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CD5-dependent CK2 activation is critical for the maintenance of B-1a B cells

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CD5-DEPENDENT CK2 ACTIVATION IS CRITICAL FOR THE MAINTENANCE OF B-1a B CELLS

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CD5-DEPENDENT CK2 ACTIVATION IS CRITICAL FOR THE MAINTENANCE OF B1-a B CELLS

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ABSTRACT

CD5 has classically been shown to act as a negative regulator of antigen receptor signaling, however recent evidence has discerned that the CD5 molecule contains a previously undefined cytoplasmic domain which constitutively binds inactive CK2 and facilitates its activation through CD5 ligation. With the development of a mouse model which contains a micro-deletion knock-in form of CD5 which lacks the amino acids necessary to facilitate this CD5-CK2 interaction, it is now understood that CD5 plays a more significant role in cellular physiology than previously appreciated. T cells from this mouse model show increased AICD and dysregulation in T helper subset polarization.

In mice CD5 expression is limited to only a few different cellular populations, one of which is the innate-like B-1a B cell population. This population has been shown to play a major role in the early natural antibody responses to TI-II antigens, and is a driving force in the clearance of many commonly encountered pathogens. The studies in this dissertation demonstrate a new role for CD5 in B-1a mediated signaling cascades and physiologic responses to T-independent antigens.

In these studies we evaluated the impact of the CD5-CK2 signaling domain on shaping the peritoneal cavity cellular distribution and the functional consequences stemming from the loss of this signaling domain on normal B-1a B cells. In addition, by

applying what we determined in normal B-1a B cells, we elucidated the role of CD5-CK2 signaling in maintaining B-1a B cell dominated idiotypic responses, specifically with regard to anti-PC responses to *S. pneumoniae*. Overall, these studies determine a new and previously undefined essential role for the CD5-CK2 signaling axis in maintaining B-1a B cell homeostasis through regulating essential cellular signaling pathways necessary for proliferation, growth, and regulation of immune-dominant responses.

DEDICATION

I would like to dedicate this dissertation in memory of my father, Marvin L. Cashman, Jr., who lost the battle with Parkinson's disease. You are missed.

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DISCUSSION

LIST OF ABBREVIATIONS

AICD	Activation induced cell death
BCR	B cell antigen receptor(s)
BSA	bovine serum albumin
Cdc37	cell division cycle 37 protein
CK1	casein kinase I
CK2	casein kinase II
CK2BD	casein kinase II binding domain
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
HRP	horseradish peroxidase
Ig	immunoglobulins
IRF	Interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
IκB	inhibitor of κB
KLH	keyhole limpet hemocyanin
KSR1	Kinase suppressor of Ras 1
LPS	lipopolysaccharide
MEK	MAPK/ERK kinase
mRNA	messenger ribonucleic acid
NFAT2	Nuclear factor of activated T-cells 2
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells

PC	phosphorylcholine
PMA	phorbol 12-myristate 13-acetate
PtC	phosphatidyl choline
S.p.	*Streptococcus pneumoniae*
SH2	Src Homology 2
SHP-1	Src homology region 2 domain-containing phosphatase-1
SLE	Systemic lupus erythematous
STAT3	Signal transducer and activator of transcription 3
TCR	T cell antigen receptor
TdT	Terminal deoxynucleotidyl transferase
TI-II	Thymus independent-type II
TNP	2,4,6-trinitrophenol
tRNA	transfer ribonucleic acid

INTRODUCTION

B1-a B cells

The immune system is made up of a myriad of cellular populations that reside in tissues and circulate throughout the body. All of these different cell types work together toward one common goal; the protection of an organism from foreign pathogens (i.e. viruses, bacteria, toxins, fungi, or helminthes). One broad and evolutionarily consistent form of protection is the production of immunoglobulin (Ig), which form the antibody pool necessary to recognize and opsonize foreign pathogens and target them for neutralization (Reviewed in (1, 2)). B cells are the only class of immune cells that have the ability to produce immunoglobulins.

B cells are a heterogeneous population that can be subdivided into many different subsets based on phenotypic surface antigen expression and functional role within the immune response. One such population of B cells is the B-1a B cell, which has a classic phenotype of IgM^{hi} IgD^{lo} $CD11b^{+/int}$ $B220^{lo}$ $CD5^{+}$ (3)(4). In adult mice, the B-1a B cell population is primarily found within serous cavities such as the peritoneal and pleural cavities, and as a small percentage within the spleen. Although peritoneal and splenic B-1a B cells are commonly grouped together under the B-1a classification, there are distinct differences between these two populations. The most notable differences are the lack of constitutively phosphorylated/activated STAT3, the inability to proliferate following PMA (phorbol ester) stimulation, reduced surface IgM expression, and the lack of CD11b in splenic B-1a B cells as compared to their peritoneal counterparts (5, 6). Interestingly, splenic B-1a B cells display higher Notch family member and IL-10 gene expression than the peritoneal subset.

Although there are a number of differences between peritoneal and splenic B-1a B cells, they share very similar roles in the immune response. It has been proposed that splenic B-1a B cells may result from peritoneal B-1a migration into the spleen during inflammatory responses and with subsequent division become additive to the resident splenic pool to reinforce early IgM reactivity to pathogens (7). This would also argue that through peritoneal activation, B-1a B cells in the spleen are essentially a memory B cell pool that awaits further re-stimulation with their cognate antigen.

Origins of B-1a B cells

Within the peritoneal cavity of mice three distinct primary B cell populations can be discerned using flow cytometric staining; The afore mentioned B-1a population with an IgM^{hi} IgD^{lo} $CD11b^{+/int}$ $B220^{lo}$ $CD5^{+}$ phenotype, a closely related B-1b "sister" population with an IgM^{hi} IgD^{lo} $CD11b^{+/int}$ $B220^{lo}$ $CD5^{-}$ phenotype, and a conventional/B2 B cell expressing an IgM^{int} IgD^{hi} $CD11b^{-}$ $B220^{hi}$ $CD5^{-}$ phenotype (8). Although these three populations reside in the same anatomical niche they do not respond to the same antigens or impact the immune response in the same manner, nor do they have the same developmental pathways. B-1 B cells, particularly B-1a B cells, are unique in their development.

A conventional B cell arises during neonatal development as a result of sequential stage maturation within the bone marrow (9). These cells then migrate from the bone marrow and wait to find their antigens within the periphery. B-1a B cells are unique in their development because unlike conventional B cells they have been shown to be generated during fetal development, with major sites of lymphopoiesis within the day nine

embryonic yolk sac and intra-embryonic para-aortic splanchnopleura in mice, and in the fetal omentum and fetal liver in humans (10, 11). Unlike conventional B cells, which are consistently produced during life, B-1a B cells contract and migrate to serous cavities shortly after birth, where they primarily self-renew over the lifespan of the mouse (12). This self–renewing property makes the B-1a B cell population unique among B cell subsets, though adult progenitor cells have been found in both the spleen and bone marrow and can selectively give rise to B-1a B cells (13, 14). These B-1a B cells tend to vary from the fetal B-1a population in that they have the ability to express TdT which allows for increased N-region insertions and a skewing away from germline BCR reactivity (15, 16).

Antigen specificity in the B-1a population

As previously stated the B-1a population, especially fetally derived B-1a B cells, primarily respond to a restricted subset of antigens due to the expression of germline-derived B cell antigen receptors (BCR). These germline BCRs have a more restricted V_H gene usage and are usually specific for antigens that elicit a T-independent type II (TI-II) immune response (17). Despite this restriction in the responsiveness of the B-1a population their role within the early immune response is critical.

The Ig produced by this population tends to be poly-reactive in nature, permitting them to bind and opsonize a larger subset of targets than those of highly specified conventional B cell origin (18). Interestingly these immunoglobulins also exhibit weak auto-reactivity which allows them to bind both pathogen-derived antigens and self-antigens (19). This auto-reactive nature of the B-1a population has long been thought to be one of the major mechanisms of autoantibody development in autoimmune disorders such as

systemic lupus erythematous (SLE), Sjögrens syndrome, and rheumatoid arthritis (20-23). However, conclusive proof of this mechanism as a dominant determinant in autoimmune disorders has not been established.

Although the B1-a B cell population has been shown to have auto-reactive BCR expression, these BCRs are essential for the role of the population in the overall immune response. B-1a B cells act as a first line of defense against pathogens of a TI-II nature. The B-1a population has the ability to rapidly produce immunoglobulins in response to stimulation, thus reducing the bacterial or pathogenic burden until the adaptive immune response can develop (24). In many cases the innate B cell response, which includes the B-1a population, may clear an infection independently of the adaptive response. An example of this protective effect can be seen in the case of *Streptococcus pneumoniae* infections (25).

Despite the fact that B-1a B cells have a unique and early lymphopoiesis during ontogeny, even after generation this population undergoes changes in its distribution of BCR specificities. These specificities, or idiotypes, start to select for clones with broad neutralizing capacity in a process known as idiotypic dominance or idiotypic selection. In terms of *S. pneumoniae* one of the hallmark antigenic determinants is the high expression of phosphorylcholine (PC). During early neonatal development, around 6-10 days following birth, an idiotype develops with high neutralizing capacity (26). This T15 idiotype has been shown to have anti-PC specificity and is critical in the neutralization of *S. pneumoniae* (27). Interestingly this idiotype constitutes approximately 85% of the anti-PC pool in adult wild-type animals (26). Early manipulation of anti-PC responses have shown that instead of selection for the dominant neutralizing idiotypes less effective anti-

PC clones are selected for. This in turn increases susceptibility in adulthood to pathogens expressing PC due to a lack of neutralizing capacity. This phenomenon has also been seen in conserved responses against other antigenic targets as well (28).

Functional impact of B-1a B cells

B-1a B cells have been shown to have many impacts on antigen driven immunity, though they also impact the overall immune response in many different facets. B-1a B cells have been shown to be one of the major producers of natural serum IgM within the mouse. Natural IgM is the immunoglobulin that is found in germ-free mice that is produced in the absence of exogenous stimulation (29, 30). In mice, roughly 80% of the natural IgM levels have been shown to arise from B-1 B cells (31). This natural IgM tends to be anti-inflammatory in nature, binding many targets including those found in apoptotic debris and increasing phagocytic clearance (32, 33).

In addition to producing natural serum IgM the B-1a population strongly influences the production of mucosal IgA. Many groups have shown that peritoneal transfer of B-1 cells or fetal liver transplant into lethally irradiated (34) or $Rag2^{-/-}$ (35) mice resulted in the expression of donor derived IgA from the lamina propria of the gut or within the mesenteric lymph nodes. Further support for the B-1a B cell influence in IgA production comes from experiments with allotype-marked B-1 B cells. Examination of fecal bacteria from these animals revealed that 65-70% of all the bacteria were coated with B-1-derived IgA (36).

In addition to effects on systemic and mucosal immunity the B1-a population is well adapted for phagocytosis (37). This phagocytic capacity also leads to a proficient ability to present antigen to T cells. In a murine model of SLE (NZM2410-derived Sle2

congenic model) this B-1a mediated antigen presentation promotes the pro-inflammatory T_h17 subset differentiation and reveals another potential mechanism for the development of autoimmunity (38).

In a contradictory facet the B-1a B cell population is one of the primary producers of the classically anti-inflammatory cytokine IL-10 (39). In past experiments, administration of anti-IL-10 antibody resulted in the depletion of the peritoneal B1-a population and a subsequent drop of about 90% in serum IgM levels (40). This would suggest that the B-1a population is subject to an IL-10 autocrine loop, and may explain why peritoneal B-1a B cells express constitutively phosphorylated STAT3. These last two observations of B-1a B cells (IL-10 secretion and the promotion of T_h17 differentiation), along with the autoreactive nature of the population and Ig secreted from them, suggests a Janus-like role for the population in the overall immune response and shows why they are the subject of much interest and study.

CD5 and its role in B-1a B cells

As stated previously the defining surface phenotype of the B-1a B cell population is IgM^{hi} IgD^{lo} $CD11b^{+/int}$ $B220^{lo}$ $CD5^{+}$, though the only distinguishing surface characteristic that can distinguish B-1a B cells from other B cell subsets (especially the B-1b population) is the surface expression of CD5. CD5 is a 67 kDa surface glycoprotein co-receptor with three extracellular scavenger receptor cysteine-rich domains that is found on a small number of immune populations ((41), Reviewed in (42)). These populations are primarily limited to the aforementioned B-1a B cell population, as well as on thymocytes, T cells, and anergic conventional B cells subsequent to upregulation. In mice the CD5

gene is located on chromosome 19 and on chromosome 11 in humans (GenBank: *Mus musculus* Chromosome 19-NC_000085.6; *Homo sapiens* Chromosome 11-NC_000011.9).

CD5 expression was first recorded on T cells with the use of polyclonal antibodies, and later on the Ly-1^{+} B cells that would eventually be described as the B-1a B cell population (43-45). Previous reports, primarily in thymocytes, determined that CD5 expression is variable in developing populations such as in the thymus (46, 47). It was also determined that CD5 levels are proportionate to the quantitative and qualitative signaling received by thymocytes through the antigen receptor (48). As strength of signaling increases so does the level of CD5 expressed, and vice versa. It is thought that this variable nature of CD5 in thymocytes is necessary for controlling hyper-responsive TCRs and limiting the chances for autoreactivity through the development of anergy by mechanisms that will be explained later. Similar observations were also seen within B cells where TI-II immunizations, which act through the B cell antigen receptor, upregulated the expression of CD5 (49). Although CD5 expression levels are regulated within developing lymphocytes in the thymus, CD5 expression is stabilized once a thymocyte matures and becomes a peripheral T cell (48).

The functional role of CD5 as a negative regulator to antigen driven signaling was clarified in B-1 B cells from mice lacking CD5, where $CD5^{-/-}$ B-1 cells were able to activate NFκB and become highly proliferative following BCR crosslinking. These functions were not attributed to the B-1a B cell population in wild-type animals (50). However sequestration of CD5 from the BCR in wild-type mice was also able to recapitulate the $CD5^{-/-}$ phenotype. These data suggest that the negative regulatory effects of CD5 are intrinsic to activation through antigen receptor and proximity to the receptor. This is

supported by the fluorescent resonance energy transfer (FRET) experiments in human T cells, which show that CD5 and CD3 are usually within 10nm of one another (51). Further analysis of the association of antigen receptors and CD5 was seen in coimmunoprecipitaction studies where CD3, CD5, and the TCR signaling molecule, Zap-70 were found to be associated (52).

A few mechanisms for how CD5 mitigates antigen receptor signaling have been proposed. Firstly, the activation of SHP-1 through the phosphorylation of the cytoplasmic juxta-membrane tyrosine has been evaluated (53). However, this activation does not involve tyrosine phosphorylation to activate SHP-1 or the SH2 domains (54). Contradictory to the juxta-membrane tyrosine of CD5 acting as the source of negative regulation, later studies showed that anti-CD5 ligation failed to coimmunoprecipitate SHP-1 with CD5 (55). These experiments also proposed that the negative regulatory effects for CD5 originated more C-terminal in the cytoplasmic region between Y429 and L444, a region containing an ITAM-like motif with an inhibitory function.

Although CD5 has primarily been demonstrated to be a negative regulator of antigen receptor signaling, the cytoplasmic tail has shown to be much more complex than the inhibitory pseudo-ITIM-like domain alone. A previous report from our group using coimmunoprecipitation studies and a yeast two-hybrid screening system showed that CD5 was a unique surface receptor which was found to constitutively bind inactive casein kinase-2 (CK2) at S459 and S461 (56). Upon CD5 ligation, CK2 was phosphorylated and activated wherein it phosphorylated S459 and S461 and mediated downstream kinase activity (Example in Figure 1). This interaction is unique and is the first evidence of a surface receptor engaging CK2 at the plasma membrane. It was later found that another

member of the scavenger receptor cysteine rich domain family, CD163, also exhibited this interaction (57). Following the development of a mouse model lacking the CK2 associating serine residues of CD5 (Fig1; $CD5^{\Delta CK2BD/\Delta CK2BD}$) it was discovered that this CK2 binding domain was important in setting the threshold of activation on thymic and peripheral T cells and was necessary for the optimal differentiation of T_h2 and T_h17 T cell subsets from naïve T cells (58). In addition, this mutation in the CD5 cytoplasmic domain also recapitulated previous $CD5^{-/-}$ findings of reduced disease severity in the experimental autoimmune encephalomyelitis (EAE) model, and an increase in activation induced cell death (AICD).

CK2 and Associated Signaling

Among the first historical discoveries of protein phosphorylation were studies using casein as a substrate for rat liver protein isolates (59). These experiments found two kinases with the ability to phosphorylate casein, which would later be termed CK1 and CK2. CK2 is a ubiquitous and pleiotropic cytoplasmic serine/threonine kinase that has been shown to play an intrinsic role in eukaryotic signaling cascades and is an important mediator in many cellular and physiologic processes. These include, but are not limited to, apoptosis, spermatogenesis, embryogenesis, and circadian rhythms (60-62). Additionally, CK2 has been established as a pro-survival kinase with the ability to mediate the synthesis of tRNA and mRNA, and aid in DNA repair mechanisms (63, 64).

Structurally CK2 exists as a tetra-subunit holoenzyme with two regulatory β subunits and a combination of two catalytic α or α' subunits (65, 66). Once assembled and phosphorylated on the activation loop, these subunits become constitutively activated to

allow for unhindered downstream signaling (67). The β subunits have been shown to interact with the cytoplasmic tail of CD5 (56). Notably once CK2 becomes activated it interacts and phosphorylates over 300 substrate targets making it one of the most prolific kinases within the kinome (68).

Interestingly CK2 has many unique interactions that are applicable to lymphocyte and B-1a B cell biology. Prominent substrates of CK2 within this category include IκBα and IκBβ, IRF1, IRF2, NFAT2, NFκB, Osteopontin, and Akt to name a few (68). One of the more intriguing substrate interactions of CK2 is the phosphorylation of Cdc37. Cdc37 is a co-chaperone protein which normally binds Hsp90 and complexes with a myriad of target proteins and kinases positively impacting their kinase activity (69). This co-chaperone is only phosphorylated at Ser13 by CK2, which is necessary to facilitate its interaction with Hsp90 and complexed substrates (70). Taken together with the other more than 300 known substrates of CK2, the additive substrates from proteins and kinases, like Akt and Cdc37, add a complexity of interactions that make CK2 one of the most pleiotropic kinases within the body.

Rationale of dissertation study

The activation of CK2, through its cognate binding domain of CD5, alters TCR threshold for activation in T cells and leads to increased AICD and decreased EAE severity resembling the phenotype of $CD5^{-/-}$ mice (58). Through cytokine secretion and T-B cell interactions, T lymphocytes are critical for adaptive immune responses, but the other arm of the immune system is also critical for optimal protection from pathogens and diseases. This innate immune system comprises many cell types important in phagocytosis and

antigen presentation resulting in T cell activation, which links the two arms of the immune response. A particularly interesting cellular population of the innate immune system is the B-1a B cell population which has the ability to secrete Ig in response to conserved pathogen associated antigens and self-antigens, essentially acting as a hybrid population between the two arms of the immune response. Interestingly this population expresses and is defined by the surface antigen CD5, however the role CD5 in regulating this population is poorly understood.

In contrast to impact on T cells, genetic deletion of CD5 results in the loss of discrimination of the B-1a B cell population, essentially rendering the surface phenotype identical to the B-1b B cell population. This has limited the ability to determine the impact of CD5 on the B-1a population. Utilization of the CD5ΔCK2BD (developed by Sestero *et al,* 2012) and the $CD5^{-/-}$ animal model (Fig. 1) allows for the discrimination between CD5-dependent CK2 signaling and other CD5 dependent and independent effects. Therefore the aim of this study is to dissect the impact of the CD5-dependent CK2 signaling on the B-1a B cell population.

Because CK2 impacts cell survival signaling, determining the influence of the CK2BD on maintenance and physiological output of the B-1a population will be addressed within the first aim of the study. Depletion of this population significantly impacts serum IgM, but how CD5-CK2 signaling modifies the physiologic output, cellular signaling cascades, and functional activity of the B-1a population remains undetermined. The development of the CD5ΔCK2BD mouse allows for the first time to dissect the mechanistic influences of CD5 signaling domains on the B-1a B cell population.

Since B-1a B cells heavily impact the primary immune response, understanding the role of this CD5-CK2 domain on physiologic TI-II responses will be addressed. The B-1a population undergoes idiotypic enrichment and dominates the idiotypic responses to many antigenic determinates, so understanding how the CD5-CK2BD modifies innate-like immune responses to antigens such as PC and PtC is important in many facets. It is understood that modifying early idiotypic responses can skew the immune system away from dominant idiotypes increasing susceptibility to certain infections, but how CD5 influences this antigen specificity and responses have not been addressed. Also understanding this pathway's importance may shed light not only on the magnitude of the influence that CD5 exhibits on anti-idiotypic responses, but also on a mechanism that has potential to regulate complex disorders such as autoimmunity, atherosclerosis, and perhaps even B cell neoplasia.

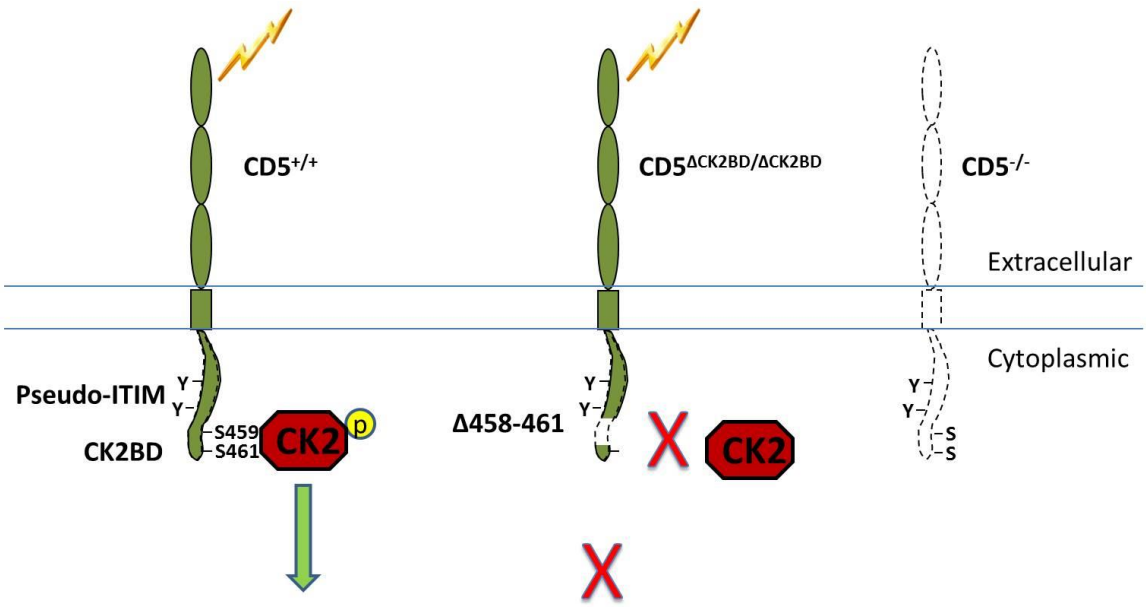

Figure 1. Activation of CK2 through CD5 is lost in CD5ΔCK2BD mice. Schematic representation of normal activation of CK2 through the CD5 receptor and how micro-deletion of cytoplasmic amino acids 458-461 of CD5 in the CD5ΔCK2BD mouse oblates this association.

CD5-CK2 SIGNALING IS ESSENTIAL FOR EFFICIENT B-1A B CELL ANTIBODY RESPONSE AND SURVIVAL

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Abstract

The expression of CD5 is the only phenotypic marker that clearly distinguishes B-1a B cells from all other B cell subpopulations. However, the question if CD5 has a function in B-1a B cells has remained unanswered. To address this question we utilized our recently developed mouse ($CD5^{\Delta CK2BD/\Delta CK2BD}$) in which the ability of CD5 to engage and activate CK2, a serine-threonine kinase, was ablated by gene targeting. The activation of the CK2 is one of the major signaling pathways triggered by CD5. Ablation of CD5-CK2 signaling had no effect on B-1a B cell numbers. In mice, the natural IgM and IgA are primarily produced by B-1a B cells and we found that the serum of $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice contained significantly lower levels of these Igs than in $CD5^{+/+}$ mice. Furthermore, T-independent type 2 immune response was also significantly diminished in the absence of CD5-CK2 signaling. From these data we inferred that CD5 through activation of CK2 qualitatively modulated BCR responses in B-1a B cells. Consistent with this prediction we found that B-1a B cells but not B-1b B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice hypoproiliferated in response to BCR but not TLR stimulation. BCR-induced phosphorylation of Cdc37, an early cell-cycle protein, as well as basal and anti-IgM induced activation of ERK was diminished in $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells. We also determined that ablation of CD5-CK2 signaling rendered B-1a B cells more susceptible to activation induced cell death. These results provide the first evidence that CD5 has an essential function in the physiology of B-1a B cells.

Introduction

B1-a B cells are a unique population of lymphocytes that retain many phenotypic and physiologic characteristics of an innate immune lineage (i.e CD11b, MHCII, CD80, CD86 expression) (1-4). This population derives in the fetal omentum, primarily self-renews, and populates serous cavities during adulthood (5, 6). The B1-a population has been shown to have germline BCR reactivity selected on highly conserved epitopes; many of which are shared between ubiquitous pathogens and self-antigens (7-9). This antigen reactivity and the production of polyreactive and weakly autoreactive low-affinity antibodies have sparked much interest in this population in the pathogenesis of many autoimmune disorders such as systemic lupus erythematous, Sjögren's syndrome, and rheumatoid arthritis (10-12). In addition to the implications in autoimmune disorders, the B1-a lymphocyte population is the primary producer of the T15 idiotypic antibody (13, 14). This antibody has been shown to cross-react between PC and oxLDL antigenic targets and shares 100 percent homology between both the murine and human antibody repertoires (15, 16).

One of the most unique characteristics of this B lymphocyte population is the expression of the subset defining phenotypic marker, CD5 (17, 18). CD5 has long been shown to be a negative regulator of both T cell and B cell antigen receptor signaling, but the overall contribution of CD5 to B1-a B cell physiology remains vague (19, 20). Previously our group reported through yeast two-hybrid screenings that in addition to the negative regulatory ITIM-like domain of CD5, another primary cytoplasmic signaling region exists that is necessary for the binding and activation of the serine-threonine kinase, casein kinase-II (CK2) (21, 22). CK2 is a ubiquitous kinase that has been shown to

mediate a myriad of downstream effects including pro-survival and repair signaling cascades, however the importance of the CK2 binding/activating domain (CK2BD) of CD5 has not been fully elucidated (23).

Recently, our group had developed a knock-in mouse which lacks the serine residues necessary for the binding CK2 to CD5 and its subsequent activation (24). In this recent study, it was found that T cells from these $CD5^{\Delta CK2BD/\Delta CK2BD}$ animals were hypo-proliferative in response to anti-CD3 treatment, while exhibiting increased AICD and reduced EAE severity as compared to $CD5^{+/+}$ controls. These data suggested that CD5 modifies the threshold of T cell responsiveness and laid the ground work for the importance of better understanding how CD5-dependent CK2 signaling alters B1-a B cell biology. In this study we examine the contributions of the CK2BD of CD5 on B1-a B cell biology and how it contributes to the physiology of the peritoneal B cell compartment and immune responses.

Materials and Methods

Mice

C57BL/6 mice (CD5WT) were purchased from NCI-Frederick Cancer Research or bred in our colony. $CD5^{-/-}$ backcrossed greater than 12 generations into C57BL/6 (CD5KO) mice were from our colony (25). The $Cd5^{\Delta CK2BD/\Delta CK2BD}$ has been recently described (24). All mice utilized within the experiments were 8-10 weeks of age, unless otherwise stated. All animals were housed and treated in accordance with National Institutes of Health and University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

Flow Cytometry

Peritoneal exudate, spleen, or bone marrow cells were stained as described previously (26). For characterization of cellular populations the following antibodies were used: CD5 (53-7.3), CD11b (M1/70), IgD (11.26C.2a) from Biolegend (San Diego, CA); CD8a (53-6.7), CD19 (eBio1D3), CD23 (B3B4), B220 (RA3-6B2), CD4 (RM4-5), GL7 (GL-7), CD93/AA4.1 (AA4.1), CD1d (1B1), Fixable Viability Dye (Cat#65-0865 or 65-0863) from eBioscience (San Diego, CA); CD138 (281-2), CD21/CD35 (7G6), CD95/Fas (Jo2) from BD Bioscience (San Jose, CA); and/or IgM (1B4B1), CD24 (91) from Southern Biotech (Birmingham, AL) All samples were analyzed using either a FACScaliber or LSRII flow cytometer (BD Bioscience).

Intracellular phospho-Flow experiments were performed as previously described for T cells with the exception of the use of 70% methanol and 15 minute stimulation with goat anti-mouse IgM F(ab')2 (Southern Biotech) (27). Antibodies utilized were as follows:

anti-phospho ERK1/2 (Thr202/Tyr204) (D13.14.4E; Cat#4370), anti-phospho Akt (S473) (D9E; Cat#4060), anti-IκBα (L35A5; Cat#5743), and anti-phospho Cdc37 (Ser13) (D11A3, Cat#8733) (Cell Signaling). All samples were analyzed using either a FACScaliber or LSRII flow cytometer (BD Bioscience).

Ig Isotype Analysis and Immunizations

Serum was collected from $CD5^{+/+}$, $CD5^{-/-}$, and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and stored at -80C until needed. An ELISA was performed using Costar® 96-well high-binding EIA/RIA plates (#3590) coated with unlabeled anti-IgM, anti-IgA, anti-IgG1, anti-IgG2b, anti-IgG2c, or anti-IgG3 as a capture reagent followed by serum and HRP-labeled isotype secondary antibodies (Southern Biotech). ELISAs were then developed with TMB substrate (Thermo Scientific), stopped with 2N H_2SO_4, and analyzed at 450nm in a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT).

Eight week old animals were immunized with one of three antigens; either 200ul of a 100 ug/ml solution diluted in PBS of 2,4,6-trinitrophenyl-LPS (TNP-LPS) or 2,4,6-trinitrophenyl-AminoEthylCarboxyMethyl-Ficoll (TNP-Ficoll) i.p.(20ug/mouse final), or a 100ug immunization of a 2,4,6-trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) emulsion in incomplete Freund's adjuvant administered i.p. at days 0, 21, and 4 months post secondary booster (Biosearch Technologies, Novato, CA). Sera from animals immunized with TNP-LPS and TNP-Ficoll was collected at days 0, 5, 10, and 15 post immunization. TNP-KLH mice had serum collected at days 0, 5, 12, 21, 28, 35, 42, 141, and 148 post immunization. Serum samples were stored at -80C until analysis by ELISA

utilizing TNP(21)-BSA and/or TNP(5)-BSA in the case of TNP-KLH samples as a capture reagent followed by serum incubation. HRP labeled anti-IgM or anti-IgG secondary antibodies were then incubated to determine anti-TNP specific titers. TMB substrate and colorometric analysis were then performed as for the isotype ELISAs.

Proliferation Assays

In vitro stimulations were performed by culturing peritoneal exudates and splenocytes for 72 hrs in RPMI-1640 (supplemented with 10% FBS, 2-ME, and100U/ml Pen-Strep) with 1ug/ml anti-IgM F(ab')2, 10ug/ml anti-IgM F(ab')2 (Southern Biotech), or 10ug/ml LPS (Sigma-Aldrich, Cat#L-5024). Proliferation in gated populations of B-1a, B-1b or B2 B cells was measured by determining the incorporation BrDU that was added for the last one hour of culture (26).

Ca^{++} Flux Analysis

Peritoneal exudates and splenocytes were isolated from $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and surface stained to enable gating for B-1a B cells. Calcium mobilization was determined by flow cytometry following stimulation with 10ug/ml goat anti-mouse IgM F(ab')2 using a protocol described previously (24). Cells remained at 37C throughout the stimulation utilizing a water pump with a modified FACS tube holder.

Apoptosis and Cell Cycle Analysis

Mice were injected with 10 µg (cell cycle) or 25ug anti-IgM F(ab')2 (Annexin V) in 200ul PBS i.p. 48 later cell cycle analysis using propidium iodide (PI) and Annexin V positivity within B-1a gated cells was determined (25, 28).

Results

B-1a B cell numbers are not altered in CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice.

As previously reported, peripheral T cell populations in proportion and number were unaltered in CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice (Supplemental Fig 1A, (24)). To determine if ablation of the CD5-CK2 signaling pathway leads to changes in B-1a B cell numbers, we analyzed B cell populations in the peritoneal cavity of CD5$^{+/+}$ and CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice using the Herzenberg approach (Supplemental Fig. 1B, (1, 29)). We observed no difference in the expression of CD5 on B-1a B cells from CD5$^{+/+}$ mice and CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice at all ages (8 weeks to >52 weeks), a finding we recently reported for CD5 expression on T cells (Fig. 1A and data not sown; (24)). In the peritoneal cavity the proportion and absolute numbers of B-1a B cells in CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice were not different than in CD5$^{+/+}$ mice (Fig. 1B). However, we did find that CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice had increased B-1b B cells (IgM^{+}IgD^{lo}CD11b$^{+/lo}$CD5^{-}) compared to that in WT mice, the difference was significant in proportion and but not in absolute number (Fig 1C). In the spleen, CD5$^{\Delta CK2BD/\Delta CK2BD}$ mice contained fewer numbers of B10 (CD5^{+}CD1d^{hi}) B cells whereas all other B cell populations were equal to CD5$^{+/+}$ mice (Fig 1D).

Loss of CD5-CK2 signaling leads to subclass-restricted reduction in serum Ig levels

B-1a B cells contribute to majority of the serum IgM and IgA, also referred to as natural Ig, within an unimmunized mouse (30, 31). We hypothesized that CD5-CK2 signals modulates BCR signals and its ablation would lead to changes in serum Ig levels. To test for this we measured levels of IgM. IgA, IgG1, IgG2b, IgG2c and IgG3 in serum of age-

matched 8-10 week old $CD5^{+/+}$, $CD5^{\Delta CK2BD/\Delta CK2BD}$ and $CD5^{-/-}$ mice. The $CD5^{-/-}$ mice is a complete loss of CD5 function model. Serum IgM levels in $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and $CD5^{-/-}$ mice were equivalent but significantly lower than in $CD5^{+/+}$ mice (Fig. 1A). The loss of CD5-CK2 signaling had the greatest impact on IgA levels. The serum from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice contained only 19% of the IgA as that that in serum from $CD5^{+/+}$ mice (Fig. 1B). $CD5^{-/-}$ mice serum also had lower levels of IgA than in serum from $CD5^{+/+}$ mice but significantly higher than that in serum from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice. Of the IgG subclasses, only IgG1 levels were reduced by loss of CD5-CK2 signaling (Fig 1C). Here again, serum of $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice contained significantly lower levels of IgG1 than serum of $CD5^{-/-}$ mice.

CD5-CK2 signals are necessary for efficient T-independent type 2 antibody responses

The repertoire of B-1a B cells is skewed towards T-independent type 2 (TI-2) responses (32-35). The finding that $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice have normal B-1a B cell numbers but lower levels of serum IgM and IgA led us to ask if loss of CD5-CK2 signaling might lead to diminished ability to respond to TI-2 antigens. To address this question, $CD5^{+/+}$, $CD5^{\Delta CK2BD/\Delta CK2BD}$ and $CD5^{-/-}$ mice were immunized with the typical TI-2 antigen 2,4,6-trinitrophenyl-aminoethylcarboxymethyl-Ficoll (TNP-Ficoll). The anti-TNP response in $CD5^{+/+}$ mice peaked on day 10 post-immunization and by day 15 had dramatically diminished (Fig. 3A). In contrast, the anti-TNP IgM in $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and $CD5^{-/-}$ mice at the peak of TI-2 response (day 10) was significantly lower than that in $CD5^{+/+}$

mice. Notably, the anti-IgM response in $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and $CD5^{-/-}$ mice was similar at all time points.

Bruton's tyrosine kinase deficient (Btk) mice do not have B-1a B cells and are unable to mount a TI-2 response but do respond to T-independent type 1 (TI-1) antigens (36-38). This demonstrates that B-1a B cells are not the major contributors to the immune response elicited by TI-1 antigens. We therefore predicted that immune response to a TI-1 antigen should be unaltered in CD5-CK2 signaling deficient mice. To test for this we immunized $CD5^{+/+}$, $CD5^{\Delta CK2BD/\Delta CK2BD}$, and $CD5^{-/-}$ mice with the TI-1 antigen, TNP-LPS. The anti-TNP IgM response in $CD5^{+/+}$ and $CD5^{-/-}$ were identical at the three measure time points (Fig. 3B). At all time points, levels of anti-TNP IgM was marginally higher in serum of $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice, than that in either $CD5^{+/+}$ or $CD5^{-/-}$ mice but the difference was significant only on day 10. Overall these results reveal a key role for CD5-CK2 signaling in TI-2 response by B-1a B cells.

Diminished T-dependent memory B cell responses in mice lacking CD5-CK2 signaling.

Since loss of CD5-CK2 signaling attenuates T cell activation, we tested if T-dependent immune response is altered in $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (24). To test for this, mice were immunized with TNP-keyhole limpet hemocyanin (TNP-KLH) on day 0 (1^o), day 21 (2^o) and day ~141 (3^o) and sera were analyzed for levels of anti-TNP IgG to evaluate primary, secondary and "memory" Ab response, respectively. We found that in both $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and $CD5^{-/-}$ mice anti-TNP IgG levels were significantly lower during primary and "memory" responses (Fig. 3C). However, secondary responses was

unaffected by loss of CD5-CK2 signaling. High affinity anti-TNP IgG was significantly diminished in serum of $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and $CD5^{-/-}$ mice only during memory response (Supplemental Fig 2A and B).

CD5-CK2 signaling promotes BCR-induced proliferation in B-1a and B2 B cells

We recently showed that CD4 and CD8 T cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice hypoproliferated in response to TCR stimulation (24). In contrast, TCR stimulation caused $CD5^{-/-}$ T cells to hyperproliferate presumably due to loss of CD5-dependent inhibitory activity mediated by the pseudo-ITIM domain. We interrogated if CD5 may have a similar modulatory function in B-1a B cells. Peritoneal cavity cells or spleen cells from $CD5^{+/+}$, $CD5^{-/-}$, and $CD5^{CD5\Delta CK2BD/\Delta CK2BD}$ mice were cultured for 72 h in the presence or absence of anti-IgM (Fab'2) or LPS and proliferation was measured by quantitating BrdU incorporation within B-1a and B-1b cells from the peritoneum and B2 B cells from the spleen. BrDU incorporation following 1 h of pulse labeling is a measure of cells in cycle and therefore not affected by differences in cell death. For $CD5^{-/-}$ mice, the B-1b gated population also included B-1a B cells since CD5 is the only phenotypic marker that distinguishes B-1a from B-1b B cells. When cells were stimulated with 1 µg/ml anti-IgM, the proliferation of B-1a B cells from $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice were equivalent (Fig. 4 A). At a higher stimulation of 10 µg/ml anti-IgM, CD5-CK2 signaling deficient B-1a B cells proliferated significantly less than $CD5^{+/+}$ B-1a B cells. Notably, LPS-induced proliferation was equivalent between $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells, indicating that CD5-CK2 signaling modulated BCR-induced but not TLR2/4 induced cell activation (Fig. 4A). B-1b B cells from $CD5^{+/+}$ and

$CD5^{\Delta CK2BD/\Delta CK2BD}$ mice proliferated equally in response to anti-IgM or LPS, a result that is expected since these B cells do not express CD5 (Fig. 4B). B-1b B cells (B220+CD11b+), as defined by cell surface phenotype, in $CD5^{-/-}$ mice responded far more efficiently to BCR stimulation than such B cells from both $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice. However, considering that this population in $CD5^{-/-}$ mice contains both B-1a and B-1b cells, the result most likely reflects loss of CD5-dependent negative signaling as previously reported (19). The surprising finding is that these peritoneal B220+CD11b+ B cells in $CD5^{-/-}$ mice, but not splenic $CD5^{-/-}$ B2 B cells proliferated very vigorously to LPS stimulation (Fig. 4B). This result indicates that CD5 signaling may also regulate TLR signaling in B1 B cells by a mechanism independent of CK2 signaling.

Splenic $CD5^{\Delta CK2BD/\Delta CK2BD}$ B2 B cells hypo-proliferated and $CD5^{-/-}$ B2 B cells hyperproliferated compared to $CD5^{+/+}$ B2 B cells (Fig. 4C). However, proliferation of B2 B cells in response to LPS stimulation was not affected by absence of CD5 or selective ablation of CD5-CK2 signaling. We further found that Ca^{++} mobilization was similar in B-1a or B2 B cells between $CD5^{+/+}$ or $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (Fig. 4D and 4E). From this result we infer that loss of CD5-CK2 signaling on proliferation in B-1a or B2 B cells is not due to early events of BCR signaling. Just as we observed in B-1a B cells, the response to LPS was not affected by loss of CD5-CK2 signaling in B2 B cells (Fig. 4A and 4C).

Reduced active Cdc37 and ERK in B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice

Cdc37, AKT, NFκB and ERK signaling pathways are activated by CK2 and involved in promoting cell proliferation (39-42). The co-chaperone protein Cdc37 interacts with Hsp90 and is solely phosphorylated by CK2 at Ser13 to orchestrate a series of signaling events that includes formation of complexes with early G1 phase cell cycle kinases, Cdk4 or Cdk6 (39, 43, 44). The greater order of complexes comprising Cdc37, HSP90 and Cdk4 or Cdk6 allows for stable interactions with cyclin D family members or dissociation of INK4 family members to promote entry into cell cycle (44-47). In unstimulated cells, the level of Cdc37 (S13) phosphorylation was slightly lower in $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells than $CD5^{+/+}$ B-1a B cells, but the difference was not significant (Fig. 5A and Supplemental Fig S3A&D). However, following stimulation, pS13-Cdc37 levels were significantly greater in $CD5^{+/+}$ B-1a B cells than in $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells. Notably, anti- IgM stimulation induced the phosphorylation of Cdc37 in $CD5^{+/+}$ B-1a B cells and not in $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells (Supplemental Fig. S3A&D). pCdc37 promotes complexes which phosphorylate Rb and transition of G0 to mid-G1 phase (48, 49). We found that B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ following *in vivo* anti-IgM stimulation were significantly reduced in G1 phase as compared to $CD5^{+/+}$ B-1a B cells (Fig. 5B and Table 1).

The BCR of B-1a B cells is perpetually engaged by self-antigen and this leads to constitutive ERK phosphorylation (4, 50). Cdc37 promotes activation of ERK by enhancing Raf-1 activation and subsequently MEK (51-53). We therefore tested if activation of ERK would be diminished in $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells as compared to $CD5^{+/+}$ B-1a B cells. We found that the levels of active ERK (pT202/pY204) were

significantly lower in unstimulated and anti-IgM stimulated B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (Fig. 5C and Supplemental Fig. S3B).

CK2 enhances the activation of Akt kinase activity by directly phosphorylating it or indirectly by inhibiting PTEN, the phosphatase that regulates PI3 kinase (40, 54). We observed similar pS473-Akt levels in unstimulated B-1a B cells from $CD5^{+/+}$ mice and from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (Fig. 5D and Supplemental Fig. S3C). In $CD5^{+/+}$ B-1a B cells, stimulation with anti-IgM had no effect or resulted in slight reduction in pS473-Akt levels, an observation previously reported (4). However, from $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells, BCR stimulation resulted in significant increase in levels of pS473-Akt (Fig. 5D). CK2 has a function in phosphorylating IκB leading to its degradation and to facilitate activation of NFκB. We observed no differences in levels of IκB in unstimulated or anti-IgM stimulated B-1a B cells between $CD5^{+/+}$ or from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (Fig. 5E). Loss of CD5-CK2 signaling had no effect on pAKt, pCdc37 or pERK in splenic B2 B cells (Supplemental Fig. S3E-G).

Enhanced BCR-induced apoptosis in the absence of CD5-CK2 signaling

We have shown that the CD5-CK2 signaling pathway counteracts activation induced cell death in T cells, a function consistent with pro-survival activity of CK2 (24, 26, 55). To address the question if CD5-CK2 signaling has a prosurvival function in B-1a B cells, we injected 25ug of anti-IgM into the peritoneal cavity of $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice and apoptosis in B-1a B cells were analyzed 48 h later using two different approaches. Within B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ we observed significantly greater proportion of cells in sub-G1 gate, which represents the apoptotic fraction.

Following *in vivo* stimulation for 48hrs $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells exhibited a significant increase in AnnexinV positivity (Fig. 6). This finding taken with the increase in the sub-G1 (apoptotic) phase population from the *in vitro* propidium iodide stained cell cycle analysis, suggests the $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a population may enter cell cycle at a similar rate as $CD5^{+/+}$ controls since 72hr and 48hr G2 phase proportions are comparable, but are more prone to undergo activation induced cell death/apoptosis than B-1a B cells from $CD5^{+/+}$ animals, validating a mechanism for the loss of systemic natural immunity (Fig. 5B, Table 1, Fig. 6). To rule out the possibility of adult B1 progenitors compensating for the increased AICD peritoneal B cells from $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ animals were enriched by adherent cell depletion and transferred into 8 week old non-irradiated $Rag2^{-/-}$ recipient mice ($2x10^6$/mouse) (Sup. 3). The recipient animals were then aged for four weeks following transfer and then lavages were performed to determine the distribution of peritoneal B cells within the $Rag2^{-/-}$ cavities. The $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ transferred B-1a, B-1b, and B2 B cells repopulated the cavities of the recipients to equal levels suggesting the effects of increased AICD are compensated for in a B-1a population intrinsic mechanism.

Discussion

Regulation of autoreactive lymphocyte populations is an important aspect of the immune system to inhibit the development of autoimmunity. B-1a cells are a unique population in this regard because they produce autoreactive antibodies that have the potential to be both pro- and anti-inflammatory. In this study, we find that the CD5-CK2-Cdc37 signaling

axis is a critical signaling pathway regulating B-1a B cell homeostasis primarily by initiating/modulating early cell cycle and mitogenic signaling cascades. Interestingly, the CD5 driven CK2 pro-survival signals leads to potential long-term maintenance of this self-renewing population by mitigating AICD. Loss of this signaling cascade resulted in unaltered B-1a proportions and numbers within the peritoneal cavities of $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice, but exhibited not only reduced proliferative responses but reduced B cell intrinsic immune responses.

Reduced steady state serum levels and T-independent responses from mutations in the CK2BD of CD5 in this population raise interesting questions about the induction of CD5+ B cell mediated protection from pathogens and maintenance of immune tolerance. Previous studies have shown that approximately 80% of natural serum IgM is produced from the B-1a B cell population (56). These antibodies have been shown to have anti-inflammatory properties by interactions with the innate immune system within the collagen induced arthritis model (15). It would be hypothesized that overtime the loss of this population would lead to a pro-inflammatory state and predispose the animal to immune related complications. Contradictory to the impact of CD5 survival signaling in B cells, T-dependent immunizations within the $CD5^{\Delta CK2BD/\Delta CK2BD}$ mutant mice exhibited a reduction in memory response formation that suggests T cell-mediated CD5 signaling would play an important role in driving an increased inflammatory state as well. Evidence of this theory exists in a previous study by our group that revealed that $CD5^{\Delta CK2BD/\Delta CK2BD}$ T cells failed to differentiate as efficiently into the IL-17 producing T helper cell subset and in other publications that show in human PBMCs CD5 costimulation enhances the Th17 subset (24, 57).

In addition to modifying T cell-mediated immune responses, many immune cell-derived neoplasias, such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), have been shown to express CD5, and there are reports suggesting the potential use of CK2 inhibitors as treatments for such neoplasias (58-60). This association of CD5 positivity and CK2 overexpression makes for a novel and intriguing association which may lead to a potential survival mechanism for these cancer cells. This association with CD5-CK2-Cdc37-Hsp90 holds the potential to determine other possible therapeutic targets for these B cell- and potentially T cell-derived malignancies.

This study has shown that B-1a B cells from mice with a deficiency to activate CK2 in a CD5 dependent manner exhibited increased B-1b percentages, unaltered B-1a numbers, and reduced natural immunity. In addition, B cell-dependent immune responses were reduced and the $CD5^{\Delta CK2BD/\Delta CK2BD}$ B-1a B cells exhibited increased AICD. This increase in AICD can be extrapolated back to reduced CK2-Cdc37 driven cell cycle through maintaining ERK phosphorylation. All of these findings taken together show the potential of CD5 signaling to promote natural immune protection, potenitally develop autoimmunity, and give survival cues to CD5+ B cell populations.

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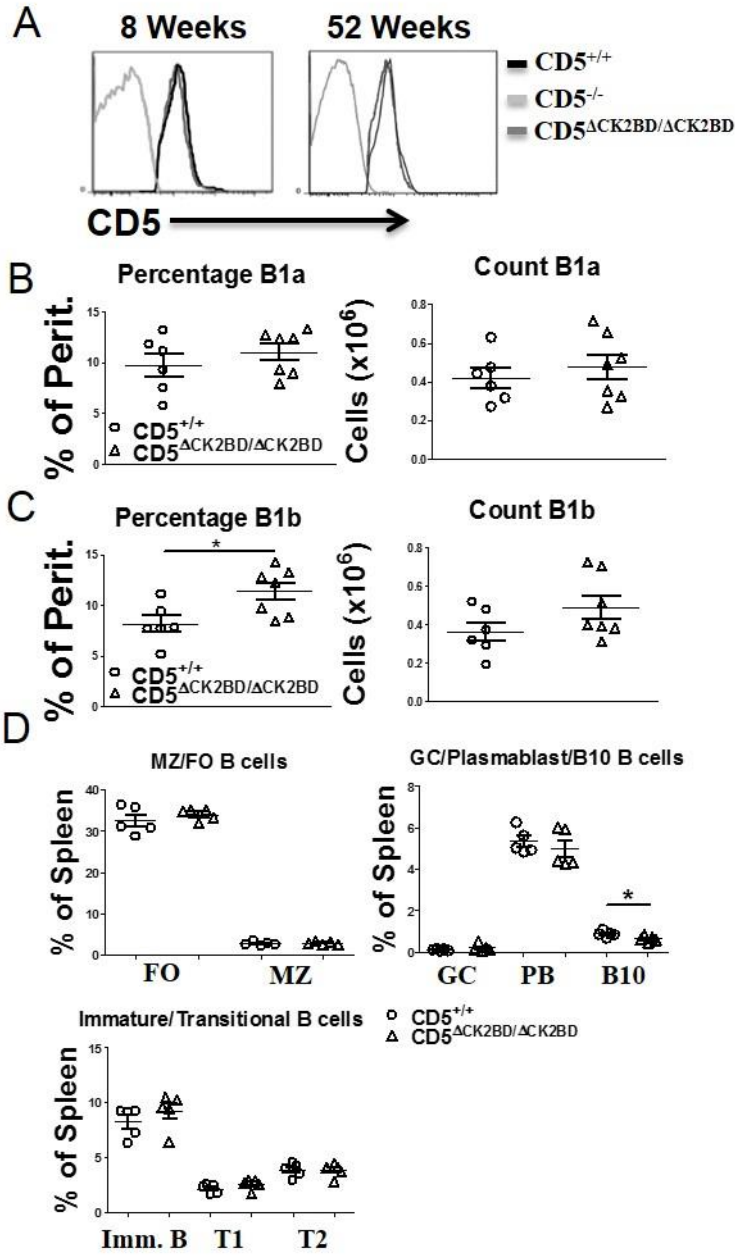

Figure 1. The CK2 binding domain of CD5 is necessary for maintenance of peritoneal B cell distribution. (**A**) CD5 surface expression on B-1a B cells ($IgM^{hi}/IgD^{lo}/CD11b^{+/-}/CD5^{+}$) from 8 week and 52 week old $CD5^{+/+}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice or B-1 (IgMhi/IgDlo) cells from $CD5^{-/-}$ mice. (**B**) Proportion and total cell number of B-1a B cells from the peritoneal cavity. (**C**) Proportion and total cell number of B-1b B cells from the peritoneal cavity. (**D**) Percentages of splenic B cell populations. Statistical analysis was performed using unpaired Student's t-test.

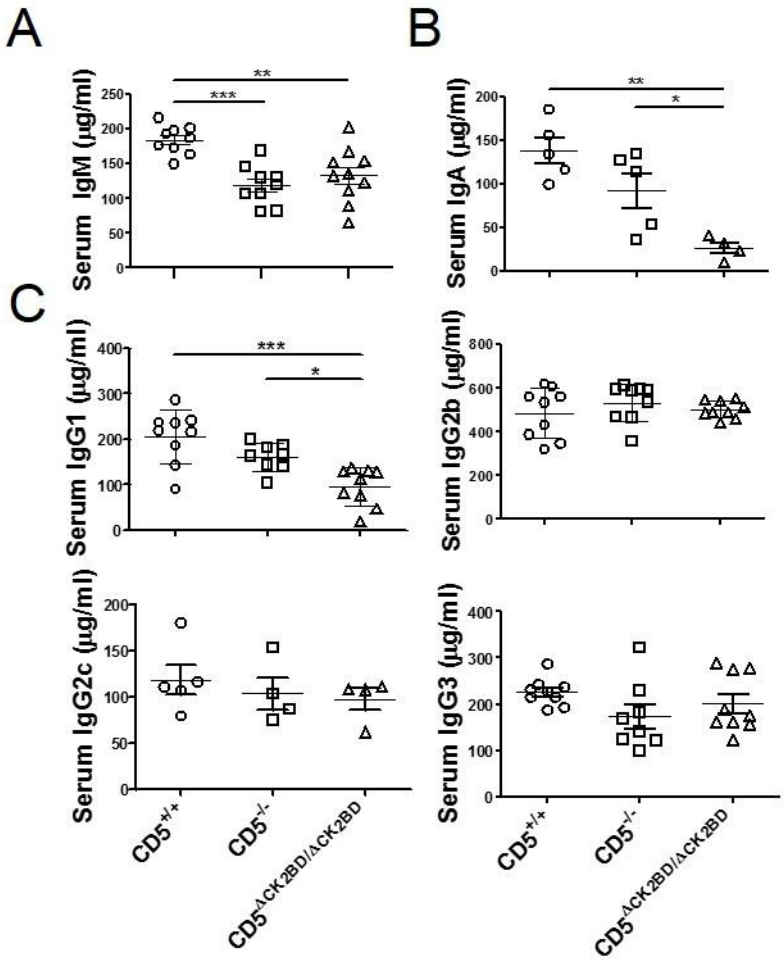

Figure 2. Reduced serum immunoglobulin levels in $CD5^{-/-}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice. (**A**) IgM concentrations in 8 week old mice (n=5/strain). (**B**) Serum IgA concentrations (n=10/strain). (**C**) Baseline gammaglobulin isotype levels (n=5-10/strain). Data is representative of replicate ELISAs from multiple serum collections. Statistics were performed using one-way ANOVA.

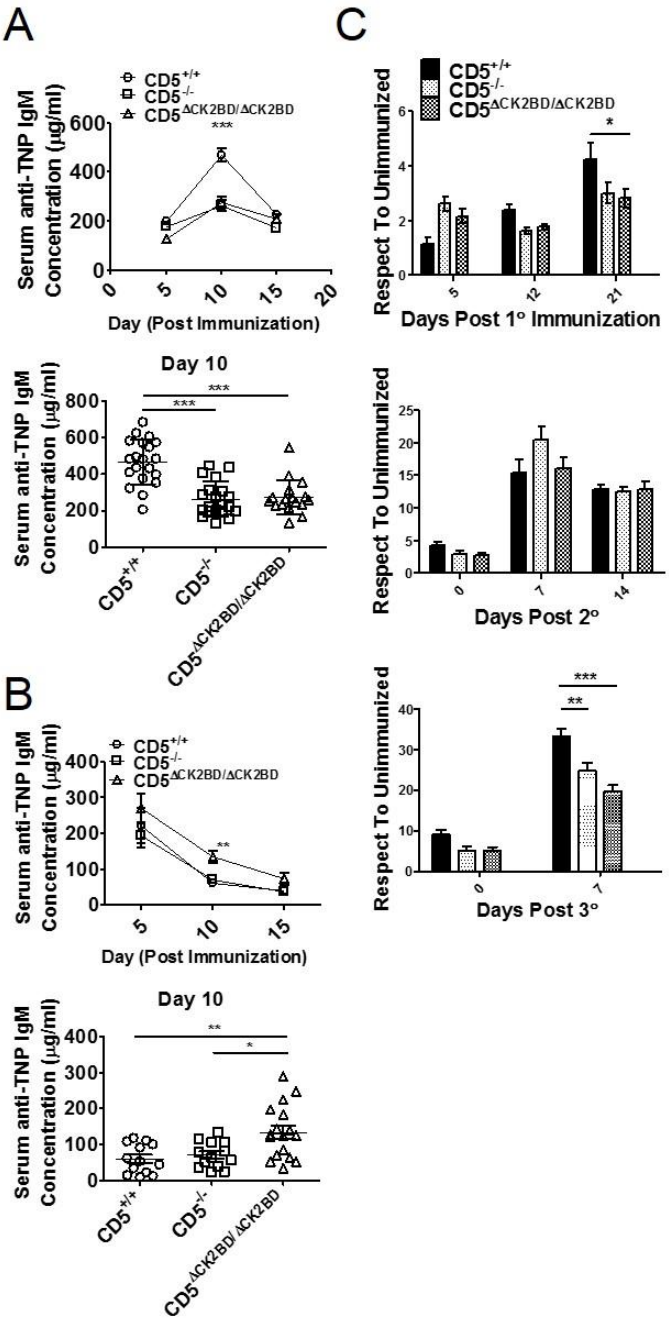

Figure 3. Mice deficient in CD5 dependent CK2 signaling exhibit diminished T-independent type-II B cell responses. (**A**) Anti-TNP IgM serum concentrations from mice immunized with 20ug TNP-Ficoll with significant time point distribution (n=15/strain). (**B**) Same as (A) using TNP-LPS as the immunogen (n=15/strain). (**C**) Primary, secondary, and tertiary anti-TNP responses following TNP-KLH immunization (n=20/strain, 2 different immunization groups of 10/strain). Statistics were performed using one-way ANOVA.

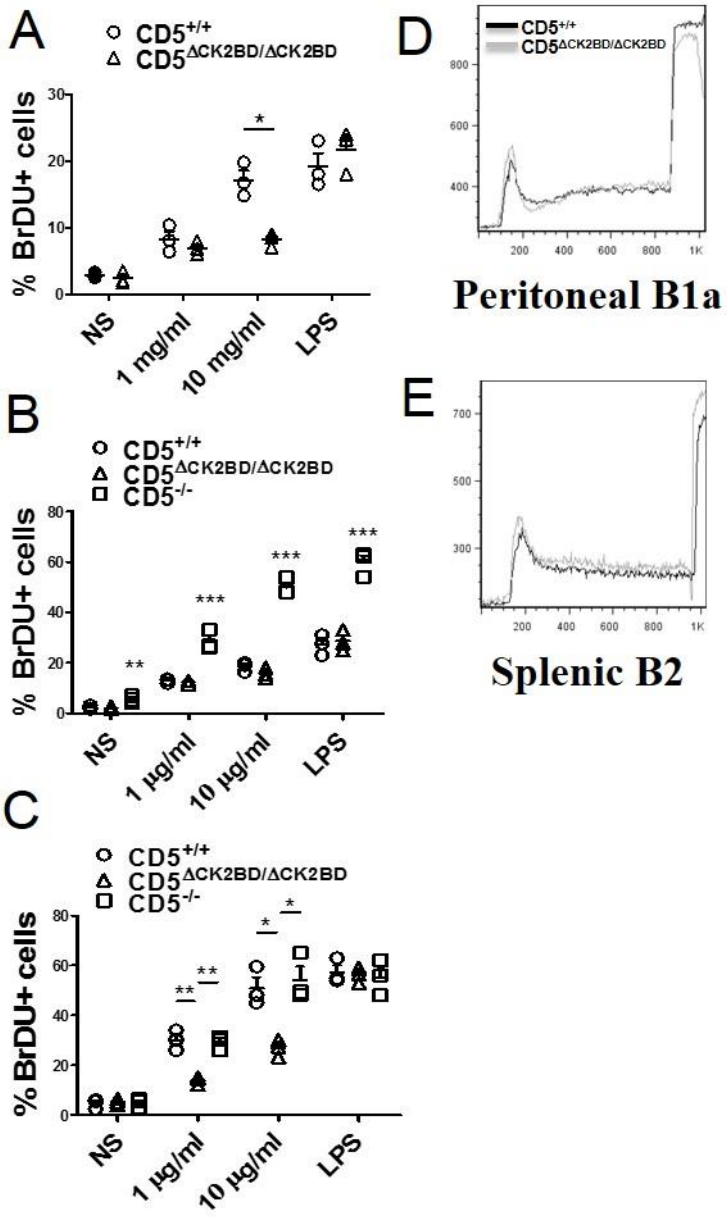

Figure 4. B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice have stunted *in vitro* proliferative capacity following antigen receptor stimulation. (**A-C**) Mixed peritoneal cavity cells cultured for 24hrs in the presence of BrDU and/or 1ug/ml goat anti-mouse IgM Fab'2, 10ug/ml goat anti-mouse IgM Fab'2, or 20ug/ml LPS and stained for (A) B-1a B cells, (B) B-1b B cells, or (C) B2 B cells. $CD5^{-/-}$ B-1b B cell (B) represent total B1 B cell gated population. (**D-E**) Ca2+ flux kinetics following goat anti-mouse IgM Fab'2 from (D) peritoneal B-1a B cells and (E) splenic B2 B cells. Statisitical analysis was performed using unpaired Student's t-test.

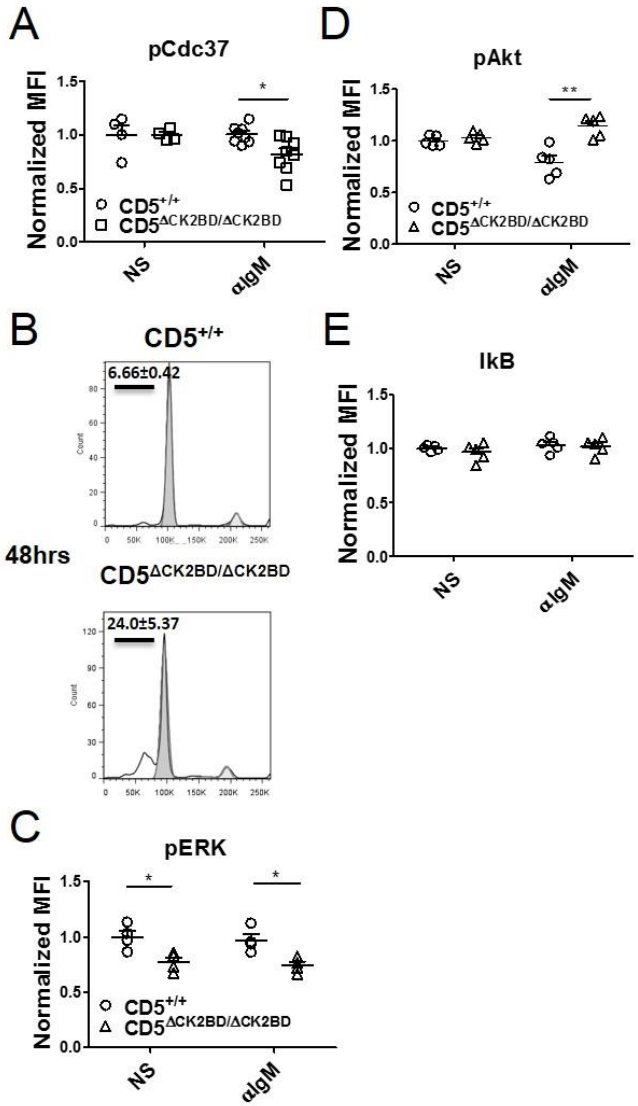

Figure 5. Reduced proliferation in B-1a B cells from $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice is attributable to reduced ERK and Cdc37 phosphorylation leading to increased apoptosis. (**A**) Normalized phospho-Cdc37 (Ser13) MFI in flow gated B-1a B cells with or without anti-IgM F(ab')2 stimulation. (**B**) Propidium iodide cell cycle analysis of the B-1a gated population following 48 or 72hrs of *in vivo* anti-IgM stimulation. (**C**) Normalized phospho-ERK1/2 MFI in flow gated B-1a B cells. (**D**) Normalized phospho-Akt Ser473 in flow gated B-1a B cells (**E**) Normalized IκB in flow gated B-1a B cells. Statisitical analysis was performed using unpaired Student's t-test.

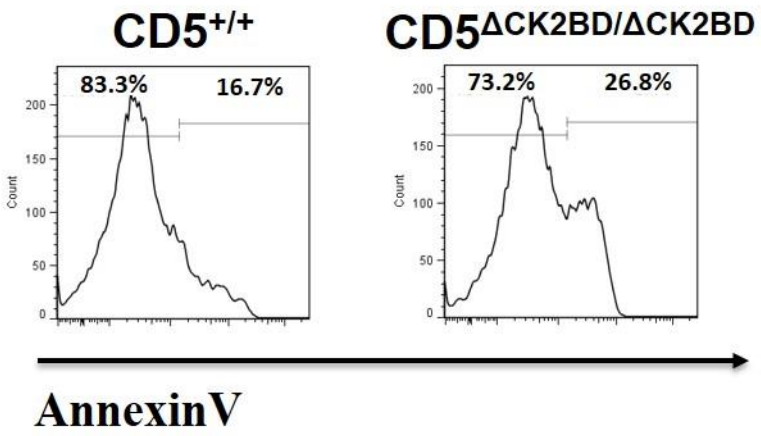

Figure 6. Increased apoptosis following antigen receptor ligation. Representative histograms of increased 48hr *in vivo* apoptosis in $CD5^{\Delta CK2BD/\Delta CK2BD}$ following 25ug anti-IgM stimulation. (n=4 mice/group)

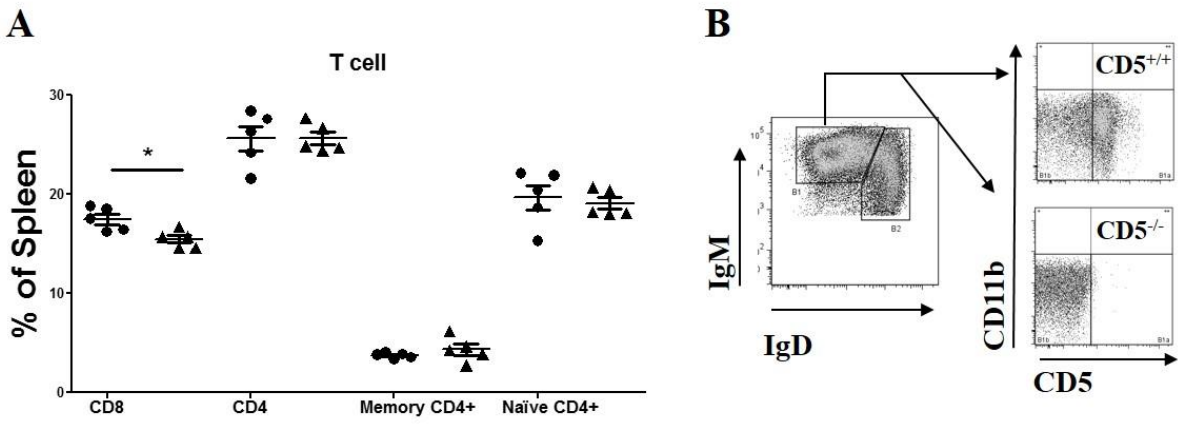

Figure S1. Unaltered T cell proportions and representative B1a gating scheme. (**A**) CD4+, CD8+, Memory CD4+ CD62Llo CD44hi, and Naïve CD4+ CD62Lhi CD44lo T cell populations from 8 week old animals (n=5) (**B**) Representative FACS gating scheme to phenotypically isolate B-1a B cells from B1-b B cells in $CD5^{+/+}$ mice as compared to a $CD5^{-/-}$ animals. Statistics are representative of Student's t-Test where * is equal to $p \leq 0.05$.

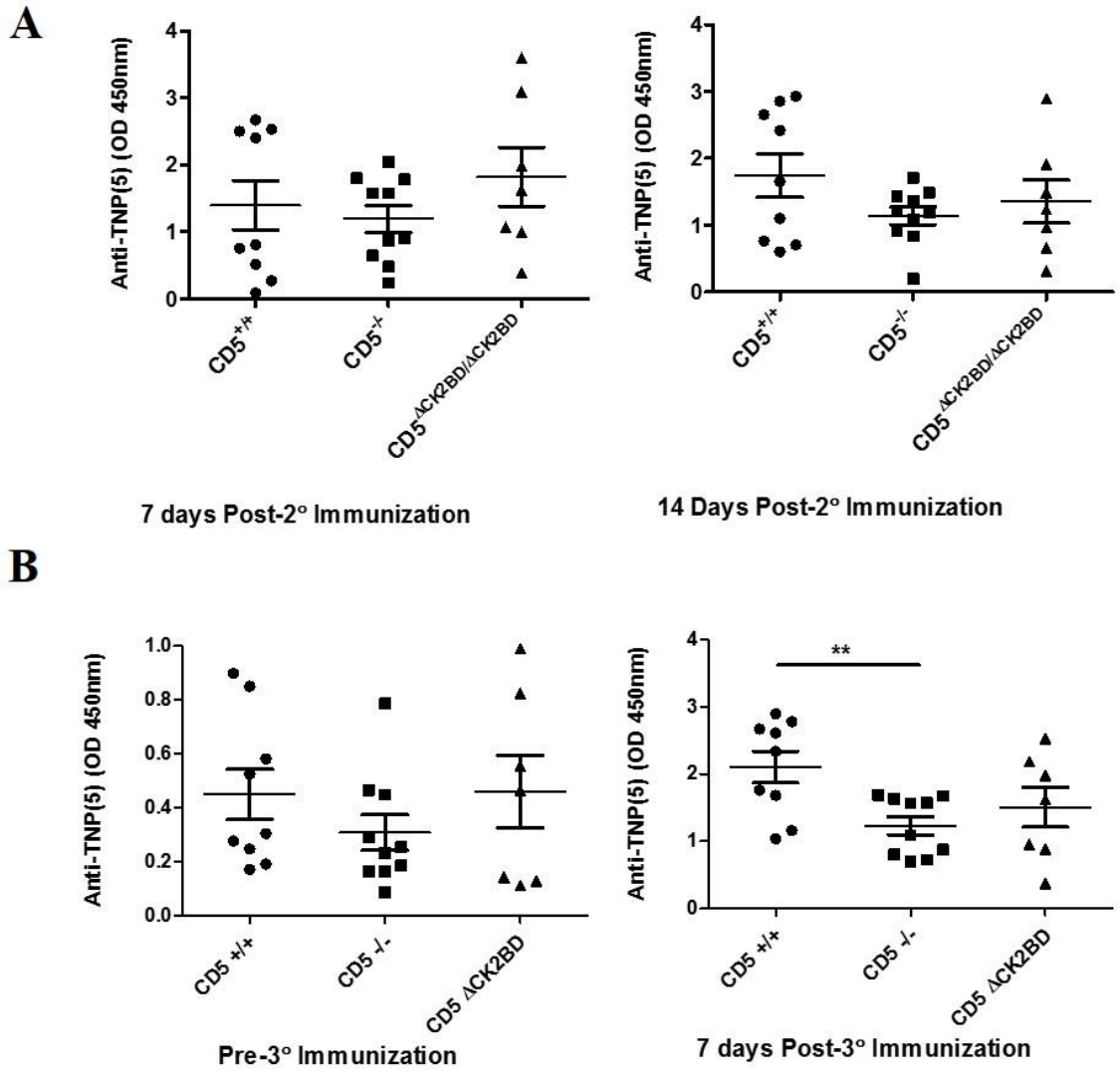

Figure S2. Reduced high affinity anti-TNP memory response titers following TNP-KLH immunization in $CD5^{-/-}$ and $CD5^{\Delta CK2BD/\Delta CK2BD}$ mice (n=7-10 animals/group). (**A**) high affinity anti-TNP(5) IgG titers at time of secondary immunization and 7 days post boost. (**B**) high affinity anti-TNP(5) IgG titers 4 months after secondary immunization and 7 days following tertiary memory immunization. Statistics are representative of one-way ANOVA where ** is equal to $p \leq 0.01$.

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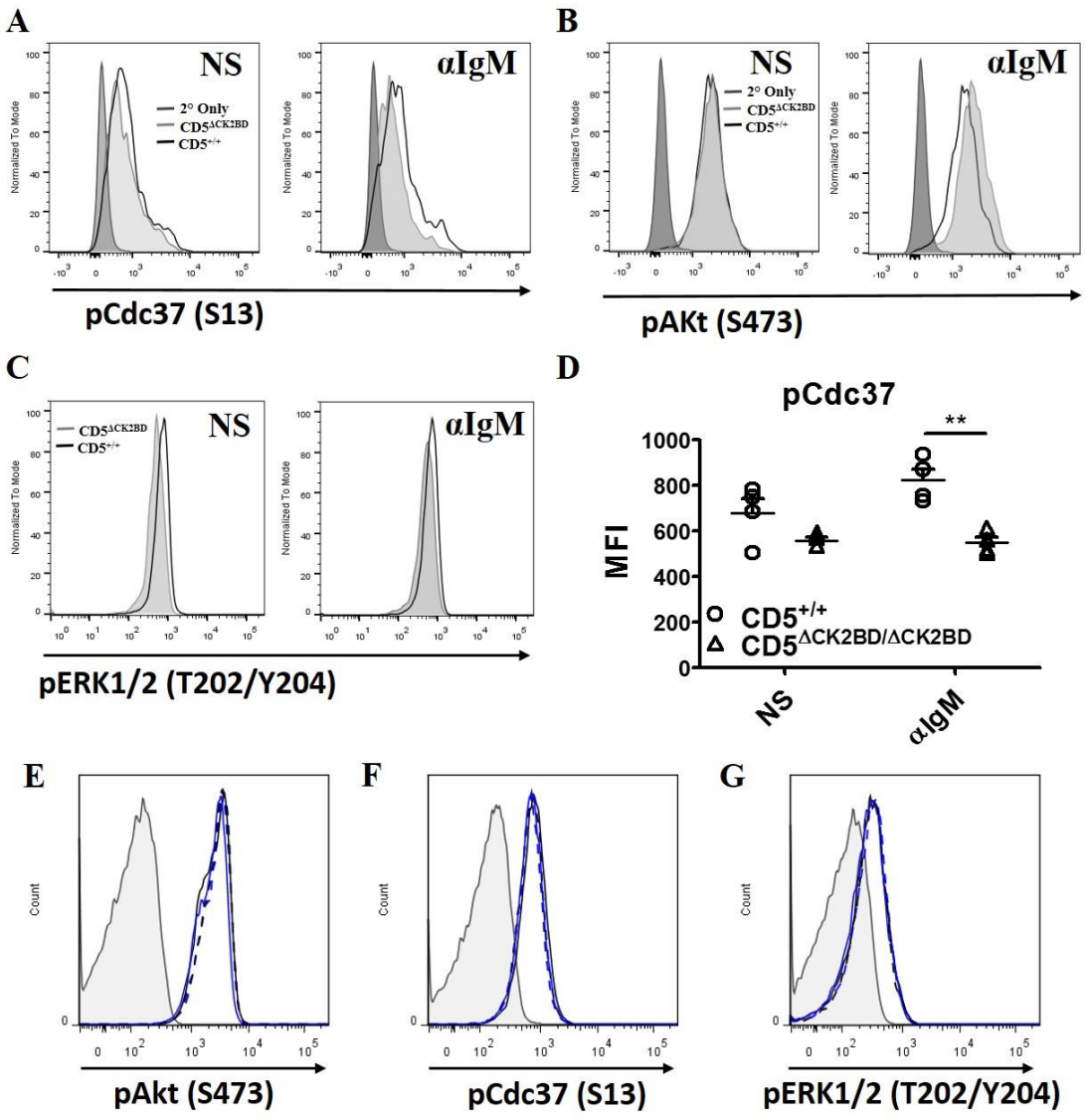

Figure S3. Splenic B cells have unaltered Akt, Cdc37, and ERK phosphorylation. (**A-C**) Representative histograms of peritoneal B-1a B cell phospho-Flow for pCdc37, pAkt, and pERK respectively with or without anti-IgM F(ab')2 stimulation (**D**) Direct MFI of pCdc37 B-1a phospho-Flow data prior to normalization. (**E**) pAkt (Ser473)phospho-Flow analysis (MFI) on splenic follicular B cells following 15 minute anti-IgM F(ab')2 stimulation. (**B-C**) Same analysis as in but determining MFI of (B) pCdc37 (Ser13) and (C) pERK1/2 (Thr202/Tyr204). Statistics are representative of Student's t-Test where ** is equal to $p \leq 0.01$.

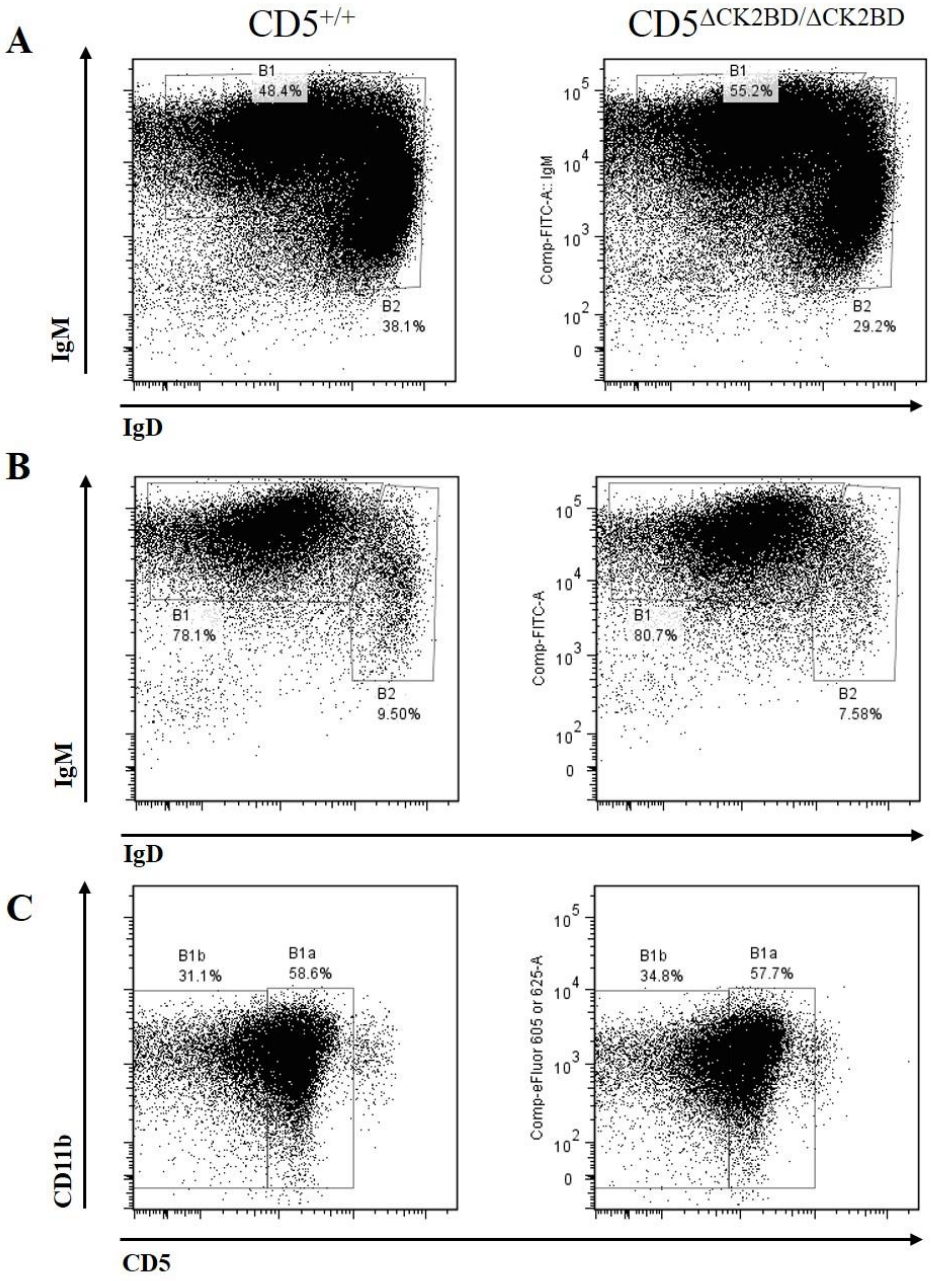

Figure S4. Unaltered B cell persistence in the absence of adult B1 progenitors. (**A**) Representative dot plot of the proportions of B1 and B2 B cells transferred into Rag2$^{-/-}$ recipient mice (n=4). (**B**) Representative plot of the B1/B2 distribution of recovered peritoneal exudate B cells after 4 weeks of repopulation. (**C**) Distribution of the B1 compartment from (B) into B1a and B1b B cells.

Tables

Table 1. Proportion of B-1a gated PI stained cell cycle stages following in vivo anti-IgM stimulation

Mouse Strain	% Sub G1	% G1	% M/S	% G2
$CD5^{+/+}$	6.66±0.42	80.2±0.92	2.25±0.13	11.0±1.2
$CD5^{\Delta CK2BD/\Delta CK2BD}$	24.0±5.37*	63.2±3.89*	4.0±0.24*	8.88±1.73

Data are presented as means ± SD (n=3 mice/group/time of stimulation)
*$p < 0.05$

CD5-DEPENDENT CK2 SIGNALING CONTROLS B-1A B CELL IDIOTYPIC SELECTION AND *S. PNEUMONIAE* RESPONSES

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Format adapted for dissertation

Abstract

$CD5^+$ B-1a B cells comprise much of the natural antibody response towards regularly encountered common pathogens and antigens. These B cell responses are evolutionarily conserved and dominate responses towards antigens such as phosphorylcholine and phosphatidyl choline. These innate-like natural antibodies derived from the B-1a population tend to represent immune-dominant idiotypic clones that arise very early in ontogeny and maintain themselves throughout the lifespan of the mouse. These clones have been shown to share a high homology to natural antibodies found within human patients. Recent evidence suggests that B-1a responses and homeostasis are controlled in large part to signaling through the recently described CK2 binding domain of CD5. In this study we aimed to examine the impact of the newly described signaling domain of CD5 (CK2BD) and its relationship to TI-II anti-PC responses towards *S. pneumoniae*, a pathogen which accounts for millions of serious infections worldwide annually in human patients. We determined that CD5-dependent CK2 signaling is critical for maintenance of B-1a anti-PC and other idiotypic responses via dysregulation of idiotypic dominant clones. This loss of idiotypic dominace in mice resulted in an increased susceptibility to pneumococcal infection.

Introduction

Pneumococcal infections account for millions of cases of pneumonia, meningitis, otitis media, and sepsis worldwide, resulting in hundreds of thousands of mortalities annually, primarily within young and old individuals (1). Animals have evolved mechanisms of early innate immune responses towards *Streptococcus pneumoniae* (*S.p.*) including a mechanism that begins to develop before the animal ever leaves the womb (2). In utero ontogeny of B cells has long been known to give rise to a predominantly B-1a B cell repertoire that enriches specific clones in a process known as idiotypic selection (3, 4). These B-1a restricted idiotypes have been shown to have responsiveness towards conserved antigens that are expressed on pathogens as well as within the host, and in terms of *S.p.* the hallmark B-1a responsive determinant is phosphorylcholine (PC) (5-7). B-1a B cells are a unique population of early innate-like B cells that shortly after birth become self-renewing and persist for the life of the animal within serous cavities (8). Interestingly, this population of B cells has been shown to produce the majority of natural IgM and IgA serum antibodies, which are poly-reactive and weakly auto-reactive in nature (9, 10). This poly-reactivity is what allows the B-1a produced antibodies to bind shared epitope targets. In mice these antibodies have been shown to be critical in the clearance/prevention of *S.p.* infections (2, 6).

A unique feature of B-1a B cells is the expression of the surface co-receptor CD5 (11, 12). CD5 has long been shown to be a negative regulator of antigen receptor signaling in B and T cells, however the impact of signaling through this receptor on B-1a B cell responses remains vague (13, 14). Previously, our group has shown through yeast two-hybrid experiments that in addition to the negative regulatory signaling domain (pseudo-

ITIM) of CD5 there is a second primary signaling domain that is necessary for the binding/activation of the serine-threonine kinase CK2 (15). In mice deficient in this domain (ΔCK2BD) we have previously shown that B-1a B cells exhibit increased AICD, diminished T-independent type II antibody responses, as well as a reduction in steady-state IgM and IgA levels, despite B-1a B cell proportions and numbers remaining relatively consistent with Wt controls (Cashman *et al*, unpublished). This increased AICD within the B-1a compartment leads us to hypothesize that B-1a B cells from ΔCK2BD mice would have diminished anti-PC responses and an increased susceptibility to *S.p.* infections.

Materials and Methods

Mice

All mice utilized within the experiments were 8-10 weeks of age, unless otherwise stated. C57Bl/6NCr mice were either bred in house or purchased from Charles River Laboratories (Frederick, MD). *Cd5*$^{-/-}$ mice were originally developed on the 129/Sv background and backcrossed for 12 generations onto the C57Bl/6 background. The *Cd5*$^{\Delta CK2BD/\Delta CK2BD}$ BAC knock-in animal was created using gene targeting techniques for the *Cd5* gene in C57Bl/6 ES cells (23). All mouse experimentation was performed under the approval and within regulations of the University of Alabama at Birmingham Institutional Animal Care and Use Committee (Birmingham, AL).

Immunizations

Animals were immunized with 200µl i.p. of 20µg PC(26)-Ficoll (Biosearch Technologies) or 1x10^{8} CFUs of heat-killed serotype 2 R36A *Streptococcus pneumoniae* in PBS. Mice were bled for serum at days 0, 5, 10, and 15. Serum was stored at -80C until ELISA could be performed.

Enzyme-linked immunosorbent assay

Costar® 96-well high-binding EIA/RIA plates (#3590) were coated overnight with PC(6)-BSA (Biosearch Technologies). The plates were then blocked with 2% porcine gelatin (Sigma-Aldrich) and serum from PC-Ficoll or R36A immunizations were then incubated for 4 hrs at ambient temperature. HRP labeled goat anti-mouse IgM (Southern Biotech) was then incubated for 1 hour at ambient temperature, and plates were developed with TMB-Substrate (eBioscience) and reactions stopped with 2N H_2SO_4. The

plates were then analyzed at 450nm in a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT).

Bacterial preparation and animal infection

Stock of serotype 2 D39 *Streptococcus pneumoniae* were maintained at -80C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) plus 10% glycerol. All cultures were generated from the same frozen stock. Cultures were grown on Remel™ TSA+ 5% sheep blood agar plates (Thermo Scientific) and a single colony was transferred to fresh THY broth and allowed to reach a ΔOD_{420}=0.2 with respect to fresh media on a BioTek Synergy HT plate reader (ΔOD_{420} of 0.2= $5x10^7$ CFU/ml). Inoculations of $2.5x10^4$ CFU were then prepared in 200ul of PBS and kept at 4C until injection. Mice were injected i.v. in the tail vein and monitored for survival for 5 days following inoculation. This procedure is similar to previously described bacteremia inducing protocols (24).

Recovery of bacteremia

Mice were bled retro-orbitally using a sterile Pasteur pipette and quickly added to Terumo™ Capiject™ lithium-heparin coated tubes. The tube coating was quickly disseminated through the blood sample and 100ul of total blood was serially diluted in PBS and plated on blood agar plates to determine circulating bacteremia levels.

Idiotypic population analysis

Spleens, bone marrow, and peritoneal exudates were taken from all mouse strains, an surface stained with anti-Id antibodies (T15 Id= anti-AB1.2 and anti-TC68; M167 Id= anti-M167 and anti-TC68; $V_H11/V_\kappa9$ Id= anti-V_H11 and anti-$V_\kappa9$ (peritoneal exudates only)). $V_H11/V_\kappa9$ idiotpyic antibodies were generously given by Dr. Richard Hardy (Fox

Chase Cancer Center, Philadelphia, PA), whereas T15 and M167 idiotpyic antibodies were generously given by the lab of Dr. John Kearney (University of Alabama at Birmingham, Birmingham, AL). Additional surface staining (Thy1-, Gr-1-)(Biolegend;eBioscience) and viability (propidium iodide) were used to isolate B cell populations using flow cytometry (FACSAria, BD Biosciences) and to determine total idiotype numbers within the tissues.

Results & Discussion

To address if CD5 dependent CK2 signaling altered B-1a B cell responses to PC, Wt, $CD5^{-/-}$, and CD5ΔCK2BD mice were immunized with 25ug of PC-Ficoll (Biosearch Technologies). Following this immunization CD5ΔCK2BD and $CD5^{-/-}$ mice exhibited a significant reduction in anti-PC titers at day 10 (Fig. 1A). To better address the influence of CD5 signaling on the B-1a anti-PC responses a subsequent immunization was performed utilizing $1x10^8$ heat-killed CFU of R36A *S.p.* per animal. Following this immunization $CD5^{-/-}$ and CD5ΔCK2BD mice exhibited reduced anti-PC IgM titers in both magnitude and duration (Fig. 1B).

Although overall anti-PC titers were diminished in CD5ΔCK2BD mice, is was necessary to ensure that this reduction was due to B-1a specific alterations. Serum from the previously described immunization was subjected to ELISA for the canonical B-1a restricted T15 idotypic specificity, which mirrored the anti-PC IgM levels in both heavy (anti-TC68) and light chain (anti-AB1.2) idiotypic titers (Fig.2A&B). The T15 idiotype is an anti-PC specific immuno-dominate idiotype which has been shown to be necessary for clearance of *S.p.*, and exhibits crossreactivity to oxLDL (2, 16). This reduction in B-1a specific anti-PC response, along with the diminished T15 idiotypic titers, suggests that CD5ΔCK2BD animals would be more susceptible to live *S.p.* infections.

To address the role of CD5 dependent CK2 signaling in B-1a mediated immunity to live *S.p.* infections, mice were inoculated intavenously with $2.5x10^4$ CFU D39 *S.p.*, the encapsulated parental strain to R36A, to determine survivability (17). Following inoculation, CD5ΔCK2BD and $CD5^{-/-}$ mice exhibited a significant increase in susceptibility to the *S.p.* infection, further proving that B-1a B cells are critical for early

clearance of this particular pathogen as well as the CD5-CK2 signaling axis being critical in maintenance of this population (Fig. 3A). These animals also exhibited a moderately increased bacteremia 48 hours following infection, further supporting the lack of clearance (Fig. 3B). Previously our group had found that CD5ΔCK2BD mice exhibit reduced basal levels of IgM and IgA, which would suggest that CD5ΔCK2BD mice would also have reduced basal anti-PC titers, however resting levels of anti-T15 idiotypic antibodies remained consistent between the CD5ΔCK2BD and Wt controls (Fig. 2A&B). This would suggest that the early B-1a mounted responses are the necessary factor to eliminate *S.p.* infections and not the resting antibody titers. To determine if this assumption is correct animals from the three strains of mice were injected intraperitoneally with 50ul of normal Wt serum or PBS and allowed to rest for 2 hours prior to i.v. infection. Wild-type animals exhibited no change in responsiveness with the addition of normal serum, clearing the pathogen easily, whereas $CD5^{-/-}$ and CD5ΔCK2BD animals showed a marked gain in survivability (Fig. 3C). This increased resistance to *S.p.* did not however match that of the Wt serum or sham injected controls. The increased survivability did show however, that starting titers of natural antibodies (NAb) can make a significant impact on the long-term outcome of this infection, and suggests that administration of NAb could support conventional therapies and biological mechanisms in the clearance of persistent *S.p.* infections. Taken together, the initial titer of natural anti-PC antibodies and induced B-1a responses are critical to maintain immunity to *S.p.* infection. These findings do however leave a gap in the determination of the mechanism of how the CD5-CK2 is affecting these responses.

As previously stated, our past work has shown that although basal and elicited B-1a antibody responses are effected by the CD5-CK2 signaling axis, the population remains relatively consistent between the CD5ΔCK2BD and Wt animals (Cashman *et al*, unpublished). Although, in our previous study B-1a B cells from CD5ΔCK2BD mice exhibited reduced *in vitro* anti-IgM elicited cell cycle entry and increased apoptosis which would suggest an *in vivo* compensation mechanism within the population to maintain numbers due to the loss of survivability. Since idiotypes such as the T15 clone and other anti-PC clones would be stimulated with high frequency, a loss of survival signaling could affect the idiotypic distribution within the peritoneal cavity, spleen, and/or during selection of the population within the fetal liver. To address whether or not this mechanism impacts the idiotypic distribution within the animal the bone marrow, spleens, and peritoneal lavage exudate were isolated and phenotypically stained for the T15 and lower frequency marginal zone restricted M167 idiotypes (18, 19). Bone marrow and peritoneal T15 and M167 numbers remained relatively consistent between all animal strains, whereas the splenic numbers for the T15 idiotype exhibited a significant reduction in both the $CD5^{-/-}$ and CD5ΔCK2BD mice as compared to Wt controls (Fig. 4A). Interestingly with this reduction in T15 numbers, the M167 idiotype exhibited a reciprocal trend with a significant increase in both the CD5ΔCK2BD and $CD5^{-/-}$ strains (Fig. 4B). These findings determine that the CD5-CK2 signaling axis is critical for maintaining the homeostatic balance that is developed early during B cell lymphopoiesis, and recapitulates previous studies in which early manipulation of the idiotypic selection process of immune-dominant clones (like the T15 idiotype) increase susceptibility to pathogens expressing their cognate immunogenic determinants (20, 21).

This also determines the mechanism in which CD5ΔCK2BD mice are more susceptible to *S.p.* infections.

Immuno-dominance of B-1a restricted idiotypes has been shown to be involved in responses to other antigens in addition to their effects on clearance of *S.p.* infections through PC responsiveness. One such example is the anti- bromelain treated autologous red blood cells ($V_H11/V_\kappa9$) idiotype which shows specificity for phosphatidyl choline (PtC) and is heavily enriched within the murine peritoneal cavity (22). To determine whether the importance of the CK2BD of CD5 in maintaining B-1a immuno-dominant clones is restricted to anti-PC responses, we analyzed peritoneal exudates from Wt and CD5ΔCK2BD animals and determined that the anti- $V_H11/V_\kappa9$ idiotype is also significantly reduced within the CD5ΔCK2BD animal (Fig. 4C). Our results show that the CD5-CK2 signaling axis is absolutely essentially in maintaining B-1a restricted idiotypic responses independent of antigen specificity. Taken together these data show that CD5 mediated effects of CK2 biology on B-1a idiotypic populations are critical in maintaining natural antibody responses towards ubiquitous pathogens and antigens.

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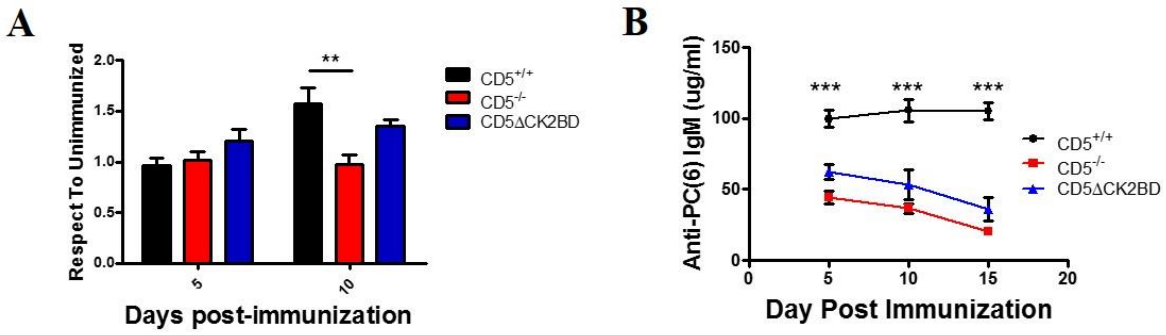

Figure 1. CD5ΔCK2BD mice exhibit diminished anti-PC responses. (**A**) IgM anti-PC titers following 20ug immunization with TI-II antigen PC-Ficoll (n=15/group). (**B**) Kinetics of IgM anti-PC titers in response to immunization with $1x10^8$ CFU heat killed R36A/mouse (n=5/group). Statistics are representative of one-way ANOVA at individual time points. **= $p \leq 0.01$ and ***= $p \leq 0.001$

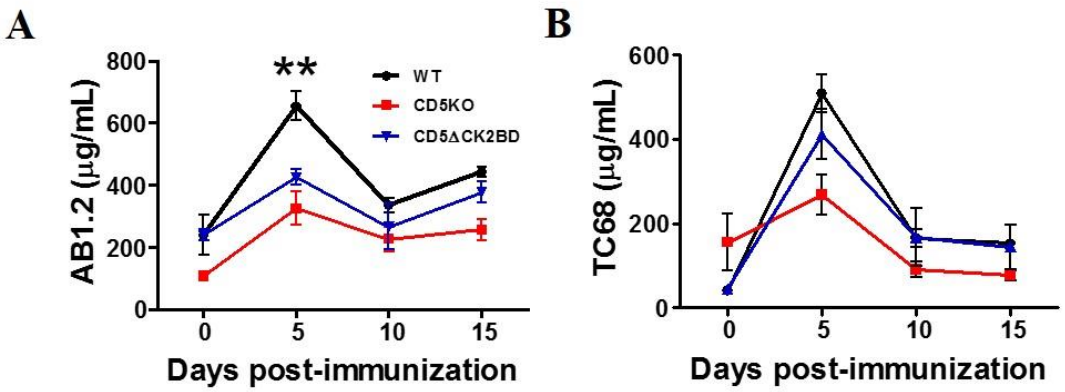

Figure 2. Serum T15 IgA levels following R36A *S. pneumoniae* immunization. Kinetics of (**A**) AB1.2 light chain titers and (**B**) TC68 heavy chain titers between mouse strains. Statistics are representative of one-way ANOVA at individual time points. **= $p \leq 0.01$

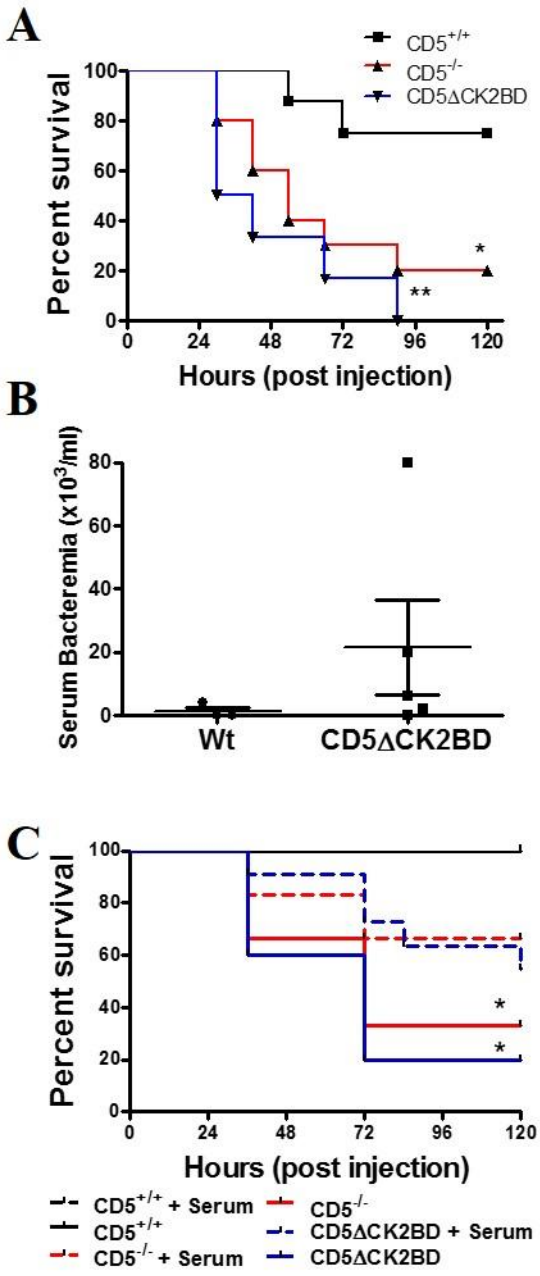

Figure 3. Increased susceptibility to *S. pneumoniae* in CD5ΔCK2BD mice can be recovered with $CD5^{+/+}$ serum. (**A**) Survival curve of mouse strains following i.v. injection of $2.5x10^4$ CFU live D39 *S. pneumoniae* (n=10/group). (**B**) Serum bacteremia of D39 infected animals 48 hours following inoculation. (**C**) Survival curve of D39 infected mice prophylactically treated with either 50µl normal $CD5^{+/+}$ serum or PBS (n=10/group). Statistics are representative of non-parametric Mantel-Cox test. *= $p \leq 0.05$, **= $p \leq 0.01$.

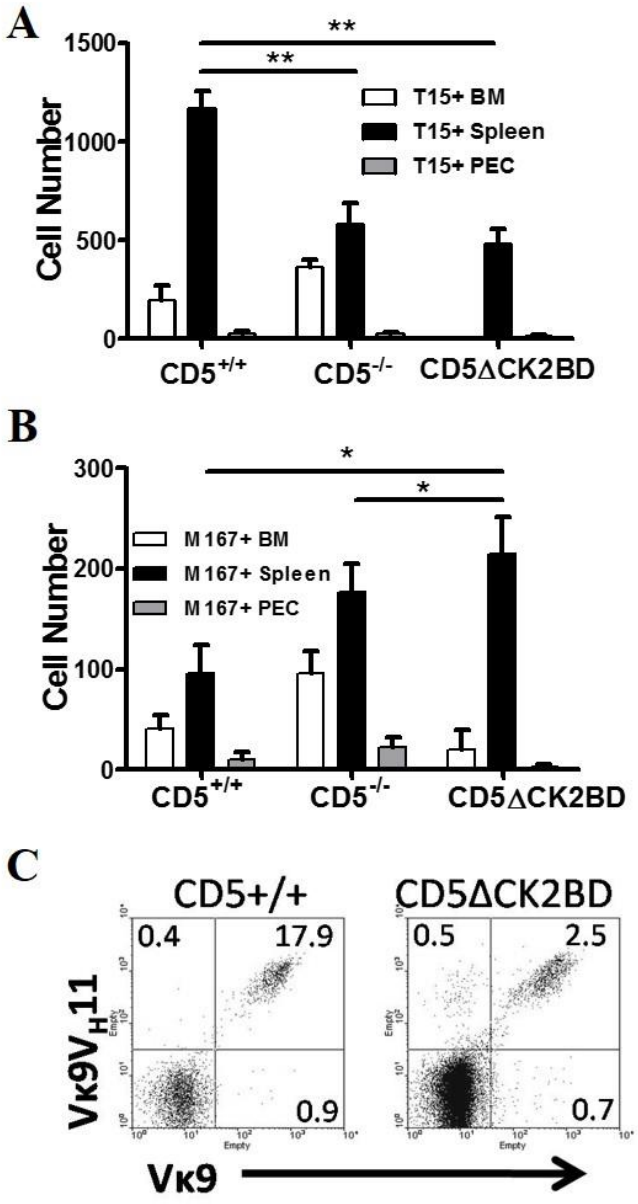

Figure 4. CD5ΔCK2BD mice exhibit reduced B-1a restricted idiotype populations. Number of (**A**) T15 Id+ B cells (n=5/group) or (**B**) M167 Id+ B cells (n=5/group) within the bone marrow (BM), spleen, and peritoneal cavity (PEC) of $CD5^{+/+}$, $CD5^{-/-}$, and CD5ΔCK2BD. (**C**) Representative flow cytometry dot plots of $V_H11/V_κ9$ Id+ B cells in $CD5^{+/+}$ and CD5ΔCK2BD peritoneal cavities. Statistics are representative of one-way ANOVA compared within the same tissue from the different animal strains. *= $p \leq 0.05$, **= $p \leq 0.01$.

DISCUSSION

This study reveals a previously undefined contribution for the CD5-dependent CK2 signaling axis to the complexity of the B-1a B cell population, and answers many questions regarding the role of CD5 in the regulation of this population. CD5 deficiency does not elicit an autoimmune-like or hyperactivated phenotype as many negative regulators of BCR signaling, but instead elicits increased AICD and causes a responsiveness similar to that observed in a conventional B cell (71)(72)(50). This phenomenon has never been explained mechanistically, but with the advent of the previously unknown interaction of CK2 with a cytoplasmic domain of CD5, it was finally determined that CD5 plays more than just a negative regulatory role within the B-1a population (56). Here we determined that loss of the CK2BD of CD5 seemed to have little impact on the maintenance of the B-1a population, resulting in similar numbers and percentages between the CD5ΔCK2BD mice and the C57Bl/6 controls. Further analysis showed that the impact of the CK2BD was more dynamic than the population numbers would suggest and that this population undergoes decreased *in vitro* proliferation in response to antigen receptor ligation and exhibits increased apoptosis following similar stimulation. This leads to a conundrum for understanding how the B-1a population within the CD5ΔCK2BD mouse could maintain similar B-1a numbers in light of increased AICD.

The B-1a B cell population has intrinsic regulatory capabilities that allow this population to sense serum immunoglobulin levels, particularly serum IgM levels, which in turn regulates the size of the population ((73); (74)). In support of this observation, adoptive transfers of peritoneal B-1 B cells into irradiated mice or B cell-depleted mice

resulted in serum IgM levels comparable to those of non-manipulated, age-matched adults (34) (75). Interestingly, even though total B-1a numbers were comparable between the CD5ΔCK2BD and wild-type controls, serum IgM and IgA levels were significantly impaired. Taken together with the findings that CD5ΔCK2BD mice exhibit increased AICD, it can be assumed that the B-1a B cell population within these mice is forced to compensate for their lack of survivability by proliferating to maintain numbers. This results in a dysregulation of peritoneal B cell homeostasis within CD5ΔCK2BD animals. This assumption is verified by the determination that B-1a B cells from the CD5ΔCK2BD mice exhibit increased *in vivo* cell cycle entry at 48 hours following antigen driven signaling. This altered homeostatic balance within the peritoneal cavity of CD5ΔCK2BD mice can also be suggested by an increase in the proportion of B-1b B cells, a population that usually remains consistent with that of the B-1a B cell population ((8)). Although the peritoneal B-1a population numbers in the CD5ΔCK2BD animals remained consistent with controls, the splenic B10 population ($CD19^+$ $CD5^+$ $CD1d^{hi}$) was significantly reduced. This alteration would suggest a possible increase in proinflammatory responses within the spleen, since B10 B cells possess an IL-10 mediated regulatory role within the spleen (76).

Considering the pleiotropic activity of CK2, narrowing the mechanism of action through which CD5-CK2 signaling would explain these data would potentially be numerous. In light of this we chose to examine the influence of the CD5-CK2 signaling axis on the most likely signaling cascades which would impact B-1a biology. As previously stated the B-1a population from CD5ΔCK2BD mice has increased cell cycle entry so understanding the role of CD5-CK2 signaling in cell cycle progression needed to

be examined. CK2 has been shown to phosphorylate Cdc37 at Ser13 aiding in its association with Hsp90 and the effective kinase activity of substrate signaling molecules (70). Phosphorylated Cdc37 levels were significantly reduced within the CD5ΔCK2BD mouse. Cdc37 is critical for facilitating the effective activity of two cyclin-dependent kinases critical for early B cell proliferation signaling, Cdk4 and Cdk6 (77-79). This reduced Cdc37 phosphorylation would suggest that B-1a B cells from CD5ΔCK2BD mice should proliferate less, which is what is seen within *in vitro* anti-IgM stimulation assays. However, *in vivo* biology suggests the opposite. This is most likely due to the dynamic turnover of the B-1a population that is occurring within the CD5ΔCK2BD peritoneal cavity.

We determined that ablation of CD5-CK2 signaling negatively impacted ERK phosphorylation within the CD5ΔCK2BD animals. Constitutive ERK activation, a hallmark phenotype seen in B-1a B cells, is a result of continuous signaling through the BCR, a process which activates and is influenced by CD5 (80) (55)). Previous studies have shown that CK2 can directly influence the ERK signaling pathway by increasing the phosphorylation of Raf isoforms by KSR1, early steps in the MEK/ERK signaling pathway (81). Interestingly, interactions of CK2 with Cdc37 positively regulate multiple myeloma signaling cascades, specifically causing increased STAT3 and ERK phosphorylation, hallmarks of B-1a signaling biology, as well as increases in the levels of anti-apoptotic proteins such as Bcl-2 (82). These data further recapitulate the importance of the CK2 and Cdc37 axes in disease states and suggest that better understanding the role of CD5 in modifying these signaling molecules may potentially lead to new therapeutic targets in many neoplasias and, possibly, autoimmune disorders (Figure 1).

This loss of ERK signaling is one of the first explanations why CD5ΔCK2BD B-1a B cells exhibit increased AICD.

In addition to reduced ERK activation, a significant increase in Akt phosphorylation in CD5ΔCK2BD B-1a B cells is noteworthy. B-1a B cells have not been shown to efficiently signal through Akt or NFκB (as evident by unchanged IκB levels), so a significant increase in phosphorylated Akt levels indicates a loss in regulation of the canonical B-1a signaling (83). This could be explained by a potential cross-interaction with the pITAM domain of CD5, or that loss of ERK activation/survival signaling is potentially compensated for by increased Akt signaling. Nevertheless, understanding how aberrant Akt activation impacts B-1a biology is a question which remains to be answered.

We further elucidated the impact of CD5-CK2 signaling on B-1a B cell biology beyond the cellular signaling cascades and found that the CD5ΔCK2BD B-1a population failed to significantly initiate TI-II immune responses towards TNP-Ficoll. This reduction was also seen within the $CD5^{-/-}$ mice, which is contradictory to previous works by Tarakhovsky *et al*. However the mice used in those experiments were maintained on the 129/Sv background instead of the C57Bl/6 which was utilized within this study (84). These data determine that CD5-CK2 signaling not only affects basal biology and signaling of B-1a B cells, but also diminishes antigen responsiveness. Failing to mount a TI-II response, a defining feature of B-1a B cells, would allow for reduced effectiveness in clearance of evolutionarily conserved pathogens.

To better understand how CD5-CK2 signaling impacts pathogen driven B-1a responses we immunized mice with PC-Ficoll and determined that TI-II driven anti-PC

responses were significantly reduced in CD5$^{-/-}$ animals with a downward trend in CD5ΔCK2BD mice. To better understand the physiological input of the CD5ΔCK2BD on B-1a driven responses, mice were immunized with heat-killed R36a *S.p.*, a pathogen expressing high levels of PC (85, 86). It was determined that CD5ΔCK2BD and CD5$^{-/-}$ mice exhibited both diminished magnitude and duration of anti-PC responses. Since B-1a B cell derived responses are critical in the clearance of pathogens such as *S.p.*, these data represented a significant role for CD5-dependent CK2 signaling in regulating immune responses to commonly encountered pathogens (27).

To test for this, animals were inoculated with live *S.p.* and it was determined that CD5$^{-/-}$ and CD5ΔCK2BD animals exhibited increased susceptibility to a live inoculum. These data verify that CD5 signaling plays a substantial role in mediating the host response towards ubiquitous and commonly encountered pathogens. *S.p.* was chosen as a working model to address this biology due to high expression of PC and potential for understanding and modulating B-1a responses to benefit in defense against this particular pathogen. *S.p.* accounts for millions of serious invasive infections worldwide, resulting in an estimated 1.6 million deaths annually as of 2002, and remains a leading cause of mortality in young and elderly patients (Reviewed in (87)). The advent of polysaccharide Pneumococcal vaccinations has diminished this mortality rate, though an increasing need for understanding the host-pathogen interaction with this bacterium remains evident (88).

In light of the increased susceptibility to *S.p.* in CD5ΔCK2BD mice it was determined that this effect may be due to multiple attributes previous described within these mice: (a) CD5ΔCK2BD animals are more susceptible because they have reduced TI-II responses to *S.p.* and/or (b) the reduced serum IgM/IgA titers within the

CD5ΔCK2BD animal decreases bacterial clearance leading to a more susceptible phenotype since B-1a derived antibodies have been shown critical for clearance of this pathogen.

To determine which or both of these mechanisms were relevant, the idiotypic T15 antibody titers were assessed following immunization with heat killed *S.p.* It was determined that T15 antibody levels were significantly reduced in both the $CD5^{-/-}$ and CD5ΔCK2BD mice, indicating anti-PC responses were significantly impaired within the response. However further examination of non-manipulated animals revealed that T15 idiotype numbers were significantly diminished in $CD5^{-/-}$ and CD5ΔCK2BD spleens prior to immunization. Interestingly with the loss of the $T15^{+}$ positive population within the spleen there was an increase within the $M167^{+}$ MZ B cell restricted idiotypic population, which suggests a complete skew of the idiotypic response away from classically dominant clones. Vakil *et al.* have previously determined that a skewing of the early idiotypic development can be seen within animals immunized neonatally with heat-killed *S.p.* and that this skew reflects a similar responsiveness and idiotypic selection to the M167 clonal phenotype as seen within the CD5ΔCK2BD animals (26) . Our data represents the first genetic based evidence that recapitulates the skewed idiotypic selection data and determines that CD5 signaling is involved in maintenance of dominant idiotypic responses. This argues for the determination that susceptibility in CD5ΔCK2BD mice is due to reduced TI-II responsiveness to *S.p.* and not natural antibody titers, yet prophylactic administration with unimmunized WT serum prior to infection was found to aid in the partial recovery of the susceptible phenotype. Clearly,

both serum antibody levels and antigen responsiveness are necessary for effective T cell independent clearance of *S.p.*

Finally we wished to determine if the influence of CD5 on idiotypic selection was limited to responsiveness to *S.p.* or could be translated to other antigenic targets as well, an effect originally suggested by the observation that premature neonatal priming also occurred within α1,3 dextran dominant idiotypes (28). We determined that the B-1a population within the peritoneal cavity of CD5ΔCK2BD animals also exhibited a significant decrease in the dominant anti-PtC idiotype, $V_H11/V_\kappa9$ idiotype (89). This would suggest that B-1a B cells from CD5ΔCK2BD animals also exhibit a decreased ability to clear damaged erythrocytes and potentially apoptotic debris, since PtC is ubiquitously expressed within all mammalian cells (Reviewed in (90) (91)).

In conclusion, this study determines that that CD5-dependent CK2 signaling axis is necessary for the development of optimal B-1a B cell mediated immune responses, basal immunity, and signaling pathways, and that removal of this signaling domain of CD5 results in increased AICD of the population. Additionally, physiologic B-1a mediated responses to commonly encountered pathogens are also diminished due to altered dominant idiotypic selection leading to increased susceptibility to pathogens such as *Streptococcus pneumoniae*. The understanding of the role of the CD5-CK2 signaling axis addressed within this study may be beneficial in aiding future therapeutic targets for clinical conditions such as $CD5^+$ B cell- dominated leukemia/lymphoma and certain autoimmune disorders

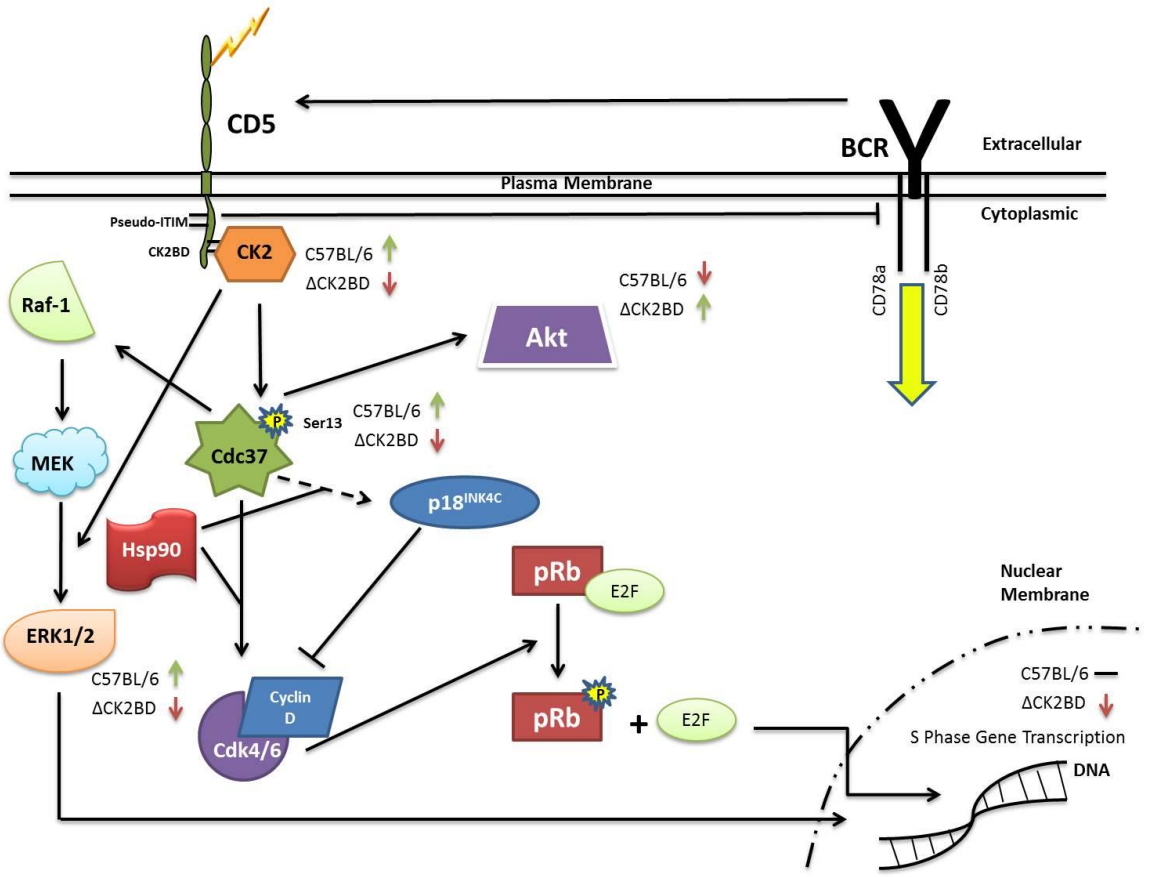

Figure 1. The effect of CK2BD deficiency on B-1a B cell signaling cascades.
CD5 driven CK2 signaling is responsible for direct phosphorylation of both the Cdc37 co-chaperone and ERK signaling molecule positively effecting either their association with cognate substrates or translocation into the nucleus, respectively. Cdc37 following CK2 phosphorylation has been shown to effect the upstream ERK signaling machinery as well as has been shown to positively influence Akt kinase activity. Interestingly, B-1a B cells from CD5ΔCK2BD mice show a significant increase in Akt phosphorylation following antigen receptor stimulation where Wt animals reduce this kinase.

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APPENDIX . IACUC Animal Usage Approval

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: October 3, 2013

TO: CHANDER RAMAN, Ph.D.
SHEL-305
(205) 934-2472

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Role of CD5 in B-Cell Development and Autoimmunity
Sponsor: NIH
Animal Project_Number: 131008261

As of October 3, 2013 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	795
Mice	B	346
Mice	C	270

Animal use must be renewed by October 2, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 131008261 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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