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Characterization Of Molecular Pathways Involved In The Production Of Class-Switched Antibodies

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ABSTRACT

This dissertation research describes two independent characterizations of molecular pathways involved in the manufacture of class-switched antibodies and differentiation of B cells involving the adaptor protein Hematopoietic SRC Homology 2 Domain-containing protein (HSH2) and the surface molecule CD19. HSH2 is dynamically expressed in different subsets of B cells, showing high expression in B1 and MZ B cells and low expression in GC B cells. Experiments involving HSH2 transgenic mice have determined that HSH2 may act as a rheostat regulating the production of class-switched antibodies. Various factors that stimulate B cell activation and differentiation induce HSH2 expression, although regulation of HSH2 has not been characterized. Here we describe a novel mechanism by which NF-κB regulates HSH2 at the level of protein turnover by negatively regulating a short-lived labile protease. HSH2 is dependent on NF-κB activity for maintenance and upregulation, while inhibition of NF-κB leads to rapid degradation of HSH2.

CD19 is a 97kD protein that interacts with the B cell receptor to amplify downstream signaling through its cytosolic phosphorylated tyrosines. CD19 knock-out studies have demonstrated the importance of CD19 signaling in an effective BCR-dependent immune response to both T-dependent and T-independent antigens. $CD19^{-/-}$ mice develop few B1 B cells, MZ B cells or GC B cells. CD19 contains several cytosolic tyrosine resi-

dues that when phosphorylated allow binding of effector proteins, thus mediating downstream signaling events. Mice with point mutations replacing tyrosines with phenylalanine at Y482 and Y513 on the cytosolic side of CD19 exhibit a similar phenotype to $CD19^{-/-}$ mice in terms of B cell development and antibody response against T-dependent and T-independent antigens. However, the importance of CD19-mediated signaling via upstream tyrosines, or whether CD19 Y482/Y513-mediated signaling is sufficient to retain a wild-type B cell subset phenotype and antigen-specific antibody response against T-dependent and T-independent antigens are not well understood. Using mice with tyrosine to phenylalanine point mutations at all positions in CD19 except 482 and 513, we demonstrate that CD19 Y82 and Y513 are sufficient for MZ and B1 B cell development but confer a skewed antigen-specific antibody response against T-dependent and T-independent antigens.

Keywords: HSH2, CD19, class-switching, NF-κB

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INTRODUCTION

B Cell Role in the Immune Response

The regulation of B cell differentiation into Antibody Secreting Cells (ASCs), memory B cells, and production of class-switched antibodies is a vital part of the adaptive immune system's ability to defend against pathogens. B cells differentiate into several subsets involved in both the innate and adaptive immune response. Marginal zone (MZ) B cells, B1a and B1b B cells have innate-like properties, while memory B cells and ASCs form the humoral adaptive immune response. The adaptive immune response depends on the ability of B cells and T cells to recognize novel pathogens that have evaded the innate immune system. Once a B cell undergoes a series of differentiation steps to produce an antibody specific for an antigen, the B cell may differentiate into an ASC or a memory B cell. The memory B cell response ensures that the host will be able to rapidly defend against reencountered pathogens, while ASCs produce large quantities of antibodies against specific pathogens. It is necessary for B cells to adapt to numerous types of antigens without producing self-recognizing antibodies. Therefore, rigorous regulatory systems have evolved to control the processes of B cell differentiation and antibody production. Problems with autoimmunity arise when B cells escape their self-recognition education and begin to recognize and produce antibodies against self-antigens.

B cell Differentiation

B cells first arise in the bone marrow from hematopoietic stem cell precursors and undergo a series of differentiation steps before they become mature B cells capable of responding to foreign antigens. Three subsets of progenitor cells expressing the B cell surface marker B220 but lacking surface immunoglobulin have been identified: pre-pro-B cells; pro-B cells; and pre-B cells (1). During the differentiation process, these B cell precursors undergo a process of arranging their immunoglobulin genes to produce an immunoglobulin protein (or antibody) unique to each B cell. This process involves arranging regions of the genes encoding the light chain and heavy chain of the immunoglobulin. The heavy chain gene locus contains variable (V), diversity (D), joining (J) and constant (C) regions. Rearrangement of the D and J regions begin in pro-B cells. Later, pro-B cells rearrange VDJ regions as they become pre-B cells making μ heavy chains (2-4). Pre-B cells undergo light chain V_L to J_L rearrangement and eventually express surface IgM (immunoglobulin with the constant region μ) as immature B cells. These cells then migrate to the spleen and lymph nodes, where they become transitional stage 1 (T1) B cells, then differentiate into mature B cells and co-express IgM and IgD (2-7).

In the bone marrow and periphery, B cells are "educated" to prevent autoreactive B cells from differentiating (1). Pre-B cells in the bone marrow require weak binding of self-antigens to survive (8). However, pre-B cells that bind strongly against self-antigens are negatively selected against, allowing only weakly-self binding B cells to migrate to the periphery. Strongly self-binding B cells may either edit their light chain VJ regions until weak self-binding occurs or they undergo apoptosis (clonal deletion). Negative

selection takes place in the bone marrow, while positive selection happens in the periphery. Positive selection depends on weak BCR signaling in periphery against self-antigens, possibly to ensure normal BCR signaling (8). When introduced to an antigen recognized by the BCR, B cells undergo further division and somatic hypermutation, leading to successive generations of B cells binding more strongly and specifically against the introduced antigen (known as clonal selection, clonal expansion and affinity maturation) (7-10).

B Cell Subsets

Follicular and Marginal Zone B Cells.

As immature B cells travel to the spleen and lymph nodes, they are considered transitional stage 1 (T1) B cells (7). After arrival in peripheral lymphoid tissue, they further differentiate into T2 B cells which either become surface $IgM^{hi}IgD^{lo}$-expressing marginal zone (MZ) B cells, or $IgM^{+}IgD^{+}$ follicular (FO) B cells (7,11).

MZ B cells exhibit innate-like immune properties by secreting natural IgM (IgM secreted without infection, which can bind with low affinity to self-antigens such as DNA), and typically undergo T cell-independent activation (12). FO B cells typically require T cell help through CD40/CD40L signaling for activation as they differentiate into germinal center (GC) B cells and undergo class-switching (rearrangement of the constant region of the immunoglobulin gene). FO B cells can express both IgM or IgD.

B1a/b Cells

It is unclear whether B1 B cells derive from T2 B cells or some previous precursor, though they arise in fetal livers of embryonic and newborn mice and are "self-renewing" throughout the lifespan of the organism (13). They are the largest population of B cells in the peritoneal cavity and are responsible for most of the IgA in the blood. B1 B cells secrete large amounts of low-affinity natural IgM. Natural IgM (alternatively known as natural antibody) is generally specific for self-antigens (such as oxidized lipids or antigens on apoptotic cells), as well as antigens specific to bacteria, viruses, or parasites. B1 B cells are classified as either B1a or B1b cells depending on the expression of CD5 (B1a) or lack of CD5 (B1b). B1 B cells have been demonstrated to be defective in CD19 signaling, and are resistant to apoptosis in response to BCR signaling (13).

Germinal Center B Cells

Germinal center (GC) B cells are derived from FO B cells as they migrate toward the T cell area of the lymph nodes or the spleen, and an antigen presented by follicular dendritic cells (FDCs) is introduced. If the BCR recognizes an antigen and the B cell receives signals from follicular helper T cells (T_{FH}), then these B cells differentiate into GC B cells (14,15). GC B cells undergo apoptosis when introduced to an antigen without T_{FH} help, as a defense against developing B cells with BCRs specific to self-antigen. As GC B cells interact with antigen presented by follicular dendritic cells (DC) in conjunction with T_{FH} cells, they differentiate into ASCs, memory B cells, or recombine the VDJ region of the immunoglobulin with a different constant region, known as "class

switching" before differentiation into ASCs and memory B cells. Class-switched antibodies confer different overall effects on the immune system, depending on the class of antibody secreted (14,15).

The purpose of the immunoglobulin is to bind to antigen, aiding the innate immune system in protection against foreign invaders. Complement proteins are better able to bind to bacteria with antibodies bound to them, and innate immune cells have receptors specific to the different classes of antibodies, mediating an immune response by cytokine release dependent on which class of antibody is bound (16). Class switching and somatic hypermutation of the variable region of immunoglobulin proteins are essential for generating high-affinity antibodies, and for mediating specific immediate responses against recurring infections (9,17). Innate immune cells release cytokines in response to different types of pathogens. Cytokines from T cells influence production of each class of antibody, and development of the B cell into a high-affinity class-switched antibody producing plasma or memory B cell depends on the strength of the interaction between the antigen and BCR (4,14,15,17), which impacts on the quality and amount of T cell help that the B cell will receive.

BCR Signaling

The BCR consists of a fully formed surface antibody, along with an Igα/Igβ pair. Igα/Igβ contain immunoreceptor tyrosine-based activation motifs (ITAM) that initiate downstream BCR signaling (18-21). Binding to antigen causes BCR crosslinking and phosphorylation of ITAM tyrosines, which recruit and activated protein tyrosine kinases (PTKs) such as Lyn, Spleen tyrosine kinase (Syk), and Bruton's tyrosine kinase (Btk)

(22,23). These PTKs lead to signal transduction through activating lipid-metabolizing enzymes such as phospholipase C-γ2 (PLCγ2) and phosphoinositide 3-kinase (PI3K) (23,24).

The PLCγ2 pathway may act with CD19 or through the B cell adaptor for PI3K (BCAP) and involves Syk recruitment to ITAMs after BCR stimulation (25,26). Syk phosphorylates B cell linker (BLNK), also known as BASH or SH2 containing leukocyte protein of 65 kDa (SLP65) (23). Phosphorylated BLNK then binds to PLCγ2 and Btk, which enables PLCγ2 to become phosphorylated and activated (23). PLCγ2 catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) into secondary messengers di-acylglycerol (DAG) and inositol 4,5,6 triphosphate (IP_3), which lead to calcium signaling and NF-AT translocation to the nucleus to aid in transcription of specific target genes (23). DAG may activate protein kinase C (PKC), which regulates further signal cascades. Other proteins regulated by PIP3 include AKT, 3-phosphoinositide dependent kinase-1 (PDK1), and Btk1 (24). These are all important signaling proteins involved in the regulation of B cell survival, apoptosis and activation.

The PI3K pathway is dependent on both BCR stimulation and CD19 involvement (25). Tyrosines on CD19 are phosphorylated by Lyn, while Syk and Btk phosphorylate BCAP (25). BCAP works synergistically with CD19 to activate PI3K to produce PIP_3 from PIP_2 (27). PIP_3 leads to downstream signals including AKT signaling. In addition, BCAP may work with BLNK to aid in the activation of PLCγ2, which in addition to activating the NF-AT pathway can also activate the nuclear factor-kappa B (NF-κB) pathway

through PKC-beta (PKCβ) activation (25). PKCβ phosphorylates CARMA1, which recruits the TGF-β activated kinase 1 (TAK1) and inhibitor of kappa B kinase (IKK) complex, which when activated leads to NF-κB signaling (23).

NF-κB Signaling

NF-κB signaling is vital for B cell activation and differentiation, and dysregulation of the NF-κB pathway can lead to cancer or problems with cell proliferation and survival (28). In addition to BCR signaling, Toll-like receptor signaling (TLR), TNF-receptor family signaling (TNFR) and BAFF receptor signaling utilize the NF-κB pathway (28-34). NF-κB is a name for a several closely related transcription factors that form either heterodimers or homodimers with each other and remain sequestered in the cytoplasm until activation allows the NF-κB duplex to translocate to the nucleus and initiate transcription (35-38). The different combinations of the NF-κB duplex can initiate transcription of specific genes, allowing cells to utilize this universal pathway to transcribe specific genes in response to the cell's environmental signals (36,38,39). NF-κB can be activated through what is known as canonical signaling (from TLR or BCR signaling through IKK complex phosphorylation and ubiquitination of inhibitor of kappa B (IκB), or non-canonical signaling (through IKKα, and NF-κB-inducing kinase, NIK) (33,36,38,39). The IKK complex consists of IKKγ (or NEMO), IKKα, and IKKβ. IKKγ acts as a scaffold for IKKα, and IKKβ allowing them to be phosphorylated, though IKKγ is not necessary for IKKα involvement in the noncanonical NF-κB pathway (40). When

activated, IKKβ phosphorylates IκB, which is then ubiquitinated and subsequently degraded by the proteasome, freeing the NF-κB dual protein complex to translocate to the nucleus and initiate transcription of specific genes depending on the NF-κB subunit pair (38).

Non-canonical NF-κB signaling depends on the processing of the NF-κB subunit precursor p100 into p52 (33,41). Signaling through the B cell activating factor (BAFF) receptor signaling, receptor activator for NF-κB (RANK), lymphotoxin beta receptor (LTβR), as well as through some TNFR superfamily members (such as CD40R) can induce NIK and IKKα into phosphorylating p100; tagging p100 for degradation into p52 (33,41). p52 forms a heterodimer with an NF-κB subunit RelB and together they translocate to the nucleus to initiate transcription of specific genes (33,37,40,41). Through these multiple mechanisms, NF-κB can be responsible for many different effects on B cells, including differentiation, class-switching, survival, or apoptosis.

HSH2

Many cell-signaling pathways utilize adaptor proteins, which lack enzymatic activity but act as scaffolds facilitating the interaction between effector proteins (18). Many adaptor proteins are expressed constitutively by subsets of B cells and affect B cell differentiation, survival, and the production of class-switched antibodies (18). One adaptor protein that seems involved in the processes of B cell differentiation and the production of class-switched antibodies is the 37kD adaptor protein Hematopoietic Src Homology 2 (HSH2).

Regulation of B cell production of class-switched antibodies is arguably the most important aspect of B cell biology, therefore understanding how B cells control the expression of HSH2 is necessary to fully comprehend the process of B cell class-switched antibody production. Previous studies have begun to decipher the pathways involved in HSH2 regulation using B cells isolated from spleen and a cell-line model for naïve B cells, WEHI-231 (42,43).

WEHI-231 cells undergo apoptosis in response to BCR signaling unless rescued by CD40 signaling; however, WEHI-231 cells which overexpress HSH2 are protected somewhat from BCR-mediated apoptosis (42). The mechanism by which HSH2 attenuates apoptosis remains unclear. BCR signaling led to degradation of HSH2 in WEHI-231 cells, and CD40 signaling seemed to stabilize HSH2 expression. However, HSH2 does not appear to affect overall BCR-induced tyrosine phosphorylation (42). HSH2-overexpressing WEHI-231 cells have increased JNK phosphorylation (42). HSH2 was found to be localized in the cytosol as well as in the mitochondria, suggesting it could play a role in protection against mitochondrial depolarization. After stimulation with anti-IgM, HSH2-overexpressing WEHI-231 cells appeared to be protected from mitochondrial depolarization over time compared to WEHI-231 cells transfected with an empty vector, as measured by $DiOC_6$ staining (42). These experiments suggested HSH2 likely played a role in the survival of B cells in response to BCR-mediated signaling and may be upregulated in response to factors signaling for survival.

Splenic B cells treated with factors leading to B cell differentiation and survival, such as IL4, CpG DNA, and lipopolysaccharide (LPS), anti-CD40 mAb, B lymphocyte stimulator (Blys or B cell activating factor belonging to the TNF family, known as

BAFF) upregulated HSH2 protein expression (43). CpG DNA and LPS stimulate TLR signaling through TLR9 and TLR4 respectively, while BAFF and anti-CD40 signal through TNF receptor superfamily members. Both families of receptors activate the NF-κB pathway (28), and experiments were conducted to determine the importance of NF-κB signaling on HSH2 expression.

Pre-treatment of B cells with NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC) and Bay 11-7082 prevented the increase in HSH2 expression in response to CpG DNA, LPS, anti-CD40 mAb, and BAFF (43). PMA treatment, which activates the NF-κB pathway through activating protein kinase C (PKC), induced HSH2 expression in treated B cells. Treatment of B cells with ionomycin to induce Ca^{2+} and activate NF-AT (an additional transcription factor in lymphocytes) failed to induce HSH2 expression (43). These experiments suggested that HSH2 expression is dependent on NF-κB activity, and that NF-κB activity is sufficient to induce expression (43). Collectively, these data suggest HSH2 expression correlates with B cell survival. Conversely, splenic B cells treated with factors known to induce apoptosis in B cells such as anti-IgM and IL-21 (44,45) showed no increase in HSH2 expression (43). It appeared that HSH2 expression is upregulated by NF-κB in response to stimuli signaling for B cell activation and survival, while factors signaling for apoptosis potentially decrease HSH2 expression. It was concluded that HSH2 possibly plays a role in B cell survival, although its exact function in B cells remained uncertain.

Early reports based on mRNA analysis suggested that HSH2 was expressed in macrophages, T cells, and B cells (46,47). However, HSH2 protein was only detected by

Western blot in B cells (43). Later studies demonstrated that HSH2 is expressed dynamically between subpopulations of B cells, with pro-B cells and pre-B cells expressing no detectable HSH2 (48). HSH2 is first expressed in T1 B cells at low amounts, with T2 B cells expressing high amounts (48). MZ and B1a/b B cells contain high amounts of HSH2 as well, while FO and GC B cells express much less (48). Among the B cell subpopulations, B1a/b B cells expressed the highest levels of HSH2, and GC B cells expressed the lowest amount (48). The differential nature of HSH2 expression among B cell subsets, as well as its upregulation in response to stimuli for B cell activation and survival, suggested HSH2 plays a major role in B cell immunity. Transgenic mice were developed to delineate the role of HSH2 in B cells, placing HSH2 in the lambda-light chain locus, causing high HSH2 expression in B cells (48). Similar to the effects seen in HSH2 overexpressing WEHI-231 cells, B cells from HSH2 transgenic (HSH2-Tg) mice were resistant to BCR mediated apoptosis and exhibited increased JNK phosphorylation (42). Although HSH2 appeared to confer protection from BCR-apoptosis, there was no overall increase in B cell populations in the periphery (42). Interestingly, there seemed to be decreased numbers of MZ, IgM^{Hi}, and T2 B cells in the spleen, despite normally high endogenous levels of HSH2 in these subpopulations (42). These results suggested a role for HSH2 in the differentiation of B cells. Further experiments seemed to confirm this, as B cell numbers were decreased in young mice 3-6 weeks old compared to WT mice (42).

The antibody response against T-independent and T-dependent antigens were also affected in HSH2-Tg mice (42). HSH2-Tg mice made significantly less antigen-specific IgM, IgG1and IgG2b serum antibodies in response to the T-dependent antigen TNP-KLH

in alum compared to WT mice (42). HSH2-Tg mice also secreted less TNP-specific IgM and IgG3 antibodies in response to the T-independent antigen TNP-LPS (42). These effects were independent of a decrease in GC B cell development, as differences in GC B cells between WT and HSH2-Tg mice were not significant, although HSH2-Tg mice appeared to develop fewer GC B cells than WT mice at each time point post-immunization (42). Analysis of transcript levels of several genes involved in GC B cell development by qRT-PCR demonstrated that HSH2-Tg mice expressed increased levels of Bach2 and decreased levels of Prdm1 (42). Bach2 represses Prdm1, while Prdm1 upregulation is required for differentiation of GC B cells into plasma cells (49). These results suggested a potential defect in the ability of HSH2-Tg mouse B cells to differentiate into plasma cells.

In summary of previous studies, HSH2 is expressed in B cells and has been linked to protection of B cells from apoptosis (42,48). HSH2 expression can be induced in B cells with a variety of B cell-activating stimuli and is most likely dependent on NF-κB activity for expression (43). HSH2 expression among subsets of B cells is dynamically regulated, and transgenic mice produce lower antibody titers compared to WT mice, possibly due to a defect the inability of GC B cells from HSH2-Tg mice to differentiate into ASCs (42). These data demonstrate that HSH2 regulation is critical a normal adaptive humoral response. The mechanism by which HSH2 expression is regulated in B cells is not known. Although NF-κB has been implicated in the regulation of HSH2, it is unknown if it affects HSH2 expression at the level of transcription, translation, or protein-turnover. Previous studies implicating NF-κB have used the inhibitors Bay 11-7082 and PDTC, whose exact mechanisms for NF-κB inhibition are uncertain, although

their common roles as antioxidants have been implicated (50-52). Because antioxidants can inhibit NF-κB activity, and redox activity may have other effects on other intracellular proteins and pathways, it is possible that the redox effects of Bay 11-7082 and PDTC may have been responsible for the inhibition of HSH2 expression seen in previous studies (43,53-57).

Here we shown that HSH2 is not regulated at the level of transcription either constitutively in B cell subsets or in B cells treated with LPS or CpG DNA to induce HSH2 expression, despite NF-κB's distinct role as a transcription factor. Inhibition of NF-κB leads to rapid degradation of HSH2 compared to inhibition of translation with cycloheximide, or inhibition of transcription with actinomycin-D.

While both PDTC and Bay 11-7082 affect the redox state of cells, inhibition of NF-κB through prevention of IκB degradation using the proteasome inhibitor MG-132 led to a decrease in HSH2 expression independent of changes in the redox status. In addition, B cells with NEMO floxed out by mx1-cre were unable to retain HSH2 expression compared to littermates. Upstream phosphorylation of proteins involved in the NF-κB pathway could potentially regulate other proteins through phosphorylation of proteins non-specific to the NF-κB pathway. However, using an NF-kB inhibitor that does not affect phosphorylation events in the NF-kB pathway still led to degradation of HSH2. Thus it appears that HSH2 expression is controlled at the level of protein turnover indirectly through NF-κB regulation of other proteins in a complicated indirect method independent of alternative phosphorylation of proteins outside the NF-κB pathway by upstream kinases. It appears that NF-κB negatively regulates a protease responsible for the degradation of HSH2.

CD19

CD19 is a 97kD protein expressed on the surface of B cells from the pre-B cell stage until the plasma cell stage. CD19 interacts with CD21, leu-1, and CD81 in lipid rafts on the surface of B cells to aid in potentiating BCR signaling (58-61). The importance of CD19 in B cell development has been demonstrated by both overexpression and knockout studies. Overexpression of CD19 kills early B cell progenitors but leads to greater numbers of mature B cells, and hypersensitive T-dependent and T-independent immune responses (62). $CD19^{-/-}$ mice contain fewer mature B cells and are unable to produce MZ, B1, and GC B cells (62-66).

CD19 mediates signaling through its nine conserved tyrosine residues on the cytoplasmic tail of CD19 (67). When the BCR and CD19 are ligated, phosphorylation of the tyrosines by Lyn occurs beginning at the furthest away tyrosines at positions 482 and 513 and continues upward through progressive amplification (68). These phosphorylated tyrosines mediate SRC and SH2 recruitment and signaling (69,70). CD19 mediates Grb2/Sos signaling at Y330/Y403, Vav/PLCγ2 signaling at Y391/Y421, and Lyn/PI3K signaling at Y482 and Y513 (71-73).

Through effector proteins binding to phosphorylated tyrosine residues on CD19, CD19 affects AKT phosphorylation (74), JNK, and MAPK activation (75). CD19 amplifies BCR activated Ca^{2+} release through association with Vav and PLCγ2 (60,72,73). Vav-mediated signaling appeared to have the most important effect on B cell signaling in Daudi cells (71). However, *in vivo* experiments suggested Lyn/PI3K had a much greater effect (26,76). CD19 mutant mice were created to express point mutations in CD19 tyrosine residues associated with effector protein binding. CD19 Y330F/Y403F, CD19

Y391F/Y421F, and CD19 Y403F/Y443F mice do not bind to Grb2, Vav, or Fyn respectively. These mice were indistinguishable from WT mice with respect to B cell differentiation, antigen-specific antibody response, and GC B cell development. However, CD19 Y482F/Y513F mice expressed a similar phenotype to $CD19^{-/-}$ mice (76). In addition to B cell development and antigen-specific response abnormalities, CD19 Y482F/Y513F mice, as well as $CD19^{-/-}$ mice, were unable to increase calcium concentration at WT levels in response to 1μg/ml anti-IgM and 5ug/ml anti-CD19 (76). CD19 Y482F/Y513F mice did not phosphorylate PI3K in response to pervanadate, demonstrating the importance of CD19 mediated PI3K signaling (76). Although PI3K may also bind to the B cell adaptor of PI3K (BCAP), and association of PI3K to BCAP may confer redundant PI3K activity (27), recent reports have also emphasized the importance of CD19-mediated PI3K activity (77). These reports suggest CD19-mediated PI3K signaling is necessary for normal B cell differentiation and antigen response.

In addition to decreased PI3K activity, the effects observed in CD19 Y482F/Y513F mice may also be due to decreased phosphorylation on upstream tyrosines (68). It is unclear if Grb2 or Vav lose the ability to bind CD19 in these mice. If these two effector proteins are unable to bind to CD19 in the CD19 Y482F/Y513F mutants, then it cannot be determined whether the phenotypes of these mice were due to inhibition of CD19 mediated PI3K signaling or due to lack of effector protein binding to CD19. To better understand the role of CD19 mediated Grb2/Sos and Vav/PLCγ2 signaling, as well as to determine if CD19 mediated PI3K signaling is sufficient to restore a WT phenotype, a new CD19 mutant strain was created. Phenylalanine replaced tyrosines on CD19 at all positions associated with effector proteins except at 482 and 513 (CD19 Y482/Y513

mice). Using these mice, we have determined CD19-mediated PI3K signaling is sufficient for normal B cell development. CD19 Y482/Y513 mice demonstrate an increased antibody response in response to T-independent antigen, but produce a delayed class-switch response against a T-dependent immunization, despite increases in IgM production. This observation allows a role for CD19 mediated Grb2 and Vav signaling in moderating the humoral immune response against T-independent antigens and in producing class-switched antibodies.

REGULATION OF THE ADAPTOR PROTEIN HEMATOPOIETIC SRC HOMOLOGY 2 IN B CELLS THROUGH A NOVEL POST-TRANSLATIONAL MECHANISM BY NF-κB

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ABSTRACT

The Hematopoietic Src Homology 2 (HSH2) adaptor protein is differentially expressed in peripheral B cell subpopulations; exhibiting high-level expression in B1 and marginal zone B cells versus low-level expression in germinal center B cells. HSH2 plays an important role in regulating the production of class switched antibodies, predominantly through its effect on B cell terminal differentiation. Specifically, studies using a mouse model revealed that enforced expression of HSH2 in the B cell lineage at levels found in B1 B cells attenuates the class switched antibody response. Thus, it is of significant interest to delineate the molecular mechanisms by which expression of HSH2 is controlled. Upregulation of HSH2 expression in response to TNF receptor family and TLR agonists occurs in an NF-κB-dependent manner, yet does not involve increased transcription of the HSH2 locus based on analysis of HSH2 mRNA levels. Additionally, maintenance of HSH2 expression is also dependent on NF-κB activation. Inhibition of NF-κB does not affect HSH2 mRNA levels, whereas it results in the rapid down regulation of HSH2 protein, with a half-life of 1 hour. In contrast, treatment of B cells with cycloheximide or actinomycin D does not alter HSH2 expression at either the mRNA or protein level even after 8 hours, demonstrating that the protein itself has a half-life significantly greater than 8 hours. These data support the conclusion that HSH2 protein expression is selectively regulated via a novel NF-κB-dependent post-transcriptional mechanism that represses the expression or activation of a protease, or both.

Introduction

The production of class-switched antibodies (Ab) following infection with microbial pathogens plays an important role in mounting an effective adaptive immune response resulting in clearance of the pathogen and the establishment of a functional memory response. Conversely, improper regulation of B cell responses can lead to the production of class-switched Abs that mediate autoimmune diseases and allergies. Therefore, regulation of B cell differentiation into class-switched Ab secreting cells (ASC) is a carefully regulated process involving numerous receptor-ligand interactions that control class switching, terminal differentiation and survival of class-switched ASCs (1-4).

Many of the receptors involved in regulating terminal B cell differentiation employ adaptor proteins to mediate signaling to downstream targets resulting in changes in gene transcription that affect the production of class-switched Abs. Recent studies have demonstrated that the Hematopoietic Src Homology 2 (HSH2) adaptor protein plays an important role in regulating the production of class-switched Abs in response to both T cell-dependent and -independent immune responses (5). HSH2 is a 37kD adaptor protein conserved between mouse and human that is expressed predominantly in B cells (6,7). Analysis of HSH2 expression in bone marrow and peripheral B cell subpopulations revealed that it is not expressed in developing B cells in the bone marrow, and is first expressed in Transitional 1 B cells. In the periphery, HSH2 is most highly expressed in B1

B cells followed by Transitional 2 B cells and then marginal zone (MZ) B cells. In contrast, HSH2 expression is significantly lower in follicular B cells and germinal center (GC) B cells. Thus, there is an inverse correlation between HSH2 expression and the propensity of a specific B cell subpopulation to undergo class-switching to produce class-switched Abs. Analysis of the humoral response in transgenic mice that express HSH2 in all peripheral B cell subpopulations at the level observed in B1 B cells, revealed a significant impairment in the production of class-switched antibodies in response to both T-independent and –dependent antigen (5). This observation supports the conclusion that high-level expression of HSH2 negatively regulates one or more processes associated with the generation of class-switched ASCs.

HSH2 is upregulated in response to several B cell activating and survival factors, including the Toll Like receptor (TLR) agonists LPS and CPG DNA, and the TNF receptor (TNFR) family agonists CD40L and BAFF (6). Although these agonists regulate B cells activation, proliferation and differentiation through distinct TLR and TNFR signaling pathways, a common feature of these receptors is that they activate NF-κB and alter expression of NF-κB target genes. Treatment of splenic B cells with the NF-κB inhibitors pyrollidine dithiocarbamate (PDTC) and Bay 11-7082 was observed to inhibit the ability of these cells to upregulate HSH2 in response to TLR and TNFR agonists, suggesting a strong link between NF-κB signaling and HSH2 expression (6). However, it was not determined whether the effect was specifically due to inhibition of NF-κB transcriptional activity, or to indirect effects of the inhibitors (8-14). In contrast to what is observed following stimulation of B cells via TLRs or TNFR family members, BCR-mediated signaling, which also activates NF-κB, does not increase HSH2 expression (6).

Previous studies suggest that Ca^{2+}-dependent signaling attenuates upregulation of HSH2 expression and this may in part explain why BCR signaling alone does not induce robust upregulation of HSH2 (6). Whereas HSH2 is highly expressed in innate-like B cell subpopulations, including B1 and MZ B cells, HSH2 is downregulated in GC B cells (5). The molecular mechanism responsible for this has yet to be elucidated. Although GC B cells are likely to receive signals that promote activation of the NF-κB pathway, it is possible that other signals override the NF-κB-mediated upregulation of HSH2 in the GC microenvironment, and that decreased HSH2 expression favors processes that lead to class switching, or differentiation of class-switched B cells, or both.

Because HSH2 expression is inversely correlated with class switched Ab production, it is important to further elucidate the mechanism that controls HSH2 expression. The specific goal of this study was to investigate the mechanism by which NF-κB regulates HSH2 expression, and to determine if HSH2 expression is regulated at the level of transcription, translation, or protein turnover in B cells. It was determined that HSH2 expression is not regulated at the level of transcription either in B cell subsets or when B cells are treated with agonists that induce signal transduction via TLRs. Therefore, it does not appear that NF-κB directly controls transcription of the HSH2 locus. It was subsequently shown that NF-κB activity is required for maintenance of HSH2 protein stability in B cells and that inhibition of NF-κB results in the rapid loss of HSH2. In vitro experiments revealed that inhibition of NF-κB leads upregulation of protease activity that degrades HSH2. In conclusion, these studies reveal that HSH2 expression is regulated via a NF-κB-dependent post-translational mechanism that involves changes in the expression, or activity of a protease, or both.

RESULTS

Differential regulation of HSH2 expression in B cell subsets does not correlate with changes in HSH2 mRNA levels.

Because HSH2 is expressed at high levels in B1 B cells and at low levels in GC B cells, and is inversely correlated with the production of class-switched antibodies (5), experiments were performed to determine if differences in HSH2 protein expression in distinct B cell subsets could be explained by changes in HSH2 mRNA levels. To compare HSH2 mRNA expression between B cell subsets, FACS sorting was used to isolate follicular (Fo), MZ, GC and B1 B cells from the spleens (Fo, MZ, and GC) and peritoneal cavity (B1) of C57BL/6 mice. B cells were then treated with Trizol reagent for mRNA extraction. The expression of HSH2 mRNA in each subset was measured using qRT-PCR. The ratio of HSH2 mRNA relative to the control mRNAs for HPRT, B2M, ribosomal 18s, or CD45 was determined and normalized to Fo B cell HSH2 mRNA expression (Fig. 1, A-D).

Although clear differences in HSH2 expression at the protein level have been shown previously such that the subpopulations can be ranked from high level to low level HSH2 expression (B1>MZ>Fo>GC) (5), the analysis failed to reveal a significant correlation with the level of HSH2 mRNA detected in the distinct subpopulations. Although there were slight variations in the amount of HSH2 mRNA observed in certain subpopulations compared to Fo B cells, depending on the control used to standardize the qRT-

PCR reaction between B cell subsets, in most instances the differences were not statistically significant and they were not consistent across the different qRT-PCR controls used (Fig. 1 A-D). As a positive control to assess the efficacy of the qRT-PCR reaction, total

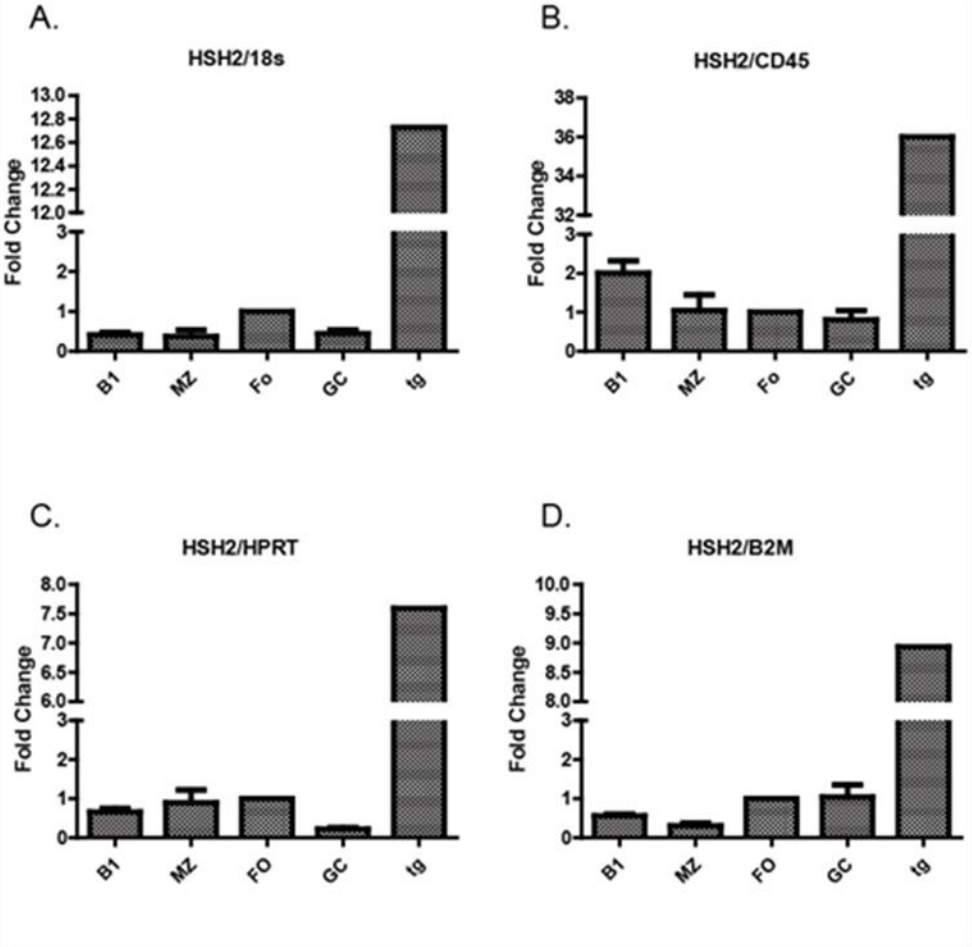

Figure 1. HSH2 mRNA levels do not correlate with HSH2 protein expression. B1 B cells were isolated from the peritoneal cavity, and marginal zone (MZ), follicular (FO), and germinal center (GC) B cells were sorted from spleens of wild-type mice. mRNA levels of HSH2 were quantified using qRT-PCR relative to 18s mRNA (A) CD45 (B) HPRT (C) and B2M (D) and normalized to Follicular B cell HSH2 mRNA levels. HSH2 mRNA from HSH2-Tg mice was used as a positive control for HSH2 primers. Bars represent means +/- SEM, n=3.

splenic B cells were isolated from HSH2 transgenic (Tg) mice and the level of HSH2 mRNA was measured by qRT-PCR (Fig.1, A-D). The results obtained support the conclusion that differential expression of HSH2 across B cell subsets is not regulated at the level of transcription.

TLR-mediated upregulation of HSH2 protein expression does not correlate with an increase in HSH2 mRNA expression.

Because differential HSH2 expression at the protein level does not correlate with changes in mRNA across different B cell subsets, experiments were performed to determine if TLR-mediated upregulation of HSH2 expression in splenic B cells occurs independent of changes in HSH2 mRNA. Splenic B cells were isolated from C57BL/6 mice and were treated either with LPS (Fig. 2) or CpG DNA (Fig. 3) to induce HSH2 expression and HSH2 protein levels were compared to HSH2 mRNA levels over time.

Following treatment of B cells with LPS, HSH2 expression steadily increased over 24 hours as measured by Western blotting using β-actin as a loading control (Fig. 2 A, B). In contrast to increased HSH2 expression at the protein level, HSH2 mRNA did not exhibit a significant change between 15 min and 24 h (Fig. 2 C). In contrast, following LPS treatment, CD69 expression increased within 4 h and reached a maximum at 12h as determined flow cytometric staining (Fig. 2 D). In parallel, CD69 mRNA increased by 4 h before decreasing back to pre-treatment levels (Fig. 2 E). Inducing HSH2 expression through TLR9 using the agonist CpG DNA produced results similar to those observed following LPS stimulation (Fig. 3). The increase in HSH2 protein expression was not associated with an increase in HSH2 mRNA expression (Fig. 3 A-C), whereas increased

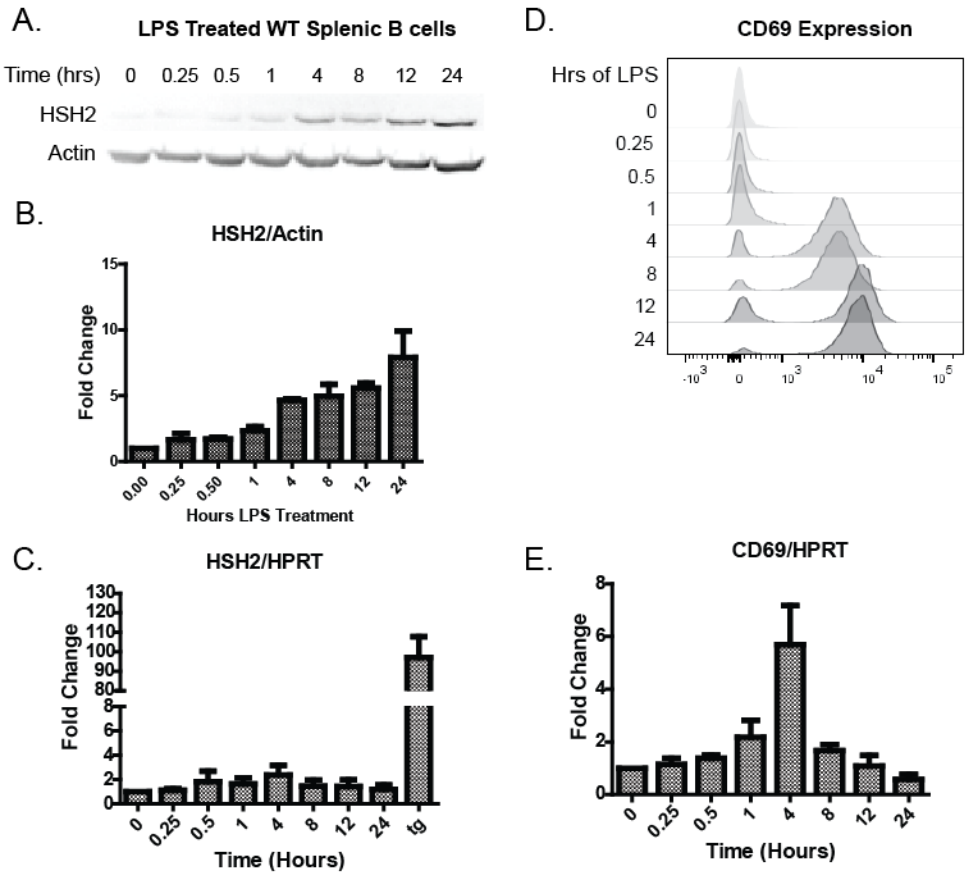

Figure 2. Induced HSH2 expression does not correlate with mRNA level in response to LPS treatment. Splenic B cells treated with 5µg/ml of LPS over 24 hours were lysed and HSH2 protein expression was analyzed using Western blot (A). HSH2 to actin ratios were measured and normalized to non-treated levels (B) and HSH2 mRNA levels relative to HPRT were measured and normalized to nont-treated levels (D). HSH2-Tg B cell mRNA used as a positive control for HSH2 primers (D). The B cell activation marker, CD69 was analyzed using flow cytometry (C), and qRT-PCR (E). Bars represent means +/- SEM. This figure is representative of 3-5 repeated experiments.

CD69 protein expression was correlated with an increase in mRNA (Fig. 3, D, E). These data suggest that increased HSH2 expression does not depend on transcription at the HSH2 locus leading to an increase in HSH2 mRNA expression.

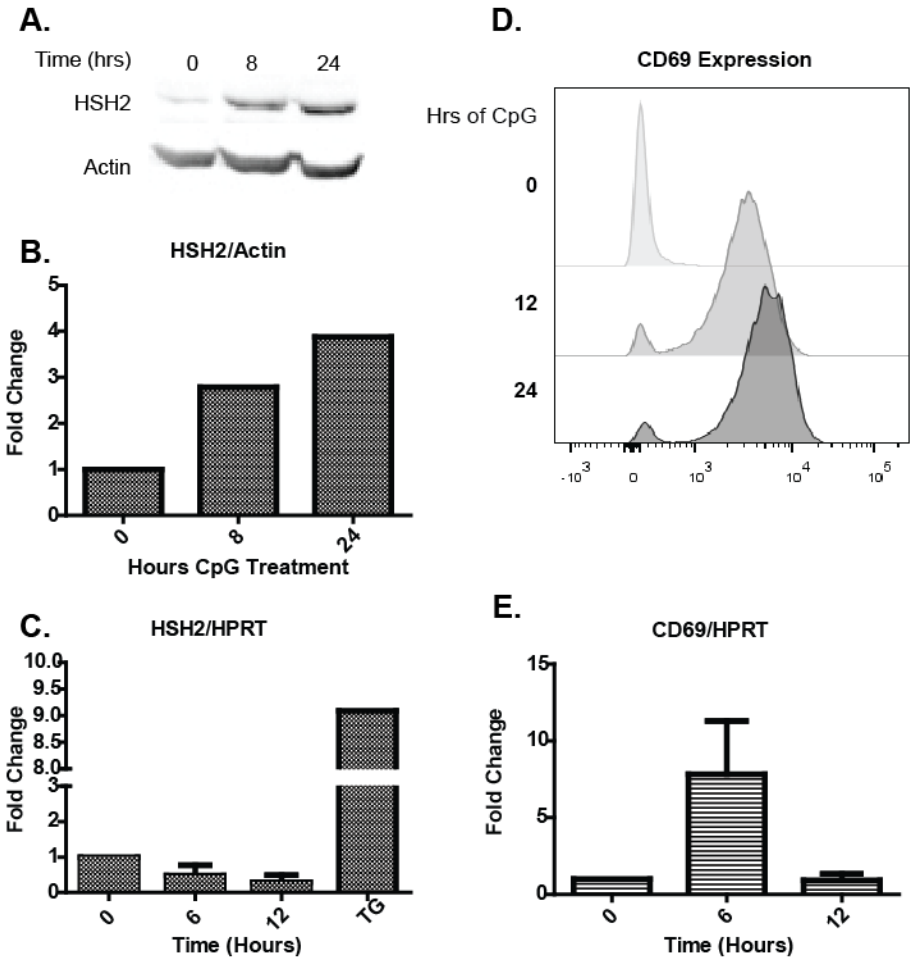

Figure 3. HSH2 Protein expression in response to CPG DNA does not correlate with an increase in HSH2 mRNA expression. Splenic WT B cells were treated with 10μg/ml CPG DNA for 24 hours, lysed at 0, 8, and 24 hours, protein expression was analyzed using Western blot (A). HSH2 levels relative to actin were measured and normalized to levels at 0 hours (B). CD69 expression for B cell activation was measured using Flow cytometry (C). HSH2 and CD69 mRNA levels were measured using qRT-PCR relative to non-treated B cells and normalized to mRNA levels at 0 hours (D, E). HSH2-Tg mouse mRNA was used as a positive control for HSH2 primers. Bars represent means +/- SEM. This figure is representative of 3 independent experiments.

Inhibition of NF-κB activity decreases HSH2 protein expression independent of HSH2 mRNA turnover.

Previous studies have shown that TLR and TNFR family members induce HSH2 expression via an NF-κB-dependent mechanism, and pretreatment of B cells with the NF-κB inhibitors PDTC and Bay 11-7082 prevents HSH2 upregulation in response to TLR or TNFR agonists (6). Yet as demonstrated in Figures 2 and 3, TLR-mediated upregulation of HSH2 expression is not associated with an increase in HSH2 mRNA, indicating that HSH2 expression may be regulated indirectly through activation of the NF-κB pathway at the translational or post-translational level (6). Relevant to this point is the fact that B1 B cells exhibit the highest level of HSH2 expression of any B cell subpopulation and exhibit elevated baseline NF-κB activity (5,15-17), yet B1 B cells do not contain levels of HSH2 message that are significantly higher than Fo or GC B cells, which express relatively little HSH2 protein (5). Thus, it is possible that NF-κB activity may be essential for promoting not only upregulation of HSH2, but also its maintenance by promoting increased translation of HSH2 mRNA or increased stability of HSH2 protein, or both. In order to determine if NF-κB activity is required for maintenance of HSH2 protein expression, experiments were performed in which NF-κB is inhibited in B cells expressing high levels of HSH2.

Splenic B cells were harvested from HSH2 transgenic (Tg) mice, in which all peripheral B cells express HSH2 at levels comparable to those observed in B1 B cells (5). In parallel, splenic B cells were harvested from C57BL/6 mice and were stimulated with LPS overnight to induce HSH2 expression. Subsequently, HSH2-expressing B cells were

incubated with the NF-κB inhibitor PDTC, cycloheximide (CXD) or Actinomycin-D

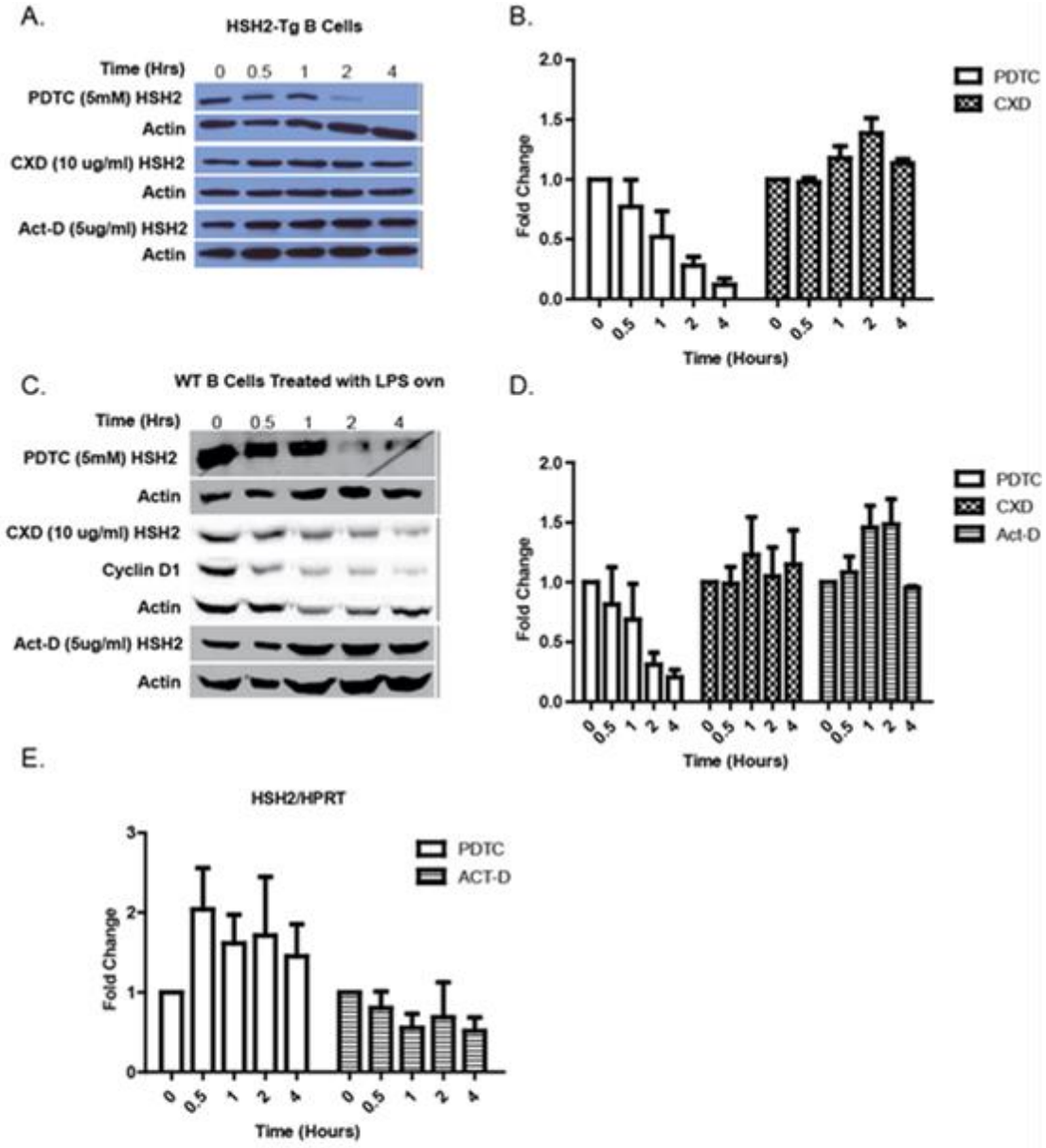

Figure 4. HSH2 expression is regulated via post-translational mechanisms. B cells from HSH2 transgenic mice were isolated from spleens and treated with PDTC (5mM), cyclohexamide (CXD, 10μg/ml) and actinomycin-D (Act-D, 5 μg/ml) over time. Cells were lysed and protein expression was analyzed with Western blot (A) and HSH2 protein to actin ratio was normalized to non-treated cells (B). This was repeated in B cells isolated from WT mouse spleens treated with 5 μg/ml LPS overnight to induce HSH2 expression before treatment with PDTC (5mM), CXD, (10 μg/ml) and Act-D, 5 μg/ml) overtime (C). HSH2 relative to actin protein levels were measured and normalized to non-treated cells (D). HSH2 mRNA was measured with qRT-PCR from PDTC and actinomycin-D treated B cells (E). Bars represent means +/- SEM. Blots and graphs are representative of 3-4 independent experiments.

(Act-D) to inhibit signaling via NF-κB, mRNA translation or transcription, respectively, for 30 min to 4 h (8,9).

Inhibition of NF-κB activity was observed to cause a rapid loss of HSH2 protein between 1-2 h in HSH2-Tg or LPS-treated B cells (Fig. 4A-D). In contrast, treatment with Act-D did not significantly alter HSH2 protein levels at 4 h (Fig. 4A-D). Similarly, treatment of B cells with CXD did not significantly affect HSH2 stability over the 4h treatment window. As a positive control for CXD, Cyclin D1 protein levels were measured and were shown to decrease significantly within 30 min (Fig. 4C). Importantly, treatment of B cells with PDTC did not cause a significant decrease in HSH2 mRNA, suggesting that NF-κB activity is not required for maintenance of HSH2 mRNA stability (Fig. 4E). These data are striking because they suggest that NF-κB-dependent transcriptional processes selectively regulate the rapid turnover of HSH2 at the protein level, whereas HSH2 is stable under conditions in which global transcription or translation are inhibited. Indeed, treatment of HSH2-Tg or LPS-treated B cells for up to 8 h with CXD or Act-D was not observed to significantly decrease the level of HSH2 detected (data not shown).

Changes in cellular Redox state do not appear to be correlated with degradation of HSH2. Although PDTC is used routinely as an NF-κB inhibitor, it is possible that the effects observed on HSH2 expression are due to off-target effects of PDTC, such as its ability to alter the Redox state of the cell (10,11). Changes in the Redox state of the cell have many implications for various signaling pathways including NF-κB, which can be activated in response to small concentrations of reactive oxygen species (ROS) (12-14,18-20)

and NF-κB in turn, influences the redox state of the cells itself (13,18). Therefore, experiments were performed to determine if other inhibitors of NF-κB exert the same effect on HSH2 expression and whether this occurs independent of alterations in the cellular Redox state.

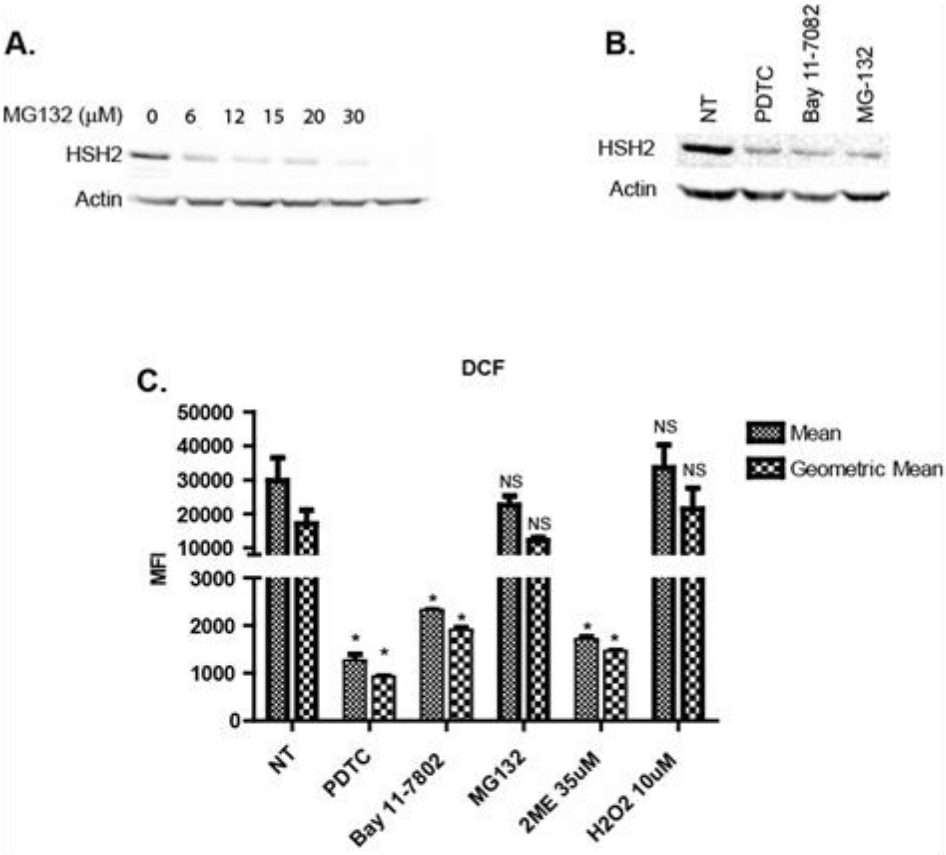

Figure 5. HSH2 degradation is independent of overall changes in the redox state of the cells. Splenocytic B cells were treated with increasing concentrations of the proteasome inhibitor MG-132 for 3 hours and cell lysates were analyzed using Western blot (A). NF-κB inhibitors PDTC (1mM), Bay 11-7082 (10μM), and MG-132 (6μM) were added to B cells isolated from WT spleens for 3 hours. Cell lysate was analyzed using Western blot (B). DCF was added to splenocytic B cells treated with or without NF-κB inhibitors and flourescence was measured by flow cytometry. Mean Flourescent Intensity and Geometric Mean Flourescent Intensity were measured and plotted in C. Statistical analysis was performed using a one-way Anova with Tukey's post-test with significance determined as $p<0.05$=* relative to non-treated samples. Bars represent means +/- SEM. Graph and blots are representative of at least 3 independent experiments.

Splenic B cells isolated from HSH2-Tg mice were treated with pharmacologic inhibitors of the NF-κB pathway, which exert their effects via different mechanisms. The proteasome inhibitor MG132, which blocks proteasome-dependent degradation of IκB, thereby inhibiting NF-κB activation (21,22), was used to treat B cells at different concentrations for 3 h after which HSH2 expression was monitored by Western blotting. As shown in Figure 5A, treatment with MG132 causes a complete loss of detectable HSH2 protein at a concentration of 30μM. Importantly, because MG132 prevents proteasome-dependent degradation of proteins, the results obtained demonstrate that turnover of HSH2 is not mediated via the proteasome. This suggests that a distinct proteolytic pathway is responsible for the rapid turnover of HSH2 following inhibition of NF-κB. Further experiments demonstrated that the NF-κB inhibitor Bay 11-7082 also causes a significant decrease in HSH2 expression when used to treat B cells for 3 h (Fig. 5B).

Because PDTC has been shown to act as an antioxidant (23) experiments were performed to determine if the different inhibitors of NF-κB exerted a common effect on the Redox state of the B cells using 2′-7′-dichlorodihydrofluorescein (DCF) (which fluoresces in an oxidative environment) (24,25). As a control, cells were treated with either H2O2, or 2-mercaptoethanol (2-ME). After treatment for 3 h, cells were loaded with 10uM DCF for 30 minutes at 37^{o}C and then analyzed by flow cytometry. PDTC was indeed observed to act as a strong antioxidant compared to untreated cells, as was the NF-κB inhibitor Bay 11-7082 (Fig. 5C). In contrast, treatment of B cells with MG132 did not affect the Redox state of the cells, yet it was equally effective in terms of causing a decrease in HSH2 expression (Fig. 5C). Moreover, treatment of B cells with 2-ME, which results in a decrease in the Redox state equivalent to that observed following treatment

with PDTC and Bay 11-7082 does not affect the expression of HSH2 (data not shown). Thus, it appears that inhibition of NF-κB results in a significant decrease in HSH2 expression independent of changes in the Redox state of the cell.

A functional NF-κB pathway is required for HSH2 expression. Although data using a range of pharmacologic inhibitors supports the conclusion that NF-κB activity is required for upregulation and maintenance of HSH2 expression, this approach does not categorically prove that degradation of HSH2 was due to the direct inhibition of NF-κB as opposed to off target effects of the particular inhibitors used. Therefore, a genetic approach was utilized to inhibit the NF-κB pathway. Mx-Cre mice, which were first described by Klaus Rajewsky (26) were bred to Nemo floxed mice (a kind gift from the laboratory of Dr. Christopher Klug, UAB) (27) to inducibly delete NEMO after treatment with INFα. Splenic B cells were isolated from $Nemo^{F/F}$, Mx1-Cre^{+} and $Nemo^{F/F}$, Mx1-Cre^{-} littermate-controls. B cells were placed in culture media with 5ug/ml LPS for 48

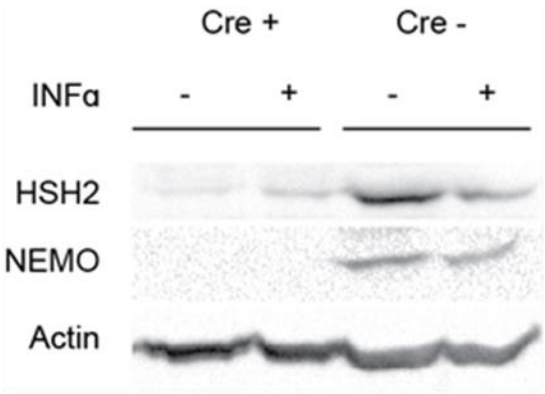

Figure 6. HSH2 expression is dependent on functional NF-κB signaling. Deletion of NEMO inhibits NF-κB. B cells from $NEMO^{F/F}$ x Mx1-Cre mice were isolated from spleens and cultured in media for 48 hours with LPS, with or without INFα. B cells from Cre-negative littermate controls were treated in a similar manner before Western blot analysis for HSH2, NEMO, and actin. Data is representative of 2 independent experiments.

hours with or without INFα, then processed for Western blot analysis to monitor HSH2 expression. To determine if NEMO had been deleted, Western blotting with anti-IKKγ mAb was performed (Fig. 6). It was observed that B cells in which NEMO had been deleted, LPS treatment failed to upregulate HSH2 expression at a level comparable to that in control B cells (Fig. 6). This finding further supports the conclusion that a functional NF-κB pathway is necessary for upregulation/maintenance of HSH2 expression.

Maintenance of HSH2 expression requires NF-κB translocation to the nucleus and changes in gene transcription. Because HSH2 expression is not directly regulated at the level of transcription, and because the IKK complex could potentially phosphorylate unknown targets that in turn regulate HSH2 stability at the protein level, experiments were performed to determine if the NF-κB inhibitors used uniformly block activation of the IKK complex thereby inhibiting downstream phosphorylation events. Splenic B cells were treated with and without the inhibitors PDTC or MG132 for 3 h prior to treatment with LPS in order to induce NF-κB activation. Downstream analysis of phosphorylation events using Western blot analysis showed that pretreatment with MG132 did not inhibit phosphorylation and activation of IKKα/β leading to subsequent phosphorylation of IκBα compared to cells treated with LPS alone (Fig. 7A). In contrast, pretreatment with PDTC blocked IKKα/β phosphorylation and activation of kinase activity based on inhibition of IκBα phosphorylation. However, both inhibitors blocked NF-κB-dependent transcription of the target genes IκBα and Myc, supporting the conclusion that both inhibitors prevent NF-κB subunit translocation to the nucleus and subsequent gene transcription (Fig. 7B). These data support the conclusion that activation of the NF-κB pathway does not promote increased stability of HSH2 at the protein level through an indirect

phosphorylation-dependent mechanism mediated by IKKα/β. Rather, NF-κB-dependent transcription indirectly regulates HSH2 expression by altering the expression of one or more proteins.

Inhibition of NF-κB results in the upregulation of protease expression or activity that in turn degrades HSH2. Studies demonstrate that HSH2 protein is rapidly degraded with a half-life between 1-2 h following inhibition of NF-κB. In contrast, HSH2 is stable for up to 6-8 h when cells are treated with CXD or Act-D to inhibit translation or transcription. Therefore, it is possible that NF-κB-dependent changes in gene transcription represses the expression or activity of a protease that targets HSH2 for degradation. To d

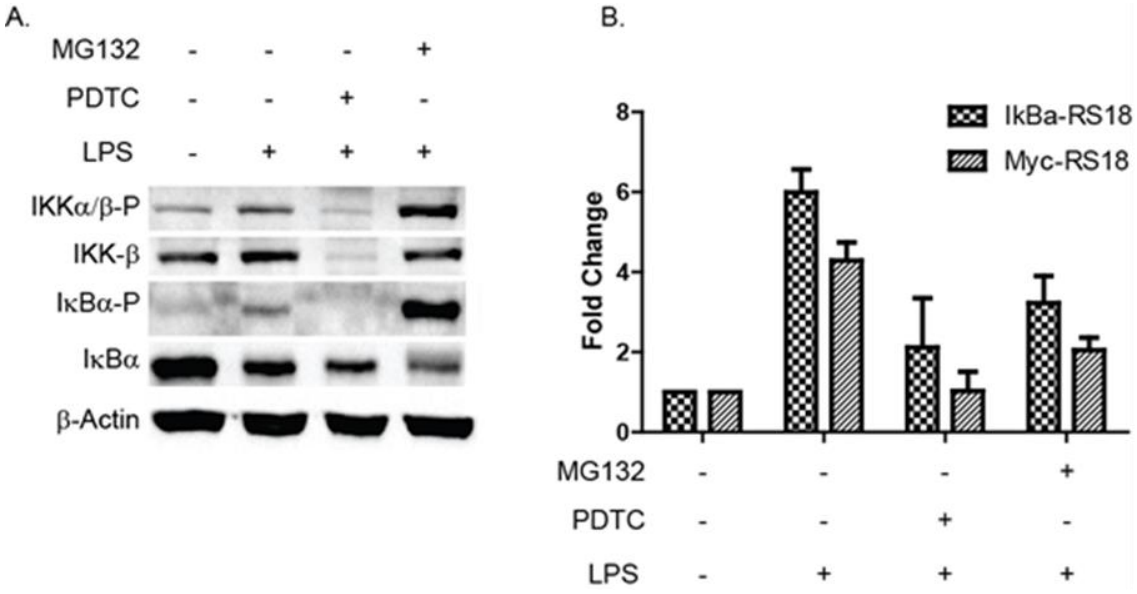

Figure 7. HSH2 maintenance is dependent on a functioning NF-κB pathway independent of upstream phosphorylation events. Although PDTC treatment inhibited IκBα phosphorylation, MG-132 had no inhibitory effect on phosphorylation events in the NF-κB pathway in response to LPS (A). Treatment with MG-132 or PDTC can prevent NF-κB activation as measured by IκBα or Myc mRNA levels relative to ribosomal subunit 18s mRNA (RS18) and normalized to non-treated cells (B). Bars in B represent means +/- SEM; n=3. Western blot is representative of 3 individual experiments.

etermine if a protease is indeed responsible for the degradation of HSH2, HSH2 was immunoprecipitated from splenic B cells isolated from HSH2-Tg mice, which has a FLAG epitope attached at the carboxyl-terminus (5) using anti-FLAG M2 magnetic beads. Immunoprecipitated HSH2 was incubated at 37°C with lysate from WT B cells, which had been treated either with or without PDTC. Protease inhibitors were added to a duplicate

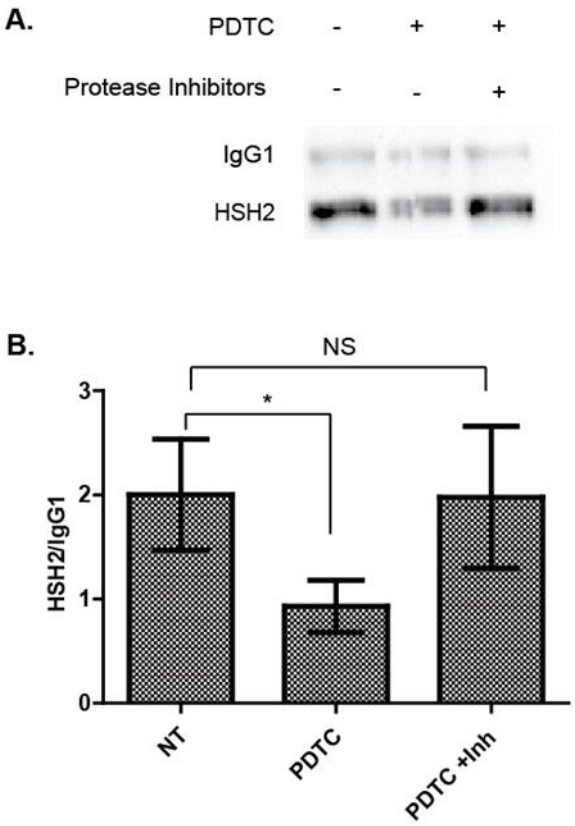

Figure 8. HSH2 degradation is dependent on a protease. HSH2 was immunoprecipitated from HSH2-Tg B cells and treated with lysate from B cells treated with or without PDTC and lysed in buffer with or without protease inhibitors. Western blot is representative of 8 independent experiments (A). Relative levels of HSH2 to IgG1 were graphed and analyzed with Graphpad Prism (B). Significance was determined using an unpaired one-tailed t-test; $p<0.05$= *, bars represent means =/- SEM.

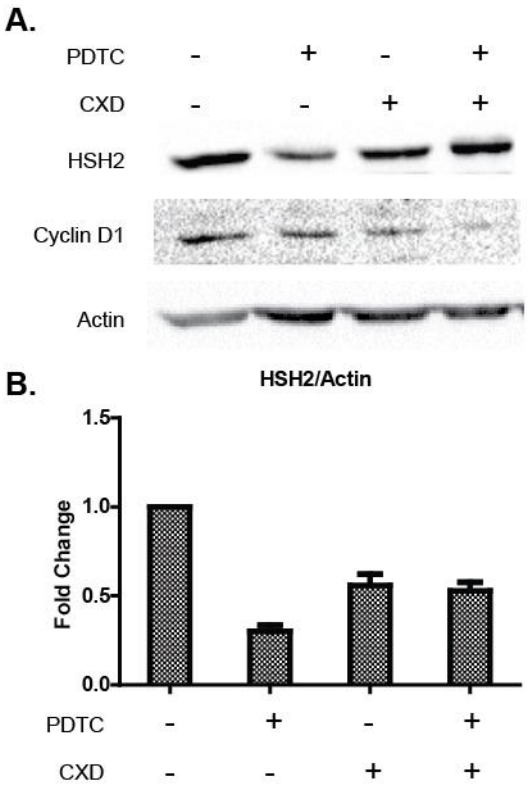

Figure 9. Cyclohexamide attenuates degradation of HSH2 from NF-κB inhibition. Western blot analysis of HSH2 in cells either non-treated (NT), PDTC treated (2mM, 3hrs), CXD treated (50μg), or pretreated with 50μg CXD before treatment with 2mM PDTC (3hrs) (A). HSH2 to actin ratios normalized to NT are depicted in B. Bar graphs represent means +/- SEM. Western blot is representative of 3 independent experiments.

sample in which PDTC treated lysate was added to HSH2 beads to determine if inhibition of protease activity would prevent HSH2 degradation in this cell-free environment. Incubation of HSH2 beads with lysate from PDTC treated B cells was observed to result in degradation of HSH2 compared to lysate from B cells treated with vehicle. Addition of protease inhibitors prevented degradation of HSH2 beads incubated with lysate from PDTC treated B cells. These data support the conclusion that inhibition of NF-κB leads to increased expression or activity of a protease that targets HSH2 for degradation.

Inhibition of translation blocks HSH2 degradation following inhibition of NF-κB.

To further confirm that inhibition of NF-κB leads to changes in gene transcription that promote upregulation of proteolytic activity that targets HSH2 for degradation, experiments were performed in which B cells were pretreated with CXD to block mRNA translation to determine if this would prevent the effect of PDTC. Splenic B cells from HSH2-Tg mice were isolated and pre-treated with 50 μg/ml CXD for one hour before addition of 2mM PDTC for an additional 3 hours. Western blotting was performed on cell lysates from B cells treated with CXD and PDTC compared to cell lysates from B cells treated with PDTC or CXD alone, or left untreated to quantitate changes in HSH2 expression (Fig. 9A). The decrease in HSH2 expression in B cells treated with PDTC alone was significantly decreased by pretreatment of cells with CXD, suggesting that inhibition of NF-κB activity alters gene expression leading to an increase in protease expression or activity or both.

DISCUSSION

Previous studies have shown that HSH2 is differentially expressed in peripheral B cell subpopulations such that B1 B cells exhibit the highest expression observed in any subpopulation followed by T2 B cells and MZ B cells (5). In contrast, Fo B cells and GC B cells express relatively low levels of HSH2. Thus, there is an inverse correlation between HSH2 expression and the propensity for a particular B cell subpopulation to undergo class switching and differentiation into class-switched ASCs. Studies in which

HSH2 expression has been manipulated in the B cell lineage to enforce expression comparable to B1 B cells in all peripheral B cell subpopulations have revealed that high level HSH2 expression significantly attenuates the production of class-switched Abs in response to challenge with T-dependent and T-independent antigens (5). These findings suggest that differential expression of HSH2 within the B cell lineage plays an important role in regulating the nature of the humoral immune response. Therefore, studies were performed to further characterize the molecular mechanisms by which HSH2 expression is regulated.

Studies presented herein demonstrate that differential expression of HSH2 at the protein level in distinct B cell subpopulations does not correlate with differences in HSH2 mRNA expression (Fig. 1). Furthermore, treatment of splenic B cells with TLR agonists, which have been shown to upregulate HSH2 expression, is not correlated with an increase in HSH2 mRNA (Fig. 2 and 3). These findings support the conclusion that changes in HSH2 expression are regulated post-transcriptionally.

The overall results indicate that although HSH2 is regulated dynamically at the cytosolic level between B cell subsets and in B cells treated with activating factors, transcription appears far less likely to be directly involved, despite the dependence of HSH2 expression on the transcription factor NF-κB. When overall transcription and translation activities are prevented with the addition of Act-D or CXD, HSH2 protein expression remains relatively unchanged compared to its expression after inhibition of NF-κB. This is likely not due to nonspecific effects of the NF-κB inhibitor, such as a change in redox or activation of caspases or other nonspecific targets as demonstrated by the use of multiple NF-κB inhibitors as well as through directly targeting the NF-κB pathway by floxing out

NEMO. Together, these data suggest very strongly that HSH2 expression is dependent on fully functional NF-κB signaling, although HSH2 does not appear regulated at the level of transcription or translation. These data also suggest that regulation of HSH2 independent of transcription or translation are not due to non-specific phosphorylation activity of the IKK complex.

Previous research has shown that HSH2 expression was dependent on the NF-κB pathway by using PDTC and Bay 11-7082 to inhibit the NF-κB pathway and by using several activators of B cells that activate the NF-κB pathway as well (6). These experiments seemed to show a direct correlation to HSH2 expression and NF-κB activity. However, BCR signaling did not induce HSH2 expression, despite simultaneous activation of NF-κB, and it has been shown that PDTC and Bay 11-7082 have effects non-specific to inhibition of NF-κB, including effects on the oxidation state as shown by changes in glutathione levels (10,11,28). Furthermore, NF-κB is active in GC B cells (14) while HSH2 is low (5). Therefore, it seemed plausible that HSH2 could be regulated by other ways than NF-κB. This seemed especially plausible after the discovery that transcriptional regulation does not appear important to the expression of HSH2.

Here we have shown that redox does not appear responsible in the regulation of HSH2, and that HSH2 is dependent on functional canonical NF-κB activity for expression. This implies that the effects of PDTC and Bay 11-7082 on HSH2 expression were due to inhibition of NF-κB and not due to nonspecific targets of these drugs. The low expression of HSH2 in GC B cells despite the activation of NF-κB in these cells indicate that degradation of HSH2 is not solely dependent on NF-κB activity, but can be employed by other pathways in GC B cells, perhaps as part of the process of preparing to

produce class-switched antibodies. However, due to the dependence HSH2 has on NF-κB activation, we suggest a model of HSH2 regulation where a protease targeting HSH2 is constitutively active but downregulated when N-FκB is activated. NF-κB could down-regulate this protease in a number of different ways, such as upregulating another protease targeting the destabilizer of HSH2, or by interfering with the transcription of that protease targeting HSH2.

This analysis concentrated on the regulation of HSH2 at the levels of transcription, translation, and protein turnover, as well as a potential mechanism regarding the role of NF-κB on HSH2 expression. Future insights into the pathways HSH2 interacts with and the proteins it binds to will further elucidate its role in the production of class-switched antibodies as well as shed further light on mechanisms by which HSH2 is regulated.

EXPERIMENTAL PROCEDURES

B cell sorting and isolation.

To analyze differences in regulation between B cell subspopulations, B cells were sorted using FACS Aria. B1 cells ($CD19^{Hi}$ IgM^{Hi} $CD23^{Lo}$ $CD11b^{Int}$) were isolated from a peritoneal lavage. Purity was measured using flow cytometry at 90-95% purity. Marginal zone and follicular cells were isolated from spleens of wild-type C57-Bl/6 mice sorting $CD19^{Hi}$ $CD23^{Lo\text{-}Int}$ $CD21^{Hi}$ for marginal zone B cells and $CD19^{Hi}$ $CD23^{Int\text{-}Hi}$ $CD21^{Lo}$ for follicular cell isolation. Germinal center B cells ($B220^{Hi}$ $CD95^{Hi}$ $Gl7^{Hi}$) were isolated from C57-BL/6 mice immunized with sheep red blood cells (Colorado Serum

Company) for 7-10 days. Spleens of immunized mice were homogenized and red blood cells were lysed using AKC red blood cell lysis buffer before staining.

Splenic B cell isolation.

For experiments not requiring specific B cell subsets, B cells were isolated through complement mediated lysis of splenocytes. Spleens from C57-BL6 mice were homogenized between glass microscope slides and treated with AKC red blood cell lysing buffer on ice for 1 minute. White blood cells were then incubated for 20 minutes at 37^{o}C with anti-Thy1.2 (clone HO13) and anti-Thy1.1 (clone T24) antibodies, 50μg/ml rabbit complement, 25M MgCl2, 1M CaCl2, and 20μg/ml DNAse in order to lyse non-B cells. Cells were centrifuged over a histopaque-1077 gradient (Sigma) at 200g. Cells were washed once with PBS, then incubated at 37^{o}C in RPMI-1640 media with 5% FBS for 30 minutes at specified concentrations and used for experiments.

Quantitative real-time PCR analysis.

To extract mRNA of B cells, isolated B cells were treated with TRIzol reagent (Life Technologies) and processed according to protocol. mRNA was extracted from B cells and used as a template for cDNA using Applied Biosystems High Capacity cDNA Reverse Transcription kit. cDNA was subjected to qRT-PCR using the following primers: HSH2 (forward (F) 5'- acttgtgccttcgctgg-3', reverse (R) 5'gagat-cctcatagt-ccacgtttg-3'); HPRT (F 5'-actgtaatgatcagtcaacggg-3', R 5'-acttcgagaggtccttttcac-3'); beta-2 micro-globulin (F 5'-ctggtctttctatatcctggctc-3' and R 5'-tgcttgatcacatgtctcgat-3'), CD69

(F 5'-ttgccttaaa-tgtgggcaagt-3', and R 5'gcaacatggtg-gtcagatgatt-3'); RS18 (F-5'-gga-gaactcacggaggatgag-3', and R 5' cgcagcttgttgtctagaccg-3'); and CD45 (F-5'-gagaatgc-ccttcttctgcct-3', and R 5'-gtctgagtgtcagctcctcc-3'). Relative HSH2 levels were calculated using B2M, HPRT, RS18 and CD45 using the 2-ΔΔCT method (29). NF-κB activity was analyzed using NF-κB targets Myc1 (F 5'-atgcccctcaacgtgaacttc-3', R 5'-gtcgca-gatgaaatagggctg-3', IκBα (F 5'-tgaaggacgagga-gtacgagc-3', R 5'-ttcgtggatgattgccaagtg-3', and CH2P1 (F 5'-tttgaatccagccaacagtctg-3', R 5'-tgatttgctcggaagtt-cacag-3) and ratioed the mRNA of the target genes to B2M or HPRT or Actin (F 5'-gctctggctcctagcaccat-3', R 5'-gccaccgatccacacag-agt-3').

Western blotting.

To analyze the content of specific proteins in cells, Western blot analysis was used. Cells were harvested from C57-Bl/6 mice and B cells were isolated as described above and lysed in M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher) at concentrations of $1x10^8$ cells/ml. B Cells from wild-type C57-Bl/6 mice were loaded at concentrations of $3\text{-}5x10^6$ cells per well. B cells isolated from HSH2 transgenic mice were loaded at concentrations of $3\text{-}5x10^5$ cells per well. Using 10% acrylamide gels, cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes and blocked with 1% milk for one hour before incubation with 3ε1 anti-HSH2 primary anti-body overnight. After extensive washing in TBS-T, membrane was then probed with goat anti-mouse IgG1-HRP secondary (Southern Biotech). Blots were washed in TBS-T and treated with pico or femto ECL (Pierce) and then exposed to film or Biorad GelDoc XR system. Blots were then stripped using RestoreTM stripping buffer (Thermofisher),

blocked in 3% milk and probed with anti-β–actin antibody (Abcam). HSH2/actin ratios were measured for analysis using NIH Image J.

For analysis of phosphorylated NF-κB pathway proteins, all antibodies (against IKKy 2658S; IKKβ 8943S; IKKα/β-P 2697S, IκBα 4814S, IκBα-P 2859S) were purchased from Cell Signaling and diluted in 5% bovine serum albumin rather than milk according to protocol. Cell lysates were processed as above except phosphatase inhibitors sodium orthovanadate (10mM) (Sigma), EDTA (1mM), and sodium fluoride (50mM) were added to M-PER lysis buffer to inhibit phosphatase activity. Protein concentration was measured using BCA Protein assay (Pierce) and equal amounts of total protein was loaded per sample.

Relative redox state analysis.

To determine if the effects of PDTC treatment on HSH2 were due to inhibition of NF-κB or by its redox affecting properties, B cells were isolated from the spleens of C57-BL6 mice according to protocol previously stated and treated with NF-κB inhibitors PDTC, Bay 11-7082, and MG-132; which have been shown to inhibit HSH2 expression; as well as H202 (an oxidant) and 2-Mercaptoethanol (an antioxidant) for relative redox state controls. After treatment for 3 hours, cells were washed 2X in ice cold PBS and treated with 10μM DCF (Molecular Probes) 30 minutes at 37oC, then washed again with PBS twice before analysis by flow cytometry on a BD-LSRII. Median fluorescent intensity was measured on the FITC channel and compared using Flowjo software (Tree-star™).

HSH2 Immuno-precipitation.

For analysis of HSH2 in a cell-free system, B cells were isolated from HSH2-Tg mice (5) as described above, lysed, and incubated with anti-FLAG-M2 magnetic beads (Sigma) overnight at 4^{o}C before incubation with lysate from wild-type B cells treated with or without 1mM PDTC. The beads bound to HSH2 were treated with the lysate at 37^{o}C for 2 hours with and without protease inhibitors (cOmplete ULTRA Tablets, Mini, EASY pack from Roche). Beads were then washed and placed in sample buffer to analyze amount of HSH2 left on the beads with Western blot.

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CD19 CYTOPLASMIC TYROSINE RESIDUES Y482 AND Y513 ARE SUFFICIENT TO PROMOTE NORMAL B CELL DEVELOPMENT AND DIFFERENTIATION BUT INDUCE A SKEWED HUMORAL IMMUNE RESPONSE

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ABSTRACT

CD19 amplifies BCR signal transduction by recruiting SH2 domain-containing proteins to its nine cytoplasmic phosphorylated tyrosine residues. Previous studies have shown that Y482 and Y513, which recruit PI3K are required for normal B cell development/differentiation and a productive T-dependent humoral response. However, it has not been determined if CD19 Y482 and Y513 are sufficient to promote normal B cell differentiation or antibody response to immunization. Furthermore, the effects of upstream CD19 tyrosine-mediated Vav/PLCγ2 and Grb2/Sos signaling are not well characterized. Using mice expressing CD19 mutated to only express tyrosines Y482 and Y513, we demonstrate that CD19 mediated Lyn/PI3K signaling is sufficient for B1 B cell development, marginal zone B cell maturation, and germinal center B cell formation. However, these mice exhibit an increased antibody response against T-independent antigens and are impaired in their ability to generate class-switched germinal center B cells during a T-dependent immune response, as well as long-lived, class-switched antibody secreting cells. These findings suggest that cross-talk between signal effectors recruited to Y482 and Y513 and those recruited to other CD19 tyrosine residues is important for determining the qualitative nature of the humoral response.

INTRODUCTION

The humoral adaptive immune system is mediated by interaction by B cells and antigen through the B cell receptor (BCR). The BCR relies on other cell surface proteins (such as CD19) and adaptor proteins to activate signaling pathways involved in controlling the B cell's response to antigen. CD19 is an important potentiator of BCR signaling in B cells (1). CD19 interacts with a signaling complex with the complement receptor CD21 (CR2), the tetraspanin member CD81 (TAPA-1), and leu-1 (2, 3). When antigen is bound to complement and recognized by the BCR, CD21 recognizes the complement protein and brings CD19 into proximity with the BCR (2, 3). Src family kinases then phosphorylate tyrosine residues on the cytoplasmic tail of CD19, allowing specific SH2 domain-containing proteins to bind to CD19 and mediate downstream signaling pathways (4).

In vivo mouse studies have illustrated the importance of CD19 mediated signaling. Mice that over-express CD19 demonstrate increased response to BCR signaling as well as develop autoantibodies and rheumatoid factor (5-7). C57-BL6 $CD19^{-/-}$ mice have difficulty developing marginal zone (MZ) B cells, B1 B cells in the peritoneal cavity, and germinal center (GC) B cells (8-12). These mice are unable to mount an effective antibody response against T-cell dependent antigens (9). Although early studies have shown T-cell independent reactions to be independent of CD19, it appears that T-cell independent antigens with low epitope density or low BCR affin-ity are affected by CD19 signaling (4, 8, 13).

CD19 signaling depends on phosphorylation of its nine cytoplasmic tyrosines (14, 15). Most of the nine cytoplasmic tyrosines in CD19 and their surrounding amino acids are conserved between mice and humans (14). These tyrosines associate with SH2 domain-containing signaling molecules important for B cell development and survival. These include Grb2 and Sos (which bind to Y330 through Grb2), Vav and Phosphlipase-Cγ2 (Y391 and Y421), Fyn (Y403, Y443), PI3-K (Y482, Y513) and Lyn (Y403, Y443, Y482, and Y513) (15-18). Together, these tyrosines with associated proteins mediate downstream signals that regulate B cell development, differentiation, and activation.

Many studies have been conducted to delineate the role of each tyrosine in vitro and in vivo. Despite the ability of CD19 to bind to several proteins essential for normal B cell activation, only Y482 and Y513 appear essential for CD19 function in mouse studies (4). Mice containing point mutations in tyrosines 482 and 513 of CD19 exhibit a similar phenotype to $CD19^{-/-}$ mice regarding T-cell independent antibody response, antigen-specific early and late GC B cell development, and the production of class-switched antibodies (4). Point mutations in CD19 affecting Grb2/Sos, Vav/PLCγ2, and Fyn signaling did not appear to greatly alter B cell responses in these mice compared to wild-type hemizygous CD19 mice (4). More recent studies have demonstrated the importance of CD19 mediated PI3-K signaling in mice (19, 20). Together these studies demonstrate the importance of CD19 Y482 and Y513 in regulating the B cell immune response. Although Y482/Y513 are essential, it not established if they are sufficient to retain a wild-type phenotype. Phosphorylation on upstream tyrosines in these mice in response to BCR ligation was diminished (4). The function of CD19 mediated Vav/PLCγ2 and Grb2/Sos signaling in B cells is uncertain.

Here we use mice homozygous for CD19 with tyrosines replaced with phenylalanine at Y330, Y360, Y391, Y403, Y421, and Y443, leaving Y482 and Y513 intact (CD19 Y482/Y513) and analyze their immune response against T-independent and T-dependent antigens. We compare their phenotype to that of wildtype mice, $CD19^{-/-}$ mice, and mice with point mutations at Y482 and Y513 (CD19 Y482F/Y513F). We show CD19 Y482/Y513 mice develop normal B cell subpopulations and GC B cell numbers compared to wild-type mice. CD18 Y482/Y513 mice show an increased response to T-independent antigens but have difficulty producing antigen-specific class-switched antibodies to T-dependent antigens. B cells grown *in vitro* from these mice skewed toward producing more IgM GC B cells and fewer IgG GC B cells compared to wild-type. Together, these data demonstrate the importance of CD19 Y482/Y513 mediated signaling and suggest a possible regulatory role for CD19 mediated Grb2/Sos and Vav/PLCγ2 signaling in producing class-switched antibodies.

MATERIALS AND METHODS

ELISAs.

To determine antibody titers, five mice from each mouse strain were immunized with 50μg/ml NP-KLH (for T-dependent responses) or 50μg/ml TNP-KLH (T-independent responses) and serum was removed at various timepoints. Flat bottomed 96 well plates (Corning cat# 9018) were coated with 50μg/ml of either TNP-BSA or NP-BSA in coating buffer (8.41g Na2HCO3/L PBS, pH 9.5) overnight at 4^{o}C, washed, and incubated with blocking buffer before incubation with serial dilutions of serum from each mouse.

Plates were washed and incubated with HRP conjugated isotype antibodies from Southern Biotech. Washed plates were then incubated at 37°C for 20 minutes with TMB before addition of stopping buffer (0.5M H_2SO_4) and analysis with a spectrometer at 450nm.

ELISPOTS.

To observe the number of ASCs, spleens and bone marrow from mice were analyzed by ELISPOT analysis. Flat-bottomed 96 well plates (Corning) were coated with goat anti-mouse IgM or anti-IgG (Southern Biotech) for incubation overnight at 4°C, washed in PBS, then incubated in PBS-gelatin mixture (porcine skin, type A; Sigma) at 37°C for 1-2 hours. After washing, plates were incubated with cells from spleens and bone marrow diluted in RPMI with 10% fetal calf serum overnight at 37°C. Plates were washed 3x with PBS-Tween and incubated with either goat anti-mouse IgM bound to alkaline phosphatase (AP) or goat anti-mouse IgG-AP for one hour at 37°C. Plates were washed 3x with PBS-Tween before incubation with 5-bromo-4-chloro-3-indolyl-phosphate substrate at 4° overnight. Plates were then washed in distilled water 3x, allowed to dry and spots counted.

Flow cytometry, gating strategy and reagents.

Spleens and peritoneal lavage were taken from 5 mice from each strain of mice (WT, CD19 Y482/Y513, CD19 Y482F/Y513F, or $CD19^{-/-}$). 3 ml PBS were injected into the peritoneal cavity of each mouse and removed for staining. Spleens were then re-

moved and homogenized, red blood cells lysed, and remaining cells stained for flow cytometry. Peritoneal lavage was stained for B2 ($B220^{+}$, IgM^{+}, $CD23^{+}$), B1a ($B220^{+}$, IgM^{+}, $CD23^{lo}$, $Cd11b^{int}$, $CD5^{+}$) and B1b ($B220^{+}$, IgM^{+}, $CD23^{lo}$, $Cd11b^{int}$, $CD5^{lo}$) cells and spleens were stained for Fo ($B220^{+}$, IgM^{lo}, $AA4.1^{lo}$, $CD23^{+}$, $CD21^{lo}$), MZ ($B220^{+}$, IgM^{+}, $AA4.1^{lo}$, $CD23^{lo}$, $CD21^{Hi}$), T1 ($B220^{+,}$ $AA4.1^{Hi}$, IgM^{Hi} $CD23^{lo}$), T2 ($B220^{+}$, $AA4.1^{Hi}$, IgM^{Hi}, $CD23^{Hi}$), and T3 ($B220^{+}$, $AA4.1^{Hi}$, IgM^{lo} $CD23^{lo}$) cells.

To analyze the kinetics of germinal center B cell development, spleens were removed from mice immunized with 50μg NP-KLH and 50μg alum at days 4,7, 10, and 14 and prepared for staining as described previously. GC's were defined as B220+ IgD^{LO} $CD95(Fas)^{Hi}$, PNA^{Hi}, and either IgM^{Hi} or IgM^{lo}. NP specificity was determined by binding to NP-PE (Biosearch Technology). T_{FH} cells were defined as $B220^{-}$, $CD4^{+}$, $PD\text{-}1^{+}$, $CXCR5^{Hi}$.

The following antibodies and reagents were used for flow cytometry: anti-B220 conjugated to Pe-Cy7, PB, APC-Cy7 (BD Pharmingen); anti IgM-PE (Southern Biotech); anti-CD23-FITC (BD Pharmingen); anti-CD11b-PB (Biolegend); anti-CD5-PE (BD Pharmingen); anti-AA4.1 APC (eBioscience); anti-CD21-PB (eBioscience); anti-IgM-APC (Southern Biotech); anti-IgD-FITC (Southern Biotech); propidium iodide (PI) (Clontech); biotinylated PNA (Vector Laboratories); Streptavidin-PB (Invitrogen); anti-CD95-Pe-Cy7 (BD Pharmingen); NP-Pe (Biosearch Technology); anti-CD4-PB (BD Pharmingen); anti-PD-1-APC-Cy7 (Biolegend); anti-CXCR5APC (eBioscience); and Count-BrightTM Absolute Counting Beads (Invitrogen).

Cell Culture.

Purified B cells (5×10^5 cells per well) were cultured in a 6-well plate on the feeder cell line CD40LB (1×10^6 cells per well) (21) in 4 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, vitamins, 10 mM HEPES and 50 μM 2-mercaptoethanol). 2 ng/ml IL-4 (R&D Systems) was added to the primary culture for 4 days, and on day 4, the cells were re-plated onto a new feeder layer and cultured with medium only or IL-4 (2 ng/ml) or IL-21 (10 ng/ml, R&D Systems) for another 2 days. Cells were cultured in a humidified atmosphere at 37°C with 10% CO2.

RESULTS

CD19 Y482/Y513 signaling alone is insufficient to create a wild-type B cell subset phenotype.

$CD19^{-/-}$ and CD19 482F/513F mice have deficient B1 and MZ B cell populations (4, 8-11). CD19 Y482/Y513 mice retain Lyn/PIK3 recruitment while CD19 Y482F/Y513F mice do not, although CD19 Y482F/Y513F mice might retain Grb2/Sos and Vav/PLCγ2 recruitment, despite diminished phosphorylation in response to BCR ligation (4). These differences in signaling may affect B cell development at transitional stages of B cell development.

To determine if CD19 Y482/Y513 mediated signaling is sufficient to retain a wild-type phenotype for B1 and MZ cell populations, wild-type (WT) mouse B cell subpopulations in the spleen and the peritoneal cavity were compared to B cell populations

of CD19 Y482/Y513 mice, CD19 Y482F/Y513F mice, and CD19$^{-/-}$ mice. Spleens were harvested from these mice and B cell populations were analyzed by flow cytometry (Fig 1A). Graphs of absolute numbers of B cell subsets are displayed in Figure 1 (Fig 1B).

Production of Fo B cells appeared unaffected by CD19 (Fig 1C), while MZ B cells were present in extremely low numbers in CD19 Y482F/Y513F and CD19$^{-/-}$ as previously described (11). CD19 Y482/Y513 mice contained MZ B cells near WT levels, demonstrating CD19 Y482/Y513 mediated signaling is sufficient for MZ B cell development (Fig 1D). No significant difference in Transitional B cell populations was detected between WT and CD19 Y482/Y513 mice, or between CD19 Y482F/Y513F and CD19$^{-/-}$ mice T1, T2, or T3 subsets (Fig 1E). T1 B cells were fewer in number in the CD19 Y482F/Y513F and CD19$^{-/-}$ mice compared to the WT and CD19 Y482/Y513 mice. Together these data demonstrate that CD19 Y482/Y513 mediated signaling is sufficient and required for MZ and T1 B cell differentiation.

B1 B cell differentiation was shown to be affected by CD19 signaling (10). To determine if CD19 Y482/Y513 signaling is sufficient for normal B cell differentiation in peritoneal cavity populations, B cells in peritoneal lavages of WT, CD19 Y482/Y513, CD19 Y482F/Y513F, and CD19$^{-/-}$ mice were analyzed with flow cytometry (Fig 2A).

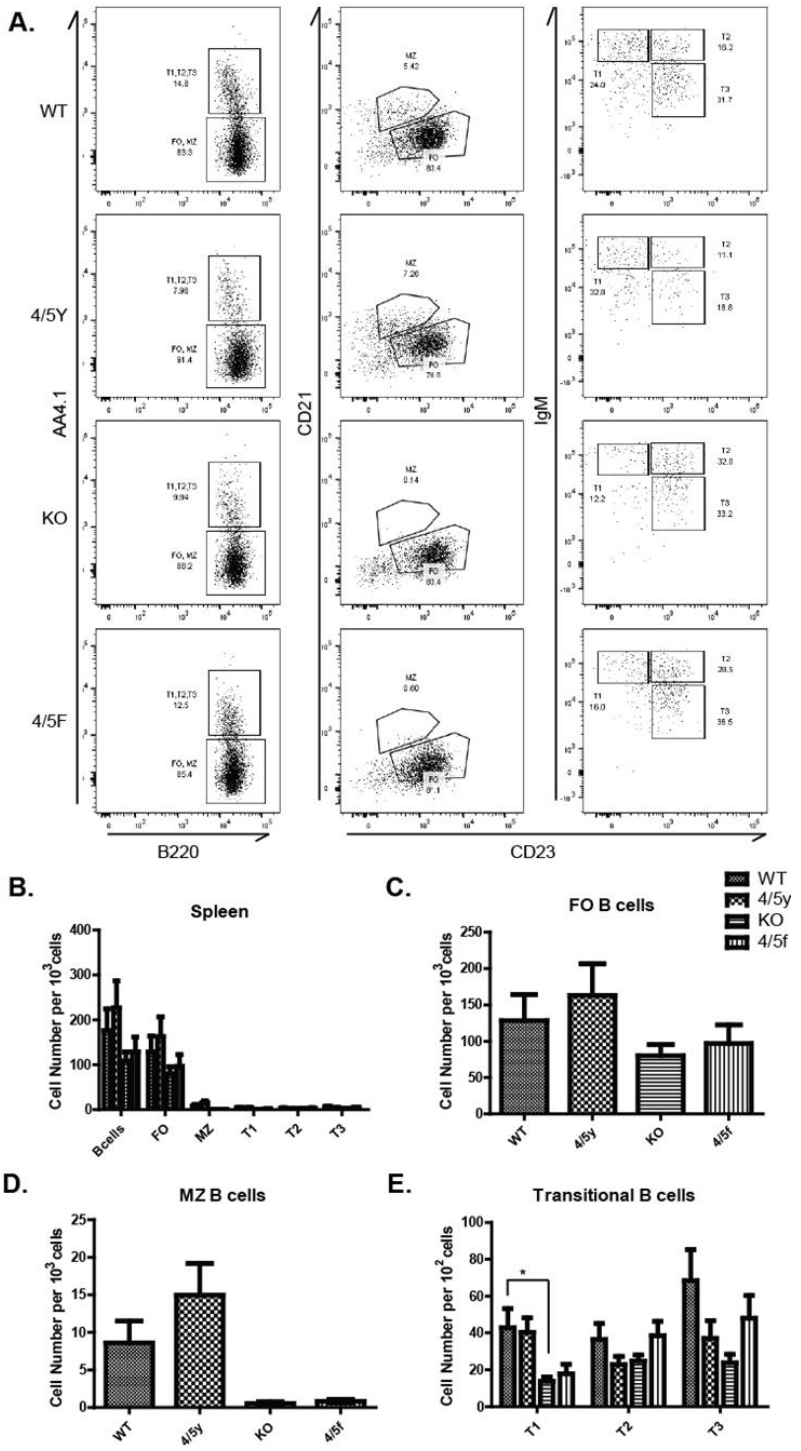

Figure 1. B cell subset differences in spleens of CD19 mutant mice. Gating strategy of live lymphocytes is shown in A. Total B cell subset cell numbers from spleens of WT, CD19 Y482/Y513 (4/5y), CD19 Y482F/Y513F (4/5f), and CD19$^{-/-}$ (KO) mice are plotted in these graphs. Total B cell subsets are in (B), with Fo B cells (C), MZ B cells (D), and transitional stage B cells (E) plotted in their respective graphs. Data is representative of two repeated experiments. Bars represent mean +/- SEM, statistics were performed using a one-way ANOVA with Tukey's post-test for comparison of groups. *=P<0.05

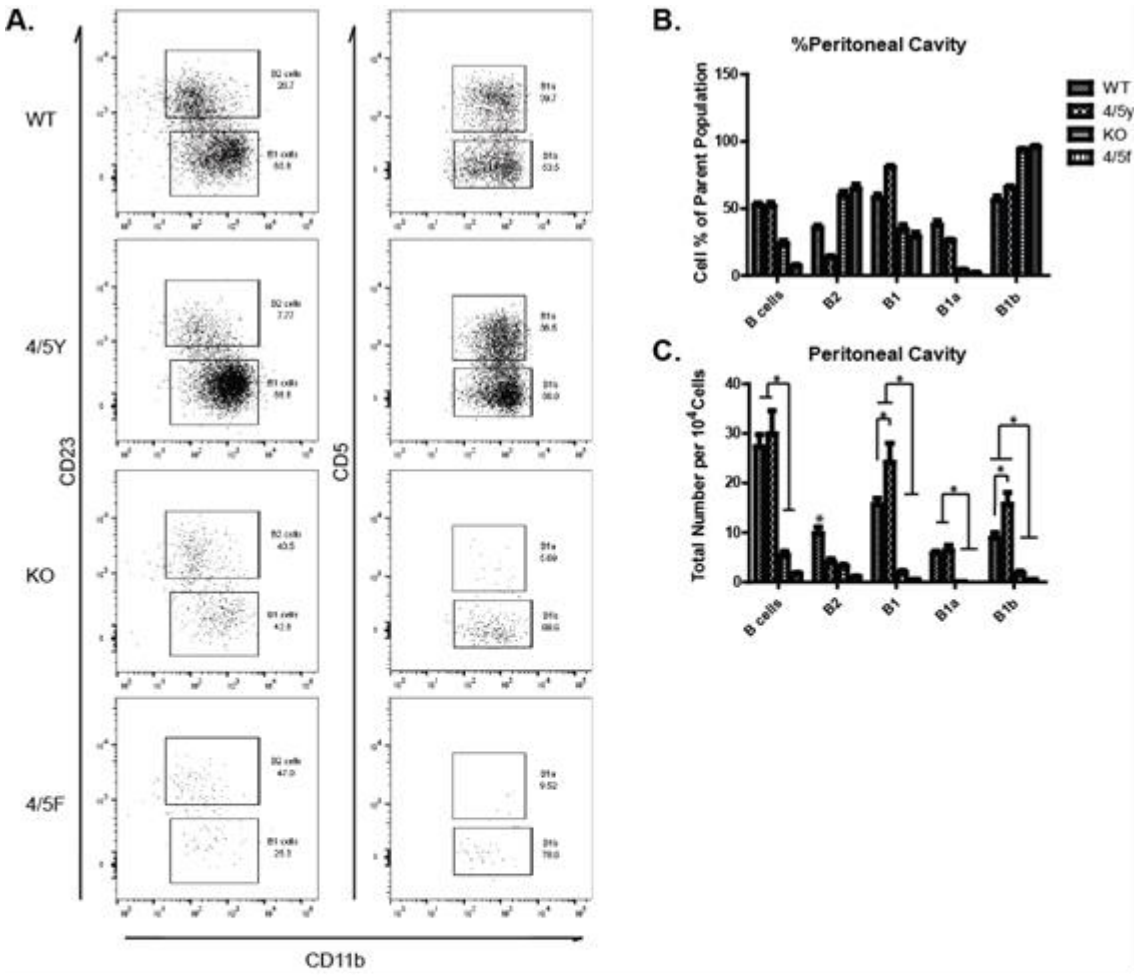

Figure 2. Differences in B cell subpopulations in the peritoneal cavity between CD19 mutant mice. Significant differences in peritoneal lavage B cell subsets between CD19 mutants. A) Representative samples of flow cytometry from peritoneal lavage of WT, 4/5Y, CD19 KO, and 4/5F mice, depicting B2 and B1 cells, B1a and B1b with percentages given in B. Absolute cell count is depicted in C. Bars represent means with SEM; significance determined by one-way ANOVA with Tukey's post-test, with $p<0.05$='*'; n=5 mice from each group; representative of two repeated experiments.

In the peritoneal cavity of these mice, CD19 Y482/Y513, CD19 Y482F/Y513F, and $CD19^{-/-}$ mice contained statistically significant fewer B2 cells than WT mice (Fig 2C). CD19 Y482/Y513 mouse phenotypes were comparable to $CD19^{-/-}$ mice in B2 cell populations, containing approximately half as many as WT mice (Fig 2B, C). CD19

482/Y513 mice contained significantly higher amounts of B1b cells than WT. These data demonstrate Y482/Y513 mediated signaling alone is sufficient to differentiate B cell populations into greater numbers of B1b B cells than in WT mice, but not peritoneal cavity B2 B cells, signifying a possibly role for Grb2/Sos and Vav/PLCg2 mediated signaling for differentiation of B2 B cells in the peritoneal cavity. The higher numbers of B1b B cells found in the peritoneal cavity of CD19 Y482/Y513 mice suggested these mice might have an altered immune response against T-independent antigens.

CD19 Y482/Y513 signaling is sufficient for a T-independent B cell antibody response.

$CD19^{-/-}$ and CD19 Y482F/Y513F mice show a diminished humoral response against T-independent antigens at low-epitope density (4). Since CD19 Y482/Y513 mice retain a normal MZ and B1a B cell population, and an increased B1b B cell population, it seemed plausible CD19 Y482/Y513 mediated signaling would be sufficient for a normal antibody response against a T-independent antigen. To determine the effects of CD19 Y482/Y513 mediated signaling in a T-independent immune response, mice from each group were immunized with 25μg TNP-Ficoll and serum was collected on days 0, 3, 5, 12, 18, and 30. Antibody titers of IgM and IgG3 were measured using ELISA (Fig 3). CD19 Y482/Y513 mice significantly increased TNP-specific IgM production in response to TNP-Ficoll immunization especially by day 18 post immunization compared to WT mice, whereas CD19 Y482F/Y513F mice and $CD19^{-/-}$ mice produced extremely low amounts of IgM, (Fig 3A). By day 30 post-immunization, CD19 Y482/Y513 mice produced significantly more IgG3 anti-body in response to TNP-Ficoll compared to WT, $CD19^{-/-}$ and CD19 Y482F/Y513F (Fig 3B).

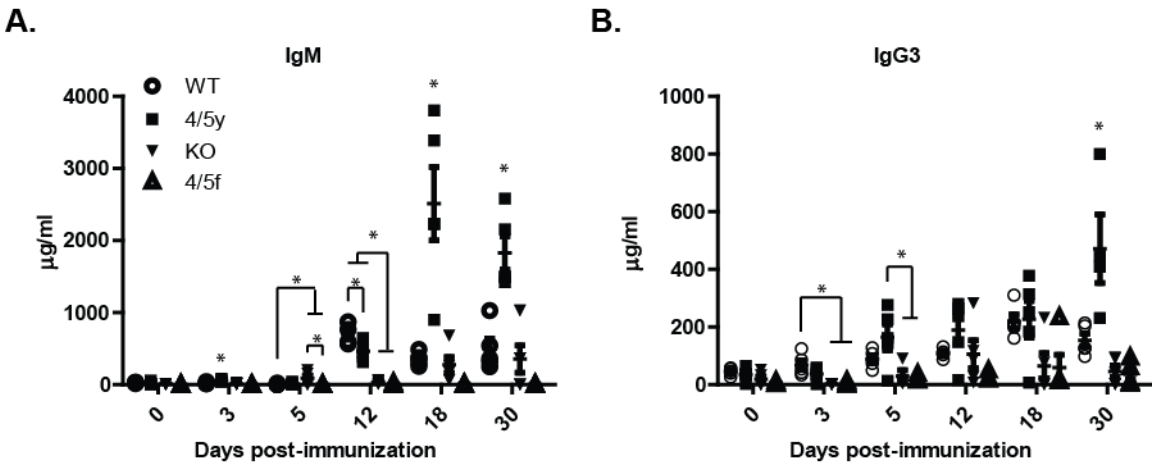

Figure 3. CD19 Y482/Y513 mice show an increased immune response to T-independent antigen. TNP-specific IgM (A) and TNP-specific IgG3 (B) antibody titers from the serum of mice immunized with TNP-Ficoll are depicted. One-way ANOVA with Tukey's post-test was used to analyze differences between groups. P<0.05='*'; n=4-5 mice from each group.

To determine if TNP-specific antibody titers correlated with ASCs in the CD19 mutants, Elispots were performed using homogenized spleen or bone marrow harvested from each group of immunized mice from days 5 and 12 post-immunization (Fig 4). CD19 Y482/Y513 mouse spleens and bone marrow contained higher amounts of IgM secreting ASCs compared to WT spleens and bone marrow (Fig 4 A, B). CD19 Y482F/Y513F and CD19$^{-/-}$ produced very few TNP specific IgM or IgG ASCs (Fig 4 A-D).

Spleens from CD19 Y482/Y513 mice contained higher amounts of IgG3 producing ASC's compared to WT, corresponding to higher IgG3 antibody titers (Fig 4C, Fig 3B). However, there was no difference in IgG3 producing ASC's in the bone marrow (Fig 4D). Together, these data suggest CD19 Y482/Y513 mediated signaling is sufficient for an antibody response to T-independent antigens.

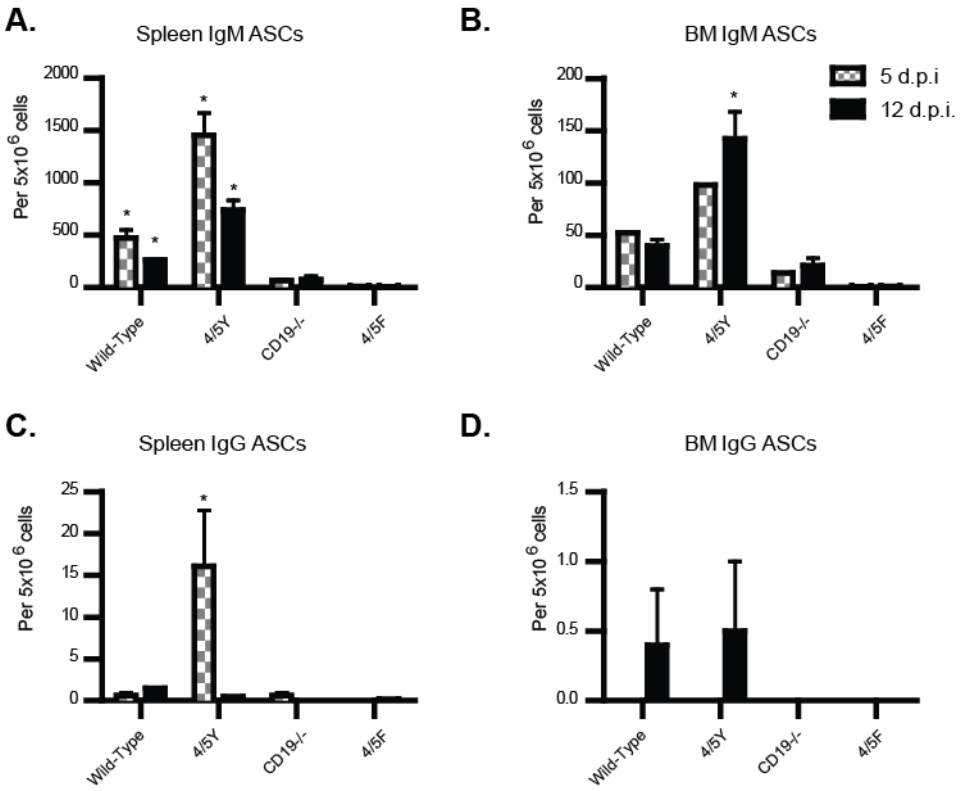

Figure 4. ELISPOT of the T-independent response of CD19 mutants. Bone marrow and spleens of mice immunized with TNP-Ficoll were harvested at days 5 and 12 and subjected to ELISPOT analysis. Numbers of IgM ASCs in the spleen (A), and the bone marrow (B) as well as IgG ASCs in the spleen (C) and bone marrow (D) are represented in graphs (A-D). Bars represent means =/- SEM. Differences of means for each day were analyzed with one-way ANOVA and Tukey's post-test; n=5, $P<0.05$='*'

CD19 Y482/Y513 mediated signaling alone leads to a skewed T-dependent antibody response.

CD19 affects the B cell response to T-dependent antigens (4, 9). To determine the effect of CD19 Y482/Y513 mediated signaling in response to a T-dependent antigen, five mice from each group (WT, CD19 Y482/Y513, CD19 Y482F/Y513F, $CD19^{-/-}$) were immunized with 50μg NP-KLH suspended in alum and reimmunized on day 28 with 50μg NP-KLH suspended in PBS. Blood was collected on days 7, 21, and 42. Serum was analyzed for antibody titers of IgM, IgG1, and IgG2b using ELISA.

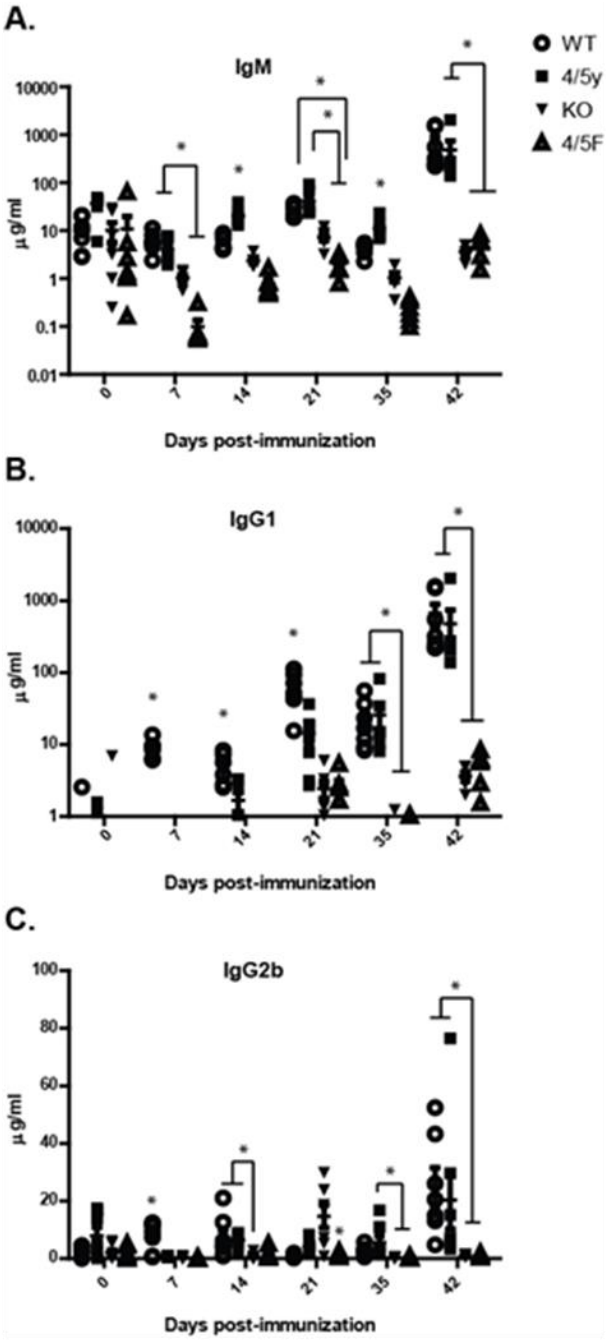

Figure 5. T-dependent antibody response of CD19 mutants. Antibody titers from mice immunized with NP-KLH were analyzed by ELISA. NP-specific IgM concentration of CD19 mutants is in A, with IgG1 and IgG2 concentration in B and C respectively. Bars represent means +/- SEM. Differences of means for each day were analyzed with one-way ANOVA and Tukey's post-test; n=7-10, P<0.05='*'.

CD19 Y482/Y513 mouse serum contained higher concentrations of IgM compared to WT mice by D14-D35 (Fig 5A). $CD19^{-/-}$ mice and CD19 Y482F/Y513F mice produced very little NP specific IgM, IgG1, and IgG2b compared to WT mice as previously reported (4).

CD19 Y482/Y513 mice appeared to have a delayed class-switched antibody response compared to WT mice. IgG1 production in WT mice was higher than in CD19 Y482/Y513 mice during the primary response (Fig 5B), however, no significant difference between WT and CD19 Y482/Y513 mice was detected during the secondary response (Fig 5B). This trend continued with the production of IgG2b, with WT mice producing significantly more IgG2b during the primary response until D21 compared to CD19 Y482/Y513 (Fig 5C). During the secondary response, IgG2b production was similar in both WT and CD19 Y482/Y513 mice (Fig 5C).

ASC numbers in response to NP-KLH immunization were observed using ELISPOT (Fig 6). Five mice from each group were sacrificed on days 7, 21, and 42, post initial immunization, with reimmunization on D28. NP-specific ASCs from spleen and bone marrow of each mouse for each timepoint were counted.

On D7, IgM ASCs in the spleen were found to be significantly higher in CD19 Y482/Y513 mice compared to WT mice (Fig 6A). CD19 Y482/Y513 mice contained higher amounts of IgM ASCs on both D7 and D42 in the bone marrow (Fig 6B). CD19 Y482F/Y513F and $CD19^{-/-}$ mice had difficulty producing any NP-specific IgM or IgG ASCs in response to NP-KLH (Fig 6A-D).

Although CD19 Y482/Y513 mice appeared to have similar numbers of IgG ASCs in the spleen compared to WT mice (Fig 6C), only about half as many IgG ASCs were

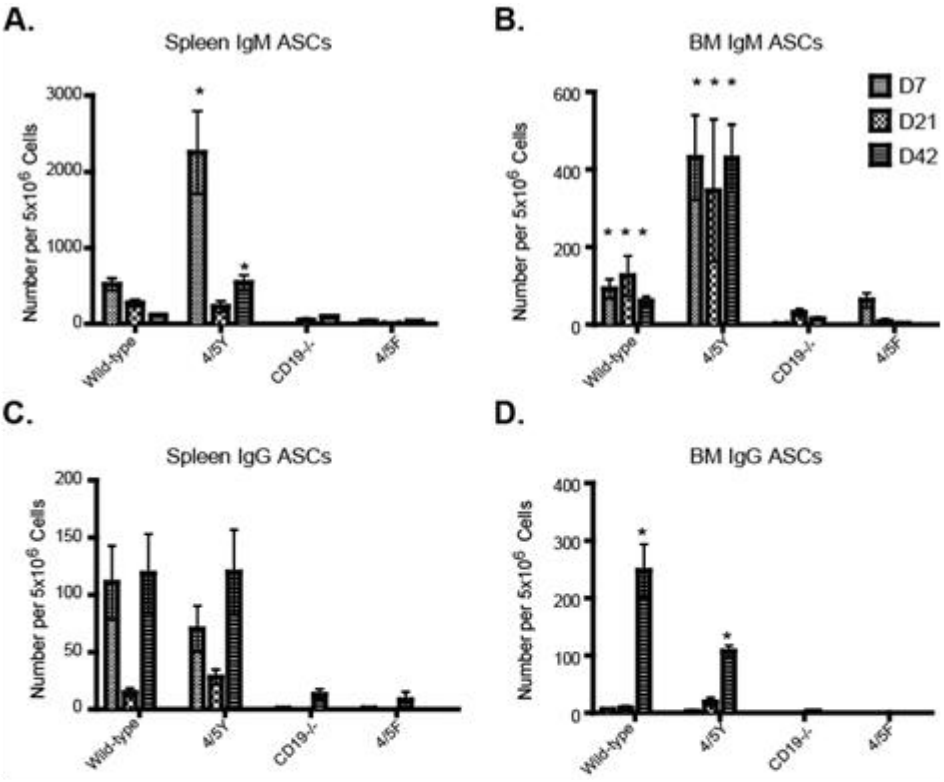

Figure 6. CD19 Y482/Y513 mice produce increased IgM ASCs in response to T-dependent antigens. To determine the effect of CD19 mutations on ASC differentiation in response to T-dependent antigens, spleens and bone marrow were harvested from five mice of each strain of WT, 4/5y, CD19$^{-/-}$, and 482F/513F immunized with NP-KLH and subjected to ELISPOT analysis. NP-specific IgM ASCs from spleen and bone marrow are depicted in A and B respectively. NP-specific IgG ASCs from spleen and bone marrow are in C and D. Statistics were determined using one-way ANOVA with Tukey's post-test for each time point. Significance was determined using $P<0.05$. Bars represent means +/- SEM; n=4-5.

found in the bone marrow of these mice compared to WT mice (Fig 6D). This suggests these plasma cells may have difficulty migrating to the bone marrow, or differentiating into long-lived plasma cells. These suggested differences might be seen in germinal center differentiation in CD19 Y482/Y513 mice.

CD19 Y482/Y513 signaling alone leads to defective class-switched germinal center B cell formation.

CD19 affects GC B cell development and the ability to produce high-affinity class-switched antibodies (4, 22-24). CD19 Y482/Y513 mice produce fewer IgG ASC's, have lower IgG1 and IgG2b titers, but higher IgM titers, suggesting a possible defect in the ability of these mice to produce class-switched GC B cells.

To determine differences in GC B cell formation between CD19 mutants, five mice of each strain for each timepoint were immunized with 50μg of NP-KLH and sacrificed at days 4, 7, 10, and 14 post immunization. Spleens were harvested and stained for NP-specific GC B cells, as well as T_{FH} cells.

By day 7 post immunization, WT mice contained higher overall GC B cells ($B220^{Hi}$, IgD^{Lo}, Fas^{Hi}, PNA^{Hi}), IgM-NP specific GC B cells ($B220^{Hi}$, IgD^{Lo}, Fas^{Hi}, PNA^{Hi}, IgM^{Hi}, NP^{+}), and IgG-NP specific GC ($B220^{Hi}$, IgD^{Lo}, Fas^{Hi}, PNA^{Hi},IgM^{Lo}, NP^{+}) B cells than any other strain analyzed (Fig 7). Overall differences in GC B cell number were not significant between WT and CD19 Y482/Y513 mice and between CD19 Y482F/Y513F and $CD19^{-/-}$ mice. CD19 Y482/Y513 mice could produce more NP-specific IgM GC B cells than CD19 Y482F/Y513F and $CD19^{-/-}$ mice, although not as many as WT mice (Fig 7B), suggesting Y482/Y513 signaling is not sufficient to imitate a WT class-switched response. However, CD19 Y482/Y513 mice produced similar amounts of NP-specific IgG GC B cells to CD19 Y482F/Y513F and CD19-/- mice, suggesting that Y482/Y513 mediated signaling is not sufficient for producing NP-specific IgG GC B cells in vivo (Fig 7C). T_{FH} cell development was not affected (data not shown).

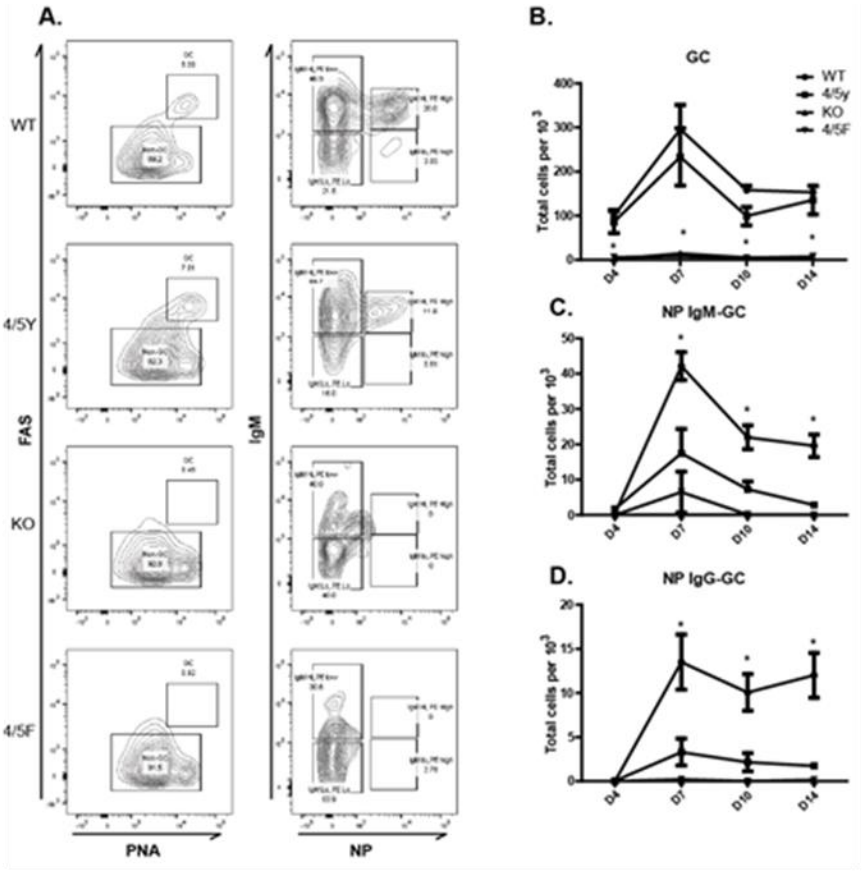

Figure 7. CD19 Y482/Y513 alone is insufficient for a normal class-switched Germinal center response. Graphs represent cell numbers of mice immunized with NP-KLH and sacrificed on days 4, 7, 10, and 14. Representative gating strategy on B220+ cells is shown in A. Total GC B cells (B), NP-specific IgM GC B cells (C) NP-specific IgG GC B cell numbers (D) are represented in the respective graphs. Each time point depicts the mean +/- SEM. Statistics were calculated using 2-way ANOVA with Bonferroni post-test between all pairs; n=4-5, $p<0.05$='*'.

It has been suggested CD19 is not required for class-switching with sufficient external help (23). To determine if CD19 Y482/Y513 affects GC B cell differentiation *in vitro*, splenocytic B cells were isolated from CD19 mutants and WT and incubated in cell culture on CD40LB cells with 2ng/ml IL-4 for four days. B cells were then either analyzed at that time or treated an additional two days with an additional 2ng/ml of IL-4, 10ng/ml IL-21, or untreated before analysis with flow cytometry for IgM or IgG1 expressing GC B cells (Fig 8).

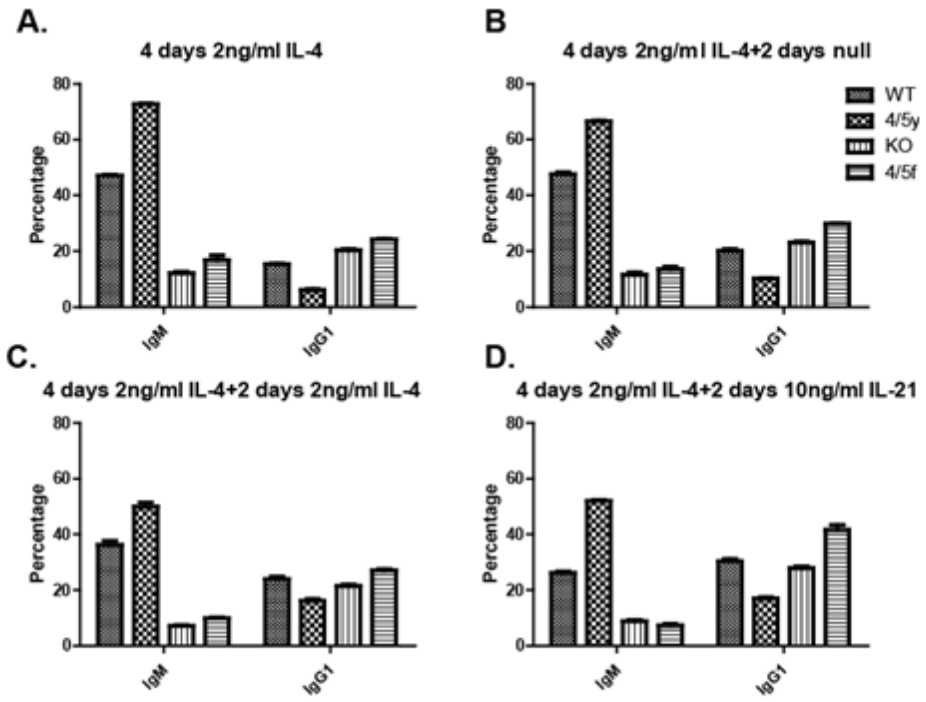

Figure 8. CD19 Y492/Y513 B cells show increased IgM and decreased IgG GC cells in vitro. B cells from CD19 mutants were isolated from spleens and treated in culture for four days in triplicate with 2ng/ml IL-4 (A). Cells were then cultured for two additional days without treatment (B), with 2ng/ml IL-4 (C), or 10ng/ml IL-21 (D) and analyzed by flow cytometry for GC B cell development ($B220^+$, FAS^+, $GL7^+$) into either IgM or IgG1 expressing GC B Cells. Bars in graphs represent means of percentages +/- SEM.

B cells from CD19 Y482/Y513 spleens developed preferentially into IgM-expressing B cells (on average 76.7%) rather than IgG1 (6.2%) after four days with 2ng/ml IL-4 treatment (Fig 8A). WT B cells developed into IgM expressing GC B cells at a rate of 47.1% and into IgG1 expressing GC B cells at 15.4%, while $CD19^{-/-}$ and CD19 Y482F/Y513F developed into IgM GC B cells at 12.1% and 15.8% respectively, and into IgG1 secreting at 20.4% and 24.3% respectively. Similar trends were observed after treatment with an additional two days in culture without extra treatment (Fig 8B), treatment of IL-4 (Fig 8C), or IL-21 (Fig 8D), although fewer IgM GC B cells percentages

were observed over time as greater percentages of IgG1 expressing GC B cells developed.

Together these data suggest that PI3K signaling mediated by Y482 and Y513 is sufficient for increased IgM GC B cell development, but is insufficient for normal IgG1 producing GC B and may suppress IgG1 GC B cell development as $CD19^{-/-}$ and CD19 Y482F/Y513F mice produce normal IgG1 GC B cells *in vitro*.

DISCUSSION

These data further demonstrate that B cells require fully functional CD19 mediated signaling for normal B cell development and production of antigen-specific class-switched antibodies. CD19 Y482/Y513 mutants appear to produce greater numbers of B1b and MZ B cells compared to WT mice. Although the increase in MZ B cell levels was not statistically significant, it may explain the enhanced response of CD19 Y482/Y513 mice to T-independent antigen and increased IgM antibody secretion in response to T-dependent antigens (Fig 1, 2, 3A). B2 cell numbers were low in the peritoneal cavity of CD19 Y482/Y513 mice, similar to CD19-/- and CD19 Y482F/Y513F mice (Fig 2). CD19 KO and CD19 Y482F/Y513F mice produced extremely low amounts of B1 cells, suggesting that CD19 Y482/Y513 mediated Lyn/PI3K signaling is required for these sub-populations to develop. It may be possible that upstream tyrosines negatively regulate B1b differentiation.

Due to these differences in MZ B cells and B1 B cells, it seems CD19 Y482/Y513 signaling might influence the T-independent immune response. After immunization with TNP-Ficoll, CD19 Y482/Y513 mice demonstrated an increased response to antigen over

time by day 18 post-immunization compared to WT mice in both IgM serum titers and by day 30 IgG3 (Fig3 A and B). These data correlated with Elispot data, which demonstrated an increase in both spleen and bone-marrow IgM-secreting cells compared to WT mice (Fig4 A and B). IgG-secreting cell numbers in CD19 Y482/Y513 mice were increased to WT mice levels in the spleen but not bone marrow over the course of 12 days post immunization (Fig4 C and D).

T-dependent responses measured in CD19 mutant mice demonstrated that IgM titers were similar in both WT and CD19 Y482/Y513 mice, with CD19 Y482/Y513 mice producing slightly higher amounts of IgM (Fig 5A). $CD19^{-/-}$ and CD19 Y482F/Y513F mice were unable to mount a significant response against T-dependent antigen (Fig 5). CD19 Y482/Y513 mice could produce IgG1 and IgG2b antibodies against the antigen, although the response was attenuated during the primary immune response (Fig 5 B, C). These data correlated with Elispot data (Fig 6), which demonstrated an increase in both spleen and bone marrow numbers of IgM-producing cells in CD19 Y482/Y513 mice compared to WT on both days 7 and 21 post-immunization (Fig 6A, B). $CD19^{-/-}$ and CD19 Y482F/Y513F mice produced few numbers of IgM and IgG-secreting cells (Fig 6A-D). CD19 Y482/Y513 mice produced similar numbers of IgG-secreting cells compared to WT mice in the spleen by day 21 post-immunization, but significantly fewer IgG ASCs were present in the bone marrow (Fig 6C, D). It is unclear why a similar number of IgG ASCs in spleens of CD19 Y482/Y513 mice and WT mice would lead to a difference in serum IgG titers. To further analyze this issue, GC B cell development was observed over time in response to a T-dependent antigen, NP-KLH (Fig 6). WT mice could produce greater numbers of NP-specific IgM and IgG GC B cells over time, despite

similar numbers in overall GC B cells (Fig 7 A, B, C). CD19-/- and CD19 Y482F/Y513F mice produced extremely low numbers of either IgM or IgG GC B cells compared to WT mice. However, $CD19^{-/-}$ and CD19 Y482F/Y513F mice could produce similar numbers of IgM and IgG1 GC B cells to WT mice in an *in vitro* system (Fig 8 A-D). CD19 Y482/Y513 mice were deficient in their ability to produce IgG1 GC B cells in response to IL-4 or IL-21 compared to WT mice, although B cells from these mice differentiated into IgM GC B cells at higher rates than WT mice (Fig 8, A-D).

It is possible that CD19 mediated Grb2/Sos and Vav/PLCγ2 signaling work in conjunction with Lyn/PI3K to both dampen the IgM response while aiding in differentiation to IgG1 expressing GC B cells in vitro. A similar pattern was observed in a T-independent immune response (Fig 3,4), with the exception that CD19 Y482/Y513 signaling appeared to increase IgG3 production. CD19 Y482F/Y513F mice express fewer IgM and IgG3 titers than $CD19^{-/-}$ mice on days 18 and 30, adding further support that upstream CD19 tyrosine-mediated signaling may depress IgM production. These results appear specific to T-independent results, however, as *in vivo* data suggest CD19 Y482/Y513 mediated Lyn/PI3K signaling is insufficient to mount an IgM response equal to WT mice in a T-dependent immune response (Fig 5). These effects may be due to the observation that CD19 Y482/Y513 mice produce more MZ and B1 B cells typically involved in innate-like B cell processes including T-independent immune responses.

Together, these data suggest 4/5y mediated signaling is sufficient to rescue KO B1 and MZ cell populations, and somewhat sufficient in rescuing an IgM GC B cell response to T-dependent antigens. Without intact Grb2/Sos and Vav/PLCγ2 signaling me-

diated by upstream CD19 tyrosines, mice seemed to respond hyperactively to T-independent antigens. This response was replicated in vitro (Fig 8) with CD40 signaling, IL-4, and IL21. With adequate external factors, $CD19^{-/-}$ and CD19 Y482F/Y513F could produce IgG1 GC B cells in similar amounts to B cells from WT mice, although they consistently produced low amounts of IgM GC B cells (Fig 8). Y482/Y513 mediated PI3K signaling is required for IgM production but is not sufficient for class switching. The possibility remains that Grb2/Sos signaling and Vav/PLCγ2 signaling may play a negative regulatory role in CD19 signaling, and may aid in class switching in vivo. More research is needed to delineate the roles of CD19 mediated Grb2/Sos and Vav/PLCγ2 signaling.

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SUMMARY AND DISCUSSION

HSH2

Although HSH2 protein expression appears dynamically regulated between subsets of B cells, HSH2 mRNA levels were not found to correlate in B cells isolated from spleens of WT C57-BL6 mice. In addition, when isolated splenocytic B cells were treated in culture with LPS to induce HSH2 upregulation, there was no corresponding upregulation of HSH2 mRNA. This lack of upregulation in mRNA levels suggested a mechanism for HSH2 regulation independent of transcription. Actinomycin-D treated HSH2-Tg and LPS overnight treated B cells had little effect on HSH2 expression compared with NF-κB inhibition by PDTC. Furthermore, inhibition of translation by cycloheximide failed to decrease HSH2 expression as rapidly as NF-κB inhibition using PDTC. NF-κB inhibition with PDTC lead to rapid degradation of HSH2 (approximately 4 hours, with a half-life of about 1.5 hours). Together, these data suggest that regulation of HSH2 at the levels of transcription and translation are less important than the regulation of HSH2 at the level of protein-turnover by NF-κB, despite the role of NF-κB as a transcription factor. The possibilities of NF-κB regulating proteins independent of translation or transcription have not been explored. It was therefore necessary to establish NF-κB as directly responsible for regulating HSH2, rather than indirect side effects of the NF-κB inhibitors used in previous studies of HSH2 regulation.

PDTC and Bay-11-7082 have previously been demonstrated to prevent HSH2 up-regulation in response to LPS, CPG DNA, and other factors involved in signaling for survival and differentiation of B cells (43). Both PDTC and Bay 11-7082 are thought to inhibit the NF-κB pathway due to their effect on the overall redox state of treated cells by behaving as antioxidants, and possibly also as oxidants depending on the dose (50-52,56). GC B cells upregulate NF-κB as well as increase oxidation due to endoplasmic reticulum stress as they prepare to produce large amounts of immunoglobulin proteins. It seemed possible that the redox state of B cells could affect HSH2 expression independent of NF-κB activity. However, treatment of B cells with MG-132, which inhibits NF-κB indirectly by inhibition of the proteasome, independent of the redox state, failed to degrade HSH2. HSH2 expression is more dependent on a functional NF-κB pathway than changes in redox state.

The use of pharmaceutical inhibitors of NF-κB contains the possibility of unknown side effects, including non-specific inhibition or activation of proteases. Therefore, it was necessary to establish the effects of NF-κB inhibition independent of pharmaceutical reagents. Using Mx-Cre X $NEMO^{F/F}$ mice (a kind gift from the Christopher Klug lab), which can be induced to cut out the NEMO (IKKγ) gene in response to TNFα, we determined that inhibition of NF-κB is sufficient to prevent HSH2 expression in response to LPS. Having established that NF-κB is directly responsible for upregulation and maintenance of HSH2 expression, it was then necessary to determine how NF-κB affects HSH2 at the level of protein turnover.

One possible mechanism through which NF-κB could affect HSH2 protein turnover involves phosphorylation by upstream kinases in the NF-κB pathway, activating or

deactivating a protease involved in degradation of HSH2. Although IKKβ regularly targets IκB proteins in the canonical NF-κB pathway, there is the possibility that IKKβ could target an unknown protein or proteins leading to degradation of HSH2. To test whether effects on upstream phosphorylation events affected HSH2 degradation, we used Western blotting on NF-κB inhibitor treated cells. PDTC treated splenic B cells were shown to have decreased upstream phosphorylation of IKKb and IkBα. However MG-132 treated splenic B cells were not affected in upstream or downstream phosphorylation of the NF-κB pathway, yet treatment of MG-132 eventually leads to degradation of HSH2. These data demonstrated NF-κB inhibition, regardless of upstream phosphorylation events in the NF-κB pathway, is sufficient for degradation of HSH2.

Global inhibition of translation and transcription leads to little change in HSH2 protein expression, while inhibition of NF-κB leads to rapid decrease in HSH2 expression. This suggested that NF-κB inhibition leads to the upregulation of protease activity involved in HSH2 degradation rather than changes in transcription or translation of HSH2 decreasing its expression. To determine if NF-κB inhibition increases the activity of a protease in the cytosol responsible for HSH2 degradation, splenic B cells were treated overnight with LPS and then treated with or without PDTC for 3 hours to induce potential protease activity. The cell lysate was then added to beads with immunoprecipitated HSH2, with or without protease inhibitors, and incubated for one hour to determine the effects of lysate alone on HSH2 degradation. Although not statistically significant, there was a decrease in HSH2 expression on immunoprecipitated HSH2 incubated with PDTC treated B cell lysate compared to non-treated lysate. This effect was rescued somewhat

by addition of protease inhibitors in the lysate. Differences in repeated experiments affecting significance may be attributed to difficulties in maintaining protease activity in a cell-free system, changes in efficacy of protease inhibitors over time, and difficulties in analyzing equivalent amounts of protein using only the precipitating antibody as the loading control between samples. Despite these challenges, there was an overall trend in decreased HSH2 degradation in response to incubation with PDTC treated lysate in a cell free system that was close to statistically significant. In addition to previous data demonstrating global inhibition of transcription and translation have little effect on HSH2 protein expression compared to inhibition of NF-κB, these data suggest that NF-κB activity is a negative regulator of protease activity.

Although NF-κB does not appear to affect HSH2 at the level of transcription or translation, and global inhibition of transcription and translation processes lead to little change in HSH2 protein expression, it is possible that NF-κB regulates a different gene or protease at the level of transcription. This would allow NF-κB to negatively regulate protease activity without requiring changes in upstream NF-κB phosphorylation. To test this hypothesis, splenic B cells were treated overnight with LPS to induce HSH2 expression before treatment with PDTC, cycloheximide, pre-treatment of cycloheximide for one hour before treatment with PDTC, or non-treated. Inhibition of translation with cycloheximide before NF-κB inhibition with PDTC protected HSH2 from degradation compared to treatment with PDTC alone, suggesting that inhibition of NF-κB upregulates a protein involved in the degradation of HSH2.

Together, these data propose a novel potential mechanism for the regulation of HSH2. It appears that NF-κB negatively regulates a short-lived labile protease responsible for the degradation of HSH2. HSH2 transcript levels do not change in response to LPS activation or NF-κB inhibition with PDTC, suggesting that HSH2 transcription is constitutive in mature splenic B cells, and likely in various B cell subsets. Activation of NF-κB downregulates the activity of a protease, allowing gradual accumulation of HSH2. Inhibition of NF-κB upregulates either a protease or downregulates an inhibitor of the protease responsible for HSH2 degradation. If NF-κB negatively regulates a protease, then it is a short-lived labile protease as it demonstrated only weak activity in a cell-free system compared to cellular processes. Alternatively, if NF-κB upregulates a repressor of this protease, then the protease could exist in the cell in an inhibited state indefinitely. The repressor would need to be rapidly degraded in the absence of NF-κB to demonstrate the effects observed on HSH2. However, in the cell-free system, one would expect more activity of a protease free from its repressor. It may be that both the repressor and the protease are labile. Evidence suggests alternative mechanisms for the regulation of HSH2 expression in B cells, as GC B cells downregulate HSH2 expression compared to other B cell subsets, while NF-κB is active, suggesting that other signals may activate the protease responsible for HSH2 degradation. Potentially, this could be from IL-21 signaling or non-canonical NF-κB signaling preventing HSH2 expression, as current experiments have failed to replicate previous studies demonstrating that non-canonical signals from aCD40 or BAFF can upregulate HSH2 (data not shown). It will be interesting to see what further studies delineate HSH2 regulation.

NF-κB prevents B cell apoptosis, and inhibition of NF-κB leads to B cell apoptosis. Previous studies suggested that HSH2 may be involved in the prevention of apoptosis in B cells (42,43). However, this still leaves many questions unanswered about the role of HSH2 in the production of class-switched antibodies. HSH2 degradation in GC B cells leads to accumulation of what appears to be a cleaved fragment of HSH2 (unpublished data), which may be involved in mediating signaling leading to the production of class-switched antibodies. It remains to be understood how HSH2 mediates the production of class-switched antibodies.

CD19

The tyrosines at positions 482 and 513 on CD19 are sufficient for B cell differentiation into B1 and MZ B cells, demonstrating the importance of CD19 mediated PI3K signaling for B1 and MZ B cell development. However, CD19 Y482/Y513 mice do not develop as many peritoneal B2 B cells as WT mice, suggesting a role for CD19 mediated Vav/PLCγ2 and Grb2/Sos signaling. Furthermore, CD19 Y482/Y513 mice responded more intensely to T-independent antigen than WT mice, allowing for the possibility that Grb2/Sos and Vav/PLCg2 signaling may moderate BRC mediated T-independent immune response. In addition, CD19 Y482/Y513 mice could produce high amounts of antigen-specific IgM and IgM-ASCs compared to WT mice but had difficulty producing antigen-specific class-switched antibodies during the primary immune response. CD19 Y482/Y513 mice could form antigen-specific IgG-ASCs in the spleen, but these ASCs were not observed in the bone marrow at the same quantities as in WT mice. Whether

these effects are due to decreased survival by CD19 Y482/Y513 mouse IgG-ASCs, or a deficiency in moderating chemokine receptors has not been determined.

It has been suggested CD19 is not required for class-switching (78,79). *In vitro* differentiation into IgG1-GC B cells was not affected in $CD19^{-/-}$ or CD19 Y482F/Y513F mice compared to WT mice, although CD19 Y482/Y513 mice were deficient percentage wise in IgG1-GC B cells. This observation is likely due to the propensity of CD19 Y482/Y513 B cells to differentiate into IgM-GC B cells, as there is no evidence to suggest CD19 Y482/Y513 mediated PI3K signaling in the absence of Grb2/Sos and Vav/PLCγ2 signaling confers increased survival in B cells.

CD19 Y330F/Y421F and Y403F/Y443F mice (deficient in Grb2/Sos and Vav/PLCγ2 signaling respectively) were not shown to have any significant effects on CD19 mediated signaling with regards to calcium signaling, B cell development or humoral immune response *in vivo* (76). It is unclear why mice unable to mediate Grb2/Sos and Vav/PLCγ2 signaling simultaneously through CD19 would express a skewed humoral immune response compared to WT mice.

It is possible that proximity to CD19 of either Grb2 or Vav is sufficient for these effector proteins to work synergistically with each other or their respective binding partners in a way CD19 Y482/Y513 B cells does not allow. Sos is active when recruited to the cell surface by Grb2 (80). Grb2 can bind to Vav through the amino terminal of the SH3 domain region of Vav to the carboxy SH3 domain of Grb2 (81,82). This binding of Grb2 to Vav may be sufficient for normal CD19 mediated signaling to occur, despite mutations in CD19.

Vav can mediate PI3K signaling by binding to PI3K products, phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), or phosphotidylinosityl-3,4,5-triphosphate ($PtdInsP_3$) through its pleckstrin homology domain. This interaction may affect PLCγ2 and downstream calcium release (83). The effector proteins associated with CD19 may interact with each other to modulate an effective immune response. These data suggest a potential regulatory role for both CD19 mediated Grb2/Sos and Vav/PLCγ2 signaling, moderating a T-independent immune response and allowing for the differentiation of B cells into IgG-GC B cells during a T-dependent immune response.

CD19 Y482/Y513 mice and HSH2-Tg mice have difficulty producing class-switched antibodies in response to T-dependent antigens. These data support the role of CD19-mediated Grb2 and Vav signaling to moderate PI3K activity, potentially decreasing IgM production and allowing for class-switched antibody production. Interestingly, HSH2 may bind to Vav (unpublished data), possibly through the polyproline domains of HSH2 to the SH3 domain of Vav. It is possible HSH2 moderates Vav through partial sequestration until HSH2 is degraded, allowing for Vav to bind to CD19 and perform Vav's observed roles in B cell signaling. This may help explain some of the effects seen in HSH2-Tg mice. Future research on HSH2 will need to explore molecular interactions between HSH2 and cytosolic proteins to determine if this is the case.

These studies further elucidate the role of two proteins, HSH2 and CD19, in the production of class-switched antibodies and B cell differentiation.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 07-Oct-2016

TO: Justement, Louis B

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 07-Oct-2016.

Protocol PI: Justement, Louis B

Title: Regulation of B Lymphocyte Survival and Differentiation by HSH2

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-09943

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 05-Oct-2016

TO: Justement, Louis B

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 05-Oct-2016.

Protocol PI: Justement, Louis B

Title: CD19 Tyrosine-Mediated Signal Transduction in Vivo

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-08614

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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