selectivity share the ability to potentiate switch to IgE. TGX115 and TGX221 inhibitors did not show an effect on IgE switch at submicromolar doses reported to selectively inhibit p110β [304]; however, an effect was observed at higher doses known to block p110δ [324]. This could indicate either that p110β is not linked to class switch or that B cells have low expression or activity levels of this isoform. Interestingly, the potent broad-spectrum PI3K inhibitors PIK-90 and PI-103 had reduced effect on IgE when used at higher doses. While this could suggest that a minimum level of PI3K signaling is required to support isotype switch, it is also known that off-target effects occur at these doses. Consistent with the latter interpretation, both of these compounds are known to directly inhibit mTOR, while IC87114 does not [306], and we found that the mTOR inhibitor rapamycin potently inhibits isotype switch. While even highly specific PI3K inhibitors such as IC87114 could indirectly inhibit mTOR through decreased Akt activity [160], B cells can also activate mTOR through PI3K-independant mechanisms [322].

Our data indicate that sufficient mTOR activity is present under conditions of p110δ-blockade to permit high levels of isotype switch.

3.4.4 Impact on the clinical usage of PI3K pharmacological inhibitors

Our results provide clear evidence that signaling via PI3Ks provide a B cell-intrinsic brake on IgE isotype switch. This PI3K-dependant regulatory mechanism is potent enough to substantially influence IgE levels generated in vivo, with removal of this "brake" allowing generation of enhanced IgE responses despite low IL-4 production. The data outline a new and unexpected immunomodulatory activity associated with multiple PI3K inhibitor compounds being developed for potential clinical use [306]. Clearly, this unexpected function of PI3K adds an additional layer of complexity that will need to be considered in efforts to target this pathway in allergic diseases.

3.4.5 Summary of main conclusions and overall significance

In summary, serum total and OVA-specific IgE levels are selectively enhanced in OVA-immunized p110δ-inactivated mice, which is opposite to the level of swich factor IL-4. In vitro studies show that p110δ is required to restrain IgE class switch recombination in a B-cell intrinsic manner. At molecular level, genetic or pharmacologic disruption of p110δ signaling leads to increased transcription of εGLTs and AID. In vivo administration of p110δ–selective inhibitor into OVA-immunized mice concurs with genetic mouse model, showing that IgE production is markedly elevated with significantly reduced Th2 cytokine production. My study also leads to a possible mechanism that p110δ signaling is involved in IgE switching by regulating the induction

of transcription factors Bcl6 and IRF4. The overall significance of this chapter is that these results uncover a novel immunomodulatory role of PI3K signaling in B cells, which challenges the ever-growing pharmaceutical development of PI3K inhibitor compounds for the treatment of cancer, chronic inflammatory diseases, infectious diseases and allergic inflammatory diseases.

Chapter 4 Roles of the PH domain-containing adaptor protein Bam32/DAPP1 in regulating germinal center responses

4-1 Specific Introduction

During B cell responses to T dependant antigens, key B cell activation and differentiation events occur within germinal centers (GCs) [27, 328-330]. The GC response is normally initiated when B cells activated by encounter with antigen and cognate T cell help migrate to the B cell follicles and begin proliferating rapidly in association with the follicular dendritic cell (FDC) network to give rise to a GC. GC B cells proliferate rapidly and undergo mutation and breakage of the chromosomal DNA in the immunoglobulin (Ig) locus while under intensive positive and negative selective pressure [331]. GC B cells are highly prone to apoptosis, but can be rescued from apoptosis in culture by stimulation through either CD40 or the BCR [332]. Maintenance of GC responses, as well as generation of memory B cells, depends on signaling via CD40L and other T cell-derived signals [42, 333-336]. Under some unusual circumstances, GC responses can be initiated in the absence of T cell help; however these GCs are short-lived and abort prematurely without giving rise to affinity maturation [43, 337], underlining the critical importance of T cell-derived signals in sustaining the GC

response through the critical selection period. Our understanding of intracellular signaling pathways that control selective survival of high affinity GC B cells is limited.

B cell adaptor molecule of 32 kDa (Bam32), also known as dual adaptor for phosphotyrosine and phosphoinositides (DAPP1), was identified during a screen for genes highly expressed in human GC cells [227] and through screens for proteins binding to phosphoinositide products of phosphoinositide 3-kinase (PI3K) [224-226]. PI3K enzymes are strongly activated by BCR ligation [338, 339], and the importance of PI3K signaling in B cell development and activation is well established [146, 147]. Bam32 binds PI3K lipid products, $PI(3,4)P_2$ and $PI(3,4,5)P_3$, in vitro [224] and is recruited to the plasma membrane in a PI3K-dependent manner upon B cell antigen receptor (BCR) ligation [227]. Bam32 phosphorylation by Src family kinases is also PI3K-dependant [225, 240]. Bam32 has been implicated in BCR signaling processes, including activation of the GTPase Rac1, and MAPKs ERK and JNK [136, 244, 246]. Bam32-deficient B cells develop normally, but show markedly impaired BCR-triggered proliferation in vitro. In line with this defect, Bam32-deficient mice showed remarkably reduced T cell-independent type 2 (TI-2) antibody responses, as assessed by either immunization with hapten-conjugated Ficoll or vaccination with the natural TI-2 antigen, bacterial capsular polysaccharide [136, 234]. However, the *in vitro* proliferative defect of Bam32-deficient B cells can be fully compensated by the addition of signals normally associated with T-cell contact, such as anti-CD40 or IL-4 [136]. Consistent with this finding, previous studies using Bam32-deficient mice showed normal T cell-dependent antibody responses, as assessed by both GC generation after 10 days immunization with potent immunogen

10% sheep red blood cells (SRBC), and antigen-specific antibody titers of selective isotypes in serum [136, 245].

The rationale of this chapter is that Bam32 is highly expressed in GC B cells, and our lab also found that Bam32 is required for optimal antigen presentation from B cells to T cells, a critical step for GC B cells to obtain T cell-derived signals *vise versa*. Furthermore, Bam32 has been shown to be required for B cell signaling and activation. Therefore, we hypothesized that Bam32 is required for GC progress or maintenance under conditions when T cell help is a limiting factor. To address this hypothesis, I re-visited the role of Bam32 in GC responses using low dose of protein antigen OVA.

4-2 Materials and Methods

4.2.1 Mice

Bam32 knockout mice were a kind gift from Michel C. Nussenzweig (Laboratory of Molecular Immunology, Rockefeller University, New York, NY, USA) and have been described previously [136]. μMT mice (Igh-6^{tm1Cgn}) were purchased from The Jackson Laboratory, and CD45.1 mice were obtained from Taconic (004007; SJL-$Ptprc^{\alpha}$). Age and sex-matched C57BL/6 mice were purchased from Charles River Canada. All animals were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg) in compliance with the guidelines established by the Canadian Council on Animal Care.

4.2.2 Primary B cell *in vitro* functional assays

1) Proliferation: Primary splenic B cells were isolated as mentioned in chapter III. B cells (2 x 10^5 in 200 μl of complete medium) were stimulated in round-bottom 96-well culture plates with titrated goat-anti mouse IgM (Jackson ImmunoResearch), purified anti-mouse CD40 Abs (BD PharMingen, San Diego, CA), LPS alone, or stimulated with αIgM + αCD40 + recombinant mouse IL-4 (Peprotech), for 72 h. In the last 18 h of incubation, cultures were pulsed with 10 μ l tritiated thymidine to give 1 μCi radiation per well. Cells were harvested to a paper filter using a cell harvester. Scintillation fluid was added to immerse the dried filter, and then the β radiation was detected by a scintillation counter to give the value of counts per minute (cpm).

2) Apoptosis: B cells (1 x 10^6/ml) were stimulated in 24-well culture plates (2 ml/well) for 3 days with different stimuli as mentioned in the previous paragraph. At the end of cultures, cells were harvested and stained with FITC-anti-Annexin V (BD Bioscience) according to the vendor's protocol. Briefly, 5 μl FITC-anti-Annexin V was added to cells suspended in 100 μl of 1×Annexin V binding buffer (BD Bioscience), and incubated for 15 min at RT. At the end of incubation, each tube was added with 400 μl 1×Annexin V binding buffer and 1 μl of 100 μg/ml DAPI (Sigma). Then, the cells were directly analyzed by a FACSCalibur flow cytometer.

3) Migration: Chemotaxis assays were conducted in 24-well plates containing Transwell inserts with 5 μm pore size (Corning). Purified B cells were stimulated with anti-CD40 (2 μg/ml) plus IL-4 (20 ng/ml) for 48 h, washed three times with migration

medium (RPMI 1640 containing 0.5% BSA), and resuspended at 10×10^6/ml in the same medium. Migration medium (600 μl) containing titrated CXCL12 was added to the lower chamber, then 100 μl re-suspended B cells was added to the upper chamber. The transwell plate was incubated at 37°C for 3– 4 h. At the end of incubation, 500 μl migration medium was added to the upper chamber. Cells in the lower and upper chambers were then collected and counted on a FACSCalibur flow cytometer. The percentage of migration was calculated as follows: lower chamber cell number/(lower chamber cell number + upper chamber cell number) × 100%.

4) Plasma cell differentiation and CSR: B cells (1 x 10^6/ml), stimulated with anti-CD40 (2 μg/ml) plus IL-4 (20 ng/ml) for 3 days in 24-well culture plates (2 ml/well), were washed and then surfaced stained with PE-labeled anti-CD138 (BD Bioscience), or intracellularly stained with anti-IgM / IgG1 / IgE as mentioned in the chapter III. After wash, cells reususpended in 400 μl FACS buffer (PBS containing 2% FBS) were acquired by a FACSCalibur flow cytometry.

4.2.3 Flow cytometric analysis of germinal center responses in vivo

1) GC B cells: Splenocyte single cell suspensions were prepared and counted, and around 1×10^6 cells were preincubated with Fc receptor blocker in FACS tubes. Then, 100 μl antibody cocktails were added: 1:200 dilution of FITC-labeled anti-GL7 (BD Biosciences) or 1:600 dilution of FITC-labeled anti-PNA (Vector Laboratories), 1:200 dilution of PE-labeled anti-Fas (Jo2; BD Biosciences), 1:400 dilution of APC-labeled anti-B220 (RA3-6B2; BD Biosciences). For mice adoptive transferred with B cells,

APC-labeled anti-CD19 (6D5; Southern Biotech) was used instead of APC-labeled anti-B220. Biotinylated-CD45.1 or CD45.2 (eBioscience) was added to discriminate the donor and recipient cells, which were further visualized with streptavidin-PE-Alexa647 (Molecular Probes).

2) GC B cell proliferation: GC B cell proliferation was determined by BrdU incorporation. BrdU (Sigma-Aldrich) in PBS (2.5 mg/mouse) was administered by i.p. injection 5h before the mice were sacrificed. Splenocytes were surfaced stained with GC markers (B220 and Fas) as mentioned above, then 1-2×10^6 cells were fixed by 2% PFA and permeabilized by 0.1% saponin buffer twice. Next, cells were treated with 1ml (100 Kunitz units/ml) deoxyribonuclease I (Sigma) diluted in DNAse buffer (0.15 M NaCl + 4.2 mM MgCl) at 37°C for 30min to digest genomic DNA and to expose BrdU. After wash, the cell pellets (20 μl volume) were mixed with 5 μl of pre-diluted FITC anti-BrdU (eBioscience), and incubated at RT for 20min. Finally, cells were washed and resuspended for flow cytometric analysis.

3) GC B cell apoptosis: After surface staining, splenocytes were further stained with CaspGlow or Annexin V to detect apoptotic cells. To stain active caspases in apoptotic cells, CaspGlowTM Fluorescein Caspase Staining kit (Biovision) was used. 1-2×10^6 cells were resuspended with 300 μl of complete medium. 1 μl of FITC-VAD-FMK was added to the cells and then incubated for 30min at 37°C incubator with 5% CO_2. After extensive washing, cells were suspended in FACS buffer and transferred to FACS tube for sample acquisition. For Annexin V staining, 1-2×10^6 cells were resuspended with 1ml Annexin V-binding buffer (BD Biosciences). 100 μl was transferred to another

FACS tube and then incubated with 5 μl FITC-anti Annexin V (BD Biosciences) and 100ng DAPI for 15min at RT, followed by adding 400ul Annexin V-binding buffer at the end of incubation. DAPI staining was detected using violet laser (405nm) excitation and the PacificBlue detection channel on FACS Canto II (BD Biosciences).

4) CSR and Plasmablasts within GCs: Splenocytes were co-stained with GC markers (B220 and Fas), Biotinylated anti-IgM, and FITC-labeled anti-IgG1 (A85-1; BD Biosciences) or FITC-labeled anti-CD138 (281-2; BD Biosciences). Biotin conjugates were visualized with streptavidin-PerCP-Cy5.5 (BD Biosciences).

5) Follicular helper T cells: Spleoncytes were stained with antibody cocktails: FITC-labeled anti-CD4, PE-labeled anti-ICOS, PE Cy7-labeled anti-B220 (negative gating), and Biotinylated anti-CXCR5 with 2nd antibody streptavidin-APC. All antibodies mentioned above are purchased from BD Biosciences. After staining, cells were acquired on FACS Canto II.

4.2.4 In vivo immunization and antibody affinity maturation assay

To measure antibody affinity maturation, WT and Bam32$^{-/-}$ mice were immunized i.p. with 2 μg NP-OVA (Biosearch Technologies) absorbed to 2 mg/ml Alum. Sera were collected at day 7, 14, 21 after primary immunization by tail bleeding. Mice were boosted 28 days later with the same dose NP-OVA. 10 days after the boosting, mice were sacrificed and blood was collected by cardiac puncture. NP-specific IgG1 and IgG2a antibody of high and low affinity were detected by ELISA with NP3-BSA (2 μg/ml) and NP20-BSA (2 μg/ml) coated plates as described earlier [36, 340]. The

quantities of NP-specific IgG1 and IgG2a in each experiment were expressed in relative units compared to a standard hyper-immune serum and the ratios of NP3-binding over NP20-binding antibodies were calculated.

4.2.5 B cell adoptive transfer experiments

Splenic B cells were purified from either WT or Bam32-deficient mice. After purification, B cells were washed with 1×PBS twice, and the cells were resuspended in 1×PBS at concentration 5-10×10^7/ml. 0.2 ml (1-2×10^7 cells) were transferred by intravenous injection into tail vein of B cell-deficient mouse (μMT). For competitive adoptive transfer experiments, splenic B cells from either WT or Bam32-deficient mice (both are CD45.2+) were mixed with WT CD45.1 B cells at 1:1 ratio, and then the mixture was adoptively transferred to μMT mice (2-4 × 10^7 per recipient). 6 days after transfer, recipient mice were i.p. injected with 10 μg OVA/alum, and GC responses were investigated 12 days after immunization.

4.2.6 Agonistic anti-CD40 antibody treatment

At day 5 and 10 after OVA immunization, WT and Bam32-deficient mice were treated with 50 μg of agonistic anti-CD40 (FGK45; Alexis Biochemicals) or Rat IgG2a isotype control (eBioscience) by i.p. injection. At day 12 after OVA immunization, GC responses were analyzed by flow cytometry and immunofluorescence microscopy.

4.2.7 Immunofluorescence microscopy analysis

Spleens from immunized mice were harvested, embedded in O.C.T. Compound (Tissue-Tek), and snap frozen in liquid nitrogen. 8-10 μm- thick frozen sections were fixed in cold acetone for 15 min and dried in air for 10 min. The slides were blocked with 5% horse serum for 30 min at RT and then were stained overnight at 4^{o}C with biotinylated anti-IgD (Southern Biotechnology Associates), biotinylated anti-IgM (Southern Biotechnology Associates) or biotinylated-anti-CD35 (BD Biosciences). After washing with PBS, the slides were stained with cocktails of antibodies: FITC-labeled anti-GL7 or PNA, FITC-labeled anti-Ki67 (BD Biosciences), or FITC-labeled anti-IgG1 (BD Biosciences), PE-anti-CD4 and strepavidin-Alexa647 (Molecular probes) for 2h at RT. After extensive washing, the slides were mounted in Prolong Gold anti-fade reagent (Molecular Probes). The sections were then viewed and the photos were taken under an inverted confocal microscope (Ultraview LCI, Perkin-Elmer Bioscience).

The mean fluorescence intensity (MFI) of CD4 staining within GC was determined by analyzing images with ImageJ software as follows. First, an irregular region was drawn around the GC area as determined by $GL7^{+}$IgD- staining. Then, "RGB stack" was selected to display only the CD4 channel (red). The MFI of CD4 within the defined region was then calculated using the "Analyze>Measure" function.

4.2.8 Statistic analysis

Statistical significance was assessed using unpaired student's T-test. $*$ indicates a p value <0.05, $**$ indicates a p value <0.01, and $***$ indicates a p value <0.001.

4-3 Results

4.3.1 Bam32$^{-/-}$ B cells are hyper-proliferative in response to T cell-derived signals

We isolated Bam32$^{-/-}$ splenic B cells to determine their proliferative capacities to signals known to be present within GC. As previously described, Bam32-deficient B cells show markedly impaired proliferative responses to BCR stimulation; however these cells proliferate robustly in response to stimulation via CD40+IL4 (Figure 4.3.1A). In fact, ligand titration experiments revealed significantly increased proliferation to either anti-CD40 or IL4, while proliferation in response to toll like receptor 4 (TLR4) ligand LPS is similar to controls (Figure 4.3.1B/C/D). Combination of BCR ligand with anti-CD40 and IL-4 led to a marked synergistic response in Bam32-deficient cells, completely overcoming the proliferative defect seen with BCR stimulation alone (Figure 4.3.1A). Thus, those *in vitro* proliferation assays indicated that Bam32 mediates negative signals when B cells are stimulated with anti-CD40 and/or IL-4. These results also suggest that Bam32$^{-/-}$ B cells may proliferate normally within GCs if there are T cell-derived signals available.

Figure 4.3.1

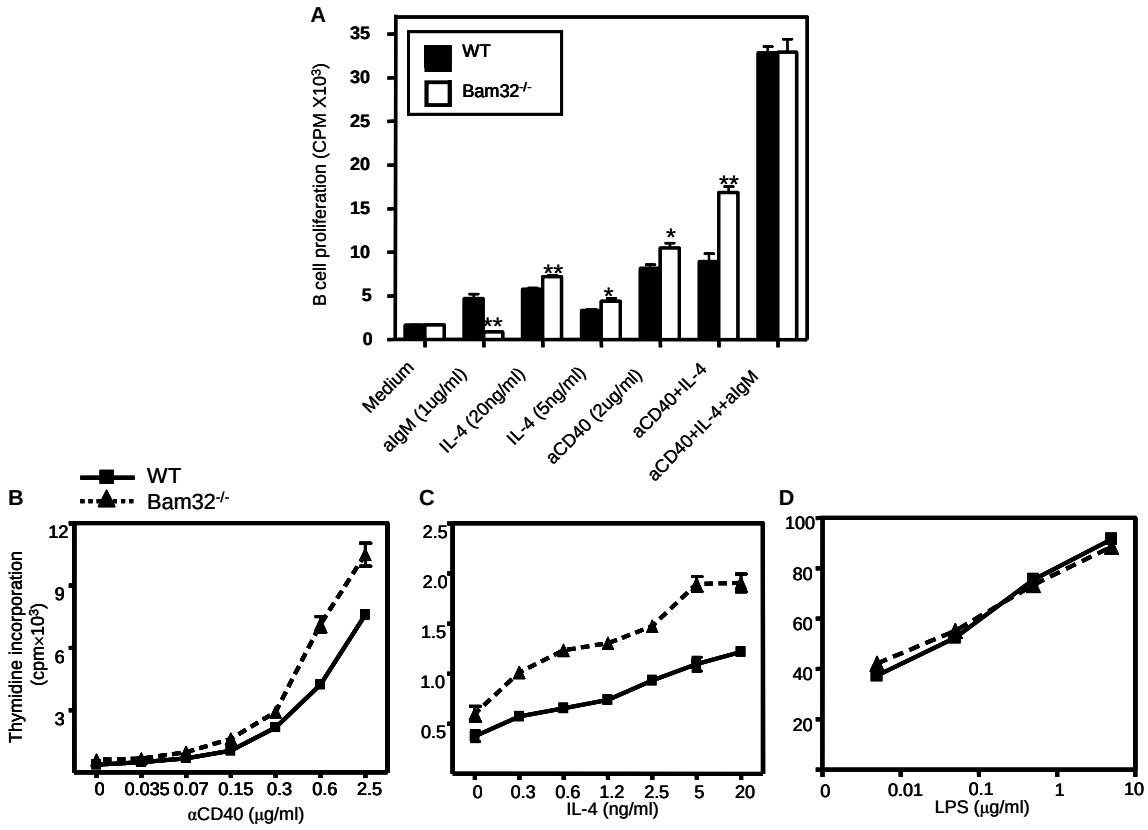

Figure 4.3.1 Bam32$^{-/-}$ B cells are hyper-proliferative in response to T cell-derived signals

(A) Purified splenic B cells were in vitro stimulated with anti-mouse IgM, αCD40, IL-4 of indicated dosage, or 2μg/ml αCD40 + 20ng/ml IL-4 with / without 1μg αIgM for 72h, H3-thymidine was added to the culture medium during the last 18h. Each bar represents the average and SEM from at least 4-6 independent experiment. (B-D) Purified splenic B cells from either WT or Bam32-deficient mice were in vitro stimulated with serial titrated dosage of αCD40 (B), IL-4 (C), or LPS (D) for 72h, H3-thymidine was added to the culture medium at the last 18h. Graphs B-D is representative of 4 independent experiments. * p<0.05; ** p<0.005 compared to wild-type.

4.3.2 Cultured Bam32$^{-/-}$ B cells show no significant defect in apoptosis, migration, CSR and plasma differentiation

We also tested other B cell activities known to be required for GC progression or to be outcomes of GC responses. Isolated splenic B cells were cultured *in vitro* with αIgM, αCD40, IL-4 alone or together to mimic stimuli available in GCs, and then the cells were co-stained with FITC labeled anti-Annexin V and DAPI for detection of apoptotic cells. We found that the percent Annexin V+DAPI- (early apoptotic) and Annexin V+DAPI+ (late apoptotic) cells were comparable between Bam32$^{-/-}$ and WT B cell cultures in the presence of the same stimulus (Figure 4.3.2A). Chemotactic responses of GC B cells towards SDF-1 (CXCL12) or BLC (CXCL13) have been demonstrated to be essential for GC organization [22]. Here, we observed that Bam32$^{-/-}$ B cells migrated towards titrated SDF-1 at similar rates to WT B cells (Figure 4.3.2C). Finally, we examined the capabilities of Bam32$^{-/-}$ B cells to become isotype-switched cells and plasma cells, which are outcomes of GC responses. Bam32$^{-/-}$ B cells underwent normal differentiation *in vitro* as assessed by antibody isotype switch from IgM to IgG1 or generation of CD138+ plasma cells (Figure 4.3.2B). In general, Bam32$^{-/-}$ B cells functioned normally in apoptosis, migration, CSR and plasma cell differentiation, when provided with oppropriate stimuli in vitro.

Figure 4.3.2

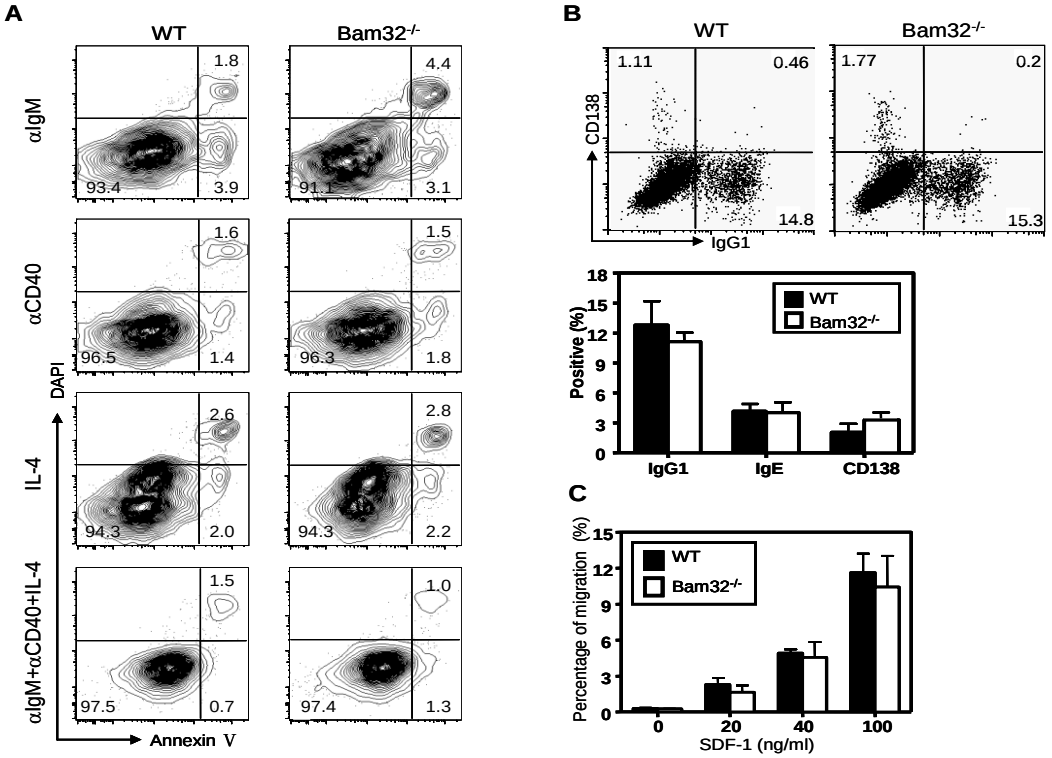

Figure 4.3.2 Bam32 is not required for apoptosis, migration, isotype switching and plasma cell differentiation in B cells

(A) Purified splenic B cells were in vitro stimulated with 1μg/ml anti-mouse IgM, 2μg/ml αCD40, 20ng/ml IL-4 alone or together for 72h, and then cells were cultivated for staining of annexin V and DAPI. Representative FACS contour graphs were shown from 2 independent experiments. (B) After 3 days' culture with 2μg/ml αCD40+20 ng/ml IL-4, B cells were intracellularly stained for IgG1 and IgE and CD138. Representative of FACS plots and the mean percentage±SEM were shown. (C) Transwell migration assay of pre-stimulated B cells with indicated concentration of SDF-1 added in the bottom well. 3h later, non-migrated or migrated cells in the upper or bottom wells were cultivated, and then counted using FACS Calibur. Each bar representative of the mean ±SEM of at least 4 mice.

4.3.3 Flow cytometric analysis revealed that germinal centers terminate early in Bam32-deficient mice immunized with T cell-dependent antigen OVA

GC B cells can be readily detected based on cell surface expression markers B220, GL7, PNA and Fas, therefore we compared the kinetics of the GC response in WT or Bam32$^{-/-}$ mice immunized with protein antigen OVA in alum using flow cytometer. B220^{+}GL7^{+}Fas^{+} GC cells, were present in similar frequencies at day 7 after immunization; however Bam32$^{-/-}$ mice showed a decline in GC cells starting at day 10 post-immunization (Figure 4.3.3A). GC B cells are strikingly absent in Bam32$^{-/-}$ spleens at day 14, a time point when the WT response reached its peak (Figure 4.3.3A). Similar reductions in GC populations at day 14 were also observed using PNA staining to identify GC cells (Figure 4.3.3B). Previous studies of Bam32-deficient mice found no obvious deficiency in GC formation after immunization with sheep red blood cells [136, 245]. Consistent with these findings, we found that immunization with 10% sheep red blood cells results in similar robust GC responses in both WT and Bam32$^{-/-}$ mice (Figure 4.3.3C); however, immunization with 1% SRBC revealed reduced GC responses in Bam32-deficient mice at day 12 post-immunization (Figure 4.3.3D). Therefore, GCs generated Bam32-deficient mice when immunized with less potent immunogen terminate prematurely as assessed by flow cytometry.

Figure 4.3.3

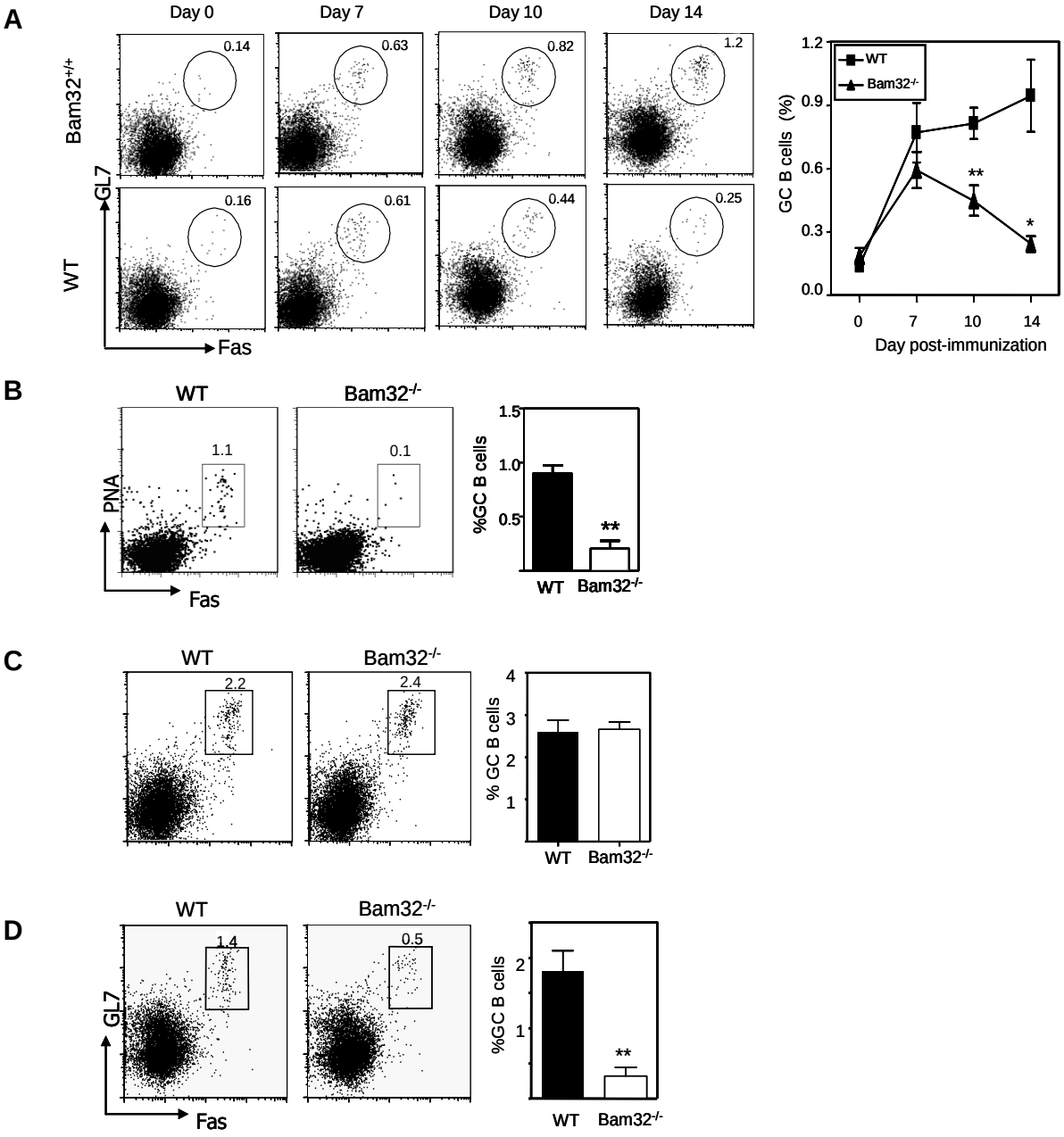
A
Day 0
Day 7
Day 10
Day 14
Bam32+/+
WT
GL7
Fas
0.14
0.63
0.82
1.2
0.16
0.61
0.44
0.25
GC B cells (%)
Day post-immunization
WT
Bam32-/-
B
PNA
1.1
0.1
%GC B cells
C
2.2
2.4
% GC B cells
D
1.4
0.5
%GC B cells

Figure 4.3.3 Premature dissolution of GCs in the spleen of immunized Bam32$^{-/-}$ mice detected by flow cytometric analysis

(A) WT and Bam32$^{-/-}$ mice were immunized with T-dependent antigen 2μg OVA/ 2mg alum. After 7, 10, or 14 days, splenocytes were isolated and stained with FITC-anti-GL7, PE-anti-Fas, and APC-anti-B220 to identify GC B cells. Representative GL7/Fas plots are shown (gated on B220+ lymphocytes). The right panel shows the percentage of GC B cells at different time points expressed as the mean ± SD of at least 4 mice per point. (B) 14 days after OVA immunization, splenocytes were stained with FITC-PNA, PE-anti-Fas, and APC-anti-B220 to identify GC B cells. Representative PNA/Fas plots are shown (gated on B220+ lymphocytes). Bar graph represents percent GC B cells expressed as the mean ± SD of 4 mice. (C) and (D) WT or Bam32$^{-/-}$ mice were immunized i.p. with 200μl 10% (C) or 1% (D) sheep red blood cells. By 12 day postimmunization, the mice were sacrificed to recover single splenic cell suspensions, which were further stained for GC B cells. Representative FACS dot plots showed GL7+ Fas+ cells gated on B220+ population, and the bar graph indicated the mean percentage ± SD of GC B cells calculated from flow cytometric analysis of at least 4 mice. * p<0.05; ** p<0.005 compared to wild-type.

4.3.4 Immunofluorescence staining confirmed that germinal centers terminate early in Bam32-deficient mice immunized with T cell-dependent antigen OVA

Immunofluorescence staining of frozen spleen sections revealed GC initiation in both WT and Bam32$^{-/-}$ mice, with normal positioning of GL7+ foci at the interface of T and B zones at day 7 post-immunization (Figure 4.3.4A, left) and no obvious difference in segregation of FDC and non-FDC zones as assessed by CD35 staining to illustrate FDCs (Figure 4.3.4B). Consistent with FACS data, smaller GCs were observable by staining of spleen sections from Bam32$^{-/-}$ mice at day 10 post-immunization; moreover, those GCs appeared abnormal, with a less cohesive core of GL7-bright cells, and a significant number of IgD+ cells interspersed within the GC area (Figure 4.3.4A, middle). By day 14, no obvious GCs were detectable in the spleens of Bam32-deficient mice, while the number and size of WT GCs was increased compared with day 7 (Figure 4.3.4A, right). Thus, immunofluorescence staining data confirmed that in Bam32-deficient mice GC responses initiated normally, but then decayed early.

Figure 4.3.4

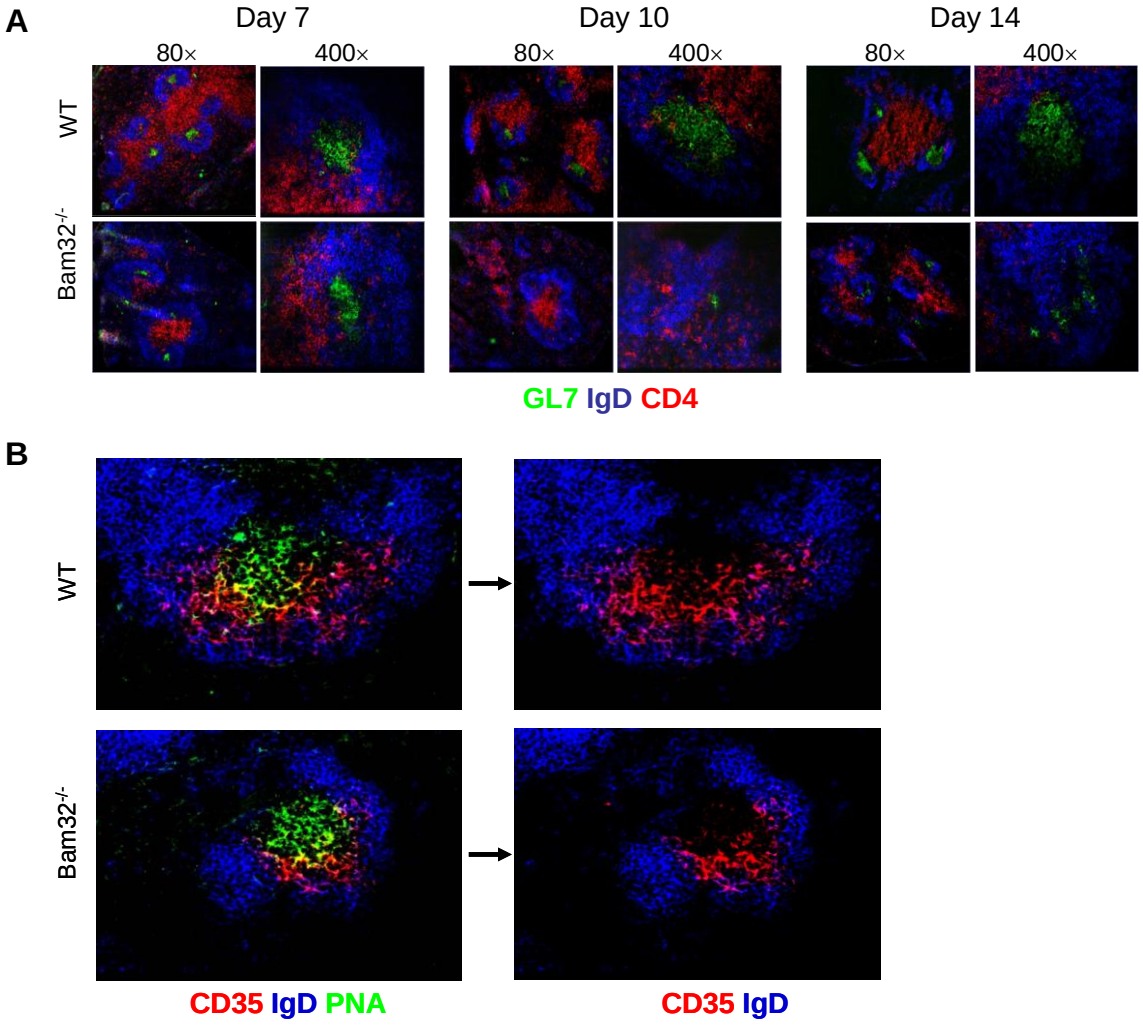

Figure 4.3.4 Premature dissolution of GCs in the spleen of immunized Bam32$^{-/-}$ mice detected by immunofluorescence staining

(A) GCs in spleen cryosections were visualized using confocal microscopy. Sections were stained with FITC-anti-GL7 (green), PE-anti-CD4 (red), and Biotin-anti-IgD with secondary Ab Avidin-Alexa647 (blue). The GC's in Bam32-deficient mice have visibly deteriorated by day 10 and are virtually absent at day 14. (B) Representative GCs from spleens collected 7 days post-immunization, stained with CD35 to identify FDCs (400×).

4.3.5 Bam32$^{-/-}$ B cell intrinsic defect leads to premature dissolution of germinal centers

In addition to B cells, Bam32 has also been observed to be expressed by murine primary T cells at relatively lower levels [338]. To explore whether Bam32 in T cells has functional effects and its deficiency in T cells may cause early decay of GCs in Bam32-deficient mice, Bam32-deficient T cell proliferation and cytokine production were examined. Purified CD3+ T cells were stimulated with plate-bound anti-CD3, IL-2 or IL-4, and then proliferation was assessed. As shown in Figure 4.3.5A, Among those stimulation, Bam32-deficient T cell proliferation was only markedly reduced in response to low dosage of anti-CD3 (TCR stimulation). In vitro re-stimulated splenocytes isolated from OVA-immunized Bam32-deficient mice secreted similar levels of IL-4, IFN-γ and IP-10 compared with that of OVA-immunized WT mice, indicating that Bam32 has no dramatic effect on T cell priming in vivo (Figure 4.3.5B). Collectively, those data suggest that Bam32 has limited function in T cells, and Bam32-deficient T cells are unlikely to be the driving cause of the GC phenotype we observed.

To directly determine whether the impairment in GC responses is due to a B cell-intrinsic function of Bam32, purified Bam32-deficient or control B cells were adoptively transferred to B cell-deficient hosts (μMT mice). Host animals receiving Bam32-deficient B cells showed significant reduction in the frequency of GC B cells generated 12 days after OVA immunization (Figure 4.3.5C). Thus, it is confident to conclude that Bam32-deficient B cell intrinsic defect leads to premature decay of the GC response.

Figure 4.3.5

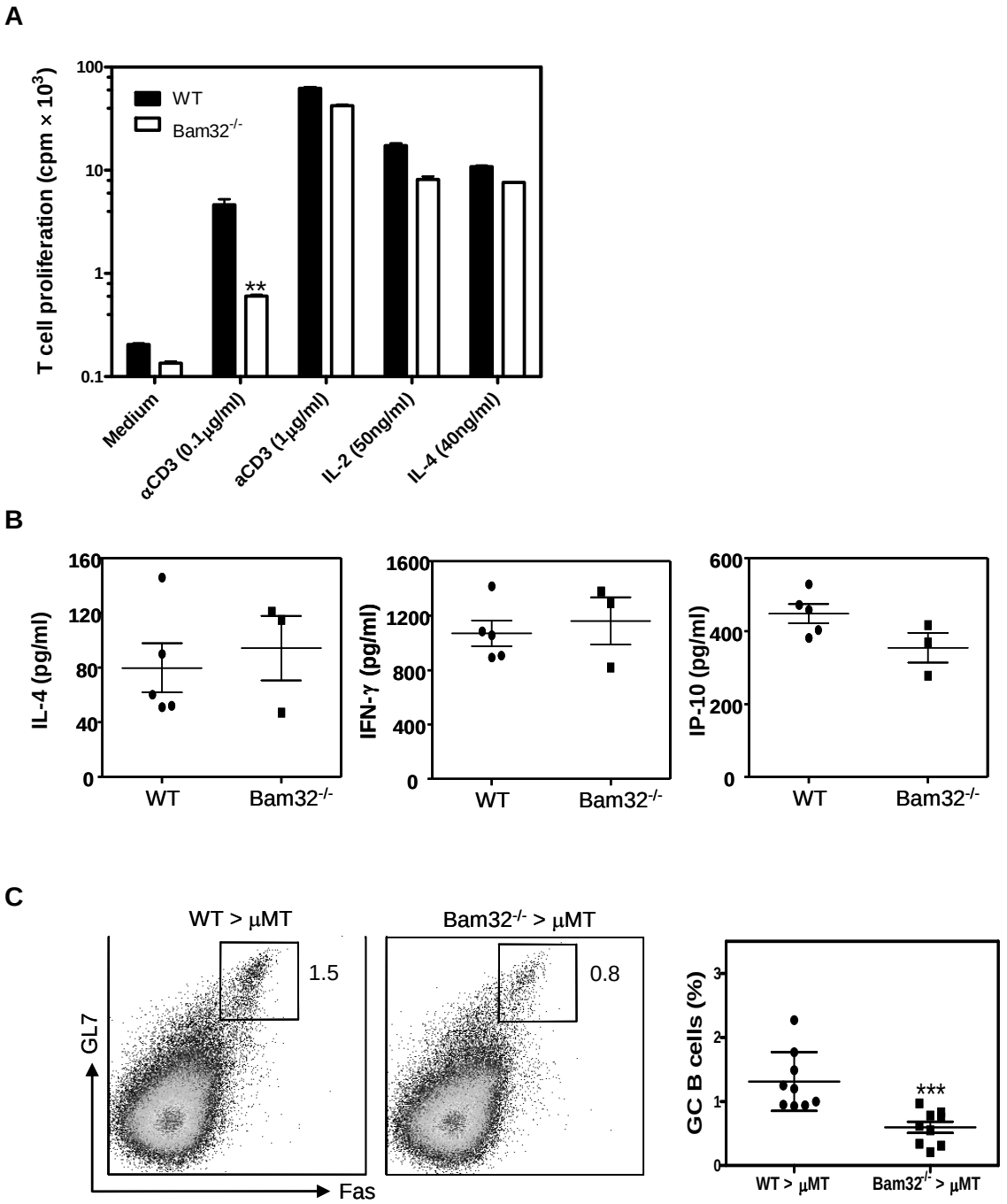
A
T cell proliferation (cpm × 10^3)
100
10
1
0.1
WT
Bam32-/-
**
Medium
αCD3 (0.1μg/ml)
αCD3 (1μg/ml)
IL-2 (50ng/ml)
IL-4 (40ng/ml)
B
IL-4 (pg/ml)
160
120
80
40
0
WT
Bam32-/-
IFN-γ (pg/ml)
1600
1200
800
400
0
WT
Bam32-/-
IP-10 (pg/ml)
600
400
200
0
WT
Bam32-/-
C
WT > μMT
Bam32-/- > μMT
1.5
0.8
GL7
Fas
GC B cells (%)
3
2
1
0
***
WT > μMT
Bam32-/- > μMT

Figure 4.3.5 Bam32$^{-/-}$ B cell intrinsic defect leads to premature dissolution of germinal centers

(A) Purified splenic T cells were in vitro stimulated with 0.1μg/ml or 1μg/ml plate-bound anti-CD3, 50ng/ml IL-2, or 40ng/ml IL-4 for 72h, H3-thymidine was added to the culture medium at the last 18h. Each bar represents the average and SEM from 3 independent experiments. (B) Spleen cells were isolated from WT and Bam32$^{-/-}$ mice after a primary OVA immunization at day 5. Then, Spleen cells were cultured with 300 μg/ml OVA and supernatants were analyzed for IL-4, IFN-γ and IP-10 by ELISA. Each dot or square represents one mouse. (C) 1.5-2.0 × 10^7 purified B cells from either wild type or Bam32-deficient mice were transferred to B cell-deficient μMT mice. At day 12 after OVA immunization, GC B cells in uMT mice were identified using flow cytometry. Representative of flow cytometric plots gated on CD19+ cells (left) and the percentages of GC B cells for individual mice (right) are shown. *** p<0.001 compared to wild-type.

4.3.6 Antibody responses in OVA-immunized Bam32$^{-/-}$ mice are partially altered

Next, we wanted to examine the T cell-dependent antibody responses in Bam32$^{-/-}$ mice, which are related to GC responses. 10 days after primary immunization of protein antigen OVA/alum, sera were collected from the mice for detection of total and OVA-specific antibodies of different isotypes. Consistent with previous studies, there was no difference of total and OVA-specific IgG1 production between WT and Bam32$^{-/-}$ mice (Figure 4.3.6). However, we found that Bam32$^{-/-}$ mice generated significantly higher level of total and OVA-specific IgM (Figure 4.3.6), which may be caused by the hyperproliferation of Bam32$^{-/-}$ B cells in response to T cell-derived signals, or due to the early crash of GCs leading to the blockage of IgM+ cells switching to other isotypes. In support of the idea of reduced class switch recombination, total and OVA-specific IgE antibodies in Bam32$^{-/-}$ mice were significantly reduced by 3-4 fold (Figure 4.3.6). In combination with the previous *in vitro* findings showing that Bam32$^{-/-}$ B cells underwent CSR as efficiently as WT B cells, those data suggest that the premature dissolution of GCs in Bam32$^{-/-}$ mice alters the production of some antibody isotypes.

Figure 4.3.6

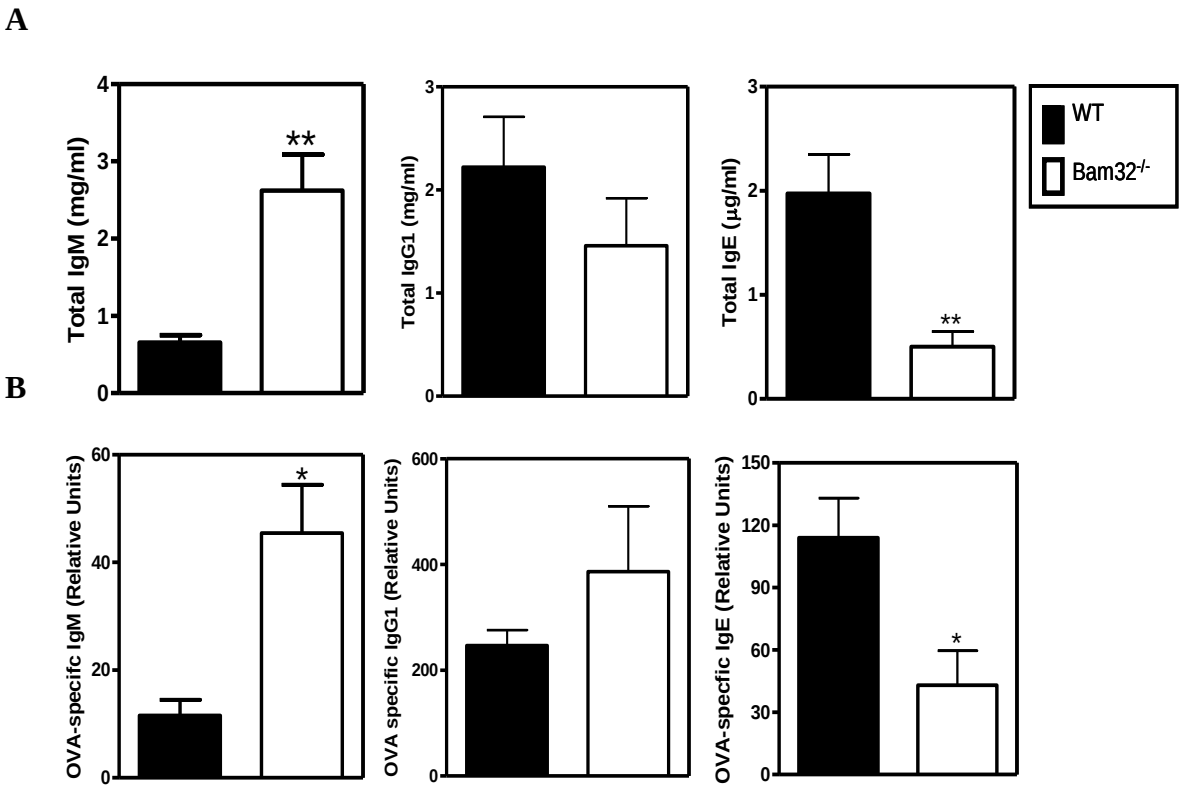

Figure 4.3.6 Antibody responses in immunized Bam32$^{-/-}$ mice are partially altered

Mice were i.p. immunized with 2 μg/ml OVA/ 2 mg/ml alum, and sera were collected 10 post-immunization. (A) Serum total antibodies IgM, IgG1 and IgE were by sandwich ELISA. (B) Levels of OVA-specific IgM, IgG1 or IgE antibodies in the sera were assessed. Each bar represents mean value of 5-8 mice and SEM. * p<0.05; ** p<0.005 (Students T-test) compared to wild-type control.

4.3.7 Affinity maturation is impaired in NP-OVA–immunized Bam32$^{-/-}$ mice

We assessed whether premature termination of GC impacts on antibody affinity maturation. Hapten-conjugated protein, NP-OVA, was used as immunogen to induce the production of anti-NP antibodies for affinity maturation analysis [36]. When ELISA plates are coated with densely NP-haptenated bovine serum albumin (NP_{20}-BSA), both high affinity and low affinity anti-NP antibodies (total anti-NP antibodies) will be detected. However, when ELISA plates are coated with sparsely NP-haptenated BSA (NP_3-BSA), only high affinity anti-NP antibodies will be detected. Thus, the ration of NP_3-binding/NP_{20}-binding antibody is correspondent to antibody affinity maturation. After NP-OVA immunization, sera were collected at one-week intervals and levels of antibody binding to NP_3-BSA or NP_{20}-BSA were determined. In wild-type mice, high-affinity NP_3-binding antibodies appeared only at later time points after immunization, resulting in an increasing ratio of NP_3-binding/NP_{20}-binding antibody over time, reaching affinity maturation ratios of 0.91 and 0.69 for IgG1 and IgG2a/c, respectively (Figure 4.3.7). Although progressive Ab affinity maturation was also detectable in Bam32-deficient mice, the ratio of NP_3/NP_{20}-binding achieved over the course of the response was significantly reduced, reaching ratios of 0.67 and 0.43 for IgG1 and IgG2a respectively (Figure 4.3.7). These results are consistent with impairment in progressive affinity selection within GC.

Figure 4.3.7

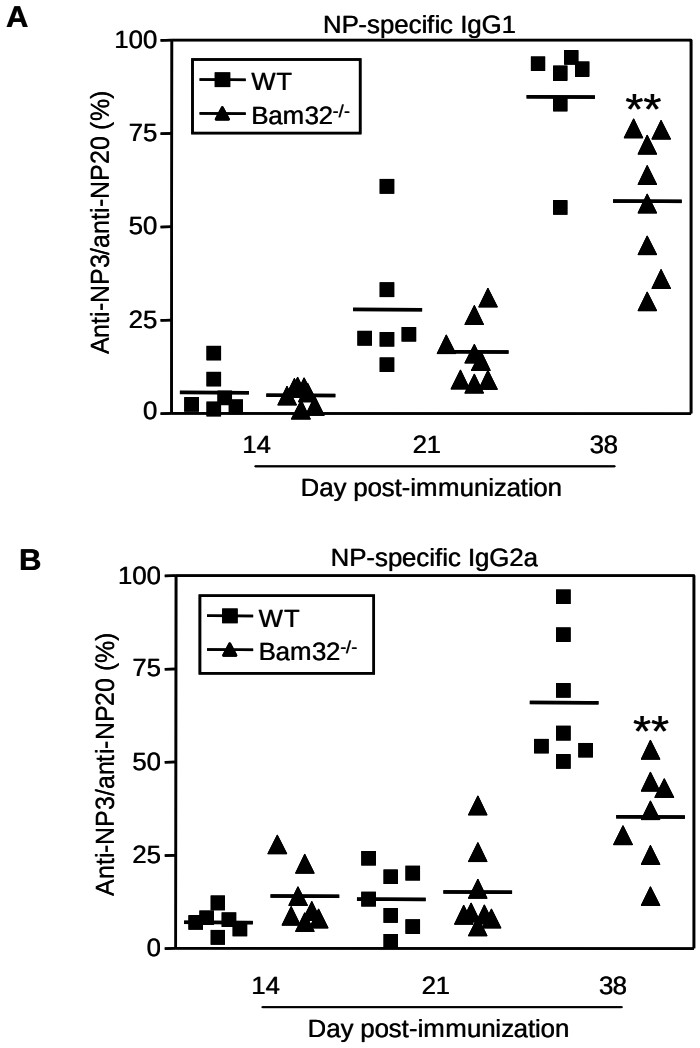

Figure 4.3.7 Reduced affinity maturation in Bam32-deficient mice

WT or Bam32$^{-/-}$ mice were immunized with T-dependent antigen NP-OVA, and boosted at day 28. At the indicated days serum were collected for measurement of low and high affinity NP-specific IgG1 (A) and IgG2a (B) Abs. Low affinity NP-specific Abs were detected using NP20-BSA-coated plates, while high affinity using NP3-BSA-coated plates. The ratio of NP3/NP20-binding, used as index of affinity maturation, is plotted for each mouse. ** p<0.005 (Students T-test) compared to wild-type control.

4.3.8 Switched IgG1+ B cells are dramatically reduced within decayed germinal centers of Bam32$^{-/-}$ mice

To further examine the role of Bam32 in B cell differentiation within germinal centers we examined B cell surface immunoglobulin isotype expression at day 10 post-immunization. Flow cytometric analysis of splenocytes revealed that Bam32-deficient GC cells showed a marked skewing towards IgM+ cells, with the frequency of IgG1-switched GC cells decreased by 3-fold on day 10 (Figure 4.3.8A). In line with the locally skewed IgM+ cells, systemic IgM levels were also increased (Figure 4.3.6). Impaired isotype switch within GC was further confirmed by immunofluorescence staining of spleen sections, directly showing that IgG1+ cells were markedly reduced within Bam32$^{-/-}$ GC (Figure 4.3.8B). In contrast, we already demonstrated that Bam32-deficient B cells were functionally competent in class switch to IgG1 when stimulated in vitro with anti-CD40+IL-4 (Figure 4.3.2B). Together these results demonstrate reduced switching to IgG1 within Bam32-deficient GC, and suggest that this may be due to lack of access to CD40 ligand and/or other switch factors within GC, rather than an intrinsic inability of Bam32-deficient B cells to switch.

Figure 4.3.8

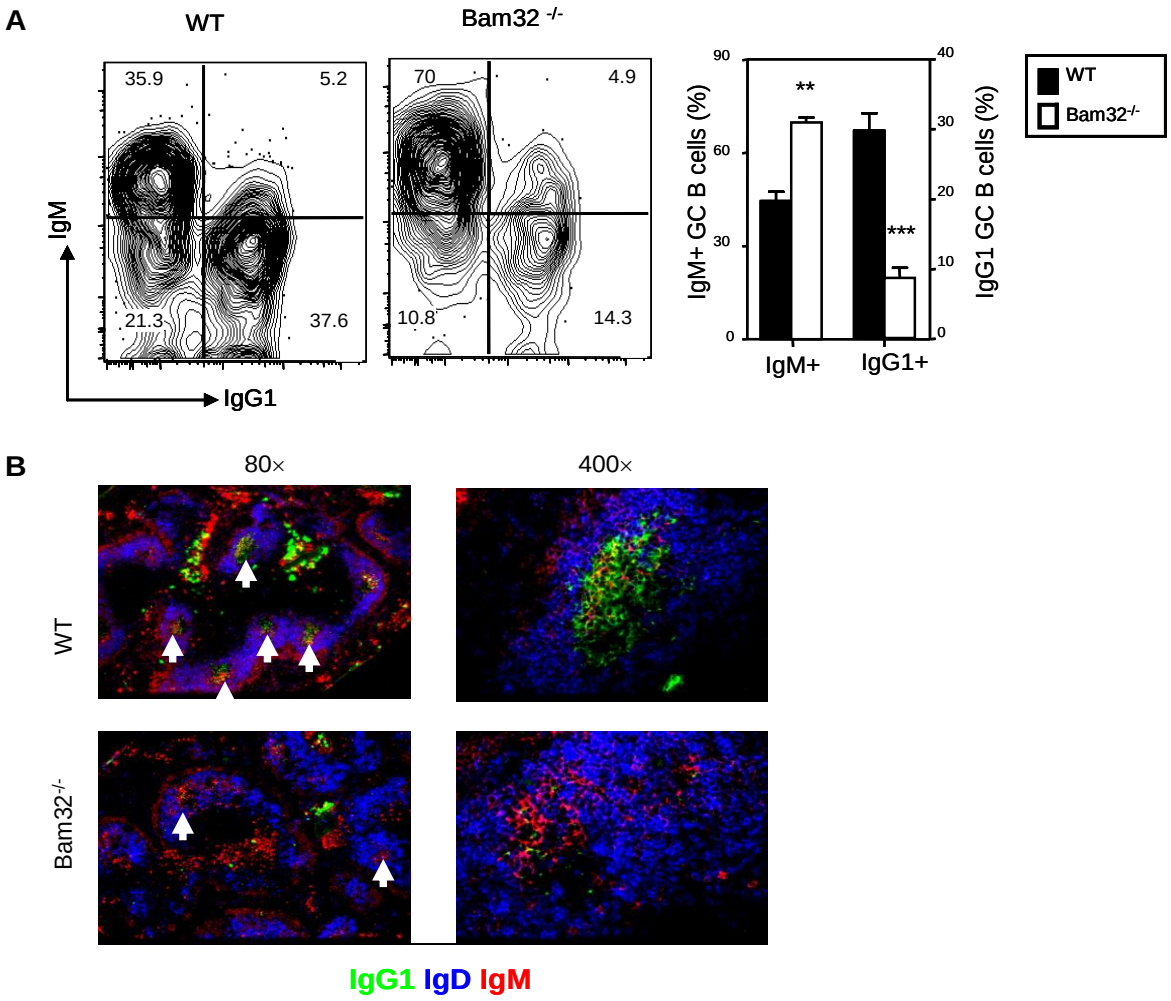

Figure 4.3.8 Bam32 regulates isotype switch within GC

(A) Expression of IgM and IgG1 on GC B cells at day 10 after OVA immunization. Representative flow contour graphs were shown, gated on B220+Fas+ GC B cells. Bar graph on the right shows the mean percentages ± SD of 6 mice per group. (B) Spleen sections harvested at day 10 post-immunization were examined by immunofluorescence staining with anti-IgG1 (green), anti-IgM (red) and anti-IgD (blue), and representative GCs from either WT or Bam32-/- mice were shown. White arrows indicate GCs. ** $p<0.005$; *** $p<0.001$ (Students T-test) compared to wild-type control.

4.3.9 Bam32$^{-/-}$ germinal center B cells proliferate normally *in vivo*

Previous *in vitro* proliferation data showed that Bam32 deficiency only leads to impaired proliferation responses to BCR stimulation, but not other T cell-derived stimuli. Thus, we investigated whether this selective defect in B cell proliferation may account for impaired GC progression in Bam32-deficient mice. To assess GC B cell proliferation in vivo, we first examined the expression of the nuclear proliferation antigen Ki67. At day 7 after immunization, both WT and Bam32 KO mice show many Ki67^{+} cells present within GCs surrounded by Ki67^{-}IgD^{+} B cells (Figure 4.3.9A, left). Similar frequencies of Ki67+ cells in the IgD- niche were found in WT and Bam32 KO spleens at day 10 post-immunization (Figure 4.3.9A, middle). At day 14, large clusters of Ki67^{+} GC cells are apparent in the WT spleen; however, at this time point clearly defined GCs are absent in Bam32 KO mice, and only a few sparsely distributed Ki67^{+} cells could be detected (Figure 4.3.9A, right).

We used BrdU labeling to quantitatively compare GC B cell proliferation at day 7 and day 10: time points corresponding to relatively normal GC initiation or the beginning of premature GC dissolution, respectively. BrdU was administered to immunized mice by i.p. injection 5h before sacrifice, and BrdU incorporation by GC B cells was determined by flow cytometry. At both day 7 and day 10, there was no significant difference in BrdU incorporation among GC B cells in WT and Bam32-deficient mice (Figure 4.3.9B). These results indicate that Bam32 is not required for GC B cell proliferation, and show that premature GC collapse in Bam32 KO does not coincide with a reduced rate of DNA synthesis among GC B cells.

In adoptive transfer experiments, μMT mice were given Bam32$^{-/-}$ (CD45.2+) and WT (CD45.1+) B cells mixed at 1:1 ratio, and then immunized to induce a GC response. Surprisingly, Bam32-deficient (CD45.2+) B cells became dominant among GC cells after immunization; however, in control groups, CD45.2+ and CD45.1+ WT cells contributed equally to the GC B cell pool (Figure 4.3.9C, left). This result suggests that Bam32-deficient B cells do not have a proliferative disadvantage, but appear to have some advantage over WT B cells in generating the initial pool of GC B cells. Interestingly, the mice receiving the mixture of Bam32-deficient and WT cells showed an overall reduction in the frequency of GC B cells present at day 12 compared with mice receiving only WT cells (Figure 4.3.9C, right). This result suggests the GCs that were initially populated with Bam32-deficient B cells later underwent premature dissolution. Together, the preceding results strongly support the conclusion that Bam32 is not required for GC initiation and GC B cell proliferation

Figure 4.3.9

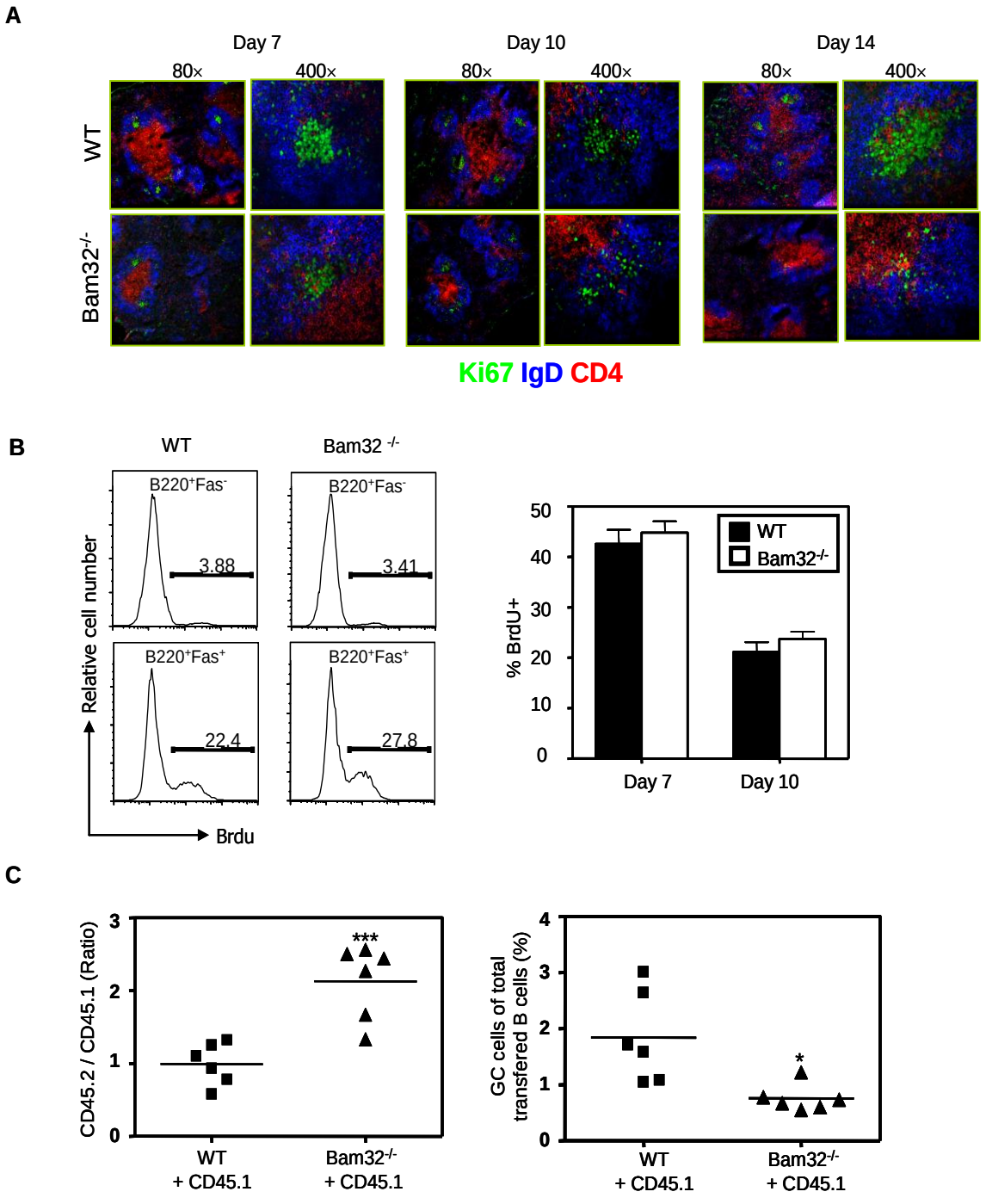
A
Day 7
Day 10
Day 14
80×
400×
WT
Bam32-/-
Ki67 IgD CD4
B
WT
Bam32 -/-
B220+Fas-
3.88
3.41
B220+Fas+
22.4
27.8
Relative cell number
Brdu
% BrdU+
50
40
30
20
10
0
WT
Bam32-/-
Day 7
Day 10
C
CD45.2 / CD45.1 (Ratio)
3
2
1
0
***
WT
+ CD45.1
Bam32-/-
+ CD45.1
GC cells of total
transfered B cells (%)
4
3
2
1
0
*
WT
+ CD45.1
Bam32-/-
+ CD45.1

Figure 4.3.9 Bam32 is not required for GC B cell proliferation

(A) Spleen sections were stained with FITC-anti-Ki67 (green) to detect the proliferating cell nuclear antigen. Representative images of sections taken at day 7, 10, 14 after OVA immunization are shown, indicating similar numbers of proliferating cells in WT and Bam32-deficient mice. (B) At 7 or 10 days after OVA immunization, WT or Bam32$^{-/-}$ mice were injected with BrdU 5h before sacrifice, and then splenocytes were stained with FITC-anti-BrdU, PE-anti-Fas, APC-anti-B220. The indicated cell populations gated on B220+ Fas+ (GC B) or B220+Fas- (Non-GC B) cells were analyzed for BrdU incorporation, and representative histograms are shown. Bar graph right shows percentage of BrdU+ GC B cells in wild type and Bam32-deficient mice, expressed as mean percentage ±SD of at least 6 mice per group. (C) Germinal centre responses in mixed B cell chimeras. Purified B cells (CD45.2+) from either wild type or Bam32-deficient mice were mixed with CD45.1+ B cells at a 1:1 ratio and transferred to μMT mice. 12 days after OVA immunization, spleen cells from μMT recipient mice were analyzed for representation of CD45.1/2 cells among GC B cells. The graph on the left shows the ratio of CD45.1+/CD45.2+ among GC B cells present in individual μMT mice receiving either WT/CD45.1 or Bam32$^{-/-}$/CD45.1 B cell mixtures. The graph on the right shows the overall percentages of GC B cells in these same mice.

4.3.10 Bam32 is required for optimal germinal center B cell survival in vivo

Initiation of apoptosis in WT and Bam32 GC cells was investigated by staining with fluorescently labeled VAD-FMK caspase inhibitor to detect active caspases. Strikingly, the frequency of cells staining with CaspGlow reagent is significantly increased in Bam32 deficient GC cells (Figure 4.3.10A/B). This increase is apparent at both day 7 and day 10 after immunization and occurs among both IgM+ and IgM- GC cell populations, arguing against selective apoptosis of the more differentiated IgM- population. Consistent with CaspGlow staining results, the frequency of Annexin V+ DAPI- cells (early apoptotic cells) among Bam32-deficient GC cells was also significantly increased (Figure 4.3.10C). Bam32-deficient B cells do not seem intrinsically prone to apoptosis, since previous data showed that *in vitro* stimulation of Bam32-deficient B cells with anti-IgM, anti-CD40, IL-4 alone or together generates comparable levels of apoptotic cells (Figure 4.3.2 A). Together these results indicate that signaling via Bam32 is required for development of mature GCs capable of promoting GC B cell survival in situ.

Figure 4.3.10

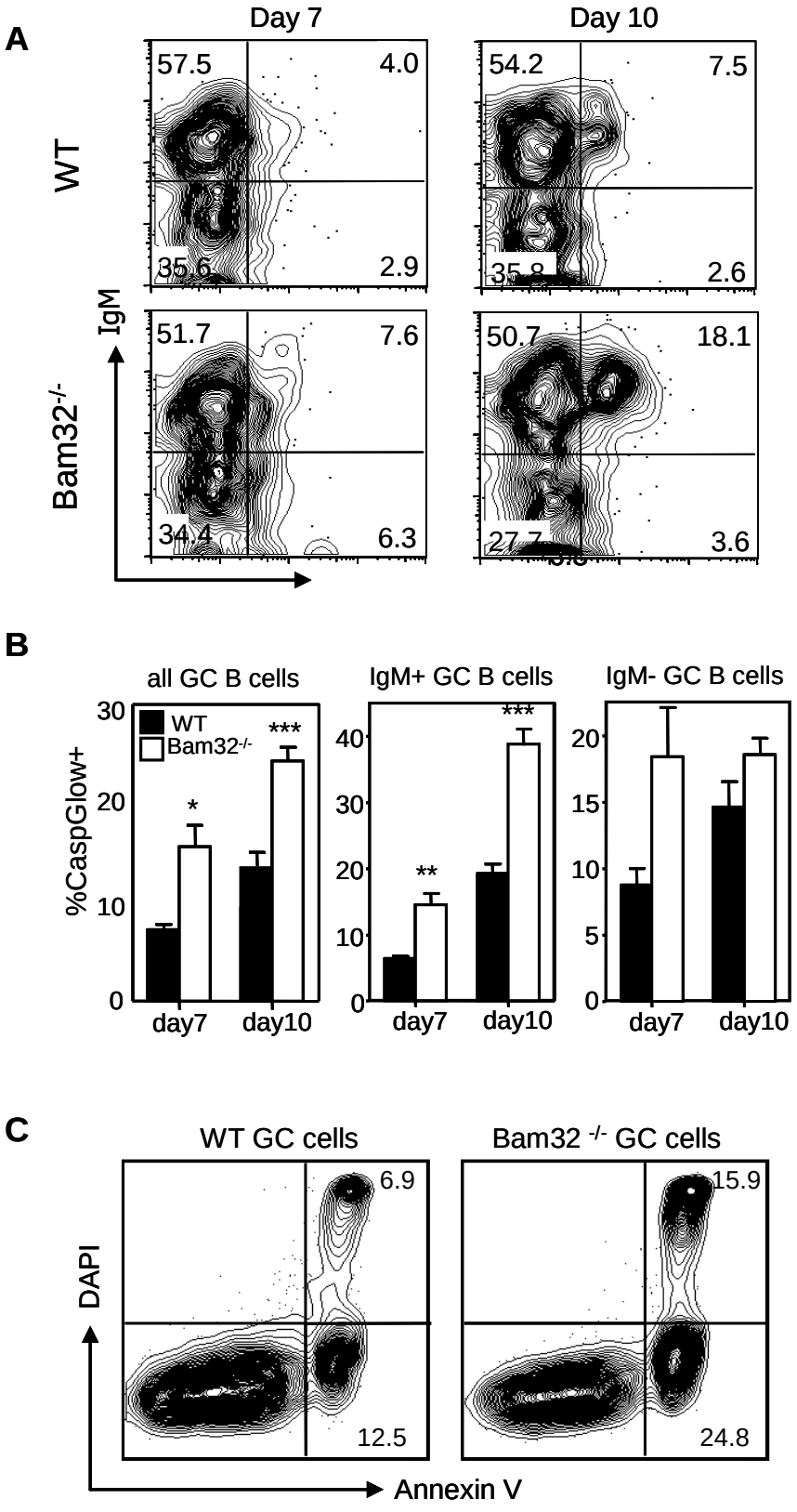
A
Day 7
Day 10
WT
Bam32-/-
IgM
57.5
4.0
35.6
2.9
54.2
7.5
35.8
2.6
51.7
7.6
34.4
6.3
50.7
18.1
27.7
3.6
B
all GC B cells
IgM+ GC B cells
IgM- GC B cells
%CaspGlow+
WT
Bam32-/-
*
**
***
day7
day10
C
WT GC cells
Bam32 -/- GC cells
DAPI
Annexin V
6.9
12.5
15.9
24.8

Figure 4.3.10 Bam32-deficiency leads to increased GC B cell apoptosis

(A) At 7 or 10 days after OVA immunization, splenocytes were surface stained to identify GC B cells and then incubated with FITC-VAD-FMK (CaspGlow) at 37 ^{o}C for 30min to detect active caspases. Representative FACS contour graphs showing IgM and CaspGlow staining splenocytes gated on B220+Fas+ GC population at day 7 and 10 post-immunization from both groups. (B) The bar graphs show percent of CaspGlow+ cells among the indicated cell populations. Data represent mean ± SD of at least 4 mice in each group. * $p<0.05$; ** $p<0.005$; *** $p<0.001$ (Students T-test) compared to wild-type control. (C) Representative FACS data showing Annexin V and DAPI staining of B220+Fas+ GC cells at day 10 post-immunization, confirming increased apoptosis in Bam32-deficient GC B cells.

4.3.11 Bam32$^{-/-}$ germinal center B cells fail to recruit or maintain CD4+ T cells within germinal centers

In the course of our confocal imaging analyses, we observed that day 10 GCs formed in Bam32-deficient mice seemed to show less staining of CD4+ cells within the PNA+ GC area. Subsequent image analysis confirmed that CD4 staining within Bam32-deficient GCs is significantly reduced (Figure 4.3.11A/B). Lack of T cell localization within Bam32-deficient GCs is not due to a global defect in development of follicular helper T cells, since similar numbers of CXCR5+ICOS+ Tfh cells are present in these mice (Figure 4.3.11D). Adoptive transfer experiments were performed to test whether deficiency of Bam32 in B cells is sufficient to cause defective T cell entry into GC. Fewer CD4+ T cells were observed within GCs formed in μmT recipients receiving Bam32-deficient B cells, compared to those receiving wild-type B cells (Figure 4.3.11C). Recent data supports the model that T cell entry into established GC is dependant on Ag-specific interactions with GC B cells [46]. The present results suggest that signals transmitted via Bam32 are required for B cell-dependant T cell entry or retention in GC.

Figure 4.3.11

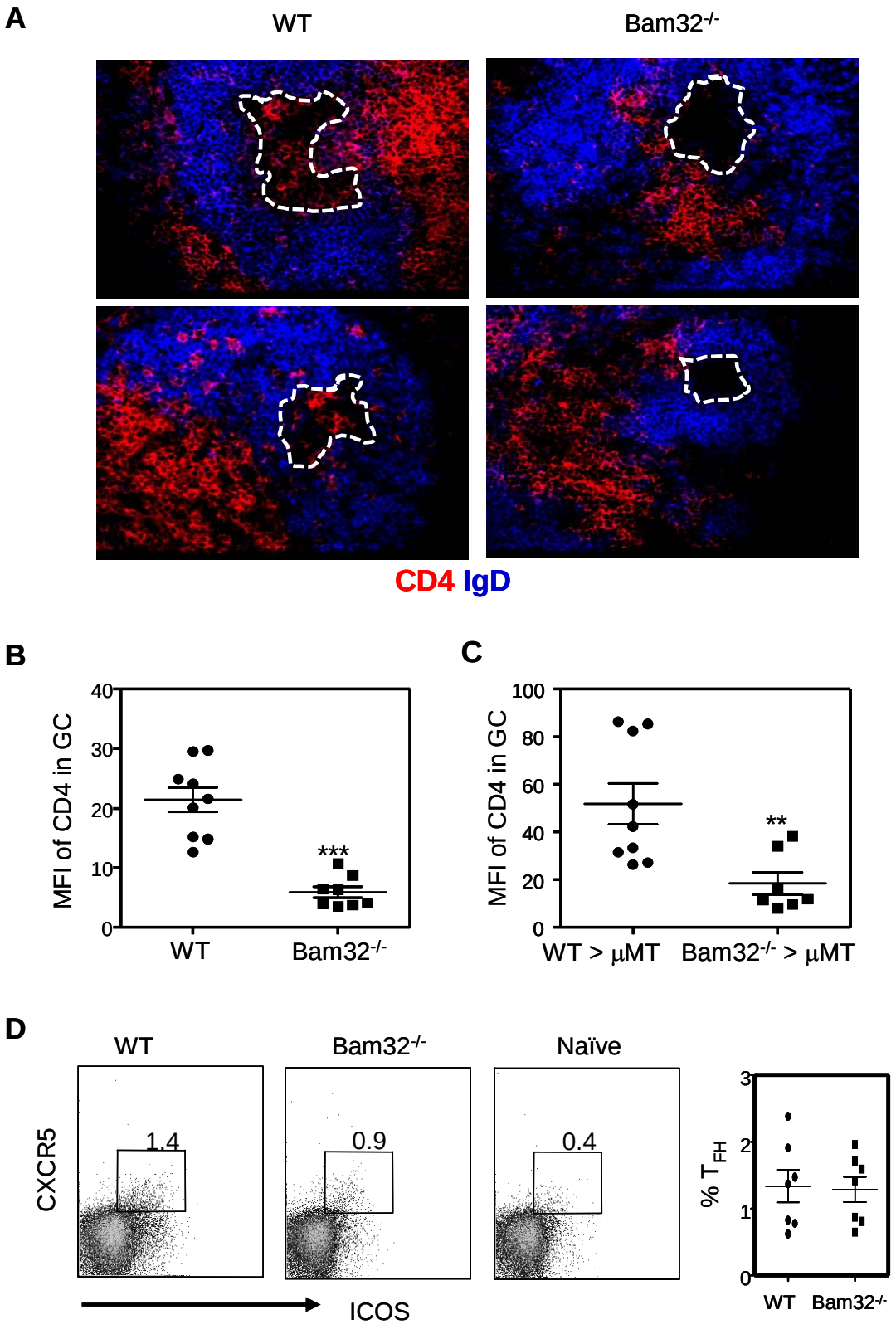
A
WT
Bam32-/-
CD4 IgD
B
MFI of CD4 in GC
40
30
20
10
0
***
WT
Bam32-/-
C
MFI of CD4 in GC
100
80
60
40
20
0
**
WT > µMT
Bam32-/- > µMT
D
WT
Bam32-/-
Naïve
CXCR5
1.4
0.9
0.4
ICOS
% TFH
3
2
1
0
WT
Bam32-/-

Figure 4.3.11 GCs formed by Bam32-deficient B cells contain fewer CD4+ T cells

(A) WT or Bam32$^{-/-}$ mice were examined 7 days post-immunization for the presence of CD4+ T cells located within GC. After capturing images of spleen sections stained with anti-PNA/CD4/IgD, the PNA+ areas were defined as regions of interest using image analysis software (PNA staining not shown to allow better visualization of CD4 staining). Representative images of sections obtained from two WT and Bam32$^{-/-}$ mice are shown. (B) The mean fluorescence intensity of CD4 staining within GC areas, quantified by image analysis. Sections from 4 mice in each group were examined, and average 2-3 GCs were counted per section based on GL7 and IgD staining. Each dot or square represents CD4 MFI cells within one GC. (C) WT or Bam32-/- B cells were transferred to μmT hosts as in Figure 1. 10 days after immunization, spleens from 4 mice in each group were sectioned and stained as in (A), and CD4 MFI within GL7+ regions was calculated using image analysis software. (D) Spleens were analyzed for T_{FH} phenotype cells, by staining with biotin-anti-CXCR5 (visualized with streptavidin-APC), FITC-anti-CD4, PE-anti-ICOS, PE-Cy7-anti-B220 and DAPI (for exclusion of dead cells in the Pacific Blue channel). The CXCR5/ICOS plots shown were gated on the CD4+B220-DAPI- singlet cell population. The right panel shows a summary of CXCR5+ICOS+ frequencies from 7 mice per genotype. ** $p<0.005$; *** $p<0.001$ (Students T-test) compared to wild-type control.

4.3.12 Agonistic αCD40 treatment can fully sustain germinal center responses of Bam32$^{-/-}$ mice

To investigate whether premature collapse of Bam32 GC responses can be rescued by an exogenous T cell-derived signal, we treated mice with agonistic anti-CD40 Ab, starting at day 5 post-immunization. By day 12, the group of Bam32-deficient animals treated with anti-CD40 Ab showed fully restored GC responses, as assessed by both flow cytometry and immunofluorescence staining (Figure 4.3.12A/B). In contrast, animals treated with a control Ab did not show restored GC responses. The overall reduction in GC responses in control Ab-treated mice may be due to FcR-mediated inhibitory effects. Anti-CD40 treatment also restored the frequency of GC cells expressing IgG1 to levels similar to wild-type mice (Figure 4.3.12C). Together these results demonstrate that the requirement for Bam32 can be completely overcome through exogenous provision of CD40 ligand during the critical period of GC progression.

Figure 4.3.12

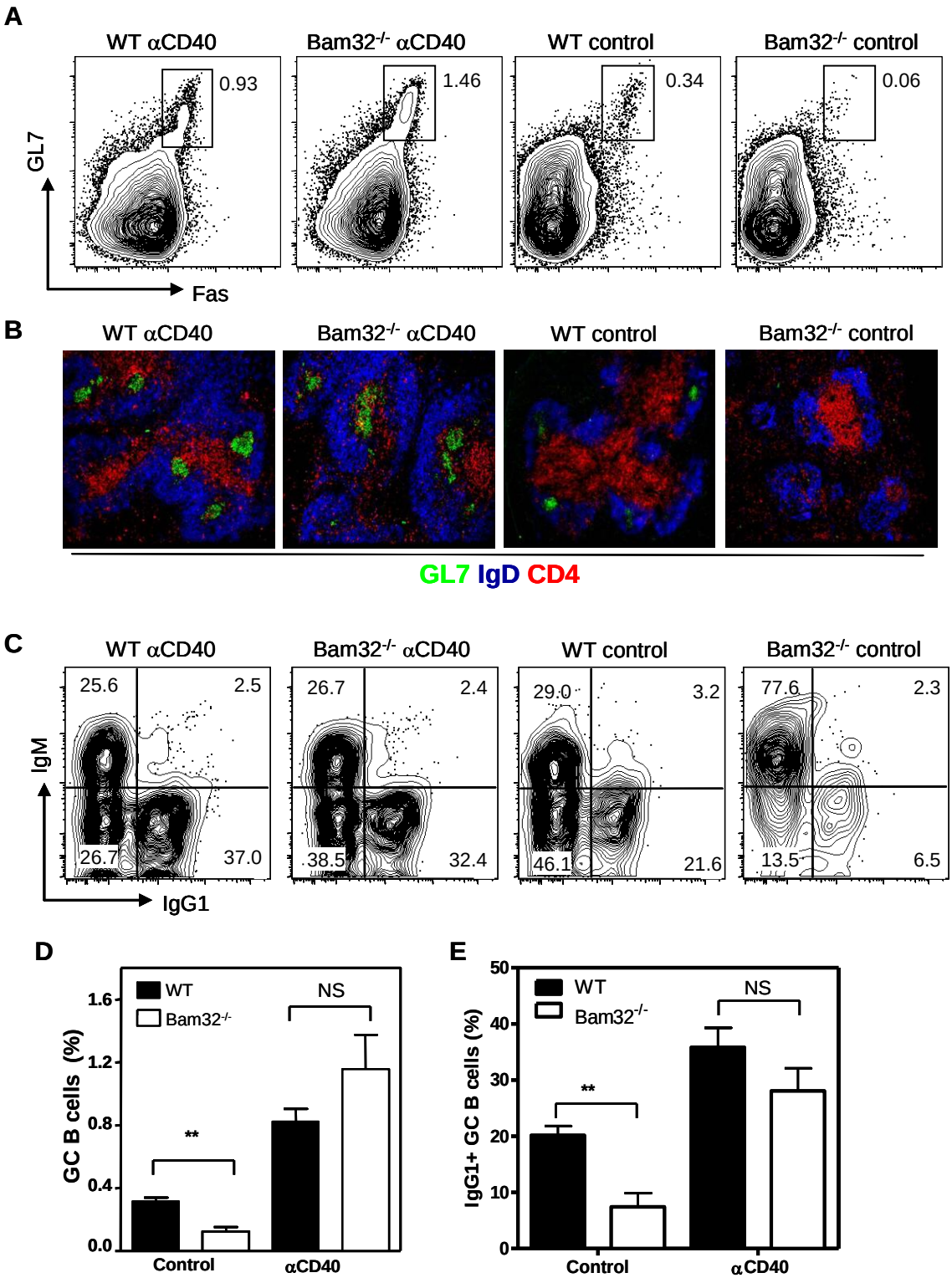
A
WT αCD40
Bam32-/- αCD40
WT control
Bam32-/- control
0.93
1.46
0.34
0.06
GL7
Fas
B
WT αCD40
Bam32-/- αCD40
WT control
Bam32-/- control
GL7 IgD CD4
C
WT αCD40
Bam32-/- αCD40
WT control
Bam32-/- control
25.6
2.5
26.7
37.0
26.7
2.4
38.5
32.4
29.0
3.2
46.1
21.6
77.6
2.3
13.5
6.5
IgM
IgG1
D
WT
Bam32-/-
NS
**
GC B cells (%)
1.6
1.2
0.8
0.4
0.0
Control
αCD40
E
WT
Bam32-/-
NS
**
IgG1+ GC B cells (%)
50
40
30
20
10
0
Control
αCD40

Figure 4.3.12 Administration of agonistic Abs to CD40 restore GC responses in Bam32-deficient mice

WT and Bam32-/- mice were treated with agonistic anti-CD40 Ab or isotype control Ab on days 5 and 10 after OVA immunization. GC responses were then assessed at day 12. (A) Frequencies of GC cells analyzed by flow cytometry. (B) Immunofluorescence staining of spleen sections to detect GC structures. (C) Frequency of switch to IgG1 assessed by flow cytometry. (D) Bar graph showing mean percentages of GC cells ± SEM, based on 4 mice per group. (E) Bar graph showing mean percentages of IgG1+ GC B cells ± SEM, based on 4 mice per group. ** $p<0.005$ (Students T-test) compared to wild-type control.

4-4 Discussion

4.4.1 Comparison with previous studies on Bam32-deficient mice

Previous studies on Bam32-deficient mice found normal induction of GC responses after immunization with sheep red blood cells [245], and our results using 10% SRBC immunization are consistent with these findings. However, premature dissolution of GCs in Bam32-deficient mice is clearly apparent when the mice were immunized with 1% SRBC or low doses of OVA protein antigen. We speculate that CD4 T cells are markedly activated during immunization with high doses of SRBC, known to polyclonally T lymphocytes through iron-containing compounds such as hemin [341]. As a consequence, T cell derived signals, such as CD40L and cytokines, are more abundant and accessible for Bam32-deficient GC B cells. Thus, driven by strong CD40 signaling, Bam32-deficient GC B cells are capable to survive and progress upon immunization of high dose of SRBC.

Previous published studies on Bam32-deficient mice indicated that type II T-independent antibody production is dramatically reduced, but T-dependent antibody responses are normal by showing hapten-specific IgM and IgG1 [136, 234]. In our study, we observed elevated OVA-specific IgM and reduced OVA-specific IgE in Bam32-deficient mice compared to WT after OVA immunization. There is no difference in systemic IgG1 responses (measured by serum titers), similar as previous results; however, local IgG1 switching in GCs is clearly decreased by day 10. To confirm there is no change in the genetic background or living environment of Bam32-deficient mice we used, we immunized the mice with type II T-independent antigen NP-Ficoll, showing markedly

reduced production of NP-IgG3, consistent with previous studies. Furthermore, the T-dependent antibody responses detected in our system are correlated with the premature dissolution of GCs we observed, except IgG1 production, which will be explained in the next paragraph. Therefore, conclusions of previous studies about no difference in hapten-specific IgM were drawn based on the detection of high affinity antibodies, which does not exclude the possibility that total or low affinity hapten-specific IgM is increased. IgE responses, which have been shown to be more closely dependent on T-derived signals [90, 340, 341], were not examined by previous studies.

4.4.2 How to reconcile the disconnection between systemic IgG1 production and GC IgG1 responses in Bam32-deficient mice

Our interpretation about the discrepancy between in vivo generation of IgG1 at comparable levels and premature dissolution of GCs in Bam32-deficient mice is that Bam32 is involved in both positive and negative signaling pathways, which may both have some effect on the antibody responses. The negative role of Bam32 in regulating B cell proliferation is supported by evidence that Bam32-deficient B cells are specifically hyper-responsive to stimulation via CD40 or IL-4. In vitro proliferation in response to these stimuli is significantly increased, and these stimuli dramatically synergize with BCR stimulation to restore proliferation. Previous studies also found that addition of IL-4 and/or anti-CD40 can restore BCR-triggered proliferation of Bam32-deficient B cells (23,26), and these published data are also consistent with the trend of increased responsiveness of Bam32-deficient B cells to these T-dependent signals. Our data indicate that, after immunization of Bam32-deficient mice with T-dependent Ag, initial B

cell proliferation in vivo is robust. In addition, in competitive adoptive transfer, Bam32-deficient cells appear to initiate a GC response more efficiently than WT cells. Although the reasons for this hyper-response to T cell-derived stimuli are not known, it is possible that this may partially obscure the effects of subsequent GC collapse. Therefore, while IgG1 switching in GCs is clearly decreased by day 10, systemic IgG1 responses are in the normal range, likely due to strong initiation of the T-dependant response and strong production of low affinity IgG1, such that the impairment in the systemic Ab response is only discernable at later time points, at the level of affinity maturation.

4.4.3 Antibody affinity maturation in Bam32-deficient mice

While there is abundant evidence that somatic hypermutation and affinity selection occur within GCs, there are a number of documented cases in which affinity maturation can occur relatively efficiently in the face of disrupted GC responses [36, 342]. This may be due to defective proliferation and/or disruption of tissue architecture in that precludes formation of robust GCs, while normal selective survival mechanisms favoring high affinity B cells may remain relatively intact. In the case of Bam32 deficiency, the opposite is true: there is no impairment in GC induction and proliferation, yet affinity maturation is less efficient. These findings are consistent with the idea that initial proliferation within GCs may occur relatively independently of affinity for Ag, with the selective threshold for GC B cell survival increasing over time with establishment of more stringent selection checkpoints. Our data show that the reduction in Ab affinity in Bam32-deficient mice is most pronounced at later time points. The inability to detect differences in affinity at earlier time points may partly reflect a technical limitation of the

assay and/or may be related to the slow turnover rates of the initial wave of low affinity serum antibodies.

4.4.4 Potential mechanism underlying the GC collapse in Bam32-deficient mice

Since Bam32 is not required for B cells to respond to T cell derived signals, we hypothesize that it is required for B cells to obtain access to these signals via Ag-mediated B:T conjugate formation. Indeed our results suggest that Bam32-deficient GC B cells are unable to promote CD4+ T cell accumulation within established GC, a function recently shown to depend on cognate B:T interactions [46]. We have found that PI3K is required for efficient BCR-mediated antigen presentation and formation of stable B:T conjugates [343], consistent with the model that signaling through this pathway promotes B:T cognate interactions within GC. My colleague Dr. Monther Al-Alwan has found that Bam32 is also required for polarized B:T cell conjugate formation and efficient BCR-mediated antigen presentation *in vitro*, likely via its role in promoting Rac activation and B cell adhesion to ICAM [248]. Thus, our working model is that Bam32 deficiency leads to impaired B:T cognate interaction within GC, limiting the amount of T cell-associated survival signals available to GC B cells. Exogenous provision of anti-CD40 restored GC progression, indicating that CD40 signaling in GC B cells can bypass the requirement for Bam32.

4.4.5 The essential roles of PI3K signaling in GC responses

Here, we demonstrated that Bam32, an adaptor molecule in the PI3K signaling pathway, are required for GC progression, possibly through regulating the availability of T cell-derived signals to GC B cells. These results differ markedly from observations made with mice lacking the PI3K docking site in CD19, which show diminished proliferation in nascent GCs and decreased serum IgG titres, in addition to subsequent GC collapse [21]. GC formation was restored in CD19-deficient mice by coinactivating PTEN (thus deregulating PI3K signaling), further suggesting that PI3K-dependent signals are key for GC responses (34). Also, these results are distinct from those observed in mice exhibiting global defects in PI3K signaling. For example, inactivation of PI3K catalytic subunit p110δ leads to nearly complete failure to generate GC cells because of impaired B cell development, proliferation and survival [160]; however, signaling via PI3K is also important for restraining AID expression and class switch recombination as indicated by our previous data in chapter III and by Dr. Omori's studies [311]. Clearly, PI3K-dependent signals are critical for both GC initiation and progression, and the present results implicate Bam32 as one of the PI3K-dependent effector molecules that comes into play, particularly during GC progression.

4.4.6 Other molecules or signaling pathways required for GC progression, but not initiation

The differential signaling requirements for germinal center initiation versus progression and selection are not well understood. Our results indicate that BCR signaling in the absence of Bam32 is sufficient to allow GC initiation and robust GC B cell proliferation,

in marked contrast with other BCR signaling deficiencies such as CD19, PI3K or BLNK deficiency. A similar phenotype of normal GC initiation but premature GC collapse was observed in CD45-deficient B cell chimeras after immunization. In that case, intrinsically poor survival of $CD45^{-/-}$ B cells seemed to account for the failure to maintain GC responses, whereas Ag presentation function was normal [344]. In contrast to Bam32-deficiency, BCR triggering of CD45-deficient B cells induced an abnormally high degree of apoptosis even in presence of CD40 ligation [344]. Thus it is likely that GC B cell survival requires collaboration of survival signals directly emanating from the BCR involving CD45 and signals provided via cognate interactions with T cells involving Bam32.

B cell-activating factor (BAFF) and BAFF receptor (BAFF-R) have also been demonstrated to be required for GC progression [346]. BAFF, belonging to TNF family, is mostly expressed on myeloid cells, such as monocytes and DCs, and BAFF-R expressed on B cells specifically binds to BAFF with high affinity [347]. Mice deficient in either BAFF or BAFF-R showed normal GC initiation followed by rapid GC collapse with SRBC immunization [346]. Although *in vitro* studies on BAFF strengthened its fundamental role in providing survival signals to B cells, both BAFF-deficient and BAFF-R–deficient GC B cells were not hyper-apoptotic based on in situ TUNEL staining [346]. Instead, BAFF deficiency leads to dissolution of FDC network and impaired immune complex trapping, which may be responsible for the GC defect described [346]. In contrast, BAFF-R deficiency results in the failure of GC B cells upregulating proliferation nuclear marker Ki67, suggesting that BAFF-R signaling is required for GC

B cell proliferation in vivo [346]. Therefore, BAFF and BAFF-R signaling regulates GC progression at multiple levels different from the role of Bam32.

4.4.7 Summary of main conclusions and overall significance

Our studies clearly demonstrated that GC responses in Bam32 deficient mice initiate normally, but they terminate prematurely upon immunization with lower doses of SRBC or OVA protein antigen. In line with this GC defect, T-dependent antibody production is partially affected by showing increased levels of antigen-specific IgM and reduced antigen-specific IgE. In addition, affinity maturation of hapten-specific IgG1 and IgG2a is impaired in these mice. The basis for premature collapse of the GC response appears to relate to increased apoptosis among GC B cells, which also correlates with decreased switch to IgG1. These phenotypes suggest that Bam32-deficient GC B cells fail to sufficiently access T cell derived signals such as CD40L, and indeed our results indicate a failure of B-dependant T cell entry into GC. The requirement for Bam32 in GC maintenance can be overcome by treatment with agonistic anti-CD40 mAb, indicating Bam32-deficient B cells are capable of mounting a sustained response when the required GC progression signals are provided exogenously. Collectively, the results reported here provide evidence that a signaling process involving Bam32 is essential for GC progression through a critical checkpoint. In contrast, Bam32 is not required for GC initiation or proliferation of GC B cells, consistent with different signaling requirements (or different thresholds) for GC initiation versus progression.

The significance of this chapter is that my study is the first study demonstrating a molecule required for GC B cells to obtain survival signals from T cells during GC progression. And thus the relatively subtle signaling defect in Bam32-deficient mice (allowing GC responses to initiate normally) provides a unique opportunity to study a relatively late GC progression checkpoint.

Chapter 5 General Discussion

During the journey of my thesis study, I started it by investigating the impact of genetic inactivation of PI3K p110δ signaling on T cell differentiation and function, and subsequently on airway inflammation and hyperresponsiveness. Then, my research focus diverted to the roles of p110δ signaling in B cell functions because of our accidental findings showing that serum IgE levels were surprisingly potentiated in immunized p110δ–inactivated mice. Concurrently, Bam32, a PI3K-dependent adaptor protein, attracted my attention since it potentially regulates germinal center responses, which are crucial for T cell-dependent humoral immunity.

Collectively, data presented in chapter II of this thesis clearly showed that genetic inactivation of p110δ leads to Th1-skewed phenotype in vivo after primary and secondary OVA/alum immunization, as indicated by robust production of IFN-γ and CXCL10 at comparable or even higher levels than that of WT mice, and markedly reduced type 2 cytokines IL-4, IL-5 and IL-13. Consequently, Th2-induced airway inflammation and airway hyperresponsiveness, hallmarks of allergic asthma, were prevented in p110δ–D910A mice after OVA sensitization and airway challenge. Therefore, p110δ signaling appears to be a key factor for the pathogenesis of allergic asthma by preferentially regulating Th2 differentiation and airway inflammation, and targeting p110δ seems to be a good therapeutic strategy in this disease setting.

During the years of my thesis work, small compounds targeting PI3Ks have been extensively studied for their therapeutic potential against different diseases. PI3K inhibitors have evolved from the first prototype inhibitors, LY294002 and wortmannin, which are toxic and show no selectivity towards isoforms, to PI3K isoform-selective inhibitors or to potent but less toxic pan-PI3K inhibitors. In this context, my work sheds light on the immunomodulatory effects of blocking PI3K activity.

Cancer is one major class of diseases that may receive therapeutic benefit through intervention of PI3K pathway. PI3K signaling regulates normal cellular responses in multiple aspects, including cell proliferation, survival, migration and differentiation [146, 147]. In human cancers, genes encoding PI3Ks or key molecules in PI3K pathway are frequently mutated or altered, leading to potentiated PI3K signaling globally or partially [345-348]. Specifically targeting mTOR, one downstream signaling node in the PI3K pathway, has been successfully used in the clinic to treat cancers [349]. Accumulating evidence suggests that selective targeting a single isoform of PI3K may be beneficial for particular tumor type [350-353]. Many PI3K inhibitors possess off-target effect on mTOR because of the structural similarity between PI3K catalytic subunits and mTOR; thus, those inhibitors may be more potent to treat cancers. Our data showed that targeting PI3Ks skews the immune responses towards type 1, known to be the most effective type against cancer [354, 355]. Thus, PI3K inhibitor treatment may result in potentially beneficial effect on anti-tumor immunity, in addition to direct action on cancer cells.

PI3Ks, especially p110δ and p110γ, also appear to be promising drug targets to treat chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and multiple sclerosis. P110δ and p110γ are mostly expressed by immune cells and they are functionally important in the activities of T cells, B cells, neutrophils, mast cells and NK cells [311]. Mice with p110δ and/or p110γ deficiency are viable and fertile [161, 163, 356], indicating that selective targeting p110δ and p110γ using small molecules is relatively safe. To date, pharmacologically blockage of both p110δ and p110γ shows promising therapeutic benefit in the animal models of rheumatoid arthritis [357, 358]. Inhibition of p110γ for the treatment of human rheumatoid arthritis is now in phase II clinical trials.

Targeting PI3Ks also hold promise for the treatment of some infectious diseases. In collaboration with Dong Liu from Dr. Jude Uzonna's lab, we found that p110δ-D910A mice are more resistant to *Leishmania major* infection compared to wild-type mice [284]. Although after *Leishmania major* infection p110δ-D910A CD4 T cells generate markedly reduced level of both Th1 and Th2 cytokines, the absence of IL-10-producing CD4+CD25+FOXP3+ regulatory T cells allows the residual IFNγ to more efficiently controll parasites and prevent pathology [284]. Thus, administration of p110δ-selective inhibitor may have beneficial effects in *Leishmania major* infection treatment. Targeting PI3Ks may also have therapeutic adavantage for the treatment of Ebola virus infection. It was demonstrated that PI3K signaling pathway in host cells was utilized by Zaire Ebolas virus (ZEBOV) to facilitate its entry into cells [359]. Inhibition of PI3Ks or PI3K downstream molecules significantly block ZEBOV intracellular trafficking, thereby

inhibiting virus replication in vivo [359]. Additionally, blockage of PI3K activity diminishes the production of proinflammatory cytokines [284, 360], which are involved in pathogenesis of Ebola virus hemorrhagic fever. Recent findings also suggest that targeting PI3K/Akt pathway is promising to treat HIV (human immunodeficiency virus) infection, since PI3K/Akt inhibition makes HIV-infected cells more susceptible to apoptosis [361, 362].

My data presented in chapter II strongly suggest that selectively targeting p110δ pathway is a promising therapeutic strategy for the treatment of allergic diseases and asthma. However, paradoxically we also found that PI3K signaling negatively regulates the production of IgE, a key player in allergic diseases and asthma. My results in chapter III showed that genetic and pharmacological blockage of p110δ leads to potentiated IgE production from activated B cells in vitro. After OVA immunization, mice treated with p110δ–selective inhibitor IC87114 showed selective elevation of antigen-specific IgE production although they produce less IgE switch factor IL-4 compared to mice treated with vehicle only. Evidence presented in chapter III demonstrated that p110δ signaling negatively regulates IgE class switch recombination in B cells through suppressing the expression of AID and εGLTs. Nuclear transcription factor Foxo1 inactivated by Akt has been suggested to positively regulate AID expression [324]; however, it is still unclear about the mechanism underlying this specific negative regulation of IgE. My data suggest that p110δ signaling downregulated the expression level of transcription factor Bcl6, which may compete with NFκB for the binding sites on the promoter regions of AID and εGLT. All together, these data suggest that PI3K signaling, especially p110δ

signaling, in B cells act as an intrinsic brake to prevent uncontrolled IgE switching. Thus, targeting PI3K pathway for therapeutic purpose in the clinic may induce higher IgE production. However, the clinical impact of any elevated IgE is unclear, since PI3K targeting is also reported to inhibit signaling via IgE high affinity receptor [307].

In the future, it will be interesting to investigate how blockage of PI3K activities in vivo leads to selectively potentiated IgE isotype switch, and where those IgE-switched cells are located in the tissues. When p110δ is inactivated, the help provided from CD4+ T cells to B cells is greatly attenuated in vivo not only because p110δ–inactivated T cells produce markedly reduced Th2 cytokines, but also because p110δ–inactivated B cells are impaired to present antigen to cognate T cells and subsequently to receive help from T cells [343]. The dramatic disconnect between T cell help and serum IgE levels in p110δ-D910A mice make us wonder whether non-B cells also come into play. For example, the PI3K dependent kinase Itk has been shown to be required for Th2 differentiation, however Itk-deficient mice exhibit elevated IgE levels [289, 363]. One recent study showed that CD4+ γδ T cell population is expanded in Itk-deficient mice, which is responsible for enhanced IgE production [364].

In vivo p110δ inhibition model provides a good opportunity to study the relationship between IgE switching and germinal center responses. Inhibition of p110δ activity for a short period at different stage of immune responses may lead to the production of IgE at different levels. As we reported in chapter III, elevated IgE levels were observed when mice were orally dosed with IC87114 throughout the primary immune responses. In

contrast, one study showed that intratracheal administration of IC87114 during airway challenge phase significantly attenuated the production of IgE in serum [300].

PI3K signaling is critical for germinal center development. Genetic blockage of PI3K activities leads to diminished, almost undetectable GC responses, accompanied with markedly impaired T-dependent antibody responses, as demonstrated by p110δ-deficient, p85α-deficient and p110δ-D910A mice [161, 207, 365]. Furthermore, BCR co-receptor CD19-mediated PI3K activation has been observed to have multifaceted roles in GC development, including GC B cell maturation, proliferation and positive selection [21]. Genetic mutation or deletion of Btk, one of the most important PI3K downstream enzymes expressed in B cells, results in reduced, but still detectable GC responses after primary T-dependent antigen immunization [366, 367]. Here, as described in chapter IV we find that Bam32, PI3K downstream adaptor molecule, may come into play by particularly regulating the progress of GCs.

Data presented in chapter IV showed that GCs initiate normally in Bam32-deficient mice after immunization with OVA/alum or low doses of sheep red blood cells, but those GCs dissipate prematurely. Even though Bam32 is also expressed in murine primary T cells and has some effect on T cell function, adoptive transfer studies indicated that intrinsic defects of Bam32-deficient B cells lead to premature dissolution of GC responses. In line with truncated GC responses, Bam32 deficiency was also associated with partially impaired serum antibody production and significantly reduced antibody affinity maturation. To further address why Bam32-deficient B cells fail to mount sustained GC

responses, in vitro and in vivo B cell functions were assessed. In vitro studies showed that BCR-induced proliferation of Bam32-deficient B cells was markedly reduced, however co-stimulation with T-cell-derived signals compensates this defect. Consistently, in vivo proliferation of Bam32-deficient GC B cells was not compromised. Further studies showed that Bam32-deficient GC B cells are more apoptotic, which may lead to premature GC dissolution, and they are impaired to swich to IgG1, suggesting lack of T cell help in situ, since Bam32-deficient B cells have no intrinsic defect in apoptosis and class switch recombination. In line with this piece of data, GCs formed by Bam32-deficient B cells contain fewer T cells, indicating that Bam32 is required for B cell-dependant T cell accumulation within established GCs. Exogenous CD40 ligand restored GC B cell numbers and switch to IgG1, indicating that Bam32-deficient B cells are competent to respond to CD40 stimulation when ligand is available. All together, those data demonstrate that Bam32 is not required for GC initiation, but rather functions in a late checkpoint of GC progression associated with T cell recruitment and GC B cell survival.

Even though there was no molecular mechanism presented in chapter IV to explain why GC responses were aborted prematurely in Bam32-deficient mice, studies performed by my colleagues Dr. Monther Al-Alwan and Sen Hou showed that Bam32 is required for optimal BCR-mediated antigen presentation to cognate T cells, which may become pivotal at the late stage of GC procession. Their further study indicated that BCR-mediated antigen internalization and antigen trafficking to the late endosomes/lysosomes are normal in Bam32-deficient B cells; however, Bam32 is required for polarized

conjugate formation between B cells and antigen-specific T cells probably by promoting BCR-induced Rac activation and cytoskeleton rearrangement [248]. All together, the working model of chapter IV is that Bam32-deficient GC B cells are impaired to present antigen to antigen-specific helper T cells during GC progression, leading to inaccessibility of T cell-derived signals, such as CD40L, to those B cells. Therefore, Bam32-deficent GC B cells are unable to recruit or sustain CD4+ T cells within GCs, and they die out quickly and fail to switch to IgG1+ cells locally at the late stage of GC progression.

In regard to this model, one of the future studies is to determine whether in vivo antigen presentation from Bam32-deficient GC B cells to antigen-specific CD4+ T cells is impaired at the late stage of GC progression. To address this point, the most direct evidence should come from in vivo imaging studies using two-photon microscopy [368, 369]. Compared to single-photon microscopy, two-photon microscopy has some advantages, such as greater tissue penetration, restricted excitation, low out-of-focus noise, and less phototoxicity [368]. This method allows cells of interest can be visualized within living tissues using fluorescent labelling techniques, such as adoptive transfer of in vitro labelled cells, i.v. injection of labeled cell-specific antibodies, and knock-in mice expressing a fluorescent protein in a specific cell lineage [368]. With these technologies, the actual frequency and duration of stable B:T cognate interaction could be measured [23, 370]. In our case, Bam32-deficient mice could be crossed to BCR-transgenic mice and then crossed to GFP mice to generate GFP transgenic mice expressing antigen-specific Bam32-deficient B cells. TCR-transgenic T cells could be purified and labeled

with different dye, such as CellTracker CMTMR. To visualize antigen uptake and presentation in GC B cells, antigen can be introduced in vivo in the form of PE immune complex as described previously [23]. 7 days after the antigen is introduced into the mice, I would predict that conjugates formed between antigen-engaged Bam32$^{-/-}$ B cells and antigen-specific T cells are fewer within GCs, and they last less than 10 min. In contrast, more conjugates are formed between antigen-loaded WT B cells and cognate T cells, and they pair for at least 10 min.

Another question elicited from this study is why Bam32 is not required for the initiation of GCs. To answer this question, there are three possibilities to be tested in the future: 1) During GC initiation stage, Bam32-deficient B cells are partially impaired to present antigen and form cognate conjugate with antigen-specific T cells; however, it is still sufficient to initiate GC responses; or 2) Bam32-deficient B cells are capable to present antigen and form cognate conjugate with antigen-specific T cells if excessive antigen-specific T cells are present; or 3) signals provided by innate cells in the inflammatory environment early on overcome the need for Bam32 to form stable B:T conjugates, but these signals diminish over time. CD40L is one possible signal, which decreases over time. To test the first two possibilities, mixture of GFP-labelled BCR-transgenic Bam32-deficient B cells and CMTMR-labelled TCR-transgenic mice (at different ratio) could be adoptively transferred into previously antigen (containing both BCR and TCR epitopes)-immunized WT mice, and then followed with two-photon microscopy technique for in vivo imaging.

In conclusion, data in this thesis demonstrate that PI3K p110δ signaling plays an important role in the pathogenesis of airway inflammation and hyperresponsiveness by promoting Th2 differentiation, and thus selectively targeting PI3Ks appears to have therapeutic benefit for the treatment of allergic diseases and asthma. However, signaling via PI3Ks, at the same time, acts as a B cell-intrinsic brake on IgE isotype switch. Therefore, the elevated IgE levels associated with in vivo administration of p110δ selective inhibitor IC87114 put a question mark on those PI3K inhibitor compounds being developed for potential clinical use, and this unexpected immunomodulatory effects need to be resolved in the future. In this thesis we also demonstrate that PI3K-regulated adaptor protein Bam32 is pivotal at the late stage of GC responses, probably by regulating B cell antigen presentation. Thus, this relatively subtle effect on GC responses in Bam32-deficient mice provides a unique model to study the impact of in vivo antigen presentation from GC B cells to antigen-specific T cells on GC progression versus initiation.

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