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Characterization of Expression and Function of Heparan Sulfate on Murine B Cells

by

Damian L. Trujillo

A dissertation submitted in partial satisfaction of the requirements for

the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate division

of the University of California, Berkeley

Committee in charge:

Professor Laurent Coscoy, Chair

Professor Eva Harris

Professor Russell Vance

Professor Astar Winoto

Abstract

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Heparan sulfate (HS) modulates many cellular processes including adhesion, motility, ligand-receptor interaction, and proliferation. Here I report that murine B cells express very little HS, and that upon either bacterial or viral infection, HS is rapidly upregulated at the surface of B cells. My thesis work first describes the molecular details of HS induction and regulation in B cells. I also describe the generation and characterization of mice that are unable to express heparan sulfate on B cells, which we used to investigate the role of HS expression on B cells *in vivo*. With this conditional knockout mouse, I show the importance of HS expression for the generation and/or maintenance of antibody producing cells during a viral infection. Finally, I show data that suggests that HS expression on B cells enhances the antibody response to antigens containing a heparan sulfate-binding domain. The work presented here provides the first detailed evaluation of HS expression on B cells. This work illustrates the importance of HS in the response to certain classes of antigens, as well as in the development of the B cell response.

I would like to dedicate this work to my parents,

Arsenio Trujillo
and
Norma Trujillo.

Mom and Dad,
I could not have done this without your support and love.

Without all the sacrifices that you made for my education,
none of this would be possible.

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Chapter 1. An introduction to heparan sulfate and B cell Biology

Chapter 1.1: An introduction to heparan sulfate biosynthesis and function

Composition of heparan sulfate

Heparan sulfate (HS) is a member of the glycosaminoglycan family of polysaccharides and is present on the surface of most mammalian cells (Skidmore et al., 2008). It is comprised of a linear repeating disaccharide, (iduronic acid and N-acetyl-glucosamine), that is post-translationally attached to a core-protein (Sugahara and Kitagawa, 2002). The conjugation of HS to a core-protein creates a heparan sulfate proteoglycan (HSPG) (Bishop et al., 2007). Upon synthesis, HS undergoes heterogeneous deacetylation, sulfation, and epimerization, creating a highly structured and negatively charged proteoglycan (Lamannaa et al., 2007).

Discovery and characterization of heparan sulfate

Between the 1930s and 1940s, a considerable amount of interest was directed towards determining the mechanism of blood clotting. Erik Jorpes' group at the Karolinska institute in Stockholm devoted considerable energy studying molecules with anti-coagulant activity. A large part of the research performed by Jorpes' group focused on determining the chemical composition of these molecules. With this goal in mind, the composition of chondroitin sulfate was first determined. Interest shifted when what would later be known as heparin, a molecule very similar to heparan sulfate, was found to have potent anti-coagulating activity (Jorpes and Gardell, 1948). In a seminal paper, Jorpes and Gardell isolated and characterized heparin from ox liver and lung. The authors did this by first precipitating both heparin and protein, degrading the protein, re-precipitating heparin with barium salt, and then characterizing the alcohol-soluble fraction. With this procedure, the authors were able to determine that this molecule with potent anti-coagulant activity was composed of equal parts glucosamine, uronic acid, acetic acid, and sulfuric acid. Although they found that there was monosulfuric compounds, they also noted the presence of di- and tri-sulfuric acids. This corresponds with the heterogeneously sulfated repeating HS disaccharide.

Heparan sulfate biosynthesis

Synthesis of heparan sulfate mainly occurs in the golgi apparatus (Figure 1.1). Synthesis begins with the formation of a tetrasacharide linker to a designated serine of a repeating serine-glycine repeat on a proteoglycan (Figure 1.1a). The addition of each of the four sugars that make up the tetrasacharide linker is catalyzed by a different family of enzymes. Xylosyl transferase 1 and 2 (XYLT1 and XYLT2) catalyze the initial enzymatic reaction in the assembly of glycosaminoglycans to the core protein with the addition of a xylose to the designated serine. The addition of xylose is thought to be the rate limiting step in proteoglycan synthesis. Next, xylosylprotein beta 1,4-galactosyl transferase polypeptide 7 (B4GALT7) catalyzes the addition of the first galactose onto the xylose-modified core protein. This is followed by addition of a second galactose by galactosylxylosylprotein 3 beta-galactosyl transferase (B3GALT6). The

final step of the tetrasacharide linker synthesis is performed by galtosylgalactosylxylosylprotein 3 beta-glucuronosyl transferase (B3GAT3). This enzyme catalyzes the addition of the final sugar, resulting in a glucuronic acid-galactose-galactose-xylose-core protein. At this point the tetrasacharide-conjugated core protein can either be modified with chondroitin sulfate or heparan sulfate.

The enzymes EXTL2 and EXTL3 next perform the addition of the first N-acetyl glucosamine, thereby dictating that the chain will be HS and not chondroitin sulfate (Figure 1.1b). Once the sacharide chain is confined to become HS, the exostosin enzymes EXT1 and EXT2 polymerize the repeating disaccharide: glucuronic acid and N-acetyl glucosamine (Figure 1.1c). The EXT enzymes are type II transmembrane glycosyl transferases essential for the chain elongation step of HS biosynthesis. EXT1 and EXT2 form a complex, and together they possess substantially higher glycosyl transferase activity than EXT1 alone. This is likely due to EXT2 acting as a chaperone for EXT1 (Busse et al., 2007). It is important to note that while EXT1 is absolutely necessary for this polymerization, HS chain elongation can occur without EXT2.

Concurrent with polymerization of the repeating disaccharide, the length of the chain is heterogeneously modified by multiple enzyme families. It is important to note that these modifications are not template driven, and many of the reactions carried out by these enzymes do not go to completion. This results in a high degree of heterogeneity in HS structural sequences. The NDST enzyme family is the first to act upon the growing chain by deacetylation of N-acetyl glucosamine, and sulfation of glucosamine (Figure 1.1d). This is followed by epimerization of glucuronic acid to iduronic acid by the GLCE enzyme (Figure 1.1d). The length of the chain is further sulfated on oxygen groups by the HS2ST, HS6ST, or HS3ST enzymes (Figure 1.1d) (Sugahara and Kitagawa, 2002).

"The Heparanome"

In addition to the regulation of HS and HSPG expression, it is becoming apparent that both the spacing and the degree to which HS chains are sulfated is also highly regulated (Lamannaa et al., 2007)(Salmivirta et al. 1996). This results in cell specific and tissue specific sulfation patterns along the HS chain. The fact that these sulfation patterns are reproducible in certain cell types and tissues is remarkable considering that HS synthesis and sulfation are non-templated processes. Structurally and functionally distinct HS sulfation patterns have become known as the heparanome, and "heparanomics" is a field dedicated to understanding the entirety of HS structures in a given organism, tissue, or cell (Lamannaa et al., 2007). This field has generated interest recently due to the association of individual sulfation patterns with the enhanced binding to soluble ligands (cytokines, growth factors, chemokines) (Bishop et al., 2007; Turnbull et al., 2001).

Analysis of HS sequence and sulfation patterns has lead to the discovery of three domain types within HS chains. The first type are S-domains, characterized by high sulfation along the HS chain, ranging in length from 2-8 disaccharides. The second domain type is transition zones, which flank S-domains and are less sulfated. The final domain type is the NA-domain, which is unsulfated. Interestingly, certain arrangements of domains have been shown to regulate cell growth, differentiation, adhesion, and migration by altering the interaction between HS and soluble ligands that bind HS (Lamannaa et al., 2007). Further work identifying sulfation patterns and their function will be important in determining the various ways HS function is regulated.

Heparan sulfate proteoglycans

Proteoglycans are defined as core proteins that can be modified with HS, chondroitin sulfate, or N- or O-linked mucin type chains (Bishop et al., 2007). For the purpose of this introduction, I will focus only on proteoglycans that can be modified with HS (HSPGs). There are 3 subfamilies of HSPGs: The first family is distinguished by the presence of membrane spanning domain, and this family is comprised of syndecans 1-4, CD44v3, and betaglycan (also known as TGFβR3) (Mythreye and Blobe, 2009; Van Der Voort et al., 2000). The second subfamily is distinct for being glycophosphatidylinositol- (GPI) anchored proteoglycans. This subfamily includes glypicans 1-6. The last subfamily of proteoglycans are those that are secreted into the extracellular matrix. This family is comprised of agrin, collagen XVIII, perlecan, and serglycin (Bishop et al., 2007). It is important to note that proteoglycans can be modified with multiple sugar chain types. For example, the syndecans are often modified with HS and chondroitin sulfate (Tkachenko et al., 2005).

Interestingly, some HSPGs are capable of signaling in both a HS-dependent and HS-independent fashion, further adding to HS-influenced cellular dynamics. One of the best-studied examples of HSPG signaling is syndecan 4. Expression of syndecan 4 is regulated during mammalian development, and it is unique amongst syndecans in that it is expressed in almost all adult tissues, albeit at low levels (Tkachenko et al., 2005; Wilcox-Adelman et al., 2002). It is thought that syndecan 4 aids in sensing the cell's extracellular environment, and as a result, modulating cell adhesion and motility. Syndecan 4 performs these tasks by interacting with adhesion molecules and binding extracellular matrix components necessary for cell migration (Tkachenko et al., 2005). In addition to this, ligated syndecan 4 is capable of activating protein kinase Cα, which contributes to coordinating leading edge and cell posterior retraction (Wilcox-Adelman et al., 2002). Recent studies evaluating syndecan 4-deficiency and syndecan 4 over-expression strongly support its role as an important factor in both cell adhesion and cell migration (Jarousse et al., 2011; Longley et al., 1999). Interestingly, HS is not constitutively attached to syndecan 4 (Longley et al., 1999). In the future it will be important to determine what processes, and to what extent, HS-attachment affects syndecan 4 function.

Unlike syndecan 4, most HSPGs are poorly characterized. For most cell surface associated HSPGs, it is unclear if these molecules have the ability to signal, or if they act as HS-bearing co-receptors. Furthermore, the importance of individual HSPGs is difficult to study due to the fact that most cells express multiple HSPGs with potentially redundant roles. Future studies focusing on individual HSPG HS-dependent and –independent functions will be important for illuminating the various roles these molecules play.

Heparan sulfate in Biology

HS is essential for gastrulation during development, and as a result, all vertebrate life (Perrimon and Bernfield, 2000). HSPGs are expressed in the extracellular matrix and on the surface of almost all mammalian cells and the HS-moieties affect many processes including metabolism, cell adhesion, cell motility, cell migration, cell localization, and ligand-receptor interaction (Bishop et al., 2007). As it is beyond the scope of this introduction to cover all the processes that HS has been shown to modulate, I will briefly illustrate some of the better-understood roles HS plays in development and physiology.

Within the last two decades, heparan sulfate has begun to be appreciated as a major player in how cells sense and respond to their extracellular environment (Turnbull et al., 2001). Work by numerous groups has lead to the notion that HS acts as a "catalyst of molecular encounters." (Lander, 1998). The importance of HS has become most evident by the generation of model organisms unable to synthesize HS. In organisms such as mice, *C. elegans*, and *Drosophila*, deletion of HS-synthesizing enzymes results in embryonic lethality due to abnormal/incomplete development (Bishop et al., 2007). Most of these defects result from the role HS plays in modulating metabolism (Reizes et al., 2001), or in the regulation of morphogen gradients (Koziel et al., 2004). Furthermore, alteration in specific HSPGs or HS expression has been shown to negatively affect specific developmental and cell growth and division pathways such as Hedgehog, Wingless, Decapentaplegic, and Fibroblast Growth Factor (FGF) (Bishop et al., 2007; Perrimon and Bernfield, 2000). Although the role HS plays in each of these signaling pathways varies, HS traditionally acts by one of four different mechanisms: (1) enhancing protein-protein interaction, (2) acting as a co-receptor, (3) sequestration of ligands (4) or through regulation of enzymatic activity (Bishop et al., 2007; Turnbull et al., 2001).

The interaction between HS and the factors antithrombin and FGF represent some of the best-studied examples of HS-mediated regulation. The binding of heparin and heparan sulfate to antithrombin has been exploited in medicine as an effective method to prevent blood clotting. Upon interaction between a distinct pentasacharide within HS and antithrombin, antithrombin undergoes a conformational change that induces its activation. This conformational change results in enhanced inactivation of proteases important for coagulation (Huntington, 2003). This method of regulation is distinct from how HS modulates FGF signaling: interaction between HS and FGF leads to a reduction in the amount of FGF necessary to induce FGF-receptor signaling. HS does this by forming a bridge that increases the likelihood of interaction between FGF and the FGF-receptor (Mohammadi et al., 2005).

Chapter 1.2: An introduction to B cell development and the humoral response

B cell development and maturation

B cell development occurs within the bone marrow (BM). Development is initiated when hematopoietic stem cells commit first to a lymphoid fate, and then to a B cell fate. BM resident stromal cells are critical for these lineage commitments due to the physical contact, and surface and secreted signals they provide to developing B cells. Examples of stromal-derived B cell signals include IL-7, SDF-1, and Stem Cell Factor (Murphy et al., 2008).

Upon commitment to the B cell fate, B cell precursors must pass through numerous checkpoints before they can exit the BM for the periphery. The first of these checkpoints is the Pro-B cell stage. During the Pro-B cell stage, developing B cells must successfully arrange the immunoglobulin (Ig) heavy chain locus. Once a successful Ig heavy chain has been produced, it is expressed on the surface of the cell as a Pre-B cell receptor (BCR), marking the beginning of the Pre-B cell stage. Expression of the Pre-BCR halts Ig heavy chain rearrangement and allows for arrangement of the Ig light chain. Upon arrangement of a functional Ig light chain, the cell expresses a functional IgM at its surface, marking the entrance into the immature B cell stage. Before immature B cells can egress from the BM, B cell autoreactivity towards self antigens within the BM (central tolerance), is assessed. Non self-reactive immature B cells are allowed to leave the BM (Murphy et al., 2008).

Once immature B cells exit the BM, they begin the process of maturing in the periphery. Maturation within the periphery ensures that self-reactive B cells are eliminated, (a process termed peripheral tolerance) (Su et al., 2004). Notably, there are two distinct stages between immature B cells and mature B cells; transitional type-1 and transitional type-2 B cells (Su et al., 2004). Peripheral B cells must first pass through these transitional stages before they can become mature B cells, of which there are three types: follicular mantle B cells, marginal zone B cells, and B-1 B cells. Relative to B cell development in the BM, little is known regarding the mechanisms necessary for B cell maturation in the periphery. It is apparent that the maturation process is dependent on B cell access to cytokines and growth factors that are present within the B cell follicles of secondary lymphoid organs (Murphy et al., 2008)(Su et al., 2004). Without B cell access to cytokines such as BAFF, B cells will enter apoptosis within days of entering the periphery. BCR signaling is also essential for maturation of peripheral B cells due to its importance for assessing peripheral tolerance (Su et al., 2004).

Sites of antigen encounter

In order to generate a robust B cell response, a B cell must encounter its cognate antigen and then present this antigen (in the form of a MHCII-peptide complex) to a T cell specific for the same antigen (Murphy et al., 2008). Given the low frequency of B and T cells specific for a particular antigen, the probability of this occurring is extremely low. Secondary lymphoid organs positioned throughout the body increase this probability of interaction by serving as a 24 hour ‘rest stop’ for lymphocytes (Batista and Harwood, 2009; Cyster, 1999; Harwood and Batista, 2008). Additionally, secondary lymphoid organs sample and concentrate antigens in the blood and lymphatic system, which allows for increased surveillance of these antigens by lymphocytes (Batista and Harwood, 2009).

B cell homing and localization

Homing of B cells to secondary lymphoid organs is the process by which B cells leave the vasculature and enter secondary lymphoid organs. This process is dependent on a number of stepwise interactions that allow B cells to interact with, and transmigrate across, specialized blood vessels called high endothelial venules (HEV). The first step in this process involves interaction of L-selectins expressed on B cells with sulfated glycans expressed on HEVs (Kawashima, 2006). This interaction induces sticking/rolling of B cells along the HEVs. B cell sticking/rolling along HEVs increases the likelihood of encountering the chemokine CCL21, which is essential for homing. The binding of CCL21 to the receptor CCR7, which is expressed on the B cell, induces surface expression of integrins that enable B cells to adhere firmly to HEVs via an integrin-ICAM interaction. This is followed by transmigration of the B cell across the HEV into the T cell zone of the lymph node (Kawashima, 2006).

The secondary lymphoid organs are organized with most B and T cells partitioned. This produces an architecture within lymph nodes where B cells are found mostly within the B cell follicle, which resides below the lymph node capsule. T cells are found mostly within the T cell zone (Phan et al., 2009a). Upon transmigration across the HEVs into the lymph nodes, B cells must migrate across the T cell zone to reach the B cell follicle. This directed migration is attributed to the CXCR5 receptor expressed on the B cell responding to the chemokine CXCL13 (also known as BLC), which is produced in high concentrations by follicular dendritic cells within the B cell follicle (Allen et al., 2007a). As T cells enter the lymph nodes from the HEVs, they find themselves retained in the T cell zone by interactions between the CCR7 receptor and its two ligands, CCL19 and CCL21 (Allen et al., 2007a; Okada and Cyster, 2006). Interestingly, B cells also express low levels CCR7 (it serves to activate integrin expression on B cells in the HEVs), however these low levels of expression are not sufficient to overcome the CXCR5-CXCL13 migration signal.

Upon activation of the BCR, B cells upregulate CCR7 2-3 fold (Okada et al., 2005). This increase in CCR7 expression leads to enhanced responsiveness to CCL19 and CCL21, causing the B cell to migrate to the T cell zone-B cell follicle border. Here, the activated B cell has an increased chance of presenting processed antigen to a cognate $CD4^+$ T cell. Complementary to this, activated $CD4^+$ T cells upregulate CXCR5, and also migrate towards the T cell zone-B cell follicle border (Allen et al., 2007a).

The B cell Response

The adaptive immune response contributes to the elimination of invading pathogens while simultaneously generating long-term protection against pathogens encountered (Murphy et al., 2008). The two dominant components that comprise adaptive immunity are the humoral- and the cellular-immune responses. While cellular immunity consists of cells directly mediating the elimination of pathogen-infected cells, humoral immunity targets extracellular pathogens or pathogens with an extracellular intermediate. This is achieved mainly through B-cell mediated production and release of antibodies that ultimately contribute to the elimination of pathogens.

The B cell response can be initiated by two distinct mechanisms: Thymus independent (TI) and Thymus dependent (TD) (Allen et al., 2007a) (Fagarasan and Honjo, 2000) (Murphy et al., 2008). Independent of T cell help, B cells can produce antibody upon encountering either B

cell-specific mitogens (lipopolysacharide) or through extensive cross-linking of the B cell receptor (by pathogens with repetitive motifs, such as viruses) (Fagarasan and Honjo, 2000) (Murphy et al., 2008). The TI response is comprised mainly of the IgM isotype. However, cytokines such as BAFF and APRIL released by dendritic cells can induce limited isotype switching (Castigli et al., 2004; Mackay and Schneider, 2009). The TI antibody response is usually generated early post-infection and is important to hamper pathogen replication in the early stages of an infection. As the TI response wanes, the TD response increases in quality (Murphy et al., 2008).

B cells can also be activated in a TD fashion. The TD B cell response necessitates interaction of a T cell and a B cell that recognize the same antigen; after an antigen binds to and signals through the BCR, the antigen is internalized and presented to a T cell specific for the same antigen (Murphy et al., 2008). B cells that have received T cell help initiate a germinal center (GC), in which they undergo antibody affinity maturation and isotype switching (from IgM to IgG or IgA) (Allen et al., 2007a; Allen et al., 2007b). These germline rearrangements improve the quality of the humoral response (Allen et al., 2007a) (Allen et al., 2007b; Phan et al., 2009a). GCs give rise to either an antibody producing plasma cell or a memory B cell (McHeyzer-Williams and McHeyzer-Williams, 2005). Importantly, these two B cell populations provide long-term protection against previously encountered antigens (McHeyzer-Williams and McHeyzer-Williams, 2005).

Critical to initiating and maintaining a B cell response is B cell localization, cell-cell interaction, and responsiveness to numerous soluble ligands (chemokines and cytokines) (Matloubian et al., 2004; Okada and Cyster, 2006; Phan et al., 2009b; Phan et al., 2007) (Phan et al., 2009a)). This is illustrated by the concentration of B cells within the lymph nodes and their chemokine-dependent migratory patterns that result in the surveying of follicular dendritic cells for antigen (Matloubian et al., 2004; Suzuki et al., 2009).

Figure 1.1

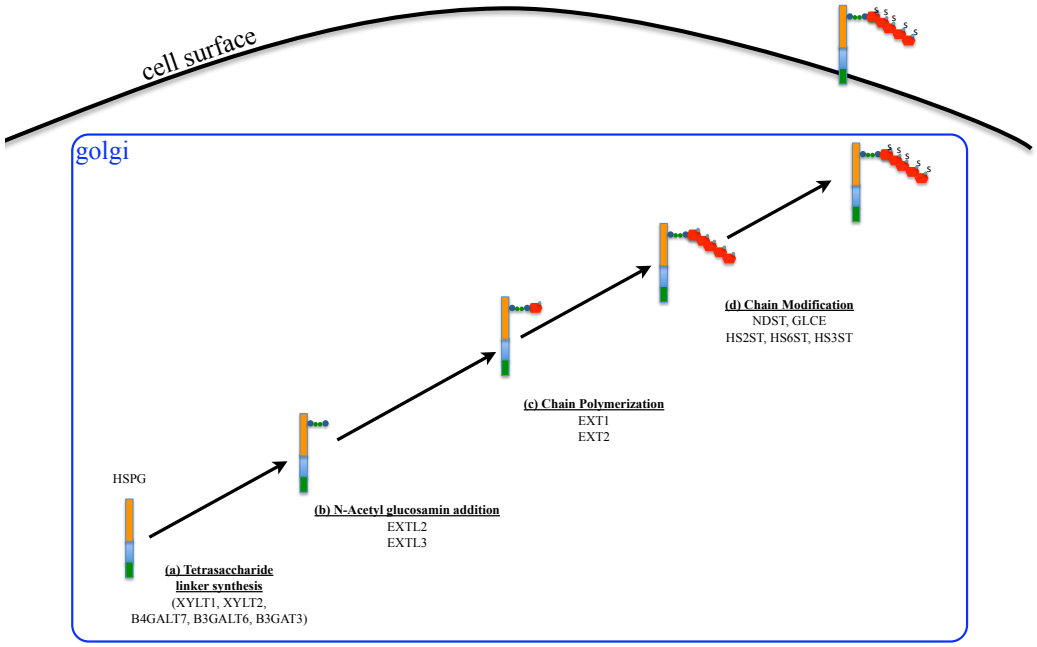


Figure 1.1. Biosynthesis of heparan sulfate. Within the golgi apparatus, heparan sulfate synthesis can be divided into four stages. In the first stage (a), a tetrasaccharide linker is attached to a heparan sulfate proteoglycan by the following enzyme families: XYLT1, XYLT2, B4GALT7, B3GALT6, and B3GAT3. This is followed by the addition of the first N-acetyl glucosamine (b), which is performed by EXTL2 and EXTL3. (c) The third step consists of EXT1- and EXT2-mediated heparan sulfate chain polymerization. Finally, the length of the heparan sulfate chain is modified by deacetylation, epimerization, and sulfation (d). These reactions are carried out by the NDST, GLCE, HS2ST, HS6ST, and HS3ST enzyme families.

Chapter 2: Virally-induced upregulation of heparan sulfate on B cells via the action of type-I interferon

Background

Heparan sulfate (HS) is a glycosaminoglycan abundantly expressed as proteoglycans on most cell surfaces and in the extracellular matrix (Bernfield et al., 1999b). Upon synthesis in the Golgi apparatus, HS is attached to a restricted group of core proteins called heparan sulfate proteoglycans (HSPG), consisting primarily of members of the syndecan and glypican families (Bishop et al., 2007). The study of various genetic mutants defective in HS biosynthesis has shown that HS plays an essential role in mammalian development and physiology (Bishop et al., 2007). HS polysaccharides mediate interactions with a wide variety of extracellular ligands including growth factors, cytokines, chemokines, enzymes, and cell-adhesion molecules (Bishop et al., 2007; Lortat-Jacob, 2009). Heparan sulfate serves as a coreceptor in many cell signaling pathways and modulates signal transduction (Lin, 2004). HS also facilitates cell adhesion to the extracellular matrix, and acts as a transporter of chemokines across cells and as a stabilizer of morphogen gradients along epithelial surfaces (Bishop et al., 2007; Lambaerts et al., 2009; Morgan et al., 2007). HS 'capture' of soluble protein ligands from three-dimensional space converts them into a two-dimensional array that enhances further molecular encounters (Lander, 1998). Thus, HS is involved in many physiological systems, orchestrating cellular processes such as growth, division, adhesion, migration, and differentiation (Bernfield et al., 1999a; Bishop et al., 2007).

HS is commonly described as a ubiquitously expressed glycosaminoglycan, but to date its expression and function have been examined mainly in adherent cells. Although many cytokines involved in immune function are known to bind HS (IL2-8, IL10, IL12, IFNγ, TNFα, MIP1β, APRIL, etc) (Bishop et al., 2007), little is known about HS contribution to lymphocyte biology. While HS molecules were shown to play a role in IL7-dependent B lymphopoiesis (Dias et al., 2005), the importance of HS chains at the surface of mature B cells is not clear. Intriguingly, HSPGs are expressed at certain stages of B lymphocyte differentiation. For example, syndecan 1 (CD138) is expressed on pre-B cells, absent in circulating and peripheral B-lymphocytes, and re-expressed upon differentiation to the plasma cell stage (O'Connell et al., 2004). Syndecan 4 is expressed on bone-marrow B cells and most mature B cell subsets (Yamashita et al., 1999). Although the expression of syndecans is tightly regulated, their function in B cell biology are not defined.

Our lab recently reported that B cell lines express little to no HS at their surface (Jarousse et al., 2008), which prompted us to examine the status of HS at the surface of naïve splenic B cells. We here report that naïve splenic murine B cells express very little HS at their surface, but that HS expression was upregulated upon a type-I interferon (IFN-I) response *in vivo*. This upregulation was essential for the signaling of APRIL, a HS-binding cytokine (Kimberley et al., 2009b) involved in class switch recombination and B cell survival (Kimberley et al., 2009b) (Schneider, 2005a). Attachment of HS to syndecan 4 and a 130 kDa HSPG accounts for the induction of HS on B cells. Altogether, our results suggest that in the context of an infection that triggers a type-I IFN response, B cell responsiveness to cytokines and other HS-binding ligands might be amplified due to an upregulation of HS surface display.

Materials and Methods

Mice and viruses

C57Bl/6 were obtained from Jackson Laboratory. Mice deficient for the interferon-α/β receptor (IFNAR KO mice) were a kind gift from Dr. Daniel Portnoy. Bone marrow from Syndecan 4 deficient mice was a kind gift from Dr. Sarah Wilcox-Adelman. Experiments were conducted with 6 to 12 week old mice in accordance with institutional guidelines for animal care and use. MHV68 was obtained from the ATCC (VR-1465) and propagated on BHK-21 cells. Smith strain MCMV (MW97.01) was a kind gift from Dr. David Raulet. MCMV was propagated on NIH3T3 cells. Virus containing supernatants were cleared of cellular debris by low speed centrifugation for 20 min. Virus was subsequently concentrated by centrifugation at 24,000 g for 2 hrs and purified over a 30% sucrose cushion by centrifugation at 85,000 g. Pelleted virus was resuspended in PBS and stored at -80 °C. Viral titers were determined on NIH3T3 cells using a standard plaque assay. BHK-21 and NIH 3T3 cells were grown in DMEM supplemented with 10% FBS and 100 Units/ml penicillin and 100 mg/ml streptomycin.

Isolation and culture of splenic B cells

Splenocytes were isolated from C57BL/6 or IFNAR KO mice and B cells were purified using the EasySep negative selection mouse B cell enrichment kit (Stem Cell Technologies). B cell purity was assessed by flow cytometry using anti CD19 or B220 antibodies (over 98% purity). Cell viability was verified by propidium iodide or 7AAD staining (over 99% viability). Cells were resuspended at a density of $2x10^6$ cells/ml (except for APRIL assay, see below) in RPMI media containing 100mM MEM non-essential amino acids, 55mM 2-Mercaptoethanol, 1 mM Sodium Pyruvate and 10 mM Hepes. When indicated, reagents were added to the media: Interferon b (IFNb) at 1000U/ml unless indicated otherwise (PBL Interferon source), anti IgM at 2μg/ml (Jackson Immunoresearch), type A and Type B CpG oligos at 3mM, LPS at 1μg/ml.

Flow cytometry

Splenocytes or purified splenic B cells were washed in PBS with 1% BSA and incubated with 1mg/ml purified rat anti-mouse CD16/CD32 (Fc block, BD Pharmingen) at 4°C for 15 min, followed with an anti HS antibody used at 1:100 (10E4 epitope, F58-10E4, from Seikagaku Corporation) for 30 min at 4^oC. The isotype control was a mouse IgM kappa (TEPC 183, Sigma). After two washes in PBS with 1% BSA, bound antibody was revealed by staining with a FITC- or PE-conjugated anti-mouse IgM^a (Igh-6a) monoclonal antibody (BD Pharmingen). When staining for TACI, PE-conjugated anti-TACI (BD 558410) was used. Monoclonal antibody KY/8.2 (BD cat. #550350) was used to stain for Syndecan 4. The isotype control for syndecan 4 was rat IgG2a (R&D Systems; Cat#MAB006). When using total splenocytes, cells were co-stained with an anti B220 or anti CD19 antibody directly conjugated with a fluorochrome. When staining cultured B cells, dead cells were excluded from the analysis by propidium iodide or 7AAD staining.

Measure of APRIL-mediated IgA production

293T cells were transfected with either PCDNA3.1 (vector control) or a vector encoding soluble APRIL (a kind gift from Dr. Kimberley Fiona) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days later, supernatant was harvested from transfected 293T cells and diluted 1:2 in RPMI that included supplements and fetal bovine serum. Diluted

supernatant was used to resuspend purified splenic B cells ($1X10^6$ cells per ml). $2X10^5$ cells/well (200ml) were plated in a round-bottom 96 well plate. Cells were left untreated, treated with either Interferon β (45 U/mL) or with 3units/ml of heparinase III (Sigma-Aldrich), or both simultaneously. Cells were cultured for six days. At days 2 and 4 post-plating, an additional 0.6 units/well of heparinase was added to all heparinase-treated samples. At 6 days post plating, supernatant from wells was collected and added to ELISA plates coated with anti-IgA antibody (Southern Biotech). IgA production was measured with an alkaline-phosphatase conjugated anti-IgA antibody. Substrate conversion (pNpP from Sigma; cat. # N2770) was monitored using a plate reader measuring at λ405nm. A standard curve using known concentrations of IgA was generated and used to determine IgA concentration (ng/ml) in the B cell supernatants.

Generation of bone marrow chimeras

Female C57BL/6 congenic mice (CD45.1+) between 6-8 weeks were lethally irradiated with 900 rads. 24 hours later, the mice were injected (iv) with $5X10^6$ bone marrow cells from syndecan deficient mice (C57BL/6 background; CD45.2+). 10 weeks post injection, reconstitution efficiency was checked by tail-vein bleeding.

Western Blot and immunoprecipitation

Purified *ex vivo* B cells were treated ± IFN-I, then were incubated ± with heparinase and chondroitinase (heparinase: Sigma cat. #H8891-50UN) (10^7 cells/ml; 1 unit/ml) (chondroitinase: Sigma cat. # C3667-10UN) (10^7 cells/ml; 0.005 Units/ml) for 3 hours at 37 degrees. Cells were then washed and lysed prior to SDS-PAGE. For immunoprecipitation, lysate was precleared with protein A/G beads (Santa Cruz Biotech cat. #sc2003), and then immunoprecipitated using an anti-syndecan 4 antibody (Abcam cat. #ab24511). The immunoprecipitate was washed prior to SDS-PAGE. After transferring to immobiline membrane (Thermo Fisher), the membrane was probed first with anti-HS (clone 3G10; Seikagaku cat. # 370260-1), and then with HRP-conjugated anti-mouse IgG. Western Blots were developed using Super Signal West Pico Chemiluminescent Substrate (Pierce biotechnology; Cat. # 34080), and exposed onto film.

Statistical Analysis

All statistical results are expressed as mean ± SEM. Statistical analysis was performed using a non-parametric Mann-Whitney test for comparison between two groups (Graphpad software). Differences were considered significant at $p<0.05$.

RESULTS

Naive B cells express low levels of surface HS

To determine the expression level of HS on naïve murine B cells, splenocytes from C57BL/6 mice were isolated, stained with a commonly used anti-HS antibody (F58-10E4, from Seikagaku Corporation) and analyzed by flow cytometry. B cells were identified using an anti-B220 antibody (similar results were obtained using an anti-CD19 antibody). The murine NIH3T3 fibroblast cell line was used as a positive control (Figure 2.1). Similar to what we observed in B cell lines (Jarousse et al., 2008), we found that the level of HS expression on the surface of splenic B cells is very low or undetectable. Likewise, Van der Voort et al. reported that HS was hardly detectable at the surface of resting human tonsillar B cells. Thus it appears that neither murine nor human naive B cells express a significant level of heparan sulfate at their surface.

HS is upregulated at the B cell surface following herpesvirus infection *in vivo*

HS participates in cell surface binding of many cytokines involved in inflammation and also plays an important role in cell adhesion and migration (Bishop et al., 2007). Interestingly, van der Voort *et al.* showed that HS was upregulated at the surface of human tonsillar B cells upon activation in culture. Thus, we speculated that HS expression might be upregulated at the surface of B cells during an immune response *in vivo,* and this upregulation might be important for B cell function. To test whether HS is upregulated on B lymphocytes in the context of an infection *in vivo*, wild-type C57BL/6 mice were infected with murine gammaherpesvirus 68 (MHV68) either by intravenous (iv) or intraperitoneal (ip) infection. Mice were sacrificed at various time points after infection and splenocytes were examined for HS expression as described above. HS is upregulated at the B cell surface as early as 12 hours after MHV68 iv infection (Figure 2.2A, left panel). Importantly, HS upregulation is observed on the entire B cell population (Figure 2.2A, middle panel). The increase in HS surface expression is also detected after ip infection (Figure 2.2A right panel). Although HS induction on the entire B cell population as early as 12 hours post infection (pi) is unlikely to be the result of a direct interaction with MHV68, we verified that HS upregulation was also observed following infection with murine cytomegalovirus (MCMV), a virus that does not target B cells. As shown in Figure 2.2B, MCMV infection also triggers an increase in HS expression at the B cell surface following either iv (left panel) or ip (right panel) infection. In all cases, the entire B cell population exhibits enhanced HS expression, suggesting the possible action of a diffusible agent such as a cytokine.

HS upregulation is mediated by IFN-I

Type-I interferon (IFN-I) constitutes the first line of host defense against viruses (Basler and García-Sastre, 2002). IFN-I plays a critical role in controlling both the acute and latent phases of MHV68 infection (Barton et al., 2005; Dutia et al., 1999). To test if IFN-I is involved in HS upregulation in B cells following MHV68 infection, mice deficient for the interferon-α/β receptor (IFNAR KO mice) were infected with MHV68 and HS expression on B cells was examined at 36 hours post infection. Whereas HS was strongly induced on B cells from infected wt mice, the level of HS expression on B cells from infected IFNAR KO mice was close to

background, comparable to that of control PBS-injected mice (Figure 2.3A), indicating a role for IFN-I in HS upregulation during an *in vivo* immune response.

To further test the role of IFN-I in HS induction, we used another model of IFN-I induction in the absence of viral infection. Polyinosine polycytidylic acid (Poly I:C) is a double-stranded RNA mimetic that is commonly used to trigger IFN-I production *in vivo* (Pugliese et al., 1980). Wild-type C57BL/6 mice were treated with poly I:C (iv injection) and HS expression was measured at the surface of splenic B cells. Enhanced HS expression was observed as early as 12 hours after poly I:C injection (Figure 2.3B left). Interestingly, the induction of HS after poly I:C injection was observed only on B cells, as the non-B cell fraction of splenocytes showed no significant HS expression (Figure 2.3B right).

To test the direct effect of IFN-I on B cells, in the absence of other cell types or cytokines, splenic B cells were purified from wt mice and cultured in the presence of recombinant IFN b for 24 hours. Surface HS was examined by flow cytometry. Culturing purified B cells for 24 hours, in the absence of IFN-I or any other stimulator, lead to an upregulation of surface HS (Figure 2.3C: media). Notably, IFN treatment results in a dramatic increase in HS expression over media alone (Figure 2.3C: IFN-I). Importantly, the induction of HS on B cells is not due to HSPG binding-Transmembrane Activator and Calcium Modulator and Cyclophilin Interactor (TACI) (Figure 2.7), as IFN-I did not induce TACI on B cells. Altogether, our results indicate that IFN-I-mediated signaling triggers upregulation of HS expression at the surface of B cells and accounts for the upregulation observed *in vivo* upon MHV68 infection. Interestingly, the increased HS on B cells upon culturing (Figure 2.3C: media) is also observed on B cells isolated from IFNAR KO mice (Figure 2.3D, right panel), indicating that signals other than IFN-I can trigger HS to be upregulated in culture.

Activation of purified splenic B cells through the B cell antigen receptor (via an anti-IgM antibody) also results in an increase in HS expression over media alone (compare Figure 2.4A and 2.4B), consistent with an earlier study on human tonsillar B cells (Van Der Voort et al., 2000). Similarly, stimulation of TLR 4 with LPS (Figure 2.4C), TLR9 with type A (Figure 2.4D) or type B (Figure 2.4E) CpG oligonucleotides leads to an upregulation of HS at the B cell surface. While this upregulation of HS is not dependent on IFN-I signaling, IFN-I does potentiate HS induction as demonstrated by the lower amount of HS observed on purified IFNAR KO B cells treated with the above stimulatory ligands (2.4A-2.4E; dashed lines). Altogether, our results indicate that a variety of stimuli normally present during the course of an infection, such as IFN-I, TLR-ligands, or molecules capable of cross-linking BCRs cause HS to be upregulated in B cells.

Syndecan 4 and a 130 kDa HSPG are modified with HS on B cells upon IFN-I treatment

We hypothesized that the increase in HS expression on B cells was due to an increase in expression of HSPGs. To investigate this possibility, we decided to examine the expression of all known HSPGs (syndecan 1-4, glypican 1-6, TGFβR3, agrin, and CD44v3). Their expression in naïve and IFN-I -treated B cells was examined by q-RT-PCR and surface staining (when commercial antibodies were available). We observed that while most HSPGs were expressed at extremely low levels or not expressed at all (data not shown), syndecan 4 was readily detectable. However, its surface expression was only slightly increased in B cells cultured in the presence of IFN-I compared to freshly isolated naïve B cells (Figure 2.5A). To determine if syndecan 4 is modified with HS, *ex vivo* and IFN-I treated B cells were treated with or without heparinase (an

enzyme that degrades heparan sulfate). Cells were then lysed and syndecan 4 was immunoprecipitated. This was followed by a western blot for HS using the 3G10 antibody (Seikagaku), which recognizes a neo-epitope that is generated upon heparinase treatment. Figure 2.5B shows that syndecan 4 from IFN-I treated B cells, but not from naïve B cells, is associated with HS (compare heparinase treated *ex vivo* and IFN-I treated lanes), indicating that syndecan 4 is modified with HS in response to IFN-I treatment.

To test if syndecan 4 is the sole HSPG that accounts for HS surface expression on B cells, we generated syndecan 4 KO bone marrow chimeras by iv injection of syndecan 4 KO bone marrow ($CD45.2^+$) into lethally irradiated congenic wt mice ($CD45.1^+$). Upon reconstitution, we confirmed that syndecan 4 was not expressed in $CD45.2^+$ B cells by RT-PCR (data not shown). B cells were purified from wt and syndecan 4 KO bone marrow chimeras and treated in culture with IFN-I. Twenty-four hours later, HS expression on B cells was assessed by flow cytometry. Surprisingly, we observed that HS induction was not affected by the absence of syndecan 4 expression on B cells (Figure 2.5C).

To take an unbiased approach in identifying HSPGs expressed on B cells, we performed a western blot for HS on lysates isolated from *ex vivo* or IFN-I treated B cells (using the 3G10 antibody). In agreement with our above results, HS expression was not detected on *ex vivo* B cells (Figure 2.5D). However, in IFN-I-treated wt B cells, we observed 3 bands corresponding to the molecular weight of approximately 130, 55 and 37 kDa. Two of these bands (55 and 37kDa) were absent in IFN-I-treated syndecan 4 KO B cells, suggesting that these two lower molecular weight bands correspond to syndecan 4. This is in agreement with the molecular weight of HS-modified syndecan 4 observed in Figure 5B. The presence of a 130 kDa band in IFN-I-treated wt and syndecan 4 deficient B cells suggests that HS moieties are also carried by an additional high molecular weight HSPG. Interestingly, culturing of B cells in the absence of IFN-I induced only HS-modified syndecan 4 (Figure 2.8). Only when B cells were cultured in the presence of IFN-I, or isolated from a poly I:C injected mouse, were both syndecan 4 and the 130 kDa HSPG induced. These data indicate that various stimuli can induce HS-modified syndecan 4, but IFN-I signaling induces HS modification of both syndecan 4 and the 130 kDa HSPG.

HS expression on B cells is essential for APRIL-mediated signaling

Given the role of HS in receptor signaling, we hypothesized that HS induction at the B cell surface increases responsiveness to HS-binding cytokines (Bishop et al., 2007). To address this hypothesis, we analyzed the signaling of A Proliferation Inducing Ligand (APRIL), a member of the tumor necrosis factor family that has been shown to bind HS (Ingold et al., 2005; Kimberley et al., 2009b). APRIL promotes B cell survival and IgG to IgA isotype switching (Kimberley et al., 2009a). We treated purified B cells with soluble APRIL (produced by transfection of 293T cells) or with control media (supernatant from pcDNA3.1-transfected 293T cells), in the presence or absence of IFN-I and heparinase either individually or concomitantly. We then assessed APRIL signaling by measuring secreted IgA. As shown in Figure 2.6A, we found that heparinase treated B cells produced less IgA than untreated controls. IFN-I treatment, which we show induces HS expression, increased APRIL-mediated IgA production. Simultaneous addition of heparinase and IFN-I drastically reduced APRIL-induced IgA production. Importantly, B cell responsiveness to APRIL, via HS upregulation, was enhanced upon IFN-I signaling, as IgA production was reduced in IFNAR KO B cells (Figure 2.6B). Altogether, these results indicate that IFN-I-induced HS expression on B cells is important for *in*

vitro APRIL responsiveness.

Figure 2.1

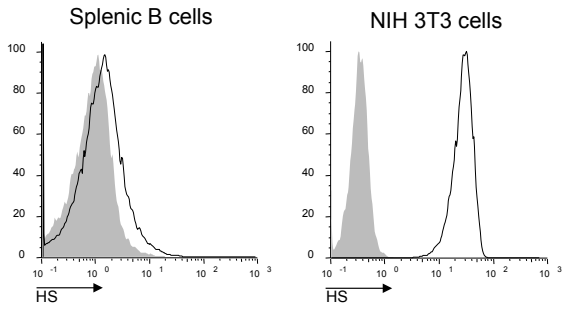


Figure 2.1. Naive B cells express very little HS at their surface. Freshly isolated murine splenocytes were stained with anti-HS antibody and an anti-B220 antibody. The histogram shows the level of HS on B220-positive cells (left panel). NIH3T3 cells were tested for HS expression as a control (right panel). Shaded histogram: isotype control. Y axis is percent of maximum. One representative experiment is shown from a total of more than ten repeats, where 1-3 mice were used per repeat.

Figure 2.2

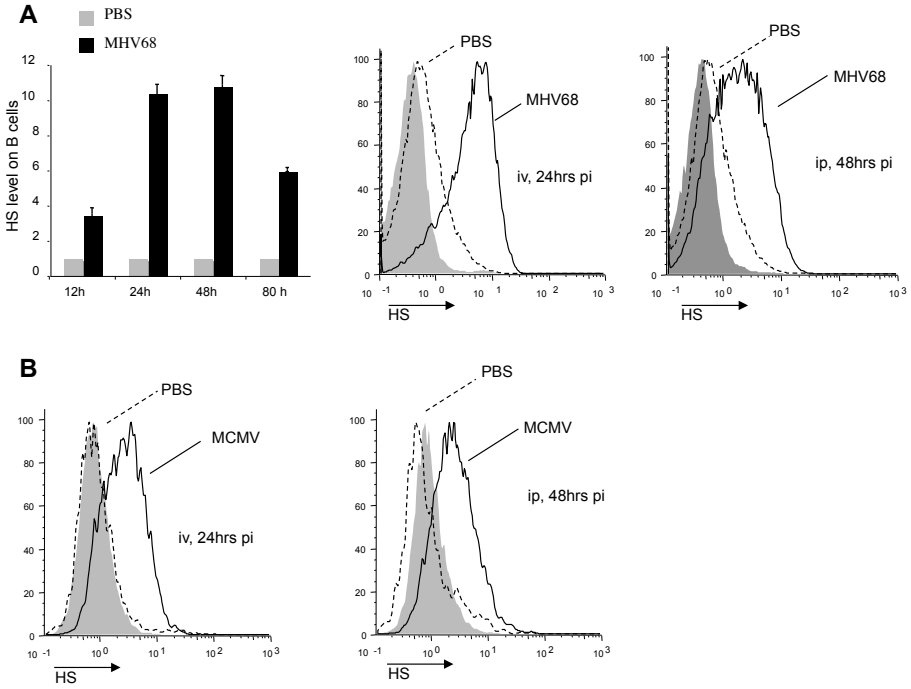


Figure 2.2. **HS is induced on the surface of splenic B cells after infection of mice with MHV68 or MCMV. (A)** C57BL/6 mice were injected with 5x10^5 pfu of MHV68, or PBS as a control. Mice were euthanized at different time points and HS expression was analyzed on B cells as before. Left panel: Mice were injected intravenously (iv). Results are expressed as a fold-induction over PBS-injected mice (PBS control set to 1). Middle panel (iv, 24 hours post infection [pi]). Right panel: Mice were injected intraperitoneally (ip); (ip, 48 hrs pi). **(B)** C57BL/6 mice were injected intravenously (left panel) or intraperitoneally (right panel) with 5x10^5 pfu of MCMV, or PBS as a control, and HS expression on splenic B cells was analyzed as before after 24 hours (iv) or 48 hours (ip). Y axis for all histograms is percent of maximum. Graph (A) and histograms (A and B) depict one representative experiment using 5-10 mice that was repeated at least 3 times.

Figure 2.3

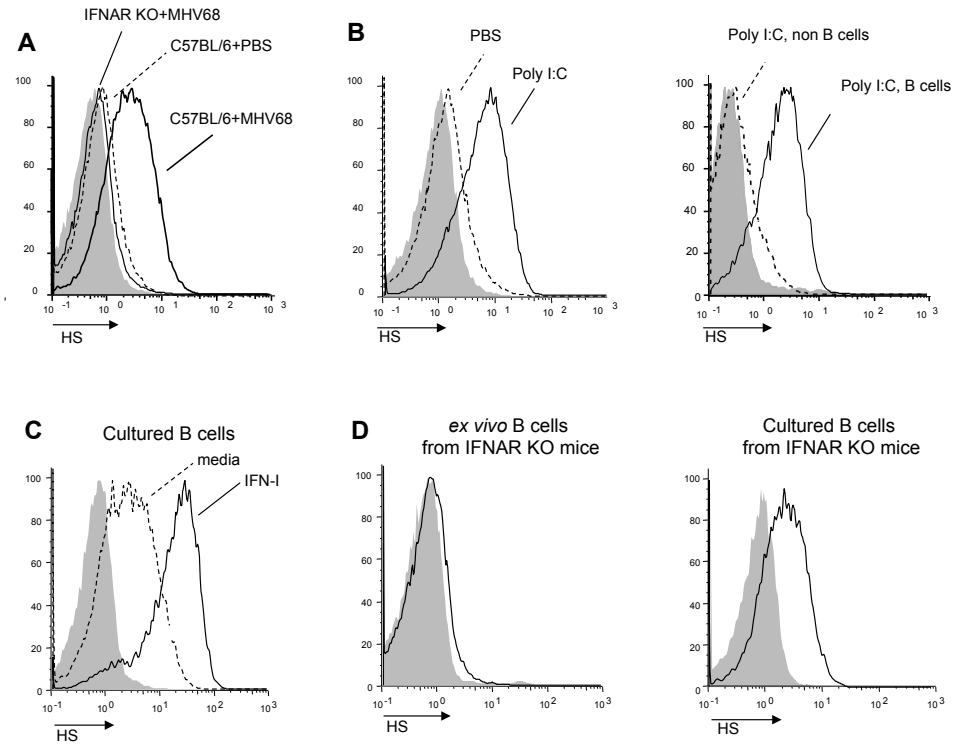


Figure 2.3. IFN-I triggers HS upregulation at the B cell surface. (**A**) C57BL/6 mice or Type-I interferon receptor deficient mice (IFNAR KO) were injected iv with 5x10^5 pfu of MHV68 and examined as before for HS expression on B cells after 36 hours. (**B**) C57BL/6 mice were injected with 200μg of poly I:C and analyzed as before for HS expression on B cells ($CD19^+$) or non B cells ($CD19^-$) 12 hours later. (**C**) B cells were purified from C57BL/6 mice and incubated for 24 hours in media± IFN β (1,000U/ml). HS expression was analyzed by flow cytometry as before. (**D**) Splenic B cells isolated from IFNAR KO mice were analyzed for HS expression either immediately after isolation (left panel) or after 24 hours in culture (right panel). In A, B, C and D: shaded histograms represent an isotype control. Y axis for all histograms is percent of maximum. All histograms are representative experiments using 3-10 mice that were repeated at least 5 times.

Figure 2.4

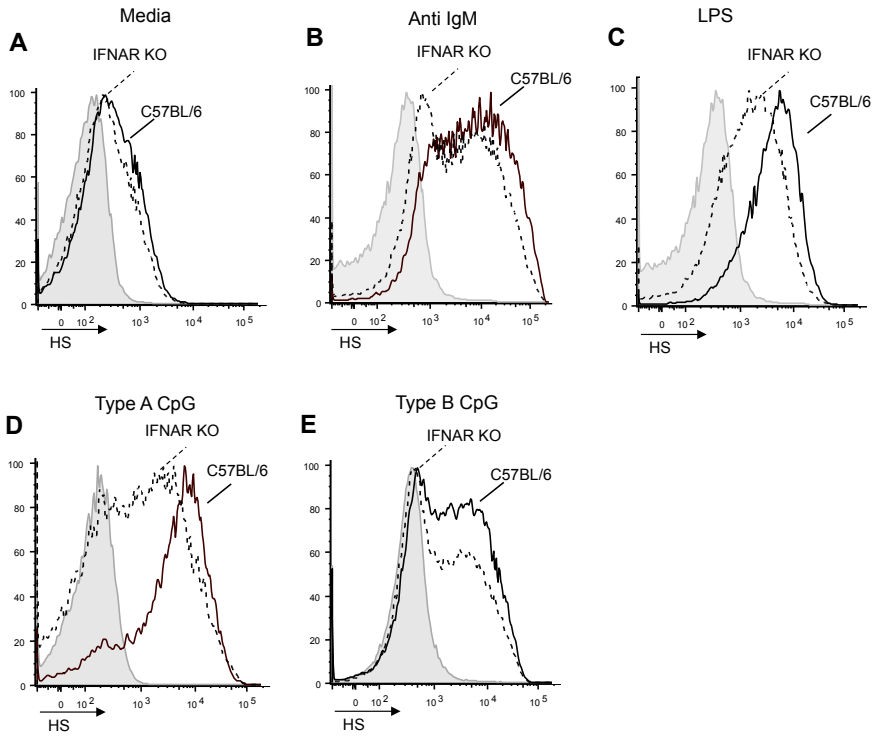


Figure 2.4. Both BCR- and TLR- mediated signaling upregulate HS on B cells. Splenic B cells were purified from C57BL/6 or IFNAR KO mice and incubated for 24 hours with **(A)** Media alone **(B)** anti IgM antibody (2μg/ml), **(C)** LPS (1μg/ml), **(D)** type A CpG oligo (5μg/ml), or **(E)** type B CpG oligo (5μg/ml). Shaded histograms show staining with an isotype control. Y axis for all histograms is percent of maximum. All histograms are representative experiments, using 1-2 mice, and were repeated at least 5 times.

Figure 2.5

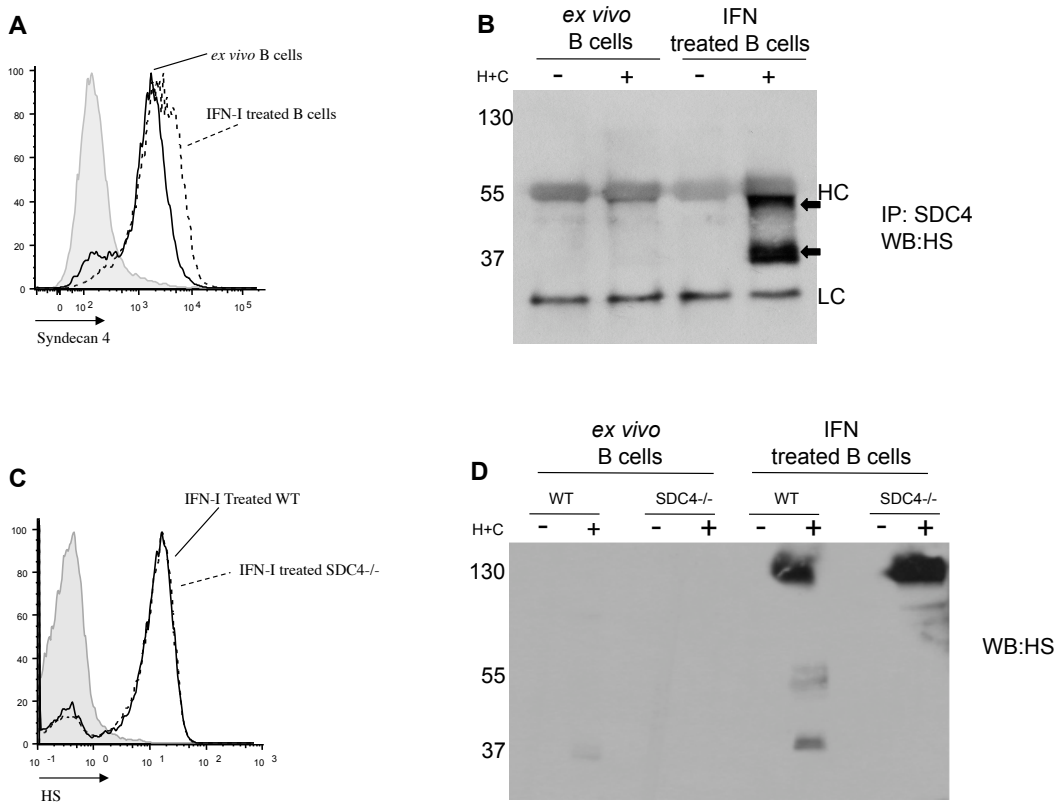


Figure 2.5. **Syndecan 4 is modified with HS on B cells upon IFN-I treatment. (A)** Purified B cells from C57BL/6, either *ex vivo* (solid line) or treated with IFN-I for 24 hours (dashed line) were stained for Syndecan 4 and analyzed by flow cytometry. **(B)** Purified B cells, either cultured for 24 hours in the presence of IFN-I or isolated immediately *ex vivo,* were treated ± heparinase III and chondroitinase before being lysed and immunoprecipitated for syndecan 4. Immunoprecipitates were then run on a SDS-PAGE gel before blotting for HS (3G10). Arrows indicate both syndecan 4 bands. **(C)** Splenic B cells were purified from either C57BL/6 (solid line) or syndecan 4-/- BM chimeras (dashed line) and cultured in the presence of IFN-I. 24 hours post IFN-I treatment, cells were harvested and stained for HS (populations gated on CD45.2^{+}). **(D)** Purified *ex vivo* or IFN-I-treated B cells were treated ± heparinase and chondroitinase prior to lysis and western blot for HS (clone 3G10; Seikaguku). Isotype controls are represented in A and B by shaded histogram. Y axis for all histograms is percent of maximum. Numbers on blot indicate kDa molecular weight marker. Heavy chain (HC) and light chain (LC) are indicated in 5B. Histograms and western blot are representative experiments, using 1-2 mice, and were performed at least 3 times.

Figure 2.6

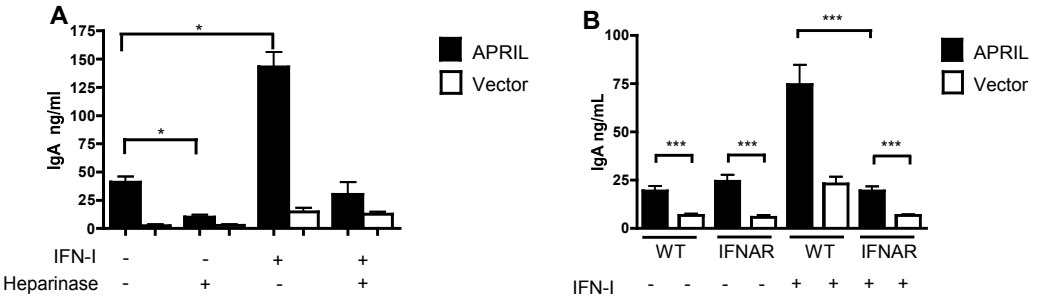


Figure 2.6. IFN-I treatment increases B cell responsiveness to APRIL in an HS-dependent manner. Purified splenic wt or IFNAR KO B cells were incubated with supernatant from APRIL- or pcDNA3.1-transfected 293T cells. B cells were either left untreated, or were treated with heparinase III, IFN-I, or both. IgA production was measured after 6 days of culturing by ELISA. Both graphs are experimental averages from 2-3 experiments where 1-2 mice of specified genotype were used per experiment.

Figure 2.7

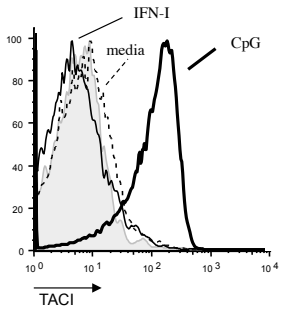


Figure 2.7. HS expression on B cells is not due to TACI binding HS-bearing proteins. Purified splenic B cells were cultured for 24 hours in media alone (dashed line), IFN-I (thin solid line), or CpG (thick solid line). After 24 hours, B cells were stained for TACI with PE-conjugated anti-TACI. Isotype control is represented as shaded histogram. Y axis is percent of maximum. Histogram is a representative experiment, using 1-2 mice, repeated at least 3 times.

Figure 2.8

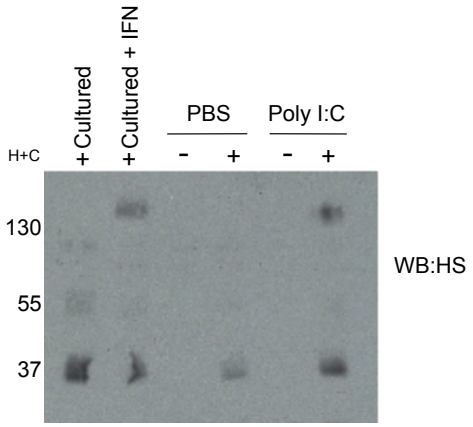


Figure 2.8. IFN treatment of B cells or injection of poly I:C induce both SDC4 and 130 kDa HSPG, while culturing of B cells in the absence of IFN only induces SDC4.
Purified splenic B cells were cultured ± IFN-I for 24 hours before being treated with heparanase and chondroitinase prior to lysis (lanes 1 and 2). B cells were purified from mice injected (iv) with either PBS (lanes 3 and 4) or poly I:C (lanes 5 and 6) 24 hours prior. B cells were then treated ± heparanase and chondroitinase prior to lysis. Lysates were then run on a 10% SDS-PAGE gel and blotted for heparan sulfate using the 3G10 antibody. Western blot is a representative experiment repeated twice.

Discussion

Here we present evidence that HS expression is tightly regulated in mature B cells *in vivo*: whereas HS is barely detectable in naive splenic B cells, its expression was upregulated by the action of IFN-I in mice. Additionally, we observed that HS expression was induced upon stimulation of murine splenic B cells via TLR-dependent pathways as well as BCR stimulation. Importantly, we did not observe an increase in HS expression on the non B cell population following viral infections or poly I:C injection (Figure 2.3B). This does not appear to be due to an inability of these cells to express HS since *ex vivo* activation of monocytes and CD4 T lymphocytes increases HS expression at the cell surface (Bobardt et al.; Jones et al., 2005; Saphire et al., 2001). Altogether, these results indicate that HS is poorly expressed on naive B cells, but its expression can be upregulated on lymphocytes upon exposure to a variety of stimuli normally present during the course of an infection. To our knowledge, our study is the first to show that HS is upregulated on immune cells *in vivo*. Our results are likely to be applicable to other species; van der Voort *et al.* demonstrated that activation of *ex vivo* B cells, via BCR or CD40, enhances HS expression on human tonsillar B cells (Van Der Voort et al., 2000). Such dynamic regulation of HS expression suggests that HS may play a critical role in regulating immune function upon infection.

We hypothesized that the induction of HS could be mediated through upregulation of HSPGs, increased synthesis of HS, or a combination of both. To investigate the mechanism of HS upregulation on B cells, we examined both transcriptional and surface expression (when commercial antibodies were available) of all known HSPGs. Of all HSPGs assessed, only syndecan 4 was expressed at significant levels. Its expression was only slightly enhanced upon treatment of B cells with IFN-I (Figure 2.5A). However, we found that the amount of HS associated with syndecan 4 was dramatically increased in B cells that had been exposed to IFN-I (Figure 5B). Interestingly, we also detected the presence of a high molecular weight HSPG (around 130KDa, Figure 2.5D). As this HSPG was only induced upon IFN-I signaling (Figure 2.8), the identity of this HSPG may give clues as to the function of HS upregulation on B cells in response to IFN-I signaling. The tendency of HSPGs to oligomerize or to run above their predicted molecular weight (Rioux et al., 2002) makes it difficult to identify the nature of this HSPG.

We were surprised to find that HS expression was similar in IFN-I treated wt and syndecan 4 KO B cells (Figure 2.5C) given that syndecan 4 is modified with HS upon IFN-I treatment (Figure 2.5B and 2.5D). One possibility is that the contribution of syndecan 4 to HS surface display is minor, compared to that of the 130 kDa HSPG. Another possible explanation is that the synthesis of the mature form of HS containing N-sulfated glucosamine residues, or its detection by the antibody, (epitope detected by the 10E4 antibody used in our flow cytometry experiments) is limiting in B cells under conditions that trigger a high level of HS (such as ex vivo IFN-I treatment, Fig 2.5C). In the absence of syndecan 4, there would thus be an increased amount of HS linked to the high molecular weight HSPG, leading to similar levels of overall surface HS in wt and syndecan 4 KO B cells.

IFN-I treatment induces an upregulation of HS modification on syndecan 4 molecules (Figure 2.5B and 2.5D), suggesting that IFN-I acts on HS biosynthesis. There are multiple steps where this may occur, as HS biosynthesis involves numerous enzymes (Bret et al., 2009). Initially, the proteoglycan is modified with a tetrasaccharide linker to which HS will be

extended. This process is initiated by the XYLT enzyme family. Upon completion of the tetrasaccharide linker, the enzymes EXTL2 and EXTL3 perform the addition of the first N-acetyl glucosamine, thereby dictating that the chain will be HS and not chondroitin sulfate. This is followed by disaccharide chain polymerization by the exostosin enzymes EXT1 and EXT2. Finally, the disaccharide chain is heterogeneously deacetylated and sulfated by the NDST enzyme family. To determine if HS synthesis was upregulated, we assessed the expression of HS synthesizing enzymes by q-RT-PCR in *ex vivo* and IFN-I-treated B cells. Although we did not see an increase in the mRNA levels of the HS-polymerizing enzymes (EXT1 and EXT2), we did see a two-fold induction in EXTL2, EXTL3, XYLT1, NDST1, and NDST2 (data not shown). Intriguingly, we also observed a 10-fold decrease in EXTL1 (data not shown). Whether or not this modest collective increase in expression of these enzymes contributes to the observed increase in surface HS remains to be determined. It is also possible that HS-synthesizing enzymes are regulated post-translationally, and this could contribute to the increased HS in IFN-I treated B cells.

A proliferation inducing ligand (APRIL), a member of the TNF family of ligands, is implicated in B cell survival, isotype switching, and T cell-independent (TI) antibody responses (Bossen and Schneider, 2006; Kimberley et al., 2009a; Schneider, 2005b). We have shown that IFN-I treatment of B cells increased IgA production in response to APRIL in a HS-dependent manner. We observed that upon culturing, HS expression at the surface of B cells spontaneously increases (Figure 2.3C: media). This likely helps explain why APRIL signaling can be measured in cultured B cells even in the absence of IFN-I or other stimulators of HS expression (Kimberley et al., 2009b).

In addition to regulating cytokine responsiveness, HS is involved in a broad variety of biological processes including cell adhesion and migration (Bishop et al., 2007). It would be interesting to determine if HS upregulation in response to IFN-I production has a direct implication on other aspects of B cell biology. For example, during an immune response, lymphocyte egress from lymphoid organs is temporarily shut down in response to several mediators including IFN-I. The role of IFN-I on lymphocyte retention is explained in part by inhibition of sphingosine 1-phosphate receptor-1 by CD69 (Shiow et al., 2006). Because HS is involved in cell adhesion, its upregulation at the B cell surface might be another key factor involved in IFN-I mediated inhibition of B cell egress from lymphoid organs. Similarly, it will be interesting to determine whether the presence of HS on B cells affects B cell movement and trafficking in secondary lymphoid organs, and ultimately, the ability of B cells to encounter and capture antigen as well as interact with T cells.

Viral infections are associated with a rapid and massive polyclonal B cell activation (Chang et al., 2007; Coro et al., 2006a; Diamond et al., 2003; Purtha et al., 2008). This early activation is thought to contribute to the production of antibodies, the induction of memory B cells, and the retention of B cells in lymphoid organs. IFN-I receptor deficiency on B cells has a profound impact on the quality and the magnitude of the early B cell response following influenza infection (Coro et al., 2006b). Interestingly, groups have suggested that IFN-I signaling in B cells *in vivo* dramatically enhances the antibody response to antigens that are of low immunogenicity, such as chicken gamma globulin (Bach et al., 2007; Le Bon et al., 2001). Because of the pleiotropic effects of IFN-I, it is difficult to ascertain the precise mechanism by which IFN-I potentiates the B cell response. However, it is tempting to hypothesize that the induction of HS on B cells may be part of early B cell activation, and this contributes to an increased antibody response. Given the number of HS-binding cytokines that have immuno-

modulatory activity (IL2-8, IL10, IL12, IFNγ, TNFα, MIP1β, APRIL(Bishop et al., 2007)), HS induction may prove critical to B cell responses.

Reijmers *et al.* (Reijmers et al., 2011) recently published a report showing that mice whose lymphocytes are impaired in their ability to produce functional HS display decreased B cell numbers and diminished B cell function. These findings differ from those of Garner *et al.* (Garner et al., 2008), who found that mice lacking HS specifically on B cells displayed only slight changes in lymphocyte development and function. These discrepancies are likely to be explained by the different systems employed. Importantly, most assays done in both the Reijmers and the Garner paper were done in conditions that would not induce the high levels of HS we observed. Thus, it would be of interest to determine how IFN-I-, TLR-, or BCR-mediated induction of HS would affect B cell function in their experimental system.

Altogether, our results suggest that IFN-I-mediated regulation of HS expression serves as a novel mechanism to increase cytokine responsiveness of B cells upon viral infection (and thus the regulation of the antibody responses). Furthermore, if IFN-I-mediated HS induction on B cells enhances their responsiveness, this may have implications in the understanding of autoimmune disorders with a B cell component. For example, Systemic Lupus Erythematosus, an autoimmune disorder characterized by elevated IFN-I and auto-reactive B cells (Bennett et al., 2003), may be better understood as a result of the work presented here.

Chapter 3: HS expression on B cells affects the generation and/or maintenance of plasma cells

Background

Heparan sulfate (HS) is a post-translational modification comprised of a repeating disaccharide (uronic acid and N-acetyl glucosamine) that undergoes heterogeneous deacetylation, sulfation, and epimerization (Sugahara and Kitagawa, 2002). This glycosaminoglycan is attached to a core-protein, creating a heparan sulfate proteoglycan (HSPG) (Skidmore et al., 2008). HSPGs are expressed on the surface of almost all mammalian cells and they modulate many cellular processes including cell adhesion, motility, migration, localization, and ligand-receptor interaction (Bishop et al., 2007; Stanley et al., 1995; Van Der Voort et al., 2000) (Skidmore et al., 2008).

HS has been shown to regulate processes necessary for the coordination of an adaptive immune response such as directed cell migration, cell-cell interaction, and responsiveness to soluble ligands (Bishop et al., 2007; Longley et al., 1999; Skidmore et al., 2008). Despite this, little attention has been directed towards the role that HS plays during an immune response. This is surprising considering the number of lymphocyte-specific chemokines, cytokines, and growth factors that contain HS binding domains (IL2-8, IL10, IL12, IFNγ, TNFα, MIP1β, APRIL, etc)(Amara et al., 1999; Borghesi et al., 1999; Jarousse et al., 2011; Lortat-Jacob, 2006; Lortat-Jacob et al., 2002; Mahtouk et al., 2006; Reijmers et al., 2011; Salek-Ardakani et al., 2000; Van Der Voort et al., 2000).

B cells are an integral component of the adaptive immune system. Through the action of surface and secreted antibodies, B cells are capable of providing both innate and adaptive protection against invading pathogens (Baumgarth, 2011; Choi and Baumgarth, 2008); (Batista and Harwood, 2009). Critical to initiating and maintaining a B cell response is B cell localization, cell-cell interaction, and responsiveness to numerous soluble ligands (chemokines and cytokines) (Matloubian et al., 2004; Okada and Cyster, 2006; Phan et al., 2009b; Phan et al., 2007) (Phan et al., 2009a). This is illustrated by the concentration of B cells within the secondary lymphoid organs and their chemokine-dependent migratory patterns that result in the surveying of follicular dendritic cells for antigen (Matloubian et al., 2004; Suzuki et al., 2009).

A recent report from our laboratory presented evidence that the expression of HS on B cells is tightly regulated (Jarousse et al., 2011). Whereas naïve B cells express little-to-no HS, HS is strongly upregulated on the B cell surface following numerous stimuli that are present during different types of infections (such as IFN-I, toll-like receptor ligands, and B cell receptor-stimulators). Jarousse et al. also presented *in vitro* evidence that HS expression on B cells can increase B cell responsiveness to the B cell-specific cytokine APRIL, and as a result, HS-expressing B cells survived longer in culture and produced more IgA antibody (Jarousse et al., 2011). Beyond these *in vitro* findings, little is known about how HS expression on B cells affects the B cell response *in vivo*.

To assess the role of HS expression on B cells *in vivo*, we have generated a B cell-specific deletion of exostosin 1 (EXT1), an enzyme necessary for HS synthesis (Busse et al., 2007). In this report, we show that expression of HS is not necessary for normal B cell development. Additionally, HS expression on B cells does not noticeably affect B cell homing, localization, or motility within secondary lymphoid organs. We also did not find any evidence that HS expression on B cells affects disease severity or mouse survival in response to influenza

infection. Similarly, HS expression on B cells did not affect clearance of influenza from the lung. Notably, we did find that HS expression on B cells plays a role in the generation and/or maintenance of plasma cells after infection with influenza.

Materials and Methods

Mice

All mice were housed and bred in pathogen-free conditions at the American Association of Laboratory Animal Care-approved animal facility at the Life Sciences Addition at the University of California, Berkeley, CA, USA. All animal experiments were approved by the Animal Care and Use Committee at the University of California, Berkeley, CA, USA. C57Bl/6 were obtained from Jackson Laboratory. Actin-CFP and ubiquitin-GFP (C57BL/6-Tg(UBC-GFP)30Scha/J) mice were a kind gift from Professor Ellen Robey. Conditional KO mice were generated by crossing $EXT1^{flox/flox}$ mice to CD19-Cre mice (both on the C57Bl/6 background) to produce EXT1 cKO ($EXT1^{flox/flox}$ CD19-Cre^{+}), and littermate controls ($EXT1^{flox/+}$ CD19-Cre^{+}). Experiments were conducted with 6 to 12 week old mice in accordance with institutional guidelines for animal care and use.

Isolation and culture of B cells

Lymphocytes were isolated from peripheral lymph nodes (inguinal, brachial, cervical, mediastinal, or axillary), the spleen, or the peritoneal cavity of WT C57Bl/6, littermate control, EXT1 cKO, Ubiquitin-GFP, or EXT1 cKO-actin-CFP mice. Briefly, a single cell suspension was isolated and treated with red blood cell lysis. For figures 1a, 3c, and 4, B cells were purified using the EasySep negative selection mouse B cell enrichment kit (Stem Cell Technologies). B cell purity was assessed by flow cytometry using anti CD19 or B220 antibodies (over 98% purity). Cell viability was verified by 7AAD staining (over 99% viability). For culturing of B cells, cells were resuspended at a density of $2x10^6$ cells/ml in RPMI media containing 100mM MEM non-essential amino acids, 55mM 2-Mercaptoethanol, 1 mM Sodium Pyruvate and 10 mM Hepes. When indicated, purified B cells were treated with Interferon b (IFNb) at 45U/ml unless indicated otherwise (PBL Interferon source). For evaluation of BM B cell subsets, BM was isolated from mice by flushing the marrow from the femurs. Bone marrow was separated from red blood cells by ficoll prior to staining.

Flow cytometry

Lymphocytes or purified splenic B cells were washed in PBS with 1% BSA and incubated with 1mg/ml purified rat anti-mouse CD16/CD32 (Fc block, BD Pharmingen) at 4°C for 15 min, prior to surface staining. B cells were detected with anti-CD19 or anti-B220 antibodies. HS was detected with the F58-10E4 clone from Seikagaku Corporation. The isotype control was a mouse IgM kappa (TEPC 183, Sigma). Bound antibody was revealed by staining with a FITC- or PE-conjugated anti-mouse IgM^a (Igh-6a) monoclonal antibody (BD Pharmingen). BM progenitor B cells were defined as B220+, CD43 high, Igm low, and IgD low. BM pre B cells were defined as B220+, CD43 low, IgM/IgD low. BM immature B cells were defined as B220+, CD43-, IgM/IgD high. Splenic immature B cells were defined as CD19+, IgM-, IgD-. Splenic Transitional type I B cells were defined as CD19+, IgM+, IgD-. Splenic transitional type II B cells were defined as CD19+, IgM+, IgD+. Follicular mantle B cells were defined as CD19+, IgM-, IgD+. B1 B cells were defined as CD19+, CD5+. Distinction of congenic CD45.1 and EXT1 cKO lymphocytes was done by staining for CD45.1 and CD45.2. For the homing

experiments, fluorescently labeled B and T cells were identified with CD3 and CD19 antibodies, respectively. Germinal center B cells were identified as CD19+, Fas+, GL7+, and IgD-. Plasma were identified as CD19+, syndecan 1+, and IgD-. For all staining procedures, dead cells were excluded from the analysis by 7AAD staining. Data were acquired on a LSRII flow cytometer (BD Biosciences) and were analyzed with FlowJo software (Treestar).

Generation of BM chimeras

Wild-type female C57BL/6 congenic mice between 6-8 weeks were lethally irradiated with 900 rads. 24 hours later, the mice were injected intraveinously with a 1:1 mixture of congenic WT CD45.1+ and EXT1 cKO CD45.2+ ($2.5X10^6$ of each genotype) bone marrow. 10 weeks post injection, reconstitution efficiency was checked by tail-vein bleeding and flow cytometry.

Homing of B cells to the spleen and peripheral lymph nodes

WT ubiquitin-GFP or EXT1cKO-actin-CFP mice were injected intraveinously with either PBS or 0.2mg Poly I:C. 24 hours later, a single cell suspension of splenocytes were isolated, and red blood cells were lysed. 30 million splenocytes of each genotype were then injected intraveinously into a naïve WT mouse. At 20 minutes or 20 hours, secondary lymphoid organs were harvested. At 20 minutes post transfer, spleens were isolated. At 20 hours post transfer, spleen, inguinal-, cervical-, brachial and axillary- lymph nodes were isolated. A single cell suspension of each organ was isolated, and the ratio of B to T cells from the WT and EXT1 cKO donor was assessed by flow cytometry.

Localization of B cells in secondary lymphoid organs

Littermate control or EXT1cKO mice were injected intraveinously with 0.2mg Poly I:C. 24 hours later, a single cell suspension of splenocytes were isolated, and red blood cells were lysed. B cells were purified using the Stem Cell Technologies kit, and labeled with either SNARF (Invitrogen) or CFSE (Invitrogen). $1X10^7$ B cells from each genotype was intraveinously injected into a naïve WT mouse. Twenty hours later, the mouse was sacrificed, the inguinal lymph nodes harvested, and cryosectioned. 10μM thick cryosections were transferred onto glass slides and imaged by microscopy.

Two-Photon imaging of B cells in the inguinal lymph nodes

B cells were purified from naïve littermate control or EXT1cKO mice, and labeled with either CFSE or SNARF before $1X10^7$ of each was transferred into a naïve WT mouse. Three hours post transfer, recipient mice were intraveinously injected with PBS or 0.2mg poly I:C. 24 hours post PBS or poly I:C, mice were sacrificed, inguinal lymph nodes isolated, and explanted Explanted lymph nodes were maintained at 37 °C and perfused with media bubbled with a 95% O_2/5% CO_2 gas mixture.

Virus and infections

Purified human influenza A/PR/8/34 (H1N1) was purchased from Advanced Biotechnologies Inc. (Cat. # 10-210-500), alliquoted, and stored at -80 degrees celsius. For infection of mice, aliquots were diluted in sterile PBS to 250 Tissue Culture Infection Dose 50 ($TCID_{50}$)/μl. Mice

were anesthetized with isoflurane, and a volume of 40ul (a total of $1X10^4$ $TCID_{50}$) was use to infect mice intranasally. Mouse survival and weight (which served as a measurement for morbidity) were monitored daily.

Titering influenza virus in the lung

At the indicated times, mice were sacrificed and both lungs were harvested in 2ml PBS. Lungs were then homogenized using a Polytron PT2100 homogenizer (Kinematica). Homogenate was than spun down, and the supernatant was used in an MDCK-agglutination assay as previously described (Cottey, Rowe, and Bender, 2001. Current Protocols). Briefly, supernatant serially diluted and added to MDCK cells for 24 hours, and then removed. 5 days later, hemagglutination of chicken red blood cells was used to determine the $TCID_{50}$.

ELISA for influenza specific antibody isotypes

14 days post influenza infection, mice were sacrificed and a terminal bleed was performed. Serum was separated using serum separator tubes (BD cat.# 02-675-188). ELISA plates were coated overnight at four degrees Celsius with heat-killed influenza (see above), diluted in PBS to 1μg/ml. Serially-diluted serum was added to the plates overnight, before detection of influenza specific antibodies with isotype specific alkaline-phosphotase antibodies. Substrate conversion (pNpP from Sigma; cat. # N2770) was monitored using a plate reader measuring at λ405nm.

Statistical Analysis

All statistical results are expressed as mean ± SEM. Statistical analysis was performed using a non-parametric Mann-Whitney test for comparison between two groups (Graphpad software). Differences were considered significant at $p<0.05$.

Results

EXT1 conditional Knock out mice exhibit normal B cell development despite an inability to express HS

To determine the significance of HS expression on B cells *in vivo*, we have generated a conditional knock-out (KO) mouse incapable of expressing HS on B cells. This was done by conditional deletion of EXT1, an enzyme necessary for HS synthesis (Busse et al., 2007). Mice with EXT1 flanked by LoxP sites were crossed to CD19-Cre^{+} mice. CD19-Cre^{+} mice express Cre-recombinase under the control of the CD19 promoter, a B cell specific surface protein that is first expressed during the pro-B cell stage of B cell development (Engel et al., 1995). Although naïve B cells express little to no HS on their surfaces, HS can be strongly induced by treatment with type I IFN (IFN-I) (Jarousse et al., 2011). As expected, when we treated B cells from littermate control mice with IFN-I (EXT1$^{fl/+}$ CD19-Cre^{+}), we found that high levels of HS expression were induced (3.1a). Upon treatment of EXT1-deficient B cells from EXT1 conditional KO (EXT1 cKO) mice (EXT1$^{fl/fl}$ CD19-Cre^{+}), very little HS expression was detected (3.1b). This data illustrates that we have generated a mouse unable to express HS selectively on B cells.

Because HS expression has been implicated in lymphocyte development (Reijmers et al., 2011) (Garner et al., 2008), we first determined if the B cell compartment of our EXT1 cKO developed normally. B cell subsets within the bone marrow (BM), (progenitor-, pre-, and immature-B cells) were present in comparable numbers to littermate controls (3.1c). Similarly, splenic B cell populations (3.1d) and B1 B cells (3.1e) were both present in similar numbers to littermate controls. Our evaluation of the B cell compartment of the EXT1 cKO mouse suggests that HS does not play a major role in B cell development.

EXT1-deficient B cells exhibit a developmental defect in a competitive context

Although no intrinsic defect in B cell development was observed in our EXT1 cKO mice, we were curious to see if EXT1-deficient B cells would develop normally in a competitive context. To address this, we used competitive BM chimeras, which provide a more stringent developmental environment that is capable of revealing small defects. A 1:1 mixture of WT congenic CD45.1^{+} bone marrow and EXT1$^{fl/fl}$ CD19-Cre^{+} CD45.2^{+}BM were transferred into a lethally irradiated WT C57Bl/6 mouse. After allowing 10 weeks for reconstitution, we isolated BM from these chimeras and quantified B cell subpopulations. We observed that relative to congenic WT populations, EXT1-deficient B cells exhibited increased progenitor and pre B cell populations, while having a decreased immature B cell population (3.2a). Similar to what was seen in the BM, splenic EXT1-deficient B cells had altered population distributions, with increased immature B cells, and decreased transitional-1 and -2 B cell populations (3.2b). The percent of follicular mantle B cells was similar between littermate controls and EXT1 cKOs. We also observed an overall defect in B cell reconstitution (3.2c). The above data suggests that HS expression plays a subtle role in B cell development that is revealed only in a competitive context. Because we observed defects in both reconstitution and B cell development in the mixed BM chimeras, we chose to perform all of the following experiments with EXT1 cKOs and littermate controls.

Lack of HS expression on B cells does not affect B cell homing or localization within peripheral lymph nodes

We next asked if other aspects of B cell biology are dependent on HS expression. HS has been shown to affect cell motility, cell-adhesion, as well as cytokine and chemokine binding (Bao et al., 2010; Bishop et al., 2007; Longley et al., 1999; Perrimon and Bernfield, 2000; Stanley et al., 1995). Because lymphocyte homing and localization are dependent on the above processes (Kawashima, 2006), we were curious if B cells lacking HS would home and localize normally. To determine if HS expression affects B cell homing, littermate control and EXT1 cKO mice were injected intaveinously (i.v.) with PBS as a control, or poly I:C, which induces expression of HS on littermate control, but not EXT1-deficient B cells (Jarousse et al., 2011). 24 hours after poly I:C treatment, splenocytes were harvested, differentially labeled with either SNARF or CFSE, mixed 1:1, and transferred into a naïve WT mouse by i.v. injection. Twenty minutes or twenty hours post transfer, recipient mice were sacrificed, and secondary lymphoid organs harvested. The number of transferred B cells relative to transferred T cells per organ was determined by flow cytometry. At both 20 minutes and 20 hours post transfer, we observed that similar ratios of B to T cells from littermate control and EXT1 cKO mice were homing to peripheral lymph nodes and the spleen of naïve mice (3.3a-3.3e).

Using a similar experimental setup, we labeled purified B cells from poly I:C treated littermate control or EXT1 cKO mice and transferred them into a naïve WT mouse. 20 hours post transfer, we assessed B cell localization within cryo-sections of peripheral lymph nodes by microscopy. We observed that both CFSE-labeled littermate control and SNARF-labelled EXT1-deficient B cells were distributed evenly throughout the B cell follicle (3.3f). The above data suggest that at the times monitored, HS expression on B cells does not affect homing, nor does it affect B cell localization within peripheral lymph nodes.

B cell motility is unaffected by the absence of HS expression

Because B cell motility within the secondary lymphoid organs is critical for the development of a humoral response (Allen et al., 2007a; Okada and Cyster, 2006; Okada et al., 2005), we were interested to see if the lack of HS expression on B cells affected motility. To address this, we fluorescently labeled purified B cells from littermate control or EXT1 cKO mice. These B cells were then transferred into a naïve WT mouse, and this recipient mouse was subsequently injected i.v. with poly I:C (to induce HS expression on littermate control B cells), or PBS as a control. 24 hours after PBS or poly I:C treatment, inguinal lymph nodes were explanted and imaged by two-photon microscopy. In the presence or absence of poly I:C, we saw no difference in B cell motility between littermate control and EXT1-deficient B cells as measured by average speed (3.4a and 3.4b), directional index (3.4c), path length, or cell turning angles (data not shown). Thus, we found no evidence that HS expression on B cells affects motility *in vivo,* neither in naïve nor in poly I:C treated mice.

Induction of HS expression on B cells after influenza infection

Having observed no defects in development, homing, localization, or cell motility in our EXT1-deficient B cells, we decided to assess the importance of HS expression on B cells for

generating a humoral response. We reasoned that although we did not detect any defects in the above processes individually, collectively, they might contribute to an impaired B cell response. To determine if HS expression on B cells plays a role in the humoral response, we chose to use mouse-adapted influenza as an infection model. Influenza was chosen because it is well established that the B cell response is critical for viral clearance and mouse survival (Barnard, 2009) (Gerhard et al., 1997) (Mozdzanowska et al., 2000).

Before determining the effects of HS expression on the B cell response, we first assessed HS expression on B cells after influenza infection. Whereas B cells from uninfected mice express little to no HS (3.5a), HS was strongly upregulated on B cells in the lung-draining mediastinal lymph node 48 hours after intranasal infection with influenza (3.5b). This high expression was transient; HS expression on B cells in the mediastinal lymph node was only slightly above background 14 days post infection (3.5c). Interestingly, when we assessed HS expression on two responding B cell populations, we noted that while germinal center B cells express low levels of HS (3.5d), plasma cells express a moderate amount of HS (3.5e). The above data demonstrate that HS is rapidly upregulated on B cells following influenza infection. Furthermore, while HS expression wanes for most B cells post infection, it is still expressed on plasma cells.

EXT1 cKO and littermate control mice exhibit similar morbidity and mortality upon influenza infection

To investigate if the regulated expression of HS on B cells following influenza infection affects influenza induced pathology or viral clearance, we infected littermate control and EXT1 cKO mice with influenza. Upon infection, both littermate control and EXT1 cKO mice exhibited similar morbidity, (measured as a function of weight loss) (3.6a). Similar percentages of both littermate control and EXT1 cKO mice also survived the infection (3.6b). We also observed that EXT1 cKO mice were able to clear influenza from the lung with similar kinetics to littermate controls (3.6c). Thus, the induction of HS expression on B cells upon influenza infection does not affect severity of pathology, nor does it affect the rate of viral clearance.

EXT1 cKO mice exhibit a defect in the number of plasma cells generated after influenza infection

Despite seeing no difference in illness severity or viral load when comparing littermate controls and EXT1 cKO mice, it is possible that responding B cell populations were affected by the absence of HS expression. We investigated this possibility by harvesting mediastinal lymph nodes and the spleen two weeks post infection, and enumerating germinal center (GC) and plasma cells by flow cytometry. While we found no difference in the number of germinal center B cells present in either the mLN or the spleen (3.7a and 3.7b), we observed a statistically significant decrease in the number of plasma cells in both the mLN and the spleen of EXT1 cKO mice (3.7c and 3.7d). The above data suggest that HS expression on B cells is important for the generation and/or maintenance of plasma cells in response to influenza infection.

EXT1 cKO and littermate control mice generate similar titers of influenza specific antibodies

Having observed a defect in the number of plasma cells present in our EXT1 cKO mice, we were curious if this translated to a reduction in influenza specific antibodies. To address this, influenza-specific antibody titers within the serum 14 days post influenza infection were quantified by ELISA. For all isotypes evaluated, we found no difference in influenza specific antibody titers between littermate control and EXT1 cKO mice (3.8a-3.8f). Because mucosal IgA has also been shown to be important for influenza restriction, we evaluated influenza-specific IgA within the lung. Similarly, we observed no difference between littermate control and EXT1 cKO mice (3.8g). The above data suggest that HS expression on B cells does not affect the titers of antibodies present 14 days post influenza infection.

Figure 3.1

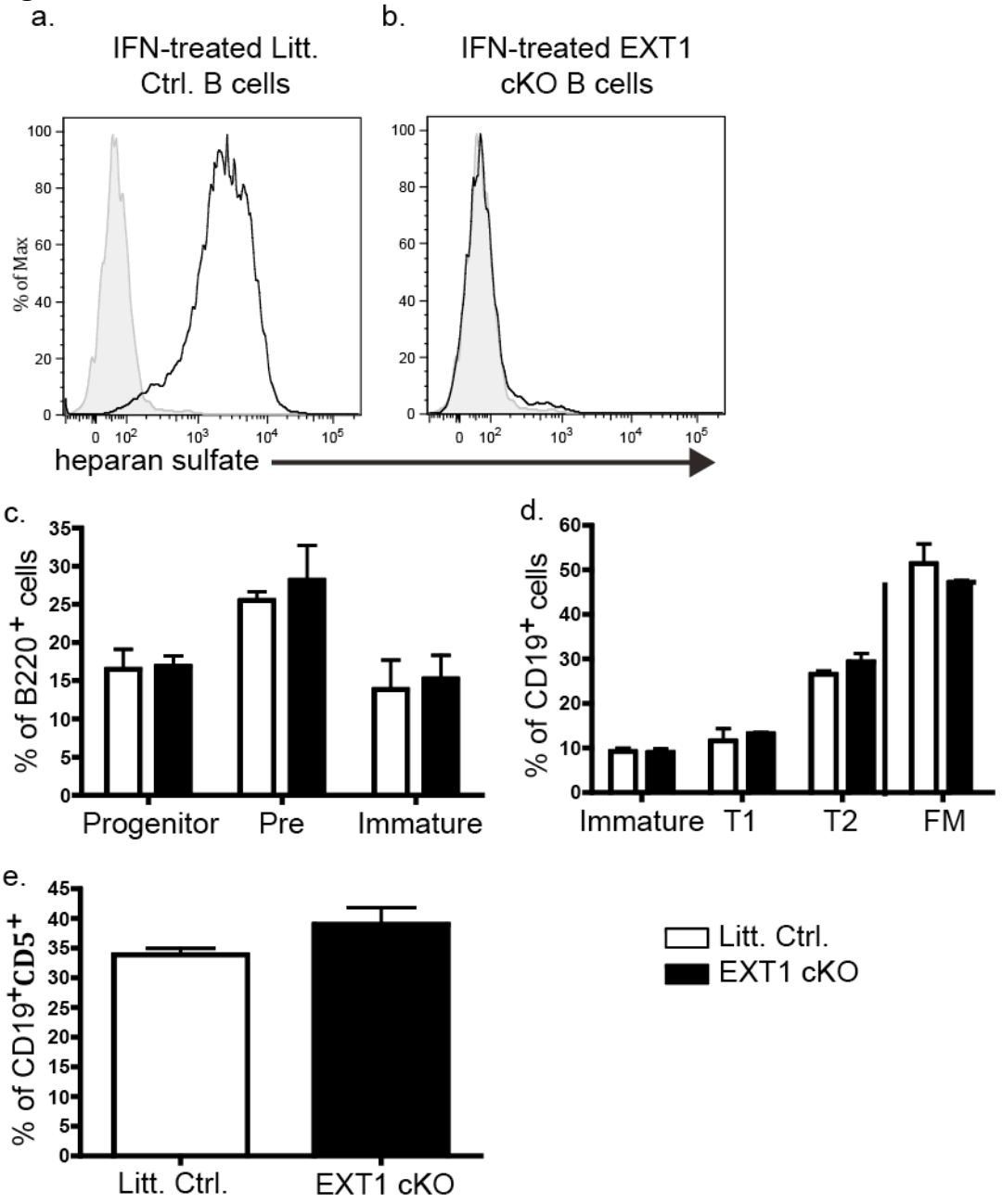


Figure 3.1. EXT1-deficient B cells do not express HS, yet they develop normally. (a) B cells from littermate control or EXT1 cKO mice were purified and cultured for 24 hours in the presence of IFN-I before HS expression was assessed by flow cytometry. Isotype controls are shown as the tinted grey histogram. Bone marrow (b), spleen (c), or peritoneal cells (d) from 6-8 week old littermate control or EXT1 cKO mice were harvested, stained for B cell populations, and analyzed by flow cytometry.

Figure 3.2

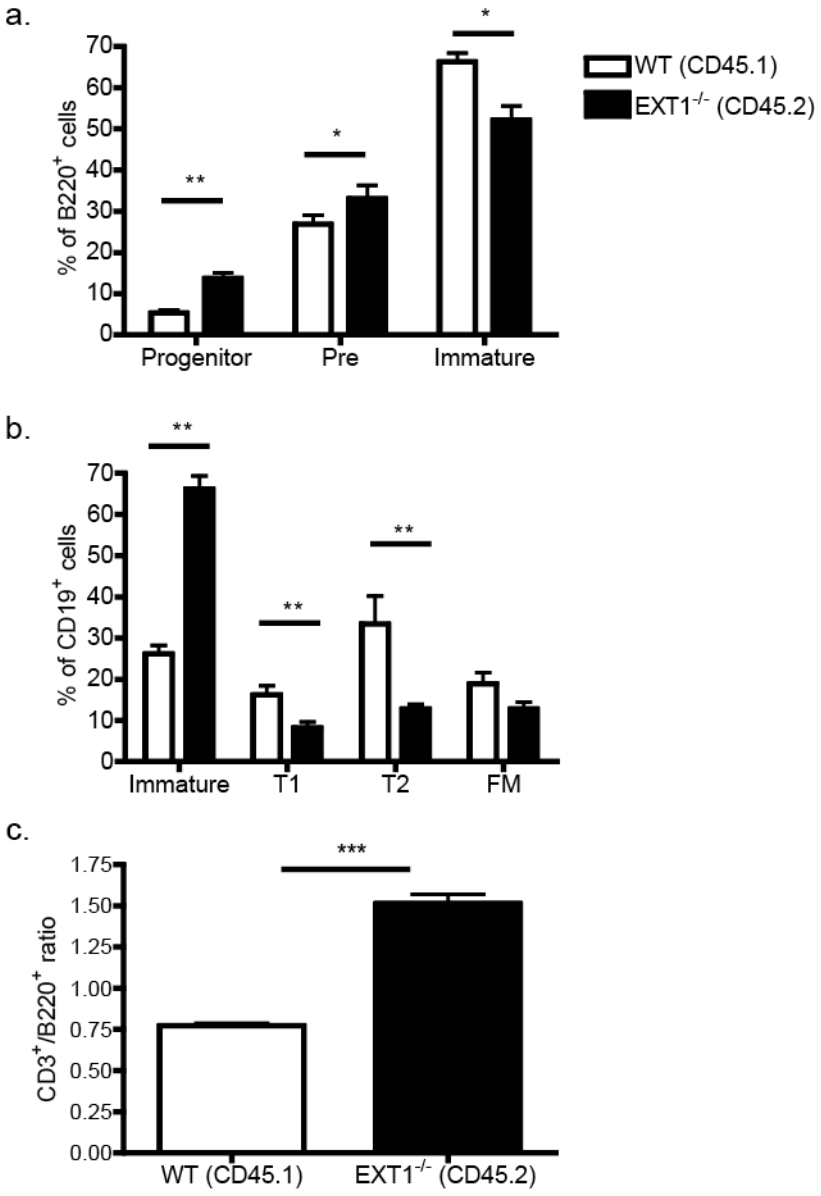


Figure 3.2. EXT1-deficient B cells exhibit a developmental defect in a competitive context. A 1:1 mixture of bone marrow from congenic WT ($CD45.1^+$) and EXT1 cKO ($CD45.2^+$) were transferred into a lethally irradiated recipient mouse. After allowing 10 weeks for reconstitution, Bone marrow (a), spleen (b), or circulating B cells (c) were harvested and stained for B cell populations. Samples were then analyzed by flow cytometry. * or ** indicate *p* values < 0.05 or 0.005, respectively.

Figure 3.3

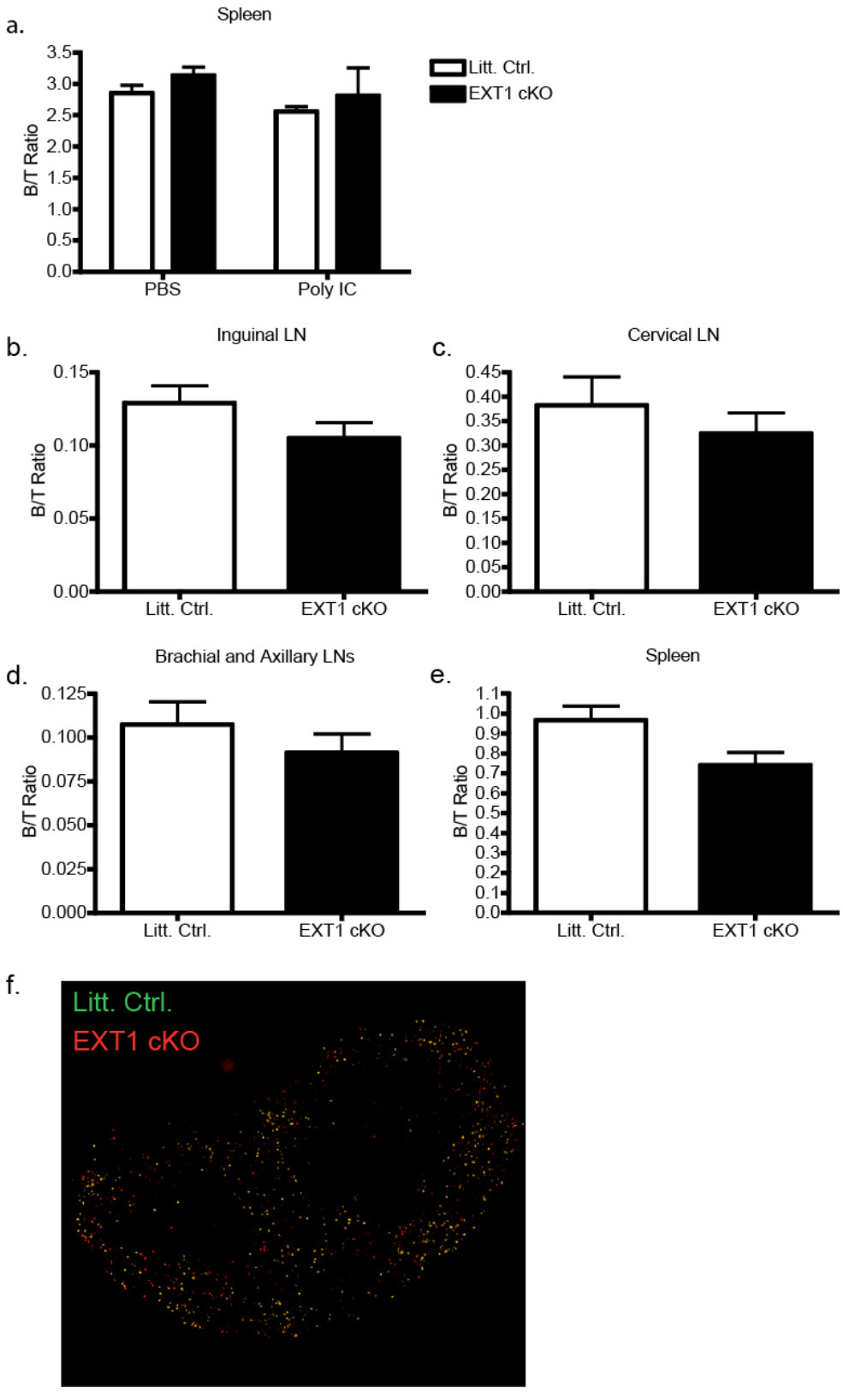

a.
Spleen
B/T Ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Poly IC
Litt. Ctrl.
EXT1 cKO
b.
Inguinal LN
B/T Ratio
0.15
0.10
0.05
0.00
Litt. Ctrl.
EXT1 cKO
c.
Cervical LN
B/T Ratio
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Litt. Ctrl.
EXT1 cKO
d.
Brachial and Axillary LNs
B/T Ratio
0.125
0.100
0.075
0.050
0.025
0.000
Litt. Ctrl.
EXT1 cKO
e.
Spleen
B/T Ratio
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Litt. Ctrl.
EXT1 cKO
f.
Litt. Ctrl.
EXT1 cKO

Figure 3.3. Lack of HS expression on B cells does not affect B cell homing or localization within peripheral lymph nodes. Splenocytes from PBS or poly I:C treated WT-ubiquitin-GFP mice or EXT1 cKO actin-CFP were harvested and transferred into naïve WT mice. Twenty minutes or twenty hours post transfer spleens (a and b) and peripheral lymph nodes (b) were isolated, and stained for CD3 and CD19. Graphs depict the ratio of transferred B cell:T cell ratio for the indicated organ. (c) Purified B cells from poly IC treated littermate control or EXT1 conditional KO were differentially labeled (littermate control with CFSE, EXT1-deficient B cells with SNARF), and transferred into a naïve recipient mouse. Twenty hours post transfer, inguinal lymph nodes were cryosectioned and imaged by microscopy.

Figure 3.4

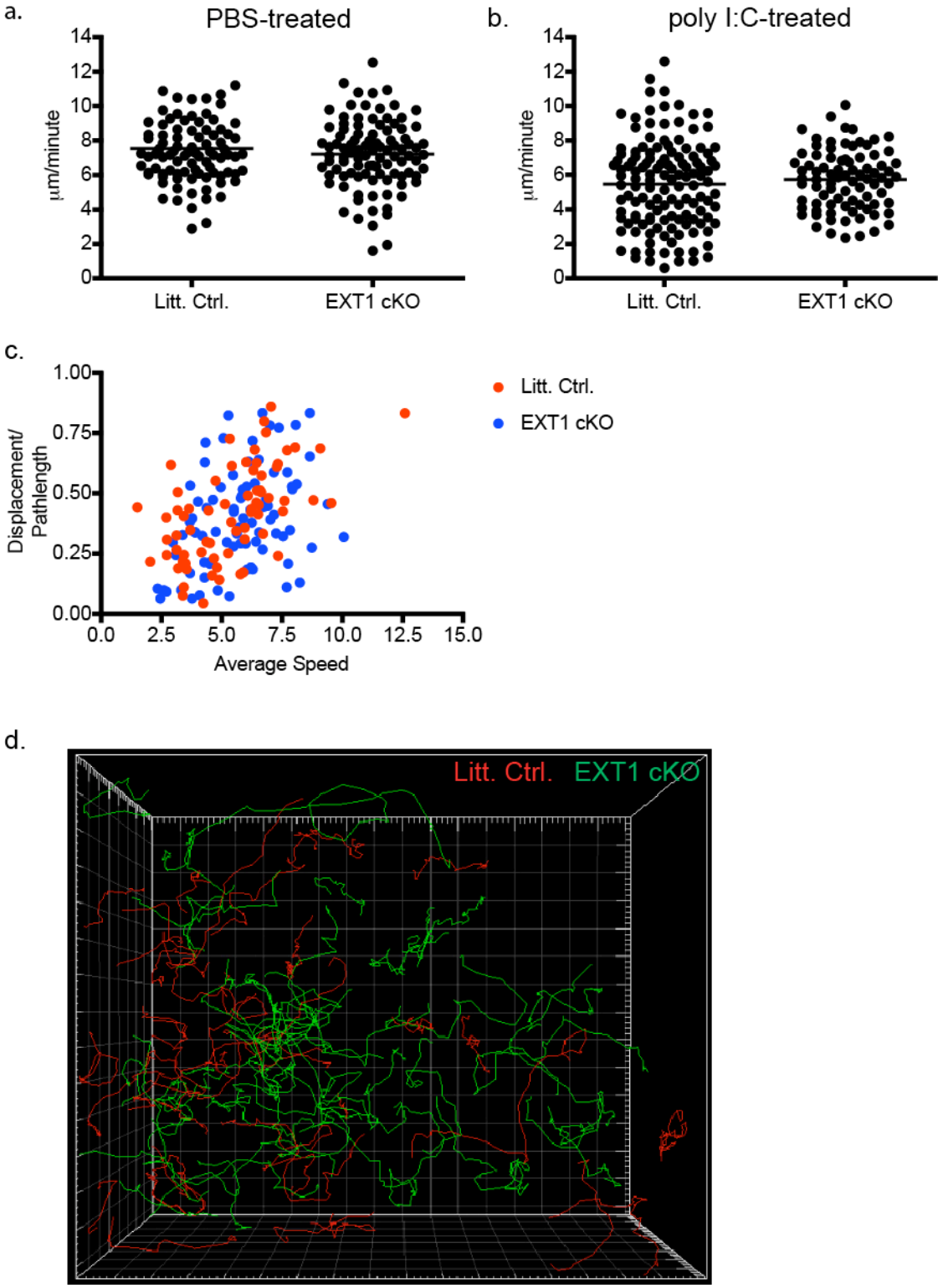


Figure 3.4 . B cell motility is unaffected by the absence of EXT1. B cells from littermate control or EXT1 cKO mice were purified and differentially labeled with SNARF or CFSE. A 1:1 mixture of these cells were transferred into a naïve mouse that was subsequently treated with either PBS as a control or poly I:C. 24 hours later, inguinal lymph nodes were explanted and

imaged by two-photon microscopy. The average speed of littermate control and EXT1-deficient mice was determined for recipient mice treated with PBS (a) or poly I:C (b). Directional path length (c) of individual B cells within a LN from a poly I:C treated mouse, either littermate control (red) or EXT1-deficient (blue), are also shown. (d) Individual tracks of individual B cells, either littermate control (SNARF-red) or EXT1-deficient (CFSE-green), from an explanted LN of a poly I:C mouse are shown.

Figure 3.5

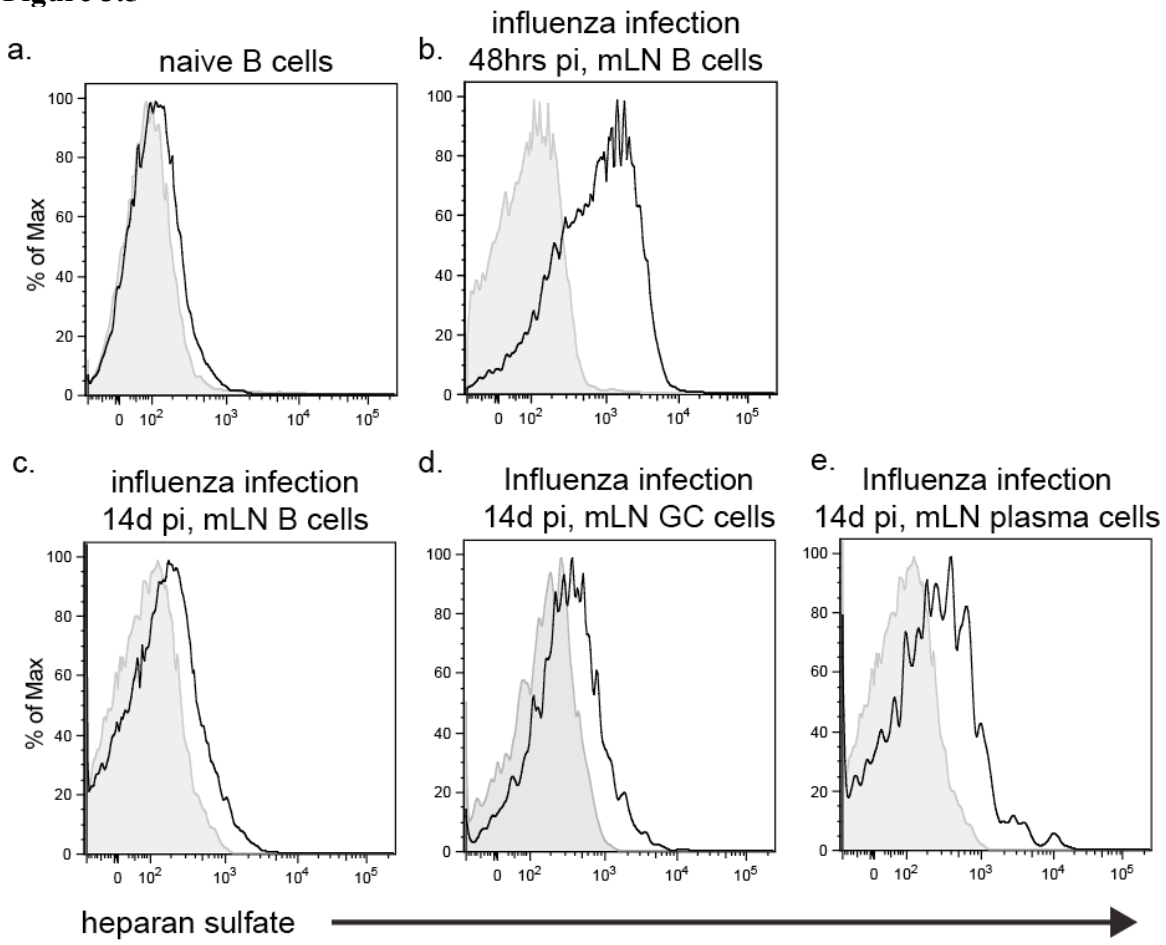


Figure 3.5. HS is induced on B cells upon influenza infection. 10 week old WT C57Bl/6 mice were either left untreated (a), or infected intranasally with $1X10^4$ $TCID_{50}$ A/PR/8 Influenza (b). 48 hours post infection, mediastinal lymph nodes were harvested, cells were stained for CD19 and HS, and HS expression on B cells was assessed by flow cytometry. ~10 week old WT C57Bl/6 mice were intransally infected with $1X10^4$ $TCID_{50}$ A/PR/8 Influenza, and 14 days later, mediastinal lymph nodes were harvested. A single cell suspension was stained for HS, total B cells (c), germinal center B cells (d), and plasma cells (e).

Figure 3.6

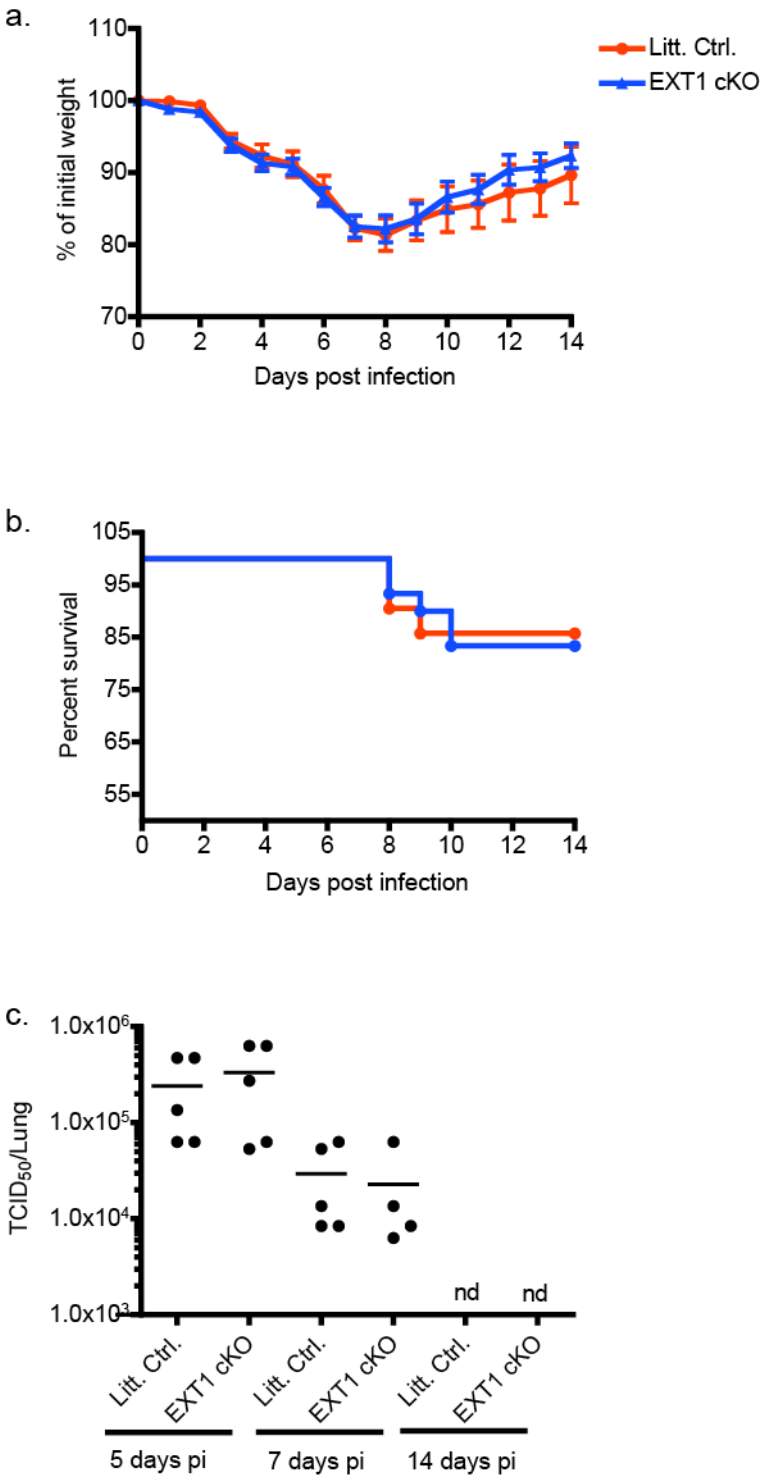

a.
110
100
90
80
70
% of initial weight
0
2
4
6
8
10
12
14
Days post infection
Litt. Ctrl.
EXT1 cKO
b.
105
95
85
75
65
55
Percent survival
0
2
4
6
8
10
12
14
Days post infection
c.
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
TCID50/Lung
nd
nd
Litt. Ctrl.
EXT1 cKO
Litt. Ctrl.
EXT1 cKO
Litt. Ctrl.
EXT1 cKO
5 days pi
7 days pi
14 days pi

Figure 3.6. EXT1 cKO and littermate control mice exhibit similar morbidity and mortality upon infection. ~10 week old littermate control or EXT1 cKO mice were intranasally infected with $1X10^4$ $TCID_{50}$ A/PR/8 Influenza. Morbidity as a function of weight loss(a), as well as mouse survival (b), was monitored for two weeks (a). Mice were also sacrificed at 5, 7, and 14 days post infection, and the titer of influenza per lung was determined using a MDCK agglutination titering assay.

Figure 3.7

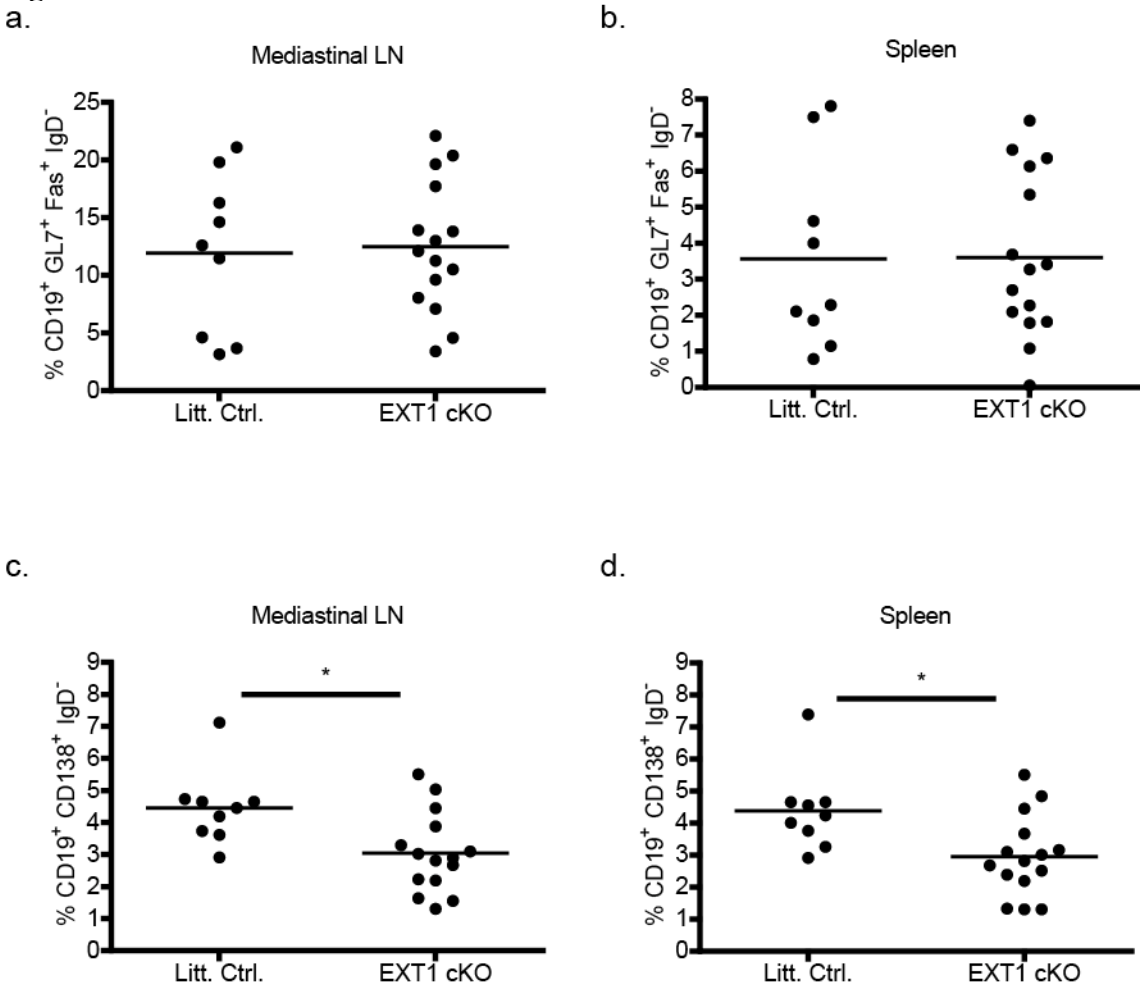


Figure 3.7. EXT1 cKO mice exhibit a defect in the number of plasma cells generated after influenza infection. 14 days after infection of ~10 week old EXT1 cKO or littermate control mice with $1X10^4$ $TCID_{50}$ A/PR/8 Influenza, mediastinal lymph nodes and spleens were harvested. A single cell suspension from both tissues was stained for either germinal centers (a and b) or plasma cells (c and d). A non-parametric student's T test (Mann-Whitney) was used to determine the *p* values, which were 0.0145 for both 7c and 7d.

Figure 3.8

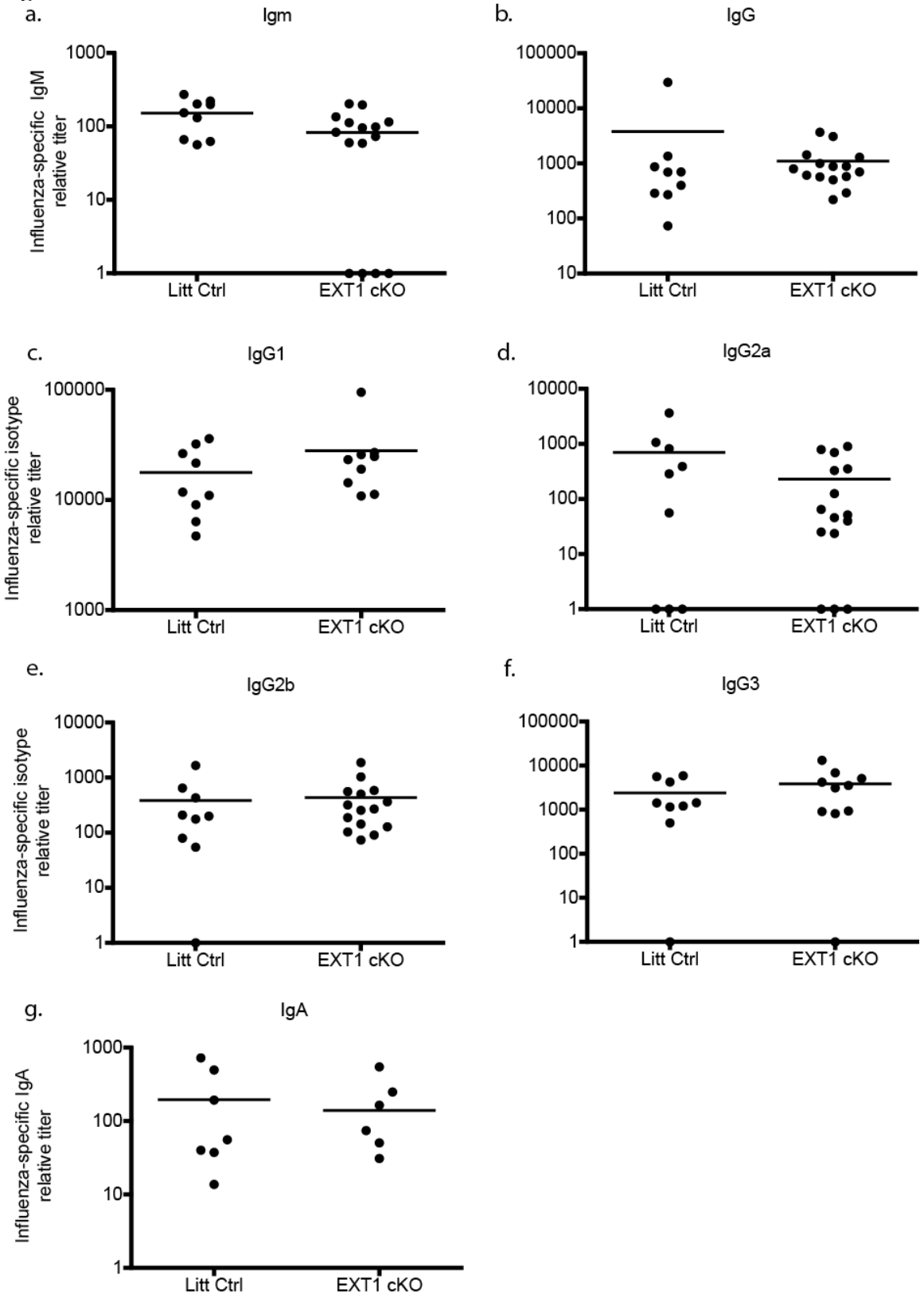

a.
Igm
Influenza-specific IgM relative titer
1000
100
10
1
Litt Ctrl
EXT1 cKO
b.
IgG
100000
10000
1000
100
10
Litt Ctrl
EXT1 cKO
c.
IgG1
Influenza-specific isotype relative titer
100000
10000
1000
Litt Ctrl
EXT1 cKO
d.
IgG2a
10000
1000
100
10
1
Litt Ctrl
EXT1 cKO
e.
IgG2b
Influenza-specific isotype relative titer
10000
1000
100
10
1
Litt Ctrl
EXT1 cKO
f.
IgG3
100000
10000
1000
100
10
1
Litt Ctrl
EXT1 cKO
g.
IgA
Influenza-specific IgA relative titer
1000
100
10
1
Litt Ctrl
EXT1 cKO

Figure 3.8. EXT1 cKO and littermate control mice generate similar titers of influenza-specific antibodies. 14 days after infection of ~10 week old EXT1 cKO or littermate control mice with $1X10^4$ $TCID_{50}$ A/PR/8 Influenza, serum or bronchiolar lavage (BAL) fluid was harvested. Serum or BAL fluid was then used in a sandwich ELISA to determine the quantity of different isotypes of influenza specific antibodies.

Discussion

Numerous studies have shown that HS is important in determining how cells sense and respond to their extracellular environment (Bishop et al., 2007) (Turnbull et al., 2001). We have previously reported that naïve B cells do not express HS, but upon viral or bacterial infection, HS is rapidly expressed (Jarousse et al., 2011). However, little is known about how HS expression on B cells affects B cell biology. To investigate the role of HS expression on B cells *in vivo*, we generated and characterized a transgenic mouse with a B cell-specific deletion of EXT1, an enzyme necessary for HS synthesis.

Development and Maturation of B cells in the absence of EXT1

Despite the fact that HS is not expressed on B cells in our EXT1 cKO mice, we did not observe any overt defects in B cell development in either the BM or in the spleen. However, a subtle role for HS expression in B cell development was revealed when we generated mixed WT/EXT1 cKO BM chimeras. The alterations in B cell subset populations observed in both the BM and spleen suggest partial blocks at the early stages in B cell development and maturation in the respective organ. In both the BM and the spleen, these partial blocks result in increased precursor B cell populations. These partial blocks suggest that the expression of HS during these stages, although not essential, aids in processes critical for B cell maturation. This observation was interesting considering that we have previously observed very low levels of HS on B cell BM subsets (data not shown). It is tempting to speculate that SDF-1, a cytokine important for B cell development in the BM that is known to have a heparan sulfate binding domain, may account for the defect observed in BM B cell development (Ma et al., 1998).

B cell behavior in the absence of EXT1

The homing of B cells to secondary lymphoid organs is key for B cell surveillance of antigens and the development of the humoral immune response (Harwood and Batista, 2008; Kawashima, 2006) (Batista and Harwood, 2009). A number of groups have recently demonstrated the importance of HS and sulfated glycan expression on endothelial cells for lymphocyte homing. Both the Fukuda and the Esko group have shown that the sulfated glycans expressed on endothelial cells serve as ligands for L-selectin expressed on lymphocytes, and interaction between L-selectin and sulfated glycans is extremely important for lymphocyte sticking/rolling along the high endothelial venules (HEV). Additionally, presentation of chemokines (such as CCL19 and CCL21), that are important for integrin-mediated adhesion and transcytosis were diminished in mice deficient for HS on HEVs (Bao et al., 2010; Wang et al., 2005). Given the importance of HS on HEVs for lymphocyte homing, it was surprising that we saw no defect in B cell homing, either immediate or accumulative, in our EXT1-deficient B cells. It remains possible that by assessing only the B cell to T cell ratio within the secondary lymphoid organs, we may be missing HS-dependent differences in B cell sticking/rolling along the HEVs. Electrostatic repulsion between HS expressed on B cells and sulfated glycans expressed on HEVs may affect B cell sticking/rolling. Alternatively, the presence of HS on B cells may alter binding of HEV-presented chemokines such as SDF-1 and CCL19, and as a result, affect integrin activation (Bao et al., 2010; Kawashima, 2006). Further studies assessing the importance of HS

expression on B cells as it relates to B cell sticking/rolling and integrin activation may reveal a role for HS in B cell homing.

Because HS has been shown to affect both chemokine-mediated cell migration (Bao et al., 2010; Bishop et al., 2007; Wang et al., 2005) and cell motility (Kaneider et al., 2003), we were quite surprised to find no defect in either motility or B cell localization in our EXT1 cKO mice. It is possible that HS expression on B cells can affect motility, however, heparan sulfate present within the extracellular matrix of the lymph node (Bao et al., 2010) may compensate for the lack of HS on our EXT1-deficient B cells. Alternatively, HS expression may affect B cell migration in a context other than that assessed here, or it may only affect B cells within certain parts of lymph nodes such as the B cell follicle-T cell zone border, where chemokine concentrations are different from those in the B cell follicle (Allen et al., 2007a) (Okada and Cyster, 2006).

To the best of our knowledge, we are the first to assess the role of HS expression on B cell localization within secondary lymphoid organs. When looking within peripheral lymph nodes (Fig 3.3c) and the spleen (data not shown), EXT1-deficient B cells appeared to be evenly distributed throughout the B cell follicle. This was surprising since T cell zone organizing chemokines CCL19 and CCL21 (also known as ELC and SLC, respectively), are known to bind HS (Okada and Cyster, 2006; Okada et al., 2005). From our data, it does not appear that the presence of HS on B cells enhances responsiveness to these chemokines, as we did not see any difference in the density of B cells at the B cell follicle-T cell zone border between littermate control and EXT1-deficient B cells. Perhaps the sulfation pattern present on B cell-HS is not optimized for interaction with these chemokines. Alternatively, upregulation of the CCL19/CCL21 receptor on B cells, as occurs upon B cell activation, may be a context in which HS affects B cell localization within the lymph node.

The B cell response to influenza in the absence of heparan sulfate expression

As was seen with murine gammaherpesvirus 68 (MHV-68) and mouse cytomegalovirus, infection of mice with influenza also induces high levels of HS on B cells early post infection (Jarousse et al., 2011) (figure 3.5b). The strong induction of HS on B cells is likely due to the robust amount of IFN-I that influenza induces in the upper respiratory tract (Coro et al., 2006b; Jarousse et al., 2011). Also similar to what was observed upon MHV-68 infection, HS expression on most B cells was transient following influenza infection. Notably however, HS expression on plasma cells was still moderately high 14 days pi. HS expression on plasma cells may be partially attributed to syndecan 1 expression, which is a known HSPG(O'Connell et al., 2004). The level of HS expression on plasma cells was particularly interesting considering the decreased number of plasma cells present in our EXT1 cKO mouse post infection (discussed further below).

Both the early T-cell independent, as well as the T-cell dependent B cell responses contribute to limiting influenza disease severity (Baumgarth et al., 2000; Mozdzanowska et al., 2000). Given the amount of HS expression induced on B cells early after influenza infection, we hypothesized that the early B cell response might be altered, and as a result, this might affect disease severity (Choi and Baumgarth, 2008). We were surprised to find that upon infection with influenza, both littermate control and EXT1 cKO mice tolerated the infection similarly. We also hypothesized that lack of HS expression might affect activation and maturation of responding B cells. Indeed, we found that the lack of HS negatively affects the number of

plasma cells present after infection with influenza. This observation suggests that while expression of HS on B cells does not affect germinal center formation, it affects either the generation and/or maintenance of plasma cells. The fact that the decreased number of plasma cells did not lead to a decrease in influenza-specific antibody titers may be explained by the generation of similar numbers of influenza specific plasma cells, which was not assessed in our quantification of total plasma cells. Alternatively, the decreased number of plasma cells in EXT1 cKO mice might be sufficient to generate similar titers of anti-influenza antibodies. The data presented here suggests that expression of HS on B cells is not necessary to respond to, or clear, the viral pathogen influenza. It will be important in the future to determine if HS expression on B cells affects either the B cell recall/memory compartment (see future directions below).

Refining our understanding of the importance of heparan sulfate expression on B cells

It is important to note that two other groups have recently investigated the role of HS expression on B cells. Garner and colleagues generated an identical EXT1 cKO mouse, however, they observed a lower efficiency of EXT1 deletion than we report here (Figure 3.1b) (Garner et al., 2008). Similar to the findings presented here, Garner and colleagues also observed no defect in B cell development, with the exception of a slight increase in the number of progenitor B cells in the BM. This discrepancy is likely explained by the different genotypes of littermate controls used ($EXT1^{fl/fl}$ $CD19Cre^{-}$ vs our $EXT1^{fl/+}$ $CD19Cre^{+}$). Upon immunization of their EXT1 cKO with the model antigens DNP-keyhole limpet haemocyanin (KLH) or DNP-Ficoll, Garner et al. observed no defect in the titers of antibodies generated (Garner et al., 2008), which is similar to what we observed upon influenza infection.

More recently, Reijmers et al. investigated the role of GLCE, an epimerization enzyme important for HS maturation (Reijmers et al., 2011). By transferring GLCE-deficient fetal liver hematopoietic stem cells into Rag-$2^{-/-}$-$\gamma_c^{-/-}$ mice (the authors could not use GLCE-null mice due to embryonic lethality of GLCE-deficiency), Reijmers and colleagues generated a mouse whose entire hematopoietic compartment is unable to express functional HS. Similar to what we observed in our competitive BM chimeras, Reijmers et al. reported a defect in B cell reconstitution and B cell development. Because the expression of functional HS on other lymphoid cells was impaired, Reijmers et al. could not attribute this defect in B cell development solely to the lack of HS expression on B cells. We saw no developmental defect in our EXT1 cKO mouse, and believe that what we observed in our BM chimeras, as well as what Reijmers et al. observed in their model, may be an over estimation of the role of HS in B cell development.

Our observation that lack of HS expression negatively affects the number of plasma cells present after influenza infection is similar to what Reijmers et al. observed after immunization with the model antigen KLH-TNP. Unique to this report is the use of a pathogen, which we show induces high levels of HS expression on B cells. Furthermore, our data allows us to definitively associate the decreased number of plasma cells to a lack of HS expression on B cells. Given the level of HS expression on plasma cells, as well as the importance of HS expression for responsiveness to the B cell specific cytokine APRIL (Jarousse et al., 2011; Reijmers et al., 2011), this result is consistent with the predicted role of HS expression on plasma cells.

Our observation that EXT1-deficiency in B cells does not affect titers of antibodies generated after influenza infection is distinct from Reijmers et al., who found that the amount of antibodies generated after immunization with the model antigen KLH-TNP was decreased. This

discrepancy may be explained by the use of a model antigen versus influenza. Alternatively, it may be due to the impaired B cell development observed in the GLCE$^{-/-}$ reconstituted Rag-2$^{-/-}\gamma_c^{-/-}$ mice.

Concluding Remarks

It is surprising that lack of HS expression on B cells does not manifest in a more striking phenotype. Perhaps induction of HS is key in certain types of infections, or perhaps it is important at a certain point in the B cell response that was not assessed here. Although we did not see any defect in the T-independent antibody response when EXT1 cKO mice were immunized with NP-Ficoll or NP-LPS (data not shown), HS expression may be key early in the immune response, when HS is most highly expressed on B cells. Alternatively, HS expression on B cells may serve to increase interactions with antigens containing HS-binding domains. Indeed, many viruses are known to encode proteins containing HS-binding domains. (Jarousse et al., 2008; Liu and Thorp, 2002; Shafti-Keramat et al., 2003). In conclusion, we find that while HS expression plays a nuanced role in B cell development, it is important for the generation and/or maintenance of antibody producing plasma cells. Further studies examining the response of EXT1 cKO to different pathogens may prove important in determining the role HS plays B cell biology.

Future Directions

We did not observe any overt defects in B cell development or in the B cell response of the EXT1 cKO. However, it remains possible that we have yet to us an appropriate pathogen, or look at a particular aspect of B cell biology, to reveal the importance of HS expression on B cells. Beyond the scope of this dissertation, multiple approaches can be taken to investigate the role of HS expression on B cells.

First, it will be important to better characterize HS expression on B cells. By determining the HS chain length, the extent of sulfation, and the composition (the heparanome) on B cells, we may be better able to infer the role of HS. Additionally, a more detailed characterization of B cell processes in the absence of HS may be informative. As was mentioned earlier, looking at the individual steps of B cell homing may reveal a role for HS expression on B cells. Also key will be determining the amount of HS expressed in the tissues in which B cells reside. This may be helpful in predicting where HS expression on B cells is likely to have the biggest impact on B cell biology.

We observed that EXT1 cKO mice had fewer plasma cells following influenza infection. In the future, it will be interesting to determine if HS affects the generation or the maintenance of plasma cells. Furthermore, identifying the HS-dependent cues (such as cytokine), that modulate the number of plasma cells will tell us how HS affects the number of responding antibody producing cells. In addition to looking at the primary B cell response to influenza, we are also interested in looking at the role of HS during the B cell recall/memory response. We are currently looking at the number of BM plasma cells and memory cells during a secondary response to influenza. We anticipate that again we will see decreased plasma cells, and we are curious as to whether this will translate to decreased influenza-specific antibodies during the recall response.

Bibliography

Allen, C.D.C., Okada, T., and Cyster, J.G. (2007a). Germinal-center organization and cellular dynamics. Immunity *27*, 190-202.

Allen, C.D.C., Okada, T., Tang, H.L., and Cyster, J.G. (2007b). Imaging of germinal center selection events during affinity maturation. Science *315*, 528-531.

Amara, A., Lorthioir, O., Valenzuela, A., Magerus, A., Thelen, M., Montes, M., Virelizier, J.L., Delepierre, M., Baleux, F., Lortat-Jacob, H.*, et al.* (1999). Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine. J Biol Chem *274*, 23916-23925.

Bach, P., Kamphuis, E., Odermatt, B., Sutter, G., Buchholz, C.J., and Kalinke, U. (2007). Vesicular stomatitis virus glycoprotein displaying retrovirus-like particles induce a type I IFN receptor-dependent switch to neutralizing IgG antibodies. J Immunol *178*, 5839-5847.

Bao, X., Moseman, E.A., Saito, H., Petryanik, B., Thiriot, A., Hatakeyama, S., Ito, Y., Kawashima, H., Yamaguchi, Y., Lowe, J.B.*, et al.* (2010). Endothelial heparan sulfate controls chemokine presentation in recruitment of lymphocytes and dendritic cells to lymph nodes. Immunity *33*, 817-829.

Barnard, D.L. (2009). Animal models for the study of influenza pathogenesis and therapy. Antiviral Res *82*, A110-122.

Barton, E.S., Lutzke, M.L., Rochford, R., and Virgin, H.W.t. (2005). Alpha/beta interferons regulate murine gammaherpesvirus latent gene expression and reactivation from latency. J Virol *79*, 14149-14160.

Basler, C.F., and García-Sastre, A. (2002). Viruses and the type I interferon antiviral system: induction and evasion. Int Rev Immunol *21*, 305-337.

Batista, F.D., and Harwood, N.E. (2009). The who, how and where of antigen presentation to B cells. Nat Rev Immunol *9*, 15-27.

Baumgarth, N. (2011). The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol *11*, 34-46.

Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L.E., Herzenberg, L.A., and Chen, J. (2000). B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J Exp Med *192*, 271-280.

Bennett, L., Palucka, A.K., Arce, E., Cantrell, V., Borvak, J., Banchereau, J., and Pascual, V. (2003). Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med *197*, 711-723.

Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., and Zako, M. (1999a). Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem *68*, 729-777.

Bernfield, M., Götte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., and Zako, M. (1999b). Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem *68*, 729-777.

Bishop, J.R., Schuksz, M., and Esko, J.D. (2007). Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature *446*, 1030-1037.

Bobardt, M.D., Saphire, A.C., Hung, H.C., Yu, X., Van der Schueren, B., Zhang, Z., David, G., and Gallay, P.A. (2003). Syndecan captures, protects, and transmits HIV to T lymphocytes. Immunity *18*, 27-39.

Borghesi, L.A., Yamashita, Y., and Kincade, P.W. (1999). Heparan sulfate proteoglycans mediate interleukin-7-dependent B lymphopoiesis. Blood *93*, 140-148.

Bossen, C., and Schneider, P. (2006). BAFF, APRIL and their receptors: structure, function and signaling. Semin Immunol *18*, 263-275.

Bret, C., Hose, D., Reme, T., Sprynski, A.-C., Mahtouk, K., Schved, J.-F., Quittet, P., Rossi, J.-F., Goldschmidt, H., and Klein, B. (2009). Expression of genes encoding for proteins involved in heparan sulphate and chondroitin sulphate chain synthesis and modification in normal and malignant plasma cells. Br J Haematol *145*, 350-368.

Busse, M., Feta, A., Presto, J., Wilen, M., Gronning, M., Kjellen, L., and Kusche-Gullberg, M. (2007). Contribution of EXT1, EXT2, and EXTL3 to Heparan Sulfate
Chain Elongation. Journal of Biological Chemistry *282*, 32802-32810.

Cardin, A.D., Weintraub, H.J. Molecular Modeling of Protein-Glycosaminoglycan Interactions. Arteriosclerosis *9*, 21-32.

Castigli, E., Scott, S., Dedeoglu, F., Bryce, P., Jabara, H., Bhan, A.K., Mizoguchi, E., and Geha, R.S. (2004). Impaired IgA class switching in APRIL-deficient mice. Proc Natl Acad Sci USA *101*, 3903-3908.

Chang, W.L., Coro, E.S., Rau, F.C., Xiao, Y., Erle, D.J., and Baumgarth, N. (2007). Influenza virus infection causes global respiratory tract B cell response modulation via innate immune signals. J Immunol *178*, 1457-1467.

Choi, Y.S., and Baumgarth, N. (2008). Dual role for B-1a cells in immunity to influenza virus infection. J Exp Med *205*, 3053-3064.

Coro, E.S., Chang, W.L., and Baumgarth, N. (2006a). Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol *176*, 4343-4351.

Coro, E.S., Chang, W.L.W., and Baumgarth, N. (2006b). Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol *176*, 4343-4351.

Cyster, J.G. (1999). Chemokines and cell migration in secondary lymphoid organs. Science *286*, 2098-2102.

Diamond, M.S., Sitati, E.M., Friend, L.D., Higgs, S., Shrestha, B., and Engle, M. (2003). A critical role for induced IgM in the protection against West Nile virus infection. J Exp Med *198*, 1853-1862.

Dutia, B.M., Allen, D.J., Dyson, H., and Nash, A.A. (1999). Type I interferons and IRF-1 play a critical role in the control of a gammaherpesvirus infection. Virology *261*, 173-179.

Engel, P., Zhou, L.J., Ord, D.C., Sato, S., Koller, B., and Tedder, T.F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity *3*, 39-50.

Fagarasan, S., and Honjo, T. (2000). T-Independent immune response: new aspects of B cell biology. Science *290*, 89-92.

Garner, O.B., Yamaguchi, Y., Esko, J.D., and Videm, V. (2008). Small changes in lymphocyte development and activation in mice through tissue-specific alteration of heparan sulphate. Immunology *125*, 420-429.

Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G., and Maiese, K. (1997). Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol Rev *159*, 95-103.

Harwood, N.E., and Batista, F.D. (2008). New insights into the early molecular events underlying B cell activation. Immunity *28*, 609-619.

Huntington, J.A. (2003). Mechanisms of glycosaminoglycan activation of the serpins in hemostasis. J Thromb Haemost *1*, 1535-1549.

Ingold, K., Zumsteg, A., Tardivel, A., Huard, B., Steiner, Q.G., Cachero, T.G., Qiang, F., Gorelik, L., Kalled, S.L., Acha-Orbea, H.*, et al.* (2005). Identification of proteoglycans as the APRIL-specific binding partners. J Exp Med *201*, 1375-1383.

Jarousse, N., Chandran, B., and Coscoy, L. (2008). Lack of heparan sulfate expression in B-cell lines: implications for Kaposi's sarcoma-associated herpesvirus and murine gammaherpesvirus 68 infections. J Virol *82*, 12591-12597.

Jarousse, N., Trujillo, D.L., Wilcox-Adelman, S., and Coscoy, L. (2011). Virally-Induced Upregulation of Heparan Sulfate on B Cells via the Action of Type I IFN. J Immunol.

Jones, K.S., Petrow-Sadowski, C., Bertolette, D.C., Huang, Y., and Ruscetti, F.W. (2005). Heparan sulfate proteoglycans mediate attachment and entry of human T-cell leukemia virus type 1 virions into CD4+ T cells. J Virol *79*, 12692-12702.

JORPES, J.E., and GARDELL, S. (1948). On heparin monosulfuric acid. J Biol Chem *176*, 267-276.

Kaneider, N.C., Egger, P., Djanani, A.M., and Wiedermann, C.J. (2003). Leukocyte motility in response to neuropeptides is heparan sulfate proteoglycan dependent. Peptides *24*, 695-700.

Kawashima, H. (2006). Roles of sulfated glycans in lymphocyte homing. Biol Pharm Bull *29*, 2343-2349.

Kim, H.-E., Kim, H.-W., and Jang, J.-H. (2009). Identification and characterization of a novel heparin-binding peptide for promoting osteoblast adhesion and proliferation by screening an Escherichia coli cell surface display peptide library. J Pept Sci *15*, 43-47.

Kimberley, F.C., Hahne, M., and Medema, J.P. (2009a). "APRIL hath put a spring of youth in everything": Relevance of APRIL for survival. J Cell Physiol *218*, 1-8.

Kimberley, F.C., van Bostelen, L., Cameron, K., Hardenberg, G., Marquart, J.A., Hahne, M., and Medema, J.P. (2009b). The proteoglycan (heparan sulfate proteoglycan) binding domain of APRIL serves as a platform for ligand multimerization and cross-linking. FASEB J *23*, 1584-1595.

Koziel, L., Kunath, M., Kelly, O.G., and Vortkamp, A. (2004). Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev Cell *6*, 801-813.

Lamannaa, W.C., c, Kalusa, I., Padvac, M., Baldwinb, R.J., Merryb, C.L., and Dierksa, T. (2007). The heparanome—The enigma of encoding and decoding heparan sulfate sulfation. 1-18.

Lambaerts, K., Wilcox-Adelman, S.A., and Zimmermann, P. (2009). The signaling mechanisms of syndecan heparan sulfate proteoglycans. Curr Opin Cell Biol *21*, 662-669.

Lander, A.D. (1998). Proteoglycans: master regulators of molecular encounter? Matrix Biol *17*, 465-472.

Le Bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F., and Tough, D.F. (2001). Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity *14*, 461-470.

Lin, X. (2004). Functions of heparan sulfate proteoglycans in cell signaling during development. Development *131*, 6009-6021.

Liu, J., and Thorp, S.C. (2002). Cell surface heparan sulfate and its roles in assisting viral infections. Med Res Rev *22*, 1-25.

Longley, R.L., Woods, A., Fleetwood, A., Cowling, G.J., Gallagher, J.T., and Couchman, J.R. (1999). Control of morphology, cytoskeleton and migration by syndecan-4. J Cell Sci *112 (Pt 20)*, 3421-3431.

Lortat-Jacob, H. (2006). Interferon and heparan sulphate. Biochem Soc Trans *34*, 461-464.

Lortat-Jacob, H. (2009). The molecular basis and functional implications of chemokine interactions with heparan sulphate. Curr Opin Struct Biol *19*, 543-548.

Lortat-Jacob, H., Grosdidier, A., and Imberty, A. (2002). Structural diversity of heparan sulfate binding domains in chemokines. Proc Natl Acad Sci USA *99*, 1229-1234.

Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA *95*, 9448-9453.

Mackay, F., and Schneider, P. (2009). Cracking the BAFF code. Nat Rev Immunol *9*, 491-502.

Mahtouk, K., Cremer, F.W., Rème, T., Jourdan, M., Baudard, M., Moreaux, J., Requirand, G., Fiol, G., De Vos, J., Moos, M.*, et al.* (2006). Heparan sulphate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma. Oncogene *25*, 7180-7191.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2005). Antigen-specific memory B cell development. Annual review of immunology *23*, 487-513.

Mohammadi, M., Olsen, S.K., and Ibrahimi, O.A. (2005). Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev *16*, 107-137.

Morgan, M.R., Humphries, M.J., and Bass, M.D. (2007). Synergistic control of cell adhesion by integrins and syndecans. Nat Rev Mol Cell Biol *8*, 957-969.

Mozdzanowska, K., Maiese, K., and Gerhard, W. (2000). Th cell-deficient mice control influenza virus infection more effectively than Th- and B cell-deficient mice: evidence for a Th-independent contribution by B cells to virus clearance. J Immunol *164*, 2635-2643.

Murphy, K., Travers, P., Walport, M. Janeway's immunobiology. Seventh edition. 2008

Mythreye, K., and Blobe, G.C. (2009). The type III TGF-beta receptor regulates epithelial and cancer cell migration through beta-arrestin2-mediated activation of Cdc42. Proc Natl Acad Sci USA *106*, 8221-8226.

O'Connell, F.P., Pinkus, J.L., and Pinkus, G.S. (2004). CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms. Am J Clin Pathol *121*, 254-263.

Okada, T., and Cyster, J.G. (2006). B cell migration and interactions in the early phase of antibody responses. Current Opinion in Immunology *18*, 278-285.

Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS Biol *3*, e150.

Paus, D., Phan, T.G., Chan, T.D., Gardam, S., Basten, A., and Brink, R. (2006). Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. J Exp Med *203*, 1081-1091.

Perrimon, N., and Bernfield, M. (2000). Specificities of heparan sulphate proteoglycans in developmental processes. Nature *404*, 725-728.

Phan, T.G., Gray, E.E., and Cyster, J.G. (2009a). The microanatomy of B cell activation. Current Opinion in Immunology *21*, 258-265.

Phan, T.G., Green, J.A., Gray, E.E., Xu, Y., and Cyster, J.G. (2009b). Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. Nat Immunol *10*, 786-793.

Phan, T.G., Grigorova, I., Okada, T., and Cyster, J.G. (2007). Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. Nat Immunol *8*, 992-1000.

Pugliese, A., Cortese, D., and Forni, G. (1980). Polygenic control of interferon production induced by poly I:C in vivo. Brief report. Arch Virol *65*, 83-87.

Purtha, W.E., Chachu, K.A., Virgin, H.W.t., and Diamond, M.S. (2008). Early B-cell activation after West Nile virus infection requires alpha/beta interferon but not antigen receptor signaling. J Virol *82*, 10964-10974.

Reijmers, R.M., Groen, R.W.J., Kuil, A., Weijer, K., Kimberley, F.C., Medema, J.P., Van Kuppevelt, T.H., Li, J.-P., Spaargaren, M., and Pals, S.T. (2011). Disruption of heparan sulfate proteoglycan conformation perturbs B-cell Maturation and APRIL-mediated plasma cell survival . Blood *117*, 6162-6171.

Reizes, O., Lincecum, J., Wang, Z., Goldberger, O., Huang, L., Kaksonen, M., Ahima, R., Hinkes, M.T., Barsh, G.S., Rauvala, H.*, et al.* (2001). Transgenic expression of syndecan-1 uncovers a physiological control of feeding behavior by syndecan-3. Cell *106*, 105-116.

Rioux, V., Landry, R.Y., and Bensadoun, A. (2002). Sandwich immunoassay for the measurement of murine syndecan-4. J Lipid Res *43*, 167-173.

Salek-Ardakani, S., Arrand, J.R., Shaw, D., and Mackett, M. (2000). Heparin and heparan sulfate bind interleukin-10 and modulate its activity. Blood *96*, 1879-1888.

Saphire, A.C., Bobardt, M.D., Zhang, Z., David, G., and Gallay, P.A. (2001). Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages. J Virol *75*, 9187-9200.

Schneider, P. (2005a). The role of APRIL and BAFF in lymphocyte activation. Current Opinion in Immunology *17*, 282-289.

Schneider, P. (2005b). The role of APRIL and BAFF in lymphocyte activation. Curr Opin Immunol *17*, 282-289.

Shafti-Keramat, S., Handisurya, A., Kriehuber, E., Meneguzzi, G., Slupetzky, K., and Kirnbauer, R. (2003). Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J Virol *77*, 13125-13135.

Shiow, L.R., Rosen, D.B., Brdicková, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540-544.

Skidmore, M.A., Guimond, S.E., Rudd, T.R., Fernig, D.G., Turnbull, J.E., and Yates, E.A. (2008). The activities of heparan sulfate and its analogue heparin are dictated by biosynthesis, sequence, and conformation. Connect Tissue Res *49*, 140-144.

Stanley, M.J., Liebersbach, B.F., Liu, W., Anhalt, D.J., and Sanderson, R.D. (1995). Heparan sulfate-mediated cell aggregation. Syndecans-1 and -4 mediate intercellular adhesion following their transfection into human B lymphoid cells. J Biol Chem *270*, 5077-5083.

Su, T.T., Guo, B., Wei, B., Braun, J., and Rawlings, D.J. (2004). Signaling in transitional type 2 B cells is critical for peripheral B-cell development. Immunol Rev *197*, 161-178.

Sugahara, K., and Kitagawa, H. (2002). Heparin and heparan sulfate biosynthesis. IUBMB Life *54*, 163-175.

Suzuki, K., Grigorova, I., Phan, T.G., Kelly, L.M., and Cyster, J.G. (2009). Visualizing B cell capture of cognate antigen from follicular dendritic cells. J Exp Med *206*, 1485-1493.

Tkachenko, E., Rhodes, J.M., and Simons, M. (2005). Syndecans: new kids on the signaling block. Circ Res *96*, 488-500.

Turnbull, J., Powell, A., and Guimond, S. (2001). Heparan sulfate: decoding a dynamic multifunctional cell regulator. Trends Cell Biol *11*, 75-82.

Van Der Voort, R., Keehnen, R.M., Beuling, E.A., Spaargaren, M., and Pals, S.T. (2000). Regulation of cytokine signaling by B cell antigen receptor and CD40-controlled expression of heparan sulfate proteoglycans. J Exp Med *192*, 1115-1124.

Wang, L., Fuster, M., Sriramarao, P., and Esko, J.D. (2005). Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. Nat Immunol *6*, 902-910.

Wilcox-Adelman, S.A., Denhez, F., Iwabuchi, T., Saoncella, S., Calautti, E., and Goetinck, P.F. (2002). Syndecan-4: dispensable or indispensable? Glycoconj J *19*, 305-313.

Yamashita, Y., Oritani, K., Miyoshi, E.K., Wall, R., Bernfield, M., and Kincade, P.W. (1999). Syndecan-4 is expressed by B lineage lymphocytes and can transmit a signal for formation of dendritic processes. J Immunol *162*, 5940-5948.

Appendix A: Enhanced Antibody Response to Antigens Containing a Heparan Sulfate-Binding Domain

Introduction

Heparan sulfate is remarkable for its ability to enhance ligand-receptor interactions. This remarkable feat is due largely to the avidity between the negatively-charged sulfated disaccharide and positively charged ligands. This interaction can serve to concentrate ligand, or alternatively, keep it in place long enough to interact with the ligand's receptor. Given the number of cellular receptor signaling pathways that are modulated by HS , many groups have spent considerable time to determining the protein domains that best interact with HS. In the late 1980s, Cardin and Weintraub (Cardin et al., 1989) described two amino acid consensus sequences that binds to HS: XBBXBX and XBBBXXBX, where X is any hydropathic amino acid, and B is a basic amino acid. With these consenses, we can now predict domains important for interacting with HS.

B cells contribute to the elimination of and protection against invading pathogens. This is largely accomplished through the action of surface and secreted antibodies. However, key to the elimination of pathogens is interaction between the B cell receptor (BCR) on the B cell and its cognate antigen. Considering the low numbers of B cells specific for any given antigen, the likely hood of this interaction occurring is small. This issue is partially addressed by the presence of secondary lymphoid organs, which serve to sample and concentrate antigens in the blood and lymphatic system, allowing for increased surveillance of these antigens by lymphocytes (Batista and Harwood, 2009). However, given the range of affinities BCRs have for their cognate antigen, this interaction may not occur if the antigen is not in sufficient quantity.

In chapter 2, we described that IFN-I signaling in B cells can induce high levels of HS expression on B cells. Here we also showed that the presence of HS on B cells increases the responsiveness to the cytokine APRIL. Interestingly, APRIL has been shown to have a heparan sulfate binding domain (Kimberley et al., 2009). Based on this observation, we were curious if the presence of HS on B cells would enhance the binding of B cells to an antigen containing a heparan sulfate binding domain. Furthermore, we were also curious if the presence of a heparan sulfate binding domain on an antigen elicits a more robust antibody response from B cells expressing HS. The work that we present in Appendix A suggests that the expression of HS on B cells may enhance binding and responding to antigens that contain a heparan sulfate binding domain.

Materials and Methods

Cloning of HSBD-HEL constructs

pPIC9K-WT HEL was a kind gift from Dr. Robert Brink. This construct was modified by the insertion of one of three HSBD between the alpha factor secretion signal and the HEL start codon. The following HSBDs were cloned with a SG di-peptide serving as a 5' linker: 1) HBP12: VRRSKHGARKDR; 2) Tat: RKKRRQRRR; 3)Rantes: VTRKNRQV. Because of the length of the HSBDs 4 primers were designed to insert each HSBD.

Expression and purification of HSBD-HEL and WT-HEL constructs

After transformation of the GS115 strain of *Pichia pastoris* (Invitrogen) by electroporation, transformants were screened for their ability to express HEL. 1500ml cultures of HEL-expressing clones were grown to an OD of ~6 in Buffered Complex Glycerol Medium (BMGY) before being induced to express WT or HSBD-HEL in 300ml Buffered Minimal Methanol media (BMM). HEL-expressing Pichia was induced for 6 days, after which, the supernatant was harvested and dialyzed against 50mM HEPES pH8.0, with an increased amount of NaCL (100-400mM) for 3 days. Dialyzed sup was filter sterilized and ran over 2 Histrap columns (GE healthcare cat. # 17-5248-02- 5 5ml columns. WT- or HSBD-HEL was eluted from the columns with 500mM imidazole, before being concentrated using Amicon 10 kDa cut-off centrifugal filter tubes (Cat. # UFC901024). Protein concentration was determined by a BCA assay, and the concentrated protein was ran on a 15% PAGE gel and stained with Coomassie Blue.

Testing HSBD-HEL binding to B cells

3 Littermate controls ($EXT1^{fl/+}$ $CD19\text{-}Cre^{+}$) and 3 EXT1 conditional knock-outs ($EXT1^{fl/fl}$ $CD19\text{-}Cre^{+}$) were injected intraveinously with 0.2mg poly I:C. 24 hours later, the mice were sacrificed and a splenocyte single cell suspension, free of red blood cells, was isolated. $1\text{-}2X10^{6}$ splenocytes from each mouse was incubated with 0, 0.01, 0.1, 0.5, 1, or 5μg of either WT-HEL, HBP12-HEL, Tat-HEL, or Rantes-HEL in PBS 1%BSA, 5% normal mouse serum, 1μg/ml Fc block for 30 minutes. Cells were then washed with PBS 1% BSA before being stained for heparan sulfate, HEL (clone HyHEL9 conjugated in house to FITC), CD19, and 7AAD. Data were acquired on a LSRII flow cytometer (BD Biosciences) and were analyzed with FlowJo software (Treestar).

Immunizations and ELISAs

In cohorts of four, WT C57Bl/6 mice were immunized intraperitoneally in the presence or absence of 0.2mg poly I:C with 10 or 100μg of either WT-HEL or HBP12-HEL. 7 days post immunization, mice were bled from the tail vein for assessing HEL-specific IgM. 14 days post immunization, all mice were sacrificed and serum was harvested using serum separator tubes (BD cat.# 02-675-188). ELISA plates were coated overnight at four degrees Celsius with HEL diluted in PBS to 10μg/ml diluted in PBS. Serially-diluted serum was added to the plates overnight, before detection of HEL specific antibodies with isotype specific alkaline-phosphotase antibodies. Substrate conversion (pNpP from Sigma; cat. # N2770) was monitored using a plate reader measuring at λ405nm.

Results

Generation of hen egg lysozyme-heparan sulfate binding domain fusion proteins.

A *Pichia pastoris* specific expression vector encoding WT hen egg lysozyme (HEL) fused to an alpha-factor secretion signal at its 5' end and a 6X His tag at its 3' end was provided by Robert Brink (1a top) (Paus et al., 2006). This construct was modified by the insertion of one of three heparan sulfate binding domains (HSBD) in between the alpha-factor secretion signal and HEL (A.1a bottom). The three HSBDs were 1) a synthetic HSBD called HBP12 (Kim et al., 2009), 2) a HSBD found within the HIV-encoded Tat protein, 3) or a HSBD within the cytokine Rantes. *Pichia pastoris* was transformed with each of the modified constructs individually, and induced to express and secrete the respective protein into the supernatant. Supernatants from HEL-HSBD or WT-HEL were harvested, dialyzed, and then further purified using Histrap columns. Purified protein was then further concentrated and quantified. From 300mls of culture, we routinely got yields between 7-15 milligrams of protein. Purity was assessed by running concentrated protein on a poly-acrylamide gel, followed by coomassie blue staining. As figure A.1b shows, rantes-HSBD-HEL protein is detectable in unpurified/unconcentrated supernatant. After purification and concentration, there is a strong ~14 kDa band present in the various concentrations of HEL-rantes-HSBD loaded.

The presence of a HSBD on HEL increases binding to heparan sulfate-positive B cells

To test if heparan sulfate (HS) expression on B cells increases B cell interaction with HSBD-containing antigens, we treated both littermate control and EXT1 cKO mice with poly I:C, which induces HS only on littermate control B cells. 24 hours post treatment, splenocytes were isolated and incubated with varying concentrations of WT-HEL, HBP12-HSBD-HEL, Tat-HSBD-HEL, or Rantes-HSBD-HEL. Cells were then stained for surface-bound HEL, HS, and the B cell marker CD19. As seen in figure A.2a, HS was strongly induced on littermate control B cells, but not on EXT1-deficient B cells.

Interestingly, HEL fused to any of the three HSBDs "stained" B cells at much higher levels than WT HEL (A.2b). Importantly, this binding of HSBD-HEL to B cells was largely dependent on HS expression, as EXT1-deficient B cells had considerably less HS bound to their surface. We also observed that certain HEL-HSBD fusion proteins bound B cells better than others (as evidenced by a higher HEL^+ mean fluorescence intensity). HBP12-HSBD-HEL bound HS^+ B cells best, followed by Rantes-HSBD-HEL. Tat-HSBD-HEL was the least efficient HSBD-HEL fusion protein at binding HS^+ B cells. The range of concentrations that bound B cells also varied between the HSBD-HEL fusion proteins. Notably, concentrations above 1ug for all HSBD-HEL protein reached a plateau in their ability to bind HS. Binding of HEL-HSBD fusion proteins was most distinct in the 0.1-0.5μg range; only HBP12-HSBD-HEL bound B cells moderately well for both concentrations. The above data suggests that the presence of a HSBD on an antigen can strongly enhance binding of the antigen to the surface of HS^+ B cells. Furthermore, the data shows that different HSBD-HEL fusion proteins bind HS^+ B cells with varying affinities.

The presence of a HSBD on HEL enhances the antibody response after HEL-immunization

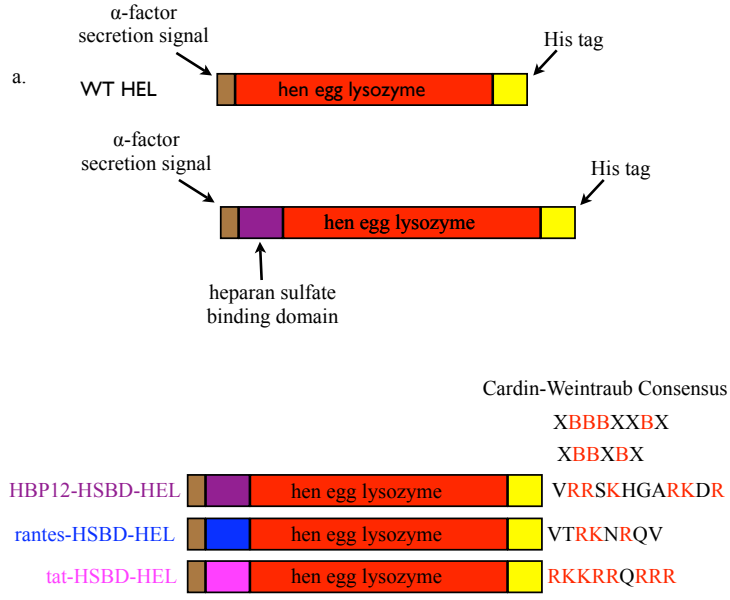
We were curious if the presence of a HSBD in a model antigen would enhance the humoral response after immunization with said antigen. To test this, WT C57Bl/6 mice were immunized with either WT HEL or HBP12-HSBD-HEL. We chose to immunize mice in the presence or absence of the adjuvant poly I:C, as poly I:C is known to induce HS expression on B cells (Jarousse et al., 2011). Upon immunization, we found that in the presence of poly I:C, HBP12-HEL generated an enhanced antibody response (Figure 3a, b , c, e, and f). We observed an increase in HEL-specific IgM, IgG, IgG2a, and IgG2b in HBP12-HEL immunized mice relative to WT-HEL. Interestingly, not all HEL-specific antibody isotypes were increased; HEL-specific IgG1 was produced at similar levels in WT-HEL and HBP12-HEL immunized mice (Figure 3d). The above data suggests that in contexts that induce HS on B cells, the presence of a HSBD in an antigen increases its immunogenicity.

Future Directions

Here we present data that suggests that antigen containing a HSBD binds to HS-expressing B cells better than antigens lacking a HSBD. Furthermore, we show that upon immunization, antigens containing a HSBD elicit a more robust antibody response. We are currently trying to show that the presence of a HSBD on an antigen increases the chance of interaction between a cognate B cell receptor and the antigen. This is being done by monitoring B cell receptor signaling via calcium release in response to cognate antigens that either contain or lack a HSBD.

We are also currently performing experiments to show that the enhanced antibody response that we show in figure A.3 is completely dependent on HS expression on B cells. This is being done by immunizing littermate control and EXT1 cKO mice with WT HEL as well as HSBD containing HEL (HBP12-HSBD-HEL). Beyond the scope of this dissertation, it will be interesting to determine if HSBDs encoded for by many viruses enhance that antibody response against said virus.

Figure A.1.


a.
α-factor
secretion signal
His tag
WT HEL
hen egg lysozyme
α-factor
secretion signal
His tag
hen egg lysozyme
heparan sulfate
binding domain
Cardin-Weintraub Consensus
XBBBXXBX
XBBXBX
HBP12-HSBD-HEL
hen egg lysozyme
VRRSKHGARKDR
rantes-HSBD-HEL
hen egg lysozyme
VTRKNRQV
tat-HSBD-HEL
hen egg lysozyme
RKKRRQRRR

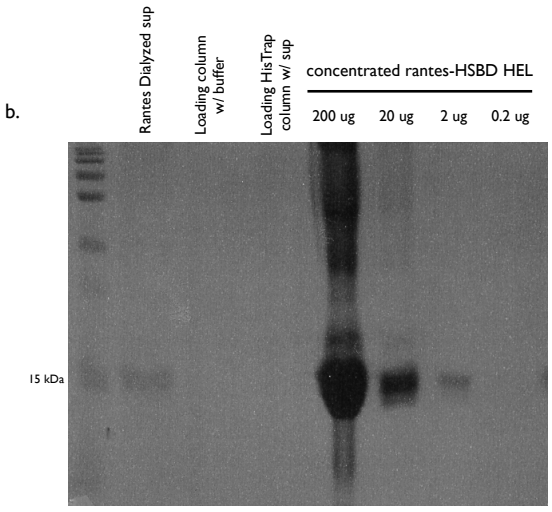

b.
Rantes Dialyzed sup
Loading column w/ buffer
Loading HisTrap column w/ sup
concentrated rantes-HSBD HEL
200 ug
20 ug
2 ug
0.2 ug
15 kDa

Figure A.1. Generation and expression of heparan sulfate binding domain-hen egg lysozme fusion proteins. 1a depicts unmodified- or heparan sulfate binding domain- (HSBD) hen egg lysozme fusion cloned into the Pichia pastoris pPIC9k expression vector to express. The alpha factor secretion signal is depicted at the 5' end with a brown box. One of three HSBDs, HBP12, rantes, or Tat, are depicted by the purple, blue, or pink box respectively. HEL is depicted as a red rectangle, while the 6X his tag is a yellow box at the 3' end of the construct. The two consensus HSBD Cardin-Weintraub sequences are shown, as well as the respective Cardin-Weintraub sequence for each HSBD. Figure A.1b shows a Coommassie stained 15% polyacrylamide gel that was loaded with (from left to right): ladder, Rantes-HEL dialyzed sup prior to purification and concentration, liquid run-off from washing the Histrap column, liquid run-off from loading the rantes sample on the column, purified and concentrated Rantes-HEL (200, 20, 2, or 0.2μg).

Figure A.2.

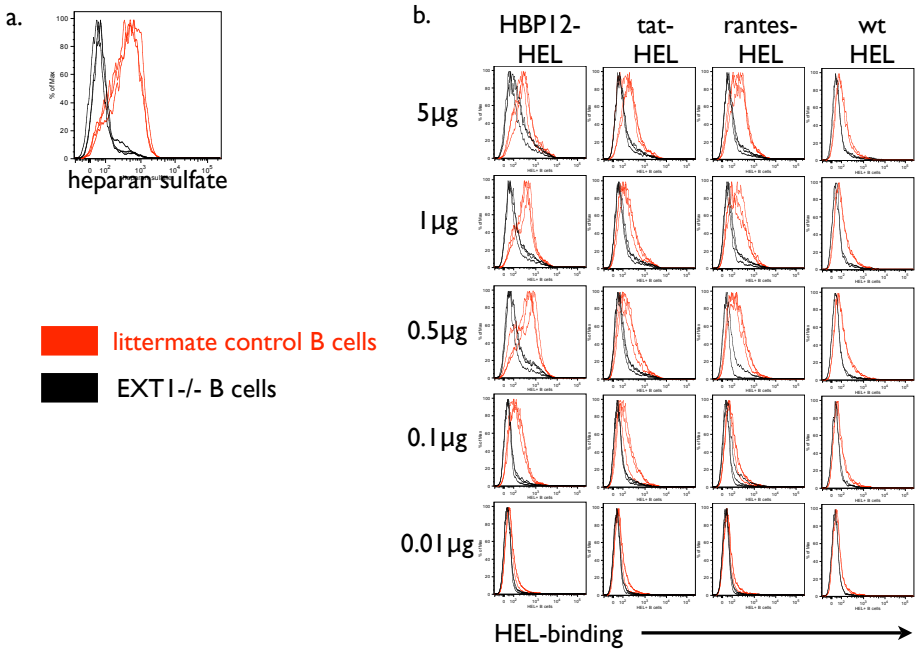


Figure A.2. Binding of HSBD-HEL fusion proteins to B cells is dependent on HS expression. A.2a shows the expression of HS on B cells from either 3 littermate control (red histograms) or 3 EXT1 cKO (black histograms) mice 24 hours post intraveinous injection of 0.2mg poly I:C. A.2b. Splenocytes from either 3 littermate control (red histograms) or 3 EXT1 cKO (black histograms) mice 24 hours post intraveinous injection of 0.2mg poly I:C were incubated with various concentrations (0.01-5µg) of WT-HEL or one of three HSBD-HEL fusion proteins (HBP12-HEL, Tat-HEL, or Rantes-HEL). B cells that "stained" positive for HEL were detected with HEL-specific antibodies by flow cytometry.

Figure A.3.

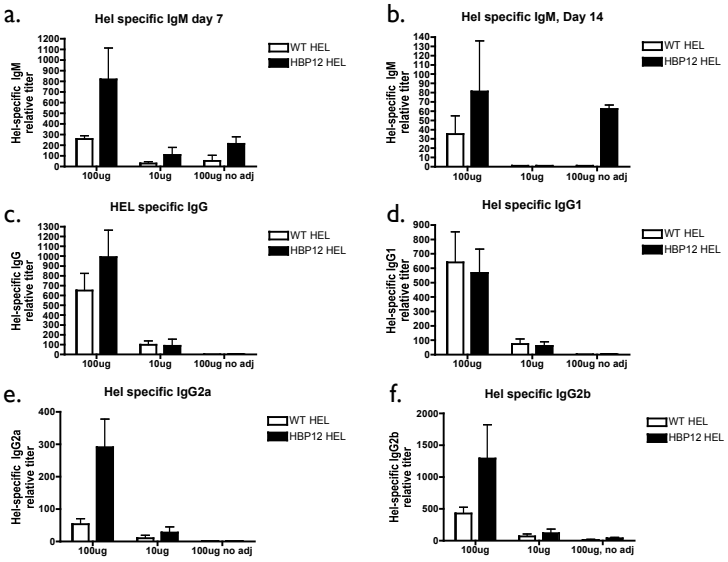


Figure A.3. Presence of a HSBD on HEL enhances the antibody response after HEL-immunization. 8-10 week old sex-matched WT C57Bl/6 mice were immunized with 10 or 100μg of either WT-HEL or HBP12-HEL in the presence or absence of 0.2mg of poly I:C as an adjuvant. At 7 or 14 days post immunization, serum was collected and HEL-specific IgM (day 7 A.3a, day 14 A.3b-A.3f), total IgG, IgG1, IgG2a, or IgG2b was determined by ELISA.