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INVESTIGATING THE ROLE OF TLR7 IN THE ACTIVATION OF AUTOREACTIVE B CELLS IN SYSTEMIC LUPUS ERYTHEMATOSUS

by

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Abstract

In systemic lupus erythematosus (SLE), auto-reactive B cells are known to be activated by combined signaling from the BCR and Toll-like receptors (TLR7 and TLR9), in a T-independent manner. While TLR7 deficiency has been shown to lead to less severe disease of common target organs, the role of this innate immune receptor in the initial activation events of SLE has yet to be explored. *In vitro* and *in vivo* experiments with the AM14 Transgenic mouse model were conducted to assess activation of auto-reactive B cells in the presence and absence of TLR7. Flow cytometry, CFSE dye, ELISA assays and ELISPOT assays were used to explore surface marker expression, proliferation, cytokine production and the quantity of auto-antibody producing B cells respectively. Results from *in vitro* experiments suggest that TLR7 is involved in generating functional auto-antibody secreting plasmablasts by inducing the up-regulation of the plasmablast marker, CD138.

INTRODUCTION

Lupus

According to the Lupus Foundation of America there are approximately 1.5 – 2 million lupus patients in the United States. Of these patients, 90% are women, primarily in the reproductive age group of 15 – 45 years. Previous studies have shown that sex hormones, released during reproductive years, contribute to susceptibility as female lupus patients have elevated levels of female sex hormones estrogen, prolactin, and gonadotropin - releasing hormone and reduced levels of the male sex hormone androgen than their normal peers (reviewed in Yacoub Wasef, 2004). Furthermore, African Americans, Latinos, Asians and Native Americans are found to be more prone to lupus than people of Caucasian descent. These vulnerable ethnic groups tend to have a high titer of auto-antibodies, the production of which is linked to certain antigen presenting molecules known to be prevalent in autoimmune diseases (reviewed in Lau et al., 2006).

During an autoimmune disease the host body is unable to differentiate between "self" and "non-self" components. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by inflammation, commonly of the skin, joint, kidney and blood (reviewed in Christensen et al., 2007). SLE patients generally experience flares or active periods following periods of remission (reviewed in Christensen et al., 2007). Physicians diagnose a patient with lupus on

the basis of 11 symptoms proposed by the American College of Rheumatology (National Institutes of Arthritis and Musculoskeletal and Skin Diseases). Once a patient has been correctly identified for 4 out of the 11 symptoms, the diagnosis is confirmed by the following blood tests: anti-nuclear antibody, anti-DNA antibody, anti-Sm antibody, and levels of serum complement. At present there is no cure for SLE. However, the disease can be minimized by non-steroid anti-inflammatory drugs, acetaminophens, corticosteroids, anti-malarials, immune-modulating drugs and anti-coagulants (National Institutes of Arthritis and Musculoskeletal and Skin Diseases).

Research in the pathogenesis of SLE is vital for the discovery of an effective cure for this chronic inflammatory disease as well as to provide insight to mechanisms of autoimmunity. This paper presents work conducted to investigate the role of a particular innate immune receptor, Toll-like receptor7 (TLR7), in the initial events that lead to disease progression of SLE in order to elucidate pathways of immune dysfunction and to determine the potentials of TLR7 in therapy.

The Immune System

The role of the immune system is to provide protection from a variety of foreign pathogens (such as bacteria, fungi, viruses and parasites) as well as dysfunctional or dangerous self (such as cancer) through an organized defense mechanism (Janeway, 1989; Matzinger, 1994). This defense mechanism

comprises the innate and the adaptive immune systems. The innate immune system provides initial defense against foreign microorganisms, while the adaptive immune system offers more specific and effective defenses as well as long term protection in the form of memory cells.

Innate Immune System

The first barrier between the host and the microbial environment is created by the skin and epithelia of the mucosal surface such as the respiratory, gastrointestinal and urogenital tracts (Parham, 2005). The mucosal epithelia produce mucins to coat pathogens, preventing their entry into host cells, and then wash the pathogens away as a preventive measure. If foreign organisms break through the epithelia, recognition occurs via germline-encoded pathogen-recognition receptors (PRR) on innate immune cells such as macrophages, neutrophils, dendritic cells (DC), eosinophils, basophils, mast cells and NK cells (reviewed in Medzhitov, 2007 and Akira et al., 2006). These PRRs have a wide specificity since they recognize common pathogen-associated molecular patterns (PAMPs), such as lypopolysaccharides (LPS) (reviewed in Barton and Medzhitov, 2002). On recognition of pathogens, innate immune cells produce soluble proteins called cytokines and chemokines (Parham, 2005). Chemokines draw other cells to the site of infection while cytokines cause a change in the behavior of cells. The release of these molecules begins the innate immune

response, manifested as inflammation in the infected tissues. Inflammation is characterized by swelling, reddening, release of heat and pain (Parham, 2005).

Toll-like receptors (TLRs) are a family of PRRs of the innate immune system known to play a key role in the recognition of pathogens in mammalian hosts (reviewed in Rifkin et al., 2005). TLRs were first discovered to provide fungal protection to *Drosophila* and at present eleven mammalian TLRs have been identified (Lemaitre et al., 1996). Structurally, TLRs comprise an extracellular domain for pathogen recognition and a cytoplasmic domain involved in signaling (Bell et al., 2003; Takeda et al., 2003). TLRs employ adaptor proteins such as myeloid differentiation factor 88 (MyD88) for their signaling pathway (reviewed in Rifkin et al., 2005). TLRs are expressed by a variety of cells such as B cells, dendritic cells (DC), macrophages, monocytes, epithelial and endothelial cells (Hornung et al., 2002).

When TLRs bind their ligand, they activate a signaling cascade leading to the expression of co-stimulatory molecules and the release of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12 (Janeway et al., 2005). The combined effect of the co-stimulatory molecules and the cytokines is able to generate an antigen-specific adaptive immune response (Janeway et al., 2005). IL-1β and IL-6 are known to activate lymphocytes by up-regulating the expression of antigen presenting and co-stimulatory molecules, IL-6 alone increases antibody production and IL-12 induces naïve T cells to differentiate into a specific type of helper T cell (Janeway et al., 2002; Janeway et al., 2005).

Adaptive Immune System

When the innate immune system is unable to clear out a pathogen, elements of the adaptive immune system are called in (with the help of the aforementioned cytokines) to complete the process within 4 – 7 days of the onset of inflammation (Janeway et al., 2005). B and T lymphocytes are the key players of the adaptive immune system. These adaptive immune cells stimulate a pathogen-specific response via their respective cell surface receptors: the B cell receptor (BCR) and the T cell receptor (TCR). These receptors specifically bind to small molecular pieces of the immuno-stimulatory substance known as antigen. The specific antigenic site at which antibody binds self or microbial antigen is called the epitope. After a successful immune response and clearance of the pathogen, these same cells provide immunologic memory to previously encountered pathogens in the form of B and T memory cells (Janeway et al., 2005).

Like the rest of the immune cells, B and T lymphocytes circulate in the blood, the lymphatic vessels and the lymphoid organs (Janeway et al., 2005). The lymphatic vessels return extracellular fluid collected from tissues to the blood. The lymphoid organs are divided into primary and secondary lymphoid organs based on function. The bone marrow and thymus are the primary lymphoid organs where development and maturation of lymphocytes occur. The spleen, lymph nodes, tonsils, adenoid, appendix and Peyer's patch are secondary lymphoid

organs where lymphocytes encounter antigen (Janeway et al., 2005). In this paper, we will focus on immune responses that take place in the spleen.

B lymphocytes

B cells develop and mature in the bone marrow before migrating to peripheral lymphoid organs via the blood (Janeway et al., 2005). Every B cell expresses many copies of one B cell receptor (BCR). The BCR, also known as the membrane immunoglobulin (Ig), comprises two heavy (H) chains as well as two light (L) chains linked via disulfide bonds into a Y shaped arrangement (see Figure 1). Both the heavy and light chains contribute to the specificity of the BCR and subsequently the ability to bind a particular antigen. The immunoglobulins are divided into five classes: α (alpha), δ (delta), ε (epsilon), γ (gamma), and μ (mu) encoding IgA, IgD, IgE, IgG and IgM respectively, based on slight differences in the portion of the heavy chain not involved in antigen binding (Janeway et al., 2005).

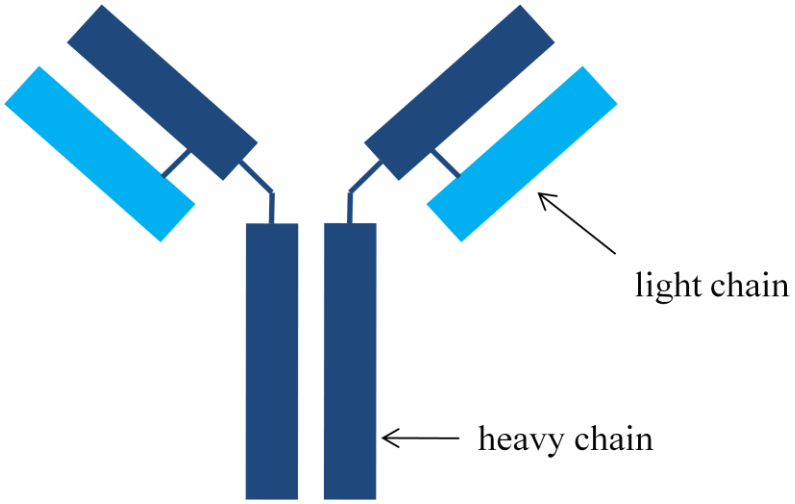


Figure 1. B Cell Receptor. The B cell receptor comprises heavy and light chains arranged in a Y shaped arrangement.

The BCR specificity is determined by random genetic recombination events that occur in the genes encoding both the heavy and the light chain, which occur separately, giving each BCR a unique specificity (Parham, 2005). Diversity in B cell specificities also comes from random joinings of different heavy and light chains together. While the surface of a B cell is covered in these BCRs, each B cell only has one kind of BCR therefore, one specificity. The vast diversity of BCRs generated on each unique B cell, however, allows for a very broad range of antigen specificities among the B cell population.

After antigen encounter (antigen binding to BCR), B cells can become activated to proliferate and differentiate into antibody producing plasma cells; antibodies are secreted forms of the BCR or membrane Ig. Variability is then introduced to the antigen-binding portions of the heavy and light chains by somatic hypermutation: point mutations or substitutions of nucleotides that may

enhance antigen-biding. The expansion and selection of increasingly specific B cells (and therefore antibodies) is called affinity maturation. B cells are tolerized in the bone marrow and the periphery to eliminate those cells that recognize components of the host body and could therefore initiate an auto-immune response (Parham, 2005).

Since random specificities of BCRs are generated so that the immune system can effectively respond to a wide array of antigens, it is important that steps are taken to avoid the production of BCRs that recognize self molecules and could generate antibodies against self. There are two steps of selection that protect against this outcome. First, in the bone marrow, when an immature B cell binds self-antigen, light chain genes undergo a new genetic rearrangement to make a new light chain, which when paired with the original heavy chain hopefully produces a BCR with a new, not self-reactive specificity. This process is known as receptor editing (Parham, 2005). If B cells are unable to generate a non self-reactive BCR they undergo clonal deletion via apoptosis, a form of cell suicide. Mature B cells that express IgM and IgD leave the bone marrow to circulate the periphery: the blood and secondary lymph organs. These B cells are considered naïve until they have encountered antigen (Parham, 2005).

In order for B cells to become activated and differentiate into plasma cells, B cells require two signals (Janeway et al., 2005). The first signal is received through cross linking of BCRs upon binding antigen. Upon binding antigen, the BCRs aggregate at this binding site allowing signaling molecules within the cell

to be in contact. The second signal is usually provided by T cells. In unique cases, B cells are activated without a signal from T cells, and antigens that can activate B cells without this 'help' are thus called T-independent antigens. T-independent responses were thought to rely on excessive cross linking of the BCR alone, but it is now understood that a second signal can be provided by PRRs such as TLRs. Therefore, a B cell that recognizes antigen through the BCR and can determine this antigen to be foreign through PRRs can be activated (Janeway et al., 2005).

B cell responses are easily visualized in the lymphoid organs of animals undergoing an immune response. The secondary lymphoid organs such as the spleen and lymph nodes are divided into the B cell zone and T cell zones (Parham, 2005). The B cell zone comprises the primary follicle and during times of an immune response, a secondary follicle, also known as the germinal center (GC), while the T cell zone surrounds these follicular regions. In the T cell zone, antigen that has been taken up by innate immune system cells (usually DCs) and presented to T cells to cause them to differentiate into antigen specific 'helper T cells'. These cells are called 'helper' because they can provide activation signals to B cells. Naïve B cells can activate specific helper T cells as well. Naïve B cells receive their first signal via BCR cross linking of antigen either in the T zone as well, or in the periphery and then they travel to the secondary lymphoid organ (Parham, 2005).

Upon antigen binding, naive antigen-specific B cells internalize their receptor and the bound antigen, degrade the antigen into peptide pieces in

endosomal compartments, and present those pieces to T cells, generating even more antigen-specific helper T cells (Parham, 2005). Helper T cells express co-stimulatory molecules that bind to receptors on B cells, supplying the second activation signal, and this cognate interaction results in the formation of a primary focus of proliferating, activated B cells. Some of these proliferating B cells, in cognate interaction with T cells, migrate from the primary focus to the GC. At the GC, proliferating B cells undergo massive proliferation, somatic hypermutation, affinity maturation and isotype switching to other Ig classes (IgG, IgE, IgA) (Parham, 2005).

T-independent B cell activation is less clearly understood, but does not require a GC, and activated B cells proliferate at the T-B border (similar to the location of the primary focus) (Fields et al., 2005a; Fields et al., 2005b**)**. These B cells do not typically undergo any kind of somatic hypermutation, affinity maturation, or isotype switch (the alteration of heavy chain portions of the BCR to change from IgM to IgG and other classes of BCR) (Fields et al., 2005a; Fields et al., 2005b**)**.

Autoimmunity

During development, self-tolerance mechanisms ensure that B and T lymphocytes are able to differentiate between "self" and "non-self" components of the host (Janeway et al., 2005). Most auto-reactive cells are rigorously deleted in selection processes before B and T cells are released from developmental

organs into the periphery. However, some auto-reactive cells do escape this selection and are present even in healthy individuals (Nemazee et al., 1983; Welch et al., 1983; Cogner et al., 1987; Decker et al., 2000). An autoimmune disease occurs when these auto-reactive cells become activated in response to self-antigens, also called auto-antigens. The probability of this occurring is heightened by certain genetic or environmental factors, but the specific causes of autoimmunity are unknown (Janeway et al., 2005). These auto-antigens may be organ specific as in the case of type I insulin-dependent diabetes mellitus, where the autoantigens are the pancreatic β cells, or systemic as in the case of systemic lupus erythematosus (SLE), where the autoantigens are nuclear components such as DNA and protein or RNA and protein (reviewed in Marrack et al., 2001).

Genetic predisposition to autoimmunity is controlled by a single gene or multiple genes, which may impact clearance of dead cells, presentation of antigen, signaling, co-stimulatory molecules, apoptosis or cytokinesis (Morel et al., 1994; Vyse et al., 1996; Harley et al., 1998; Wakeland et al., 1999). If dead cellular material is not correctly disposed, or communication between cells about antigens is altered, for example, there is an increased likelihood that an autoantigen will be 'mistaken' for a real antigen (Janeway et al., 2005).

Environmental agents such as certain drugs, heavy metal toxins and microbial infections also contribute to autoimmunity (Janeway et al., 2005). In some cases pathogens express surface antigens that resemble molecules of the host as an immune evasion strategy, which is known as molecular mimicry

(reviewed in Marrack et al., 2001). Immune cells activated in response to these pathogens will then mount an immune response on encountering similar self molecules (Janeway et al., 2005).

B cells in Systemic Lupus Erythematosus

B cells play a prominent role in the pathogenesis of SLE by secreting auto-antibodies, activating auto-reactive T cells and secreting cytokines (Shlomchik et al., 1994; Chan and Shlomchik, 1998; Chan et al., 1999a; Chan et al., 1999b; Chan et al., 1999c; Browning, 2006). Auto-reactive B cells produce antibodies in response to auto-antigens of three main types: DNA related (dsDNA or chromatin), RNA related (Smith (Sm) or ribonucleoproteins (RNP)) and immune complexes of IgG bound to DNA or RNA related antigen (Tan, 1989; Shan et al., 1994; Egner, 2000; Muro, 2005). Anti-DNA or chromatin antibodies are the most common auto-antibodies in human and murine SLE and anti-RNA antibodies are related to severe forms of the disease in humans (Kirou et al., 2005). Auto-antigens are exposed to immune cells as a result of improper clearance during a microbial response or inflammation (reviewed in Rifking et al., 2005).

Auto-antigens in SLE are believed to be secluded in the nucleus or cytoplasm but are exposed to host immune cells due to defective clearing of apoptotic cells as membrane blebs or apoptotic bodies (Voll et al., 1997; Fadok et al., 2000; Lauber et al., 2004). While undergoing apoptosis, chromatin and RNA

related molecules are modified by granule-associated proteases such as granzyme B, which could result in the formation of previously unencountered epitopes on these antigens (Casciola-Rosen et al., 1999). Furthermore, inflammation during apoptosis could also breach self-tolerance mechanisms by activating low-affinity lymphocytes (Albert et al., 1998; Inaba et al., 1998; Lu et al., 2001; Savill et al., 2002; Ma et al., 2005;).

While high-affinity self-reactive lymphocytes are eliminated during development, low-affinity self-reactive lymphocytes continue to circulate and become activated during an autoimmune response (Namazee et al., 1983; Welch et al., 1983; Cogner et al., 1987; Decker et al., 2000). Activated B cells present antigen to auto-reactive T cells, which proliferate and stimulate naïve B cells to differentiate into auto-antibody producing plasma cells thereby establishing a positive feedback loop (Chan O. T. M. et al., 1999c) (See Figure 2). These auto-antibodies accumulate in organs such as skin, blood vessel, lung and kidney causing tissue and cell damage (reviewed in Christensen et al., 2007). In the course of the response, antibodies produced by low-affinity auto-reactive B cells undergo somatic hypermutation and affinity maturation resulting in high-affinity antibodies (Wofsky et al., 1985; Shlomchik et al., 1987; Shlomchik et al., 1990; Radic et al., 1994; Peng et al., 1996a; Peng et al., 1996b).

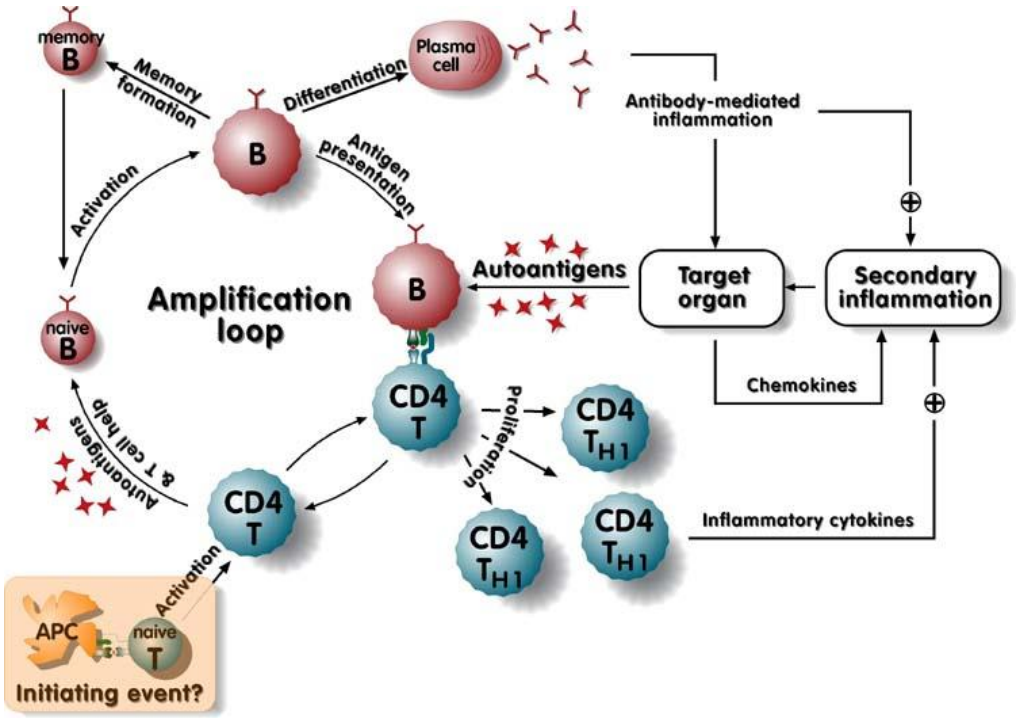


Figure 2. Positive Feedback Loop (Christensen et al., 2007). During SLE, B cells activate T cells which further activate naïve B cells to differentiate into auto-antibody secreting plasmablasts leading to amplification of auto-antibodies that accumulate at the target organs resulting in inflammation and tissue destruction.

AM14 Transgenic Mouse Model

A number of BCR transgenic (Tg) mouse models specific for DNA/ chromatin, Sm or IgG antigen have been created to explore initiation and development of B cell autoimmunity in SLE (Erikson et al., 1991; Offen et al., 1992; Tsao et al., 1992; Shlomchik et al., 1993; Santulli-Marotto et al., 1998). For this project the AM14 Tg mouse model was used. In this model, the BCR heavy chain transgene, AM14, is present on all B cells. When this heavy chain spontaneously combines with a particular light chain (the Vκ8 light chain) a BCR specific for IgG2 of allotype "a" (IgG2a) is produced (Shlomchik et al., 1993). B

cells with this specificity are known as rheumatoid factor positive (RF+), and the antibodies they secrete are called rheumatoid factor (RF) (See Figure 3).

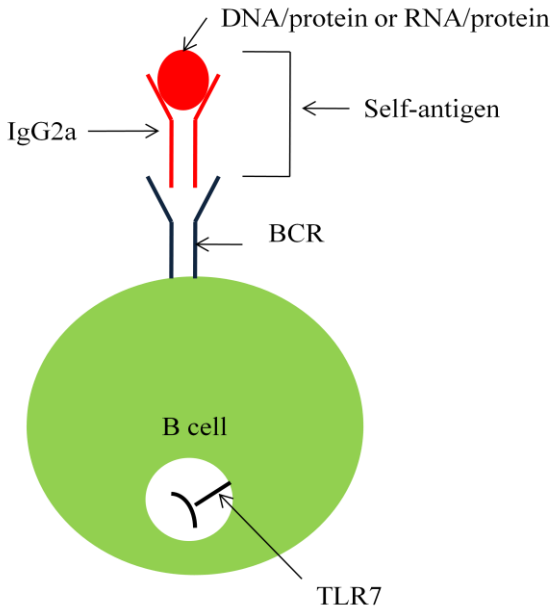


Figure 3. AM14 System. RF+ B cells bind the auto-antigen or self-antigen, which is an immune complex of DNA/ protein or RNA/ protein bound to IgG2a. The BCR will internalize the self-antigen to expose it to TLR7 residing in endosomal compartments of the B cell.

The heavy chain Tg model is a more physiologic mouse model, because RF+ B cells make up a very small population of circulating B cells in these mice, much like the low frequency of auto-reactive B cells in humans prior to disease (Shlomchik et al., 1993). This AM14 Tg mouse model is also useful in providing insight to autoimmunity during SLE as it enables the controlled administration of the antigen (IgG2a), which picks up DNA and RNA related molecules in the mice, for study of RF+B cell activation (this is possible since the AM14 Tg is an

IgM, making all B cells and circulating antibody IgM, and IgG2a something that can be introduced). RF+ B cells were first discovered in patients of rheumatoid arthritis but were later also found in other autoimmune diseases including SLE (Rose et al., 1948; Tam, 1989). Studies show that about 20% of SLE patients produce RF+ B cells (Helin et al., 1986; Howard et al., 1991; Lipsky, 2001**)**.

In this report, we used AM14 Tg mice that were crossed onto the Fas-deficient autoimmune-prone MRL/*lpr* strain in order to study initiation and progression of disease (Shlomchik et al., 1993). A lupus-like disease in the MRL/ *lpr* strain occurs due to a combination of MRL genetics (specific genes have long been under investigation) and the recessive *lpr* gene which down-regulates production of the apoptosis inducing Fas protein (Watanabe-Fukunaga et al., 1992; Watanabe et al., 1995). The MRL/*lpr* mice exhibit nephritis, arthritis, anti-RNP antibodies, anti-ssDNA and anti-dsDNA antibodies similar to human SLE patients (Eisenberg et al., 1978; Hang et al., 1982; Pisetsky et al., 1982; Kelley et al., 1985). Therefore, the AM14 Tg model on the MRL/lpr strain of mice is an ideal model in which to study B cell activation under conditions similar to human disease.

TLRs in the AM 14 Tg Mouse Model

Research with the AM14 Tg mouse model has revealed that while RF+ B cells are tolerized in normal mice, they undergo differentiation, somatic mutation and affinity maturation in autoimmune strains (Shlomchik et al., 1993; Hannum et

al., 1996; William et al., 2002; William et al., 2005a; William et al., 2005b). The auto-antigens activating RF+ B cells are immune complexes of IgG2a and chromatin or Sm released from apoptotic cells (Pancer et al., 1981; Bell et al., 1990; Emlen et al., 1992; Lau 2005). Generally, activation of B cells requires two signals: the first, provided by BCR cross linking of antigen and the second, by T cells (Parham, 2005). A recent study has shown that T cells are not required for activation and differentiation of RF+ B cells (Leadbetter et al., 2002; Herlands, unpublished data), and investigations into alternative sources of a second activation signal ensued.

Since immune complexes contain DNA and RNA antigens, TLRs were proposed as the second signal providers as many of these innate immune receptors recognize DNA and RNA components. Of the TLRs known to bind DNA and RNA components, TLR7 recognizes viral single stranded RNA (ssRNA) sequences while TLR9 recognizes bacterial CpG-DNA motifs (Hemmi et al., 2000; Diebold et al., 2004; Heil et al., 2004). TLR7 and TLR9 have now been confirmed to play a role in the onset of SLE (Leadbetter et al., 2002; Vollmer et al., 2005; Pisitkun et al., 2006; Savarese et al., 2006; Subramanian et al., 2006, Christensen, 2007). Both TLR7 and TLR9 are found in endosomal compartments of B cells, macrophages, plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (Edwards et al., 2003; Hornung et al., 2002).

These TLRs employ the adaptor protein, MyD88, in their signaling pathway and it has been shown *in vitro* and *in vivo* that RF B cells were not

activated in MyD88-deficient B cells or mice (Leadbetter et al., 2002; Herlands, unpublished data). Despite similarities in location and signaling pathway, TLR7 and TLR9 differ in their role in the clinical manifestation of SLE: TLR7 has an inflammatory role while TLR9 has a regulatory role (Christensen et al., 2006; Christensen et al., 2007). In MRL/lpr mice, TLR7 deficiency results in less severe disease of the kidney and skin, a decrease in the weight of spleen and lymph nodes, lower B cell activation, lower auto-antibody titers and reduced but detectable levels of cytokines known as type I interferons (IFN-I) (Christensen et al., 2006).

Numerous studies have shown that IFN-I: interferon-alpha (IFN-α) and interferon-beta (IFN- β) are involved in the disease progression of SLE. Human SLE patients have higher titers of IFN-I and mice that are unable to respond to IFN-α or IFN-β experience reduced form of the disease (Hooks et al., 1979; Santiago-Raber et al., 2003; Braun et al., 2003; Hron et al., 2004). IFN-1, secreted by TLR7 and TLR9 expressing plasmacytoid DCs, induce B cells to differentiate into plasma cells that secrete inflammatory antibodies such as IgG2a and IgG3 without T cell help (Le Bon et al., 2001; Lund et al., 2003; Poeck et al., 2004; Liu 2005; Uematsu et al., 2005 Savarese et al., 2006).

While both anti-DNA and anti-RNA antibodies induce pDC production of IFN-I, anti-RNA antibodies are more effective (Lovgren et al., 2004; Barrat et al., 2005). TLR7 activation releases anti-RNA antibodies, which enhance IFN-I production causing severe human and murine SLE (Kirou et al., 2005;

Christensen et al., 2006). IFN-α , in turn, increases TLR7 signaling in B cells thus releasing more anti-RNA antibodies contributing to exacerbated disease (Bekeredjian-Ding et al., 2005). TLR7 has also been shown to induce the secretion of other pro-inflammatory cytokines such as IL-6, IL-12p70 and CCL2 in spleen monocytes and DCs (Horii et al., 1993; Huang et al., 1996; Tesch et al., 1999; Ronnblom et al., 2001, Crow et al., 2004; Pawar et al., 2006). Although TLR7 is known to be central to the disease progression of SLE, the role of this innate immune receptor in the initial activation events of B cells are yet unknown.

Plasmablast Differentiation in the AM14 Tg Mouse Model

In the spleen, RF+ B cells are located in groups in the T zone – red pulp border where they undergo somatic hypermutation and are hardly present in GCs (William et al., 2002). These RF+ B cells are of two types: plasmablasts expressing low levels of the surface marker CD22 ($CD22^{low}$) and activated B cells expressing high levels of CD22 ($CD22^{high}$); CD22 is expressed on B cells but is down regulated on activated B cells (William et al., 2005). Unlike plasma cells, plasmablasts are proliferating cells that secrete antibodies (Janeway et al., 2005). These $CD22^{low}$ cells express high levels of CD138 (plasmablast marker), CD44 (activation marker), and interact with DCs, possibly for survival (Garcia De Vinuesa et al., 1999; Balazs et al., 2002; William et al., 2002). The $CD22^{high}$ cells are believed to be the precursor population of $CD22^{low}$ cells as $CD22^{low}$ cells are

short-lived (undergo apoptosis faster) but mutate more than $CD22^{high}$ cells (William et al., 2005).

A recent study has shown that TLR7 and 9 are vital for activation of RF+ B cells and their differentiation to functional antibody secreting plasmablasts (Herlands et al., manuscript submitted for publication). While TLR7-deficient mice have similar frequencies of $CD22^{low}$ cells as TLR7-sufficient mice, the number of functional antibody secreting plasmablasts was reduced in the absence of TLR7 (Herlands et al., manuscript submitted for publication). Furthermore, the absence of TLR7 led to higher expression of CD45, a B cell marker which is low on plasma cells and extrafollicular plasmablasts (Ardavin et al., 1999; Shapiro-Shelef et al., 2003; William et al., 2005). In light of this work, I hypothesize that TLR7 activates RF+ B cells to differentiate to functional antibody secreting plasmablasts by inducing changes in the expression of surface markers and stimulating the release of cytokines.

MATERIALS AND METHODS

Mice

The AM14 heavy chain transgenic (Tg) mice (Shlomchik et al., 1993), used in the *in vitro* and *in vivo* experiments, were backcrossed at least 10 generations onto the MRL/*lpr* background (William et al., 2002). The AM14 TLR7$^{-/-}$ mice were prepared as described (Christensen et al., 2006). All mice were housed under specific pathogen free conditions at Yale University and transported to Mount Holyoke College the day prior to experiments. For the *in vitro* experiment, 6-16 week old mice were used. For the *in vivo* experiment, 8-10 week old mice were used.

Anti-Chromatin Hybridomas

The IgG2a and IgG2b anti-chromatin hybridomas, PL2-3 and PL2-8 were originally obtained from Mark Monestier (Losman et al., 1993). PL2-3 and PL2-8 ascites were generated in peritoneum of RAG-/- mice (mice unable to make their own antibodies) at Yale University and the concentration of PL2-3 antibodies in this ascites was determined by IgG anti-chromatin ELISA by the laboratory of Mark Shlomchik (Yale University).

TLR ligands

The TLR ligand used in the *in vitro* experiments was CpG-S ODN 1826, called "CpG" in this paper, the sequence of which is 5'TCCATGACGTTCCTGACGTT-3'. For the *in vivo* experiment, the TLR7 inhibitor, IRS 661, was used, the sequence of which is 5'TGCTTGCAAGCTTGCAAGCA-3'. Both oligonucleotides were purchased from Oligos Etc., Inc.

Antibody Reagents

4-44biotin (anti-AM14 idiotype antibody) was prepared in Shlomchik laboratory at Yale University as described (Shlomchik et al., 1993). The other antibodies used were anti-CD22 Alexa Fluor 680 (Clone Cy34.1, donated by Mark Shlomchik, Yale University), anti-CD22 Alexa Fluor 647 (Clone Cy34.1, donated by Mark Shlomchik, Yale University), CD-22 FITC (Clone Cy34.1, donated by Mark Shlomchik, Yale University), anti-CD86 PeCy7 (Southern Biotech), anti-CD38 FITC (eBioscience), anti-CD138 PE (Bio Pharmingen), anti-CD44 PeCy7 (eBioscience), anti-CD16/32 (eBioscience) and anti-IgM (Chemicon International). Streptavidin (Invitrogen) and propidium iodide (PI) (donated by Mark Shlomchik, Yale University**)** were also used. For *in vitro* stimulation, anti-IgM $F(ab')_2$ (Jackson Immuno Research) was used in coordination with CpG. Streptavidin (SA) PE and SA Alexa 647 (Invitrogen) were used as secondary reagents for the detection of biotinylated reagents, namely 4-44 biotin.

Isolating Splenocytes

Tissue cultures from AM14 WT and AM14 TLR7$^{-/-}$ spleens were prepared in the hood under sterile conditions, using sterilized materials. The rubber end of a syringe was used to extract cells through a cell strainer in 2mL complete media (500mL RPMI, 5mL L-glutamine, 10mL HEPES, 1mL Gentamycin (antibiotic), 50mL Fetal calf serum (FBS) and 0.5mL 50M 2-ME). The strainer was washed twice (with washes being collected and added to cells) and the cell suspension was transferred to a 15mL sterile conical flask. The flasks were centrifuged (Sorvall Legend RT) at 1200rpm for 8 minutes at room temperature.

The supernatant was discarded and the resulting pellet was resuspended in 5mL Red Cell Lysis Buffer (eBiosciences) for 5 minutes then diluted in 10mL media before centrifuging at 1200rpm for 8 minutes at room temperature. The Red Cell Lysis Buffer was used to exclude red blood cells from our analysis. The supernatant was discarded and the cells were diluted in 1mL media. To count the cells on a hemocytometer (American Optical) using a light microscope (Nikon, Eclipse TS100), 10µL of the cells were diluted in a mixture of 80µL PBS and 10µL Trypan Blue (Life Technologies). The viability of the cells was calculated and confirmed to be above 85% for all experiments.

Labeling with CFSE

To detect proliferation in the *in vitro* experiments, half the cells were labeled with Vybrant Carboxy Fluoroscein Diacetate Succinimidyl Ester (CFSE) dye (Molecular Probes). Some PBS was pre-warmed to 37^{o}C and CFSE (previously stored at –20^{o}C) was brought to room temperature. 10µL aliquots of 10mM CFSE in dimethyl sulfoxide (DMSO) were prepared. One aliquot of 10uL CFSE+DMSO was diluted 1:1000 in PBS for a 10uM solution. The cells were brought to 100x10^{6} cells/mL in PBS. 10uM CFSE solution was added 1:1 to cells and incubated for 15 minutes at 37^{o}C. The cells were centrifuged at 1200rpm for 8 minutes at room temperature along with non-CFSE labeled cells and washed two times.

In vitro Experiments

Cell Culture

For *in vitro* experiments, CFSE and non-CFSE labeled cells were brought to 2x10^{6} cells/mL in IL-2 media at a concentration of 10 pg/mL in media. 2000uL aliquots of CFSE labeled cells were placed in each well of a 6 well plate (Becton Dickinson) with 2 wells per condition: CpG and anti-IgM F(ab')$_2$, PL2-3 hybridoma and PL2-8 hybridoma (Figure 4). A separate 6 well plate was prepared for non-CFSE labeled cells in an identical manner. 23µL of PL2-3 at 4.40 mg/mL;

125µL of PL2-8 at 0.80 mg/mL; 6.5µL of CpG at 0.62 mg/mL and 2.5µl of anti-IgM $F(ab)_2$ at 1.2 mg/mL was added to respective wells. The plates were incubated (Nuaire, NU-4850) for 2 days at 37^{o}C with 5% CO_2.

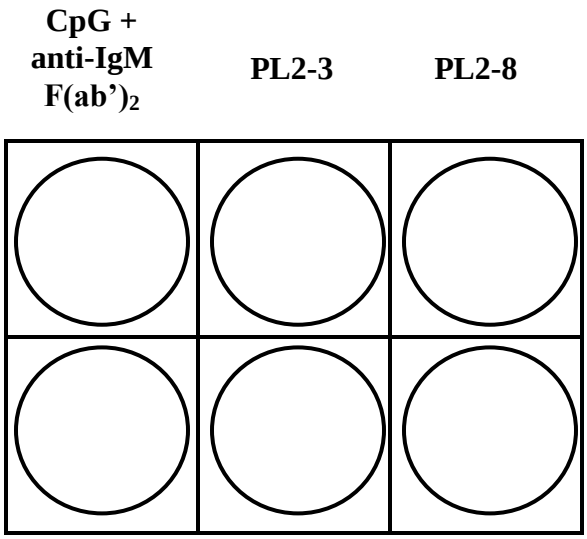


Figure 4. Representative 6 well plate used to incubate cultures treated with CpG and anti-IgM $F(ab')_2$, PL2-3 hybridoma and PL2-8 hybridoma at 37^{o}C for two days. Different plates were used for culture from AM14 WT and AM14 TLR7$^{-/-}$ mice.

Cultures were harvested by removing both CFSE labeled and non-CFSE labeled plates from the incubator and using a pipette to mix each culture well before collecting separately. 500µL non-sterile media was used to wash all the wells of the same culture and collected with the rest of the cells for that culture. These cells were centrifuged at 1200rpm for 8 minutes at 8^{o}C. 100µL of staining

media (SM: 3% FCS, 0.05% sodium azide in 1x PBS) was added to pellets from each condition and transferred to FACS tubes.

FACS Staining and Analysis

Cells from harvest of *in vitro* cultures were centrifuged at 1500rpm for 3 minutes at 8^{o}C. Supernatants were discarded and the pellets were vortexed. Fc receptors were blocked with anti-CD16/32 antibodies to prevent non-specific binding of antibodies during staining. 1µL of anti-CD16/32 antibody was added to 50µL SM per staining condition and vortexed before incubating on ice for approximately 10 minutes. 100µL of cold SM was added and the cells were centrifuged at 1500rpm for 3 minutes at 8^{o}C.

For *in vitro* experiments, two staining mixtures containing primary antibodies, were prepared for CFSE and non-CFSE labeled cells. CFSE labeled cells were stained with stain 1 comprising PI, 4-44biotin, SA 647, CD22 Alexa Fluor 680 and CD86 Pe-Cy7. Non-CFSE labeled cells were stained with stain 2 comprising CD38 FITC, CD138 PE, PI, 4-44biotin, SA 647, CD22 Alexa Fluor 680 and CD44 Pe-Cy7. For both stains, PI was only added immediately before placing tubes in the LSRII. Each stain was prepared in 50uL SM per FACS tube with the appropriate amount of antibody (Table 1).

Table 1. Amount per reaction of the different antibodies used to detect surface markers of interest.

Antibody	**Amount per reaction (µL)**
4-44bi	0.25
CD22 Alexa Fluor 680	0.10
CD22 Alexa Fluor 647	0.10
CD22 Alexa Fluor 488	0.25
CD86 Pe-Cy7	0.25
CD38 FITC	0.10
CD138 PE	0.20
CD44 Pe-Cy7	0.25

50µL of each single color control: CFSE, FITC, PE, PI, CD4 Alexa 647, CD45 Alexa 680 and Pe-Cy7 were also prepared. Cells were resuspended in 50µL of respective stains and incubated on ice, in the dark, for 20 minutes along with single color controls. The cells were washed with 100µL of SM followed by centrifugation at 1500rpm for 3 minutes at 8^{o}C. The experimental cells (all cells except the single color controls) were vortexed and incubated on ice with the secondary antibody, SA Alexa Fluor 647 or SA PE diluted in SM 1:1000, for 15 minutes in the dark. To the single color controls, 200µL of SM rather than the secondary antibody was added.

FACS tubes containing the experimental cells and the single color controls were washed with additional 200µL SM and centrifuged at 1500rpm for 3minutes at 8^{o}C two times. Finally, 200µL SM was added to each FACS tube, the tubes were placed on ice and covered with foil while being transported to University of Massachusetts at Amherst, for FACS analysis on the LSRII (BD Biosciences).

For each FACS tube, 400µL PI diluted 1:200 in SM was made and a few drops of this solution were added to each tube before placing on the LSRII. The FACS data were analyzed with Flow Jo version 7.2.4 software (Tree Star Inc.).

ELISA Assay

ELISA assays was used to quantify the serum level of cytokines in AM14 WT and AM14 TLR7$^{-/-}$ mice. Mouse IL-10, TNF-α and IFN-γ ELISA OPTia kits (BD Sciences) were used. For each cytokine, 100µL of Capture Antibody at 1:250 in Coating Buffer was added to each well in a 96 well plate (Corning Incorporated) (Figure 5). The plate was incubated at 37^{o}C for an hour. The wells were washed three times with Wash Buffer (PBS with 0.05% Tween). Each well was blocked with 200µL Assay Diluent (PBS with 10% FBS) and incubated at room temperature for an hour. The wells were washed three times and 100µL serial dilutions (1:3) of the standards as well as serum from cultures treated with CpG and anti-IgM F(ab')$_2$, PL2-3 hybridoma and PL2-8 hybridoma were added to respective wells of the plate in duplicate (Figure 5). The plate was incubated at 37^{o}C for an hour.

The plate was washed five times with Wash Buffer and 100µL Working Detector (Detector Antibody and Streptavidin-Horseradish Peroxidase) was added to each well in concentrations specified by each kit. The plate was incubated at room temperature for an hour. The plates were washed seven times and 100µL of

the Substrate Solution, which causes a color change in the presence of HRP, was added to each well and the plate was incubated for 30 minutes at room temperature in the dark. 50µL of Stop Solution, to stop color development, was added to each well and the plate was read at 450nm – 570nm within 30 minutes on the VERSAmax plate reader (Molecular Devices). SOFTmax Pro version 4.0 software (Life Sciences) was used to analyze the data.

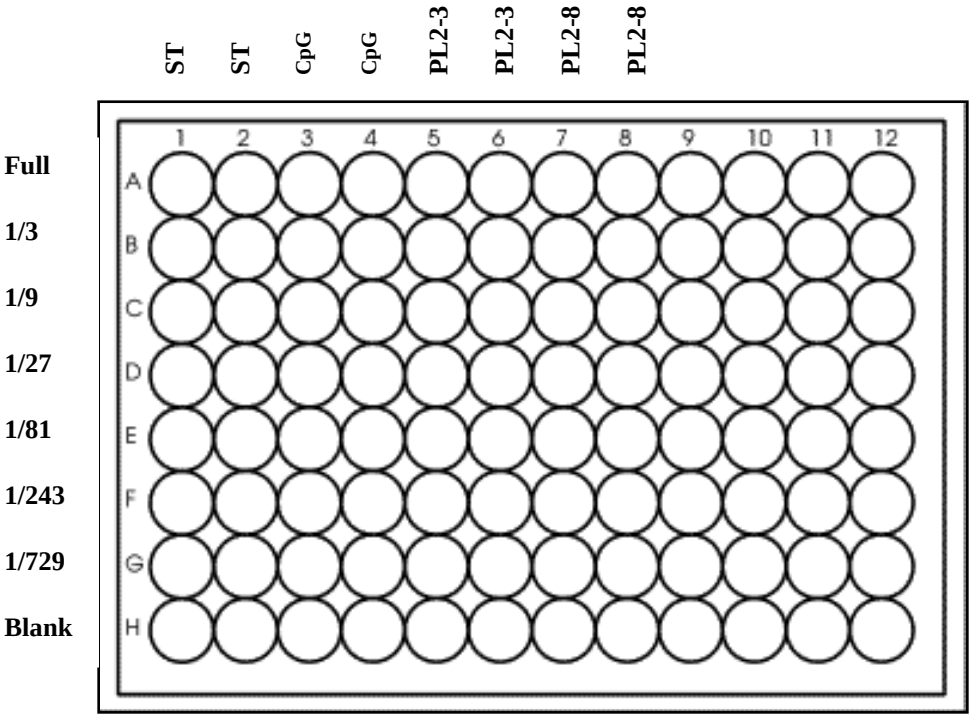


Figure 5 (Therapeutic Immunology Group). Representative 96 well plate for cytokine ELISAs of supernatants from cultures treated with CpG and anti-IgM $F(ab')_2$, PL2-3 and PL2-8 hybridomas. Different plates were used for cultures from AM14 WT and AM14 $TLR7^{-/-}$ mice.

In vivo experiment

Injections and harvest

AM14 WT mice were separated in four experimental groups: PL2-3 only (n=3), PL2-3 and IRS661 (n=3), IRS661 only (n=1), and PBS only (n=1). The dosage of appropriate injections was 100µL of IRS661 at a concentration of 50µg/ 100µL, 500µL of PL2-3 at a concentration of 1 µg/µL, and 100uL of PBS. Mice were injected with PL2-3 and IRS661 on alternate days, starting with IRS661, for a total of three days each. The PBS only mouse received injections for six days. After the seventh day, the mice were sacrificed.

Spleens were removed from the mice, on the bench, and placed in petri dishes containing 5mL RPMI (2.5% FCS, L-glutamine, HEPES, Gentamycin). The bottom of these petri dishes had been serrated. The spleen was disrupted in the petri dish using the butt of a syringe. The splenocytes were pipetted up and down with Pasteur pipettes and the cell suspension was transferred to a 15mL conical flask. The debris was allowed to settle for 2-3 minutes and the supernatant was transferred to a new 15mL conical flask and the cells were spun at 1200rpm for 8 minutes at room temperature. The supernatant was discarded, the cells were disrupted and 5mL Red Cell Lysis Buffer at room temperature was added for 5 minutes. The cells were pipetted up and down with Pasteur pipette and clumps of dead cells were removed. To stop the reaction, 10mL of RPMI was added and the cells were spun at 1200rpm, for 8 minutes at room temperature. The supernatant

was discarded and the cells were resuspended in 1mL of RPMI. The cells were counted on a hemocytometer and prepared for FACS as previously described.

FACS Staining and Analysis

For *in vivo* experiments, only one staining mixture was used comprised of 4-44biotin, SA-647, CD22-AlexaFluor 488, and CD138 PE, PI (concentrations included in Table 1) As with cells from the in vitro experiment, PI was added immediately before placing on the LSRII and otherwise stains were prepared in 50µL SM per FACS tube with the appropriate amount of antibody. Staining was completed as previously described for *in vitro* experiments.

ELISPOT Assay

To quantify the number of AM14 antibody secreting B cells, 96 well plates (Immulon 4) were coated with anti-IgM at 5 ug/mL at 40uL per well and incubated overnight. The plates were blocked with 100uL per well of PBS/1%BSA and incubated in 37^{o}C for one hour. The plates were washed thrice with Elisa Wash Buffer (EWB) and twice with dH_20 (distilled water). 200uL warm media was added to each well. 100uL of cells at $7.5x10^6$ cells/mL in warm RPMI were added to each top well in triplicate (Figure 6) then serially diluted (1:3) down the wells by taking 100µL from the top well and mixing it into the

well below, then taking 100µL of that well (after mixing) and mixing it into the well below, and discarding the last 100µL.

The plates were spun down at 1200rpm, for 3 minutes at room temperature and incubated for 4.5-6 hours in 37^{o}C with 5% CO_2. The plates were washed as before and 50µL per well of 4-44-bi at 1:1000 in PBS/1%BSA was added and incubated over night at 4^{o}C. The plates were washed as before and 35µL SA-AP at 1:1000 in PBS1%BSA was added to each well and allowed to incubate at 37^{o}C for an hour. The plates were washed and developed with 40µL per well of BCIP/ agarose mixture. For the BCIP/ agarose mixture, 132µL of BCIP stock (Sigma), stored with dessication at -20^{o}C, was added to 20mL of Tris/NaCl. 3% low-melt agarose was prepared and melted, and both BCIP and agarose were brought to 55^{o}C before mixing 1mL agarose for every 5mL BCIP/TrisNaCL solution. 40µL of this solution was added to each well. The plates are allowed to cool for 20-30 minutes at room temperature so the agarose mixture would solidify and the plates developed overnight at 4^{o}C. Spots, representative of RF antibody secreting cells, were counted approximately 16-24 hours later.

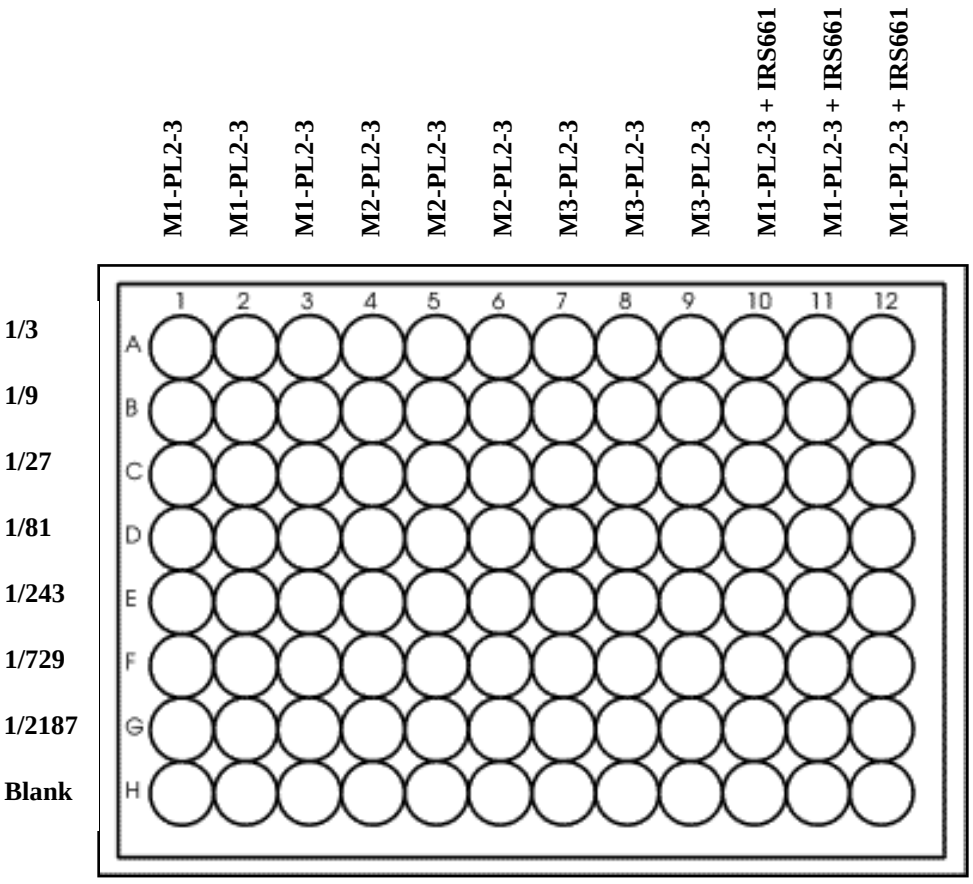


Figure 6 (Therapeutic Immunology Group). Representative 96 well plate for ELISPOT analysis of cells from mice from four different conditions: PL2-3 hyrbidoma only (n=3) and PL2-3 and IRS661 (n=3), IRS661 (n=1) only and PBS (n=1) only.

For each mouse, one row was selected and the number of spots in each of the three wells of that row was counted using a light stereo microscope with a high intensity illuminator (Olympus, SZ-ST). The count was readjusted to spots per million cells (Table 2). Since 100uL of $7.5x10^6$ cells/mL was added to each of the top wells, which already contained 200uL of media, the new concentration of the top well was $7.5x10^5$ cells in 300µL. 100µL of cells at the new concentration

was added to the second well and diluted down. The final concentration of the top well was 5 $x10^5$ cells in 200µL so the count from the top wells had to be multiplied by a factor of 2 to obtain spots per million cells.

Table 2. Values used to adjust the count from each row to spots/ million cells.

Row	**Multiplication**
1	X 2
2	X 6
3	X 8
4	X 54
5	X 162
6	X 486
7	X 1458

RESULTS

In vitro Experiment

FACS Analysis

In order to determine the role of TLR7 in early activation of RF+ B cells, *in vitro* culture experiments were set up and harvested 48 hours later. In these experiments, splenocytes from AM14 WT and AM14 TLR7$^{-/-}$ mice were subjected to three different culture conditions. As a positive control, cells were incubated with CpG, an oligonucleotide which stimulates B cells through TLR9, and anti-IgM F(ab')$_2$, which stimulates B cells through the AM14 BCR. As an experimental condition, the cells were cultured with the anti-chromatin PL2-3 hybridoma, a type of IgG2a (Losman et al., 1993). And as a negative control, the cells were cultured with PL2-8, a type of IgG2b, which does not bind the AM14 BCR (Losman et al., 1993).

Upon harvest, 2 days after incubation, cells were stained and analyzed to look at the number of RF+ B cells, and the level of expression of the following cell surface markers on those RF+ B cells: CD22, CD38, CD44, CD86 and CD138 using flow cytometry. These well-known proteins have altered expression levels which can be used to determine the level of activation and differentiation status of B cells, and will be described in detail below. Comparisons were then made for cells from the different culture conditions from the same mice as well as between AM14 WT and AM14 TLR7$^{-/-}$ mice.

Flow cytometry is a technique which can be used to identify and count cells that pass through a laser beam (Janeway, 2005). The cells of interest are tagged with fluorophore linked antibodies for surface proteins. As individual cells pass through the laser beam, they scatter light and the fluorophore on the antibody is excited and fluoresces. Both of these activities are detected and can be interpreted. The scattered light provides information on the size and complexity or granularity of the cell while the fluorescence emission can be used to determine the number of cells expressing the corresponding surface marker (Janeway, 2005).

The level of expression of cell surface markers of interest on AM14 B cells was determined by making three gates. First, the lymphocyte (B and T cell) population was selected from the entire cell population of each culture (Figure 7A). In this scatter plot, the x-axis represents Forward Scatter (FSC), which determines the size of the cell and the y-axis represents Side Scatter (SSC), which determines the structural complexity of the cell. The B cell gate was created on the basis of previously known size and complexity of this cell type. In addition to selecting B cells, which are very small, plasmablasts, which tend to be larger and more complex, and are of interest to us were also included.

Next, live cells within the lymphocyte population (Figure 7B) were determined by propidium iodide (PI). PI is a fluorophore that stains DNA and RNA in dead cells and is membrane impermeable in viable ones (Suzuki et al., 1997; Moore et al., 1998; Lecoeur, 2002). Third, AM14 B cells from the live cell population were selected using the 4-44 anti-idiotype antibody (Figure 7C). This

antibody recognizes only a BCR that contains both the AM14 heavy chain and the Vk8 light chain (and therefore RF+ B cells). From the 4-44+ B cell population, histograms of cell surface marker expression were created (Figure 7D). While data were not consistent between the two experiments for most cell surface markers, an interesting observation was made in terms of CD138, a plasmablast marker.

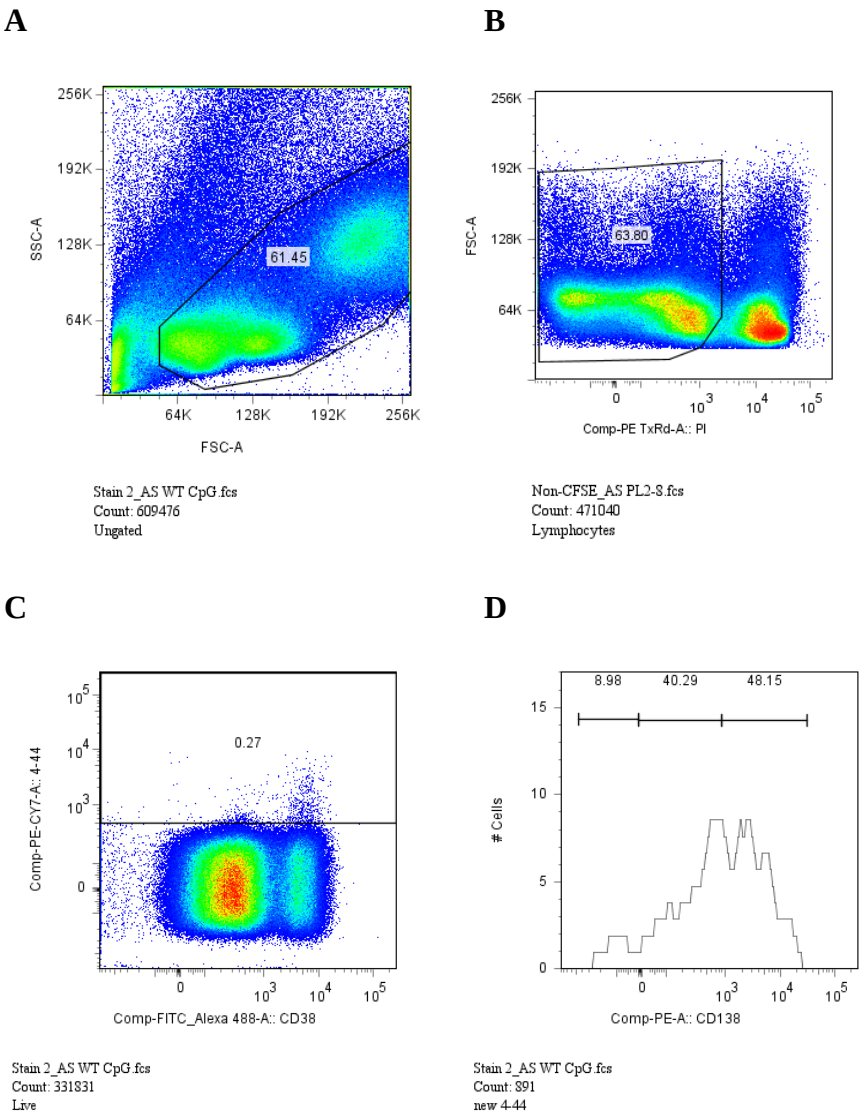
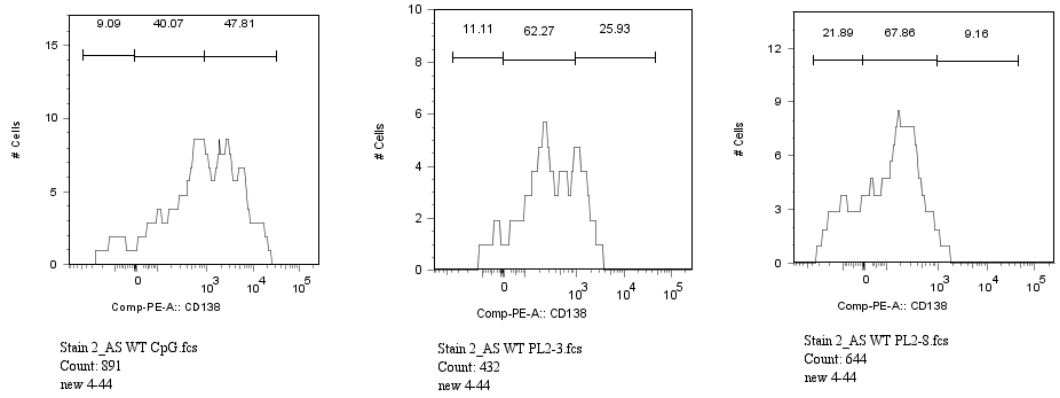


Figure 7. Gates used to select 4-44+ B cells expressing cell surface markers of interest. FACS plots of A) Lymphocyte gate in cultures treated with CpG and anti-IgM F(ab')$_2$ from AM14 WT mice, B) live cell gate in cultures treated with PL2-8 hybridoma from AM14 TLR7$^{-/-}$ mice and C) 4-44+ B cells in cultures treated with CpG and anti-IgM F(ab')$_2$ from AM14 WT mice. D) Histogram of CD138 expression on 4-44+ B cells. The three FACS plots and the histogram are representative of two experiments.

CD138 is a plasmablast marker closely linked with the antibody secreting ability of this cell type (Sanderson et al., 1989; Lalor et al., 1992; Smith et al., 1996). On the basis of levels of expression of CD138, 4-44+ B cells expressing CD138 were divided into high, medium and low groups. 4-44+ B cells from AM14 WT mice predominantly expressed medium levels of CD138 in cultures treated with PL2-3 hybridoma and PL2-8 hybridoma (Figure 8A). In cultures treated with CpG and anti-IgM $F(ab')_2$ however, the proportion of cells expressing medium and high levels of CD138 ($CD138^{high}$) was similar with a slight increase in the high levels. Cultures treated with CpG anti-IgM $F(ab')_2$ had the most 4-44+ B cells expressing high levels of CD138 followed by cultures treated with PL2-3 and PL2-8 hybridomas.

AM14 $TLR7^{-/-}$ mice mostly expressed low levels of CD138 followed by medium and high levels, in all three cultures (Figure 8B). The proportion of 4-44+ B cells expressing high levels of CD138 was similarly low in all three cultures. A comparison of cultures treated with PL2-3 hybridoma from AM14 WT and AM14 $TLR7^{-/-}$ mice revealed that WT mice had a larger percentage of 4-44+ B cells expressing high levels of CD138 than $TLR7^{-/-}$ mice (Figure 9). This pattern was also observed in cultures treated with CpG and anti-IgM $F(ab')_2$ from the two types of mice however, in cultures treated with PL2-8 hybridoma the proportion of 4-44+ B cells expressing high levels of CD138 was very similar in the two types of mice. These data imply a requirement for TLR7 in the up-regulation of CD138.

A



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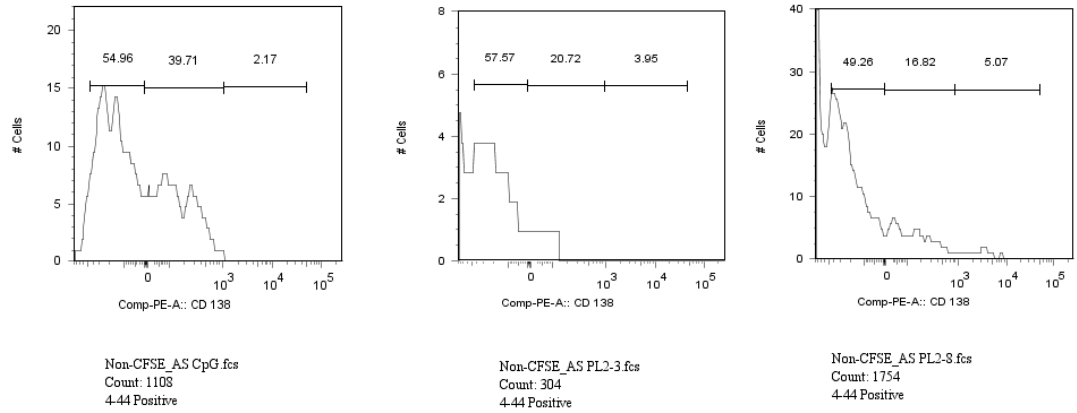

Figure 8. 4-44+ B cells up-regulate CD138 expression in cultures treated with CpG and anti-IgM F(ab')$_2$ and PL2-3 hybridoma. Histograms of CD138 expression on 4-44+ B cells in cultures treated with CpG and anti-IgM F(ab')$_2$, PL2-3 and PL2-8 hybridomas in A) AM14 WT mice and in B) AM14 TLR7$^{-/-}$ mice. Both histograms are representative of two experiments.

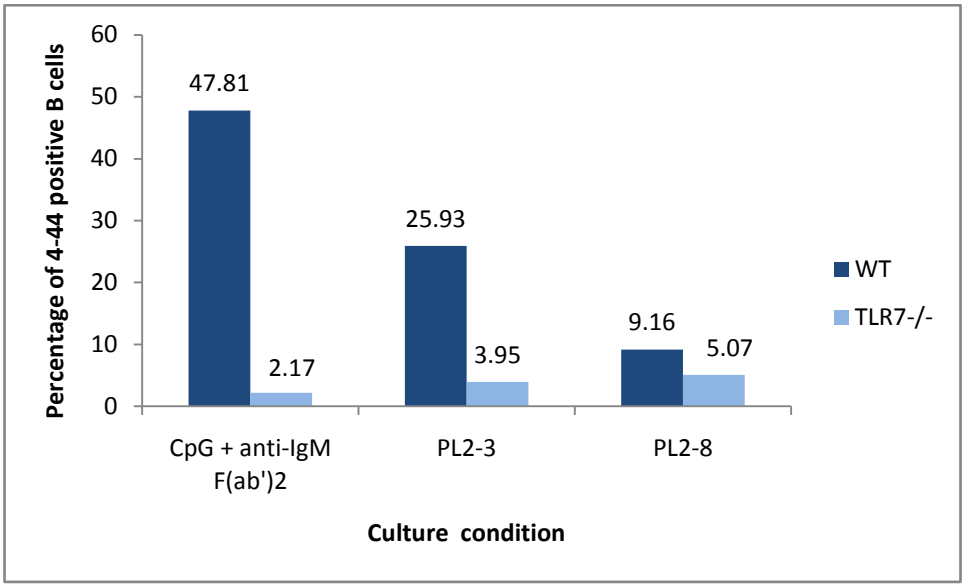

Figure 9. Cells expressing high levels of CD138. 4-44+ B cells expressing high levels of CD138 in cultures treated with CpG and anti-IgM F(ab')$_2$, PL2-3 and PL2-8 hybridoma of AM14 WT and AM14 TLR TLR7$^{-/-}$ mice (from Figure 8).

For the other surface markers either the trend could not be reproduced (as in the case of CD38, data not shown) or was unaltered between different groups and mice (as in the case of CD86). CD86, a co-stimulatory molecule expressed by antigen encountered B cells, binds the receptor CD28 on the T cells in order to activate this cell type (Janeway, 2005). On the basis of levels of expression of CD86, 4-44+ B cells were divided into high and low groups (Figure 10). Overall, there were more 4-44+ B cells expressing high levels of CD86 than low levels in all three culture conditions for AM14 WT and TLR7$^{-/-}$ mice (Figure 10). In addition, the proportion of 4-44+ B cells expressing high levels of CD86 was highest in cultures treated with CpG and anti-IgM F(ab')$_2$ followed by PL2-3 and

PL2-8 hybridoma. This trend makes some sense, as CpG is the most stimulatory and PL2-8 hybridoma the least, but the expression seems unaffected by the presence or absence of TLR7.

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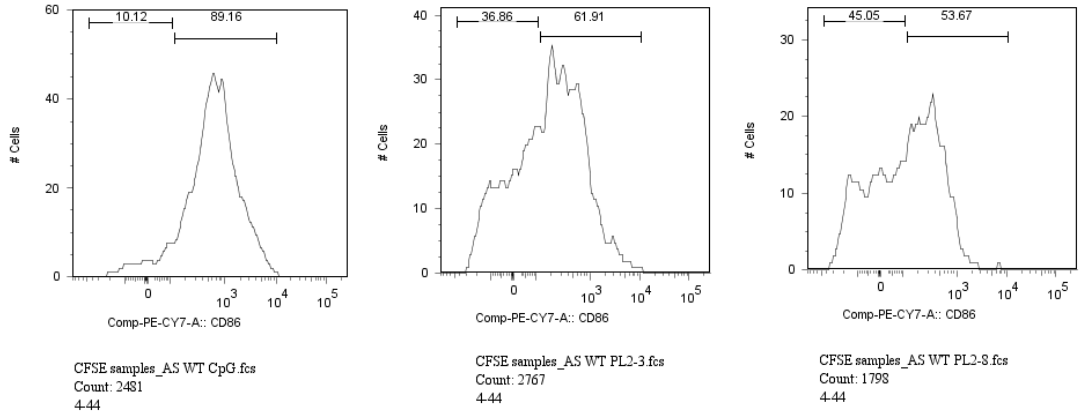

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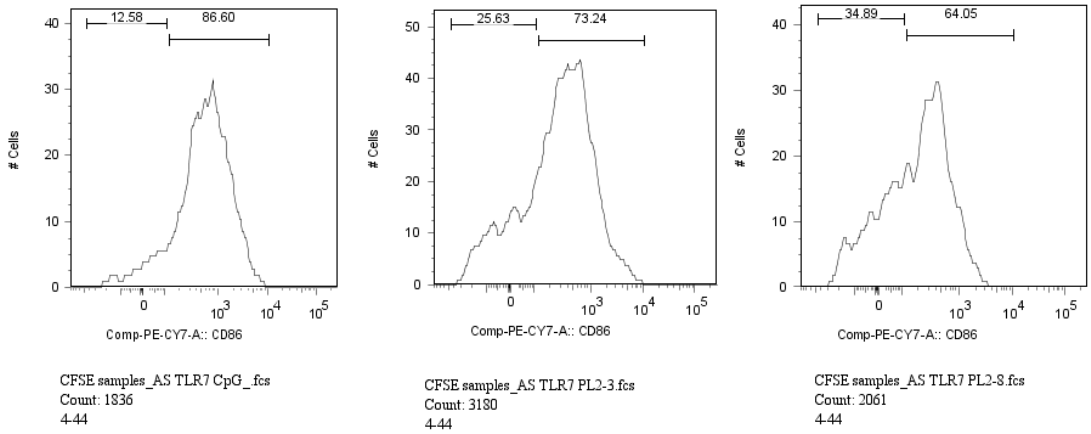

Figure 10. 4-44+ B cell expression of CD86 was unaltered in the three cultures. Histograms showing CD86 expression on 4-44+ B cells in cultures treated with CpG and anti-IgM $F(ab')_2$, PL2-3 and PL2-8 hybridomas of A) AM14 WT and B) AM14 $TLR7^{-/-}$ mice. Both histograms are representative of two experiments.

Similarly, differences in the surface marker CD22 was not observed. CD22 is an inhibitory BCR co-receptor that negatively regulates signaling through the BCR thus, CD22 is down regulated in activated B cells (reviewed in Nitschke, 2005). On the basis of levels of expression there were two populations of CD22 expressing AM14 B cells: high and low. Most of the AM14 B cells were $CD22^{high}$ in all three cultures from AM14 WT and AM14 $TLR7^{-/-}$ mice (data not shown).

Technical problems prevented learning more about the role of TLR7 in other surface marker expression. For example, All 4-44+ B cells in all conditions and all mice expressed extremely high levels of CD44 (data not shown). Since CD44 is up-regulated on activated B cells compared to naïve B cells (Camp et al., 1991), populations of $CD44^{high}$ and $CD44^{low}$ cells in various cultures were expected, but every condition lacked $CD44^{low}$ cells, which suggested the need for a different anti-CD44 antibody or the use of the antibody at a much lower titer.

Proliferation of 4-44+ B cells was also examined in AM14 WT and AM14 $TLR7^{-/-}$ mice using CFSE. CFSE is a flurorescent dye that enters live cells and then separates equally between daughter cells thus, reducing in fluorescence intensity after each cell division (Lyons et al., 1994). As expected, the proportion of 4-44+ B cells was higher in cultures treated with PL2-3 hybridoma than with PL2-8 hybridoma of AM14 WT (Figure 11A and B) and AM14 $TLR7^{-/-}$ mice (Figure 11C and D). Of the 4-44+ B cells, the majority had divided at least once

with very few that had not divided or had divided multiple times. It has previously been reported that dividing or activated cells decrease their BCR surface expression (Janeway et al., 2005). This phenomenon was also observed, as indicated by the triangular pattern inserted on the figure showing decreased 4-44 expression on dividing B cells (Figure 11C). A comparison in the proliferation of 4-44+ B cells between AM14 WT and TLR7$^{-/-}$ mice did not reveal any differences.

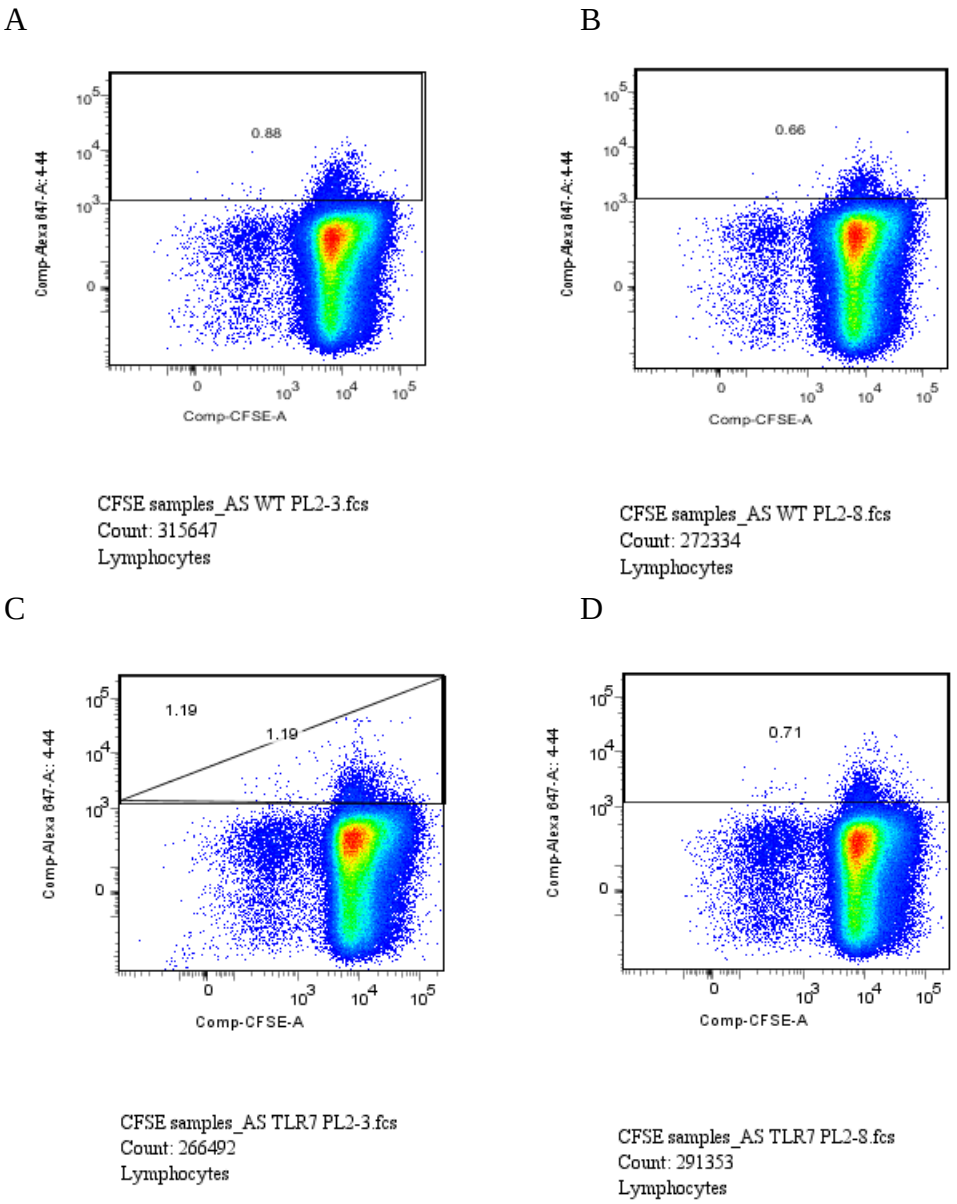

Figure 11. CFSE dye shows proliferation of 4-44+ B cells. FACS plots of 4-44+ B cells from cultures treated with A) PL2-3 and B) PL2-8 hybridomas of AM14 WT mice and C) PL2-3 and D) PL2-8 hybridomas of AM14 TLR7$^{-/-}$ mice. C) CFSE dye shows proliferation of 4-44+ B cells. All four FACS plots are representative of two experiments.

ELISA Assay

FACS analysis allowed us to look at the activation of B cells by examining their membrane-bound surface proteins. An additional way to measure activation of B cells is to examine what cytokines they secrete into the extracellular space. The quantity of cytokines in the sample can be determined by the Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is a technique used to quantify the amount of antigen (including cytokines) or antibody in a given sample (Janeway et al., 2005). The antigen is fixed on a surface and an enzyme linked antibody is added to secure the antigen. The enzyme is activated by the addition of a fluorigenic substrate and the resulting fluorescence is used to determine the amount of antigen/antibody complexes in the sample. In order to quantify the presence of secreted molecules such as cytokines, two antibodies for the cytokine are used to secure the molecule (Janeway, 2005).

ELISA assays were used to detect the presence of cytokines such as IL-10, TNF-α and IFN-γ in CpG and anti-IgM $F(ab')_2$, PL2-3 hybridoma and PL2-8 hybridoma treated culture supernatants of AM14 WT and $TLR7^{-/-}$ mice. IL-10 is an anti-inflammatory cytokine that induces B cell activation and differentiation while suppressing macrophage and T cell function (Janeway et al., 2005; De Wall et al., 1993; Rousset et al., 1992). TNF-α is a pro-inflammatory cytokine secreted by macrophages to render vascular epithelium permeable to leukocytes (Janeway et al., 2005). Cytotoxic T cells and T_H1 cells also secrete IFN-γ to induce

macrophages to destroy engulfed pathogens (Janeway et al., 2005). ELISA assays for TNF-α and IFN-γ did not yield any results. Detectable levels of IL-10 were not present in the supernatant of cultures treated with PL2-3 and PL2-8 hybridomas of AM14 WT and TL7$^{-/-}$ mice. However, in cultures treated with CpG and anti-IgM F(ab')$_2$, the concentration of IL-10 was detected and found to be similar in the two mice (Figure 12).

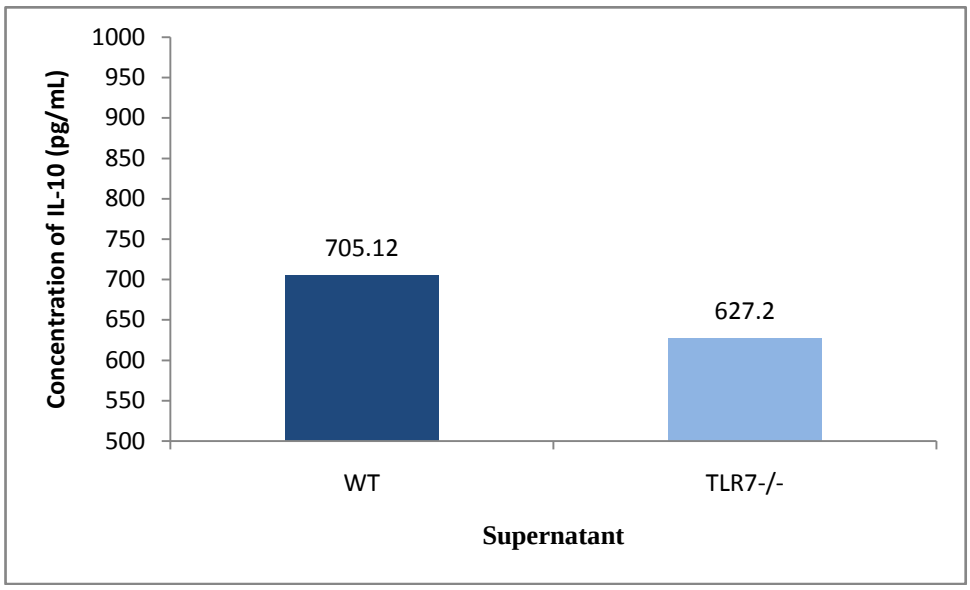

Figure 12. Differential secretion of IL-10 in AM14 WT and TLR7$^{-/-}$ mice was not observed in cultures treated with CpG and anti-IgM F(ab')$_2$. The concentration of IL-10 (pg/mL) present in the supernatant of cultures treated with CpG and anti-IgM F(ab')$_2$ of AM14 WT and TLR7$^{-/-}$ mice.

In vivo Experiment

FACS Analysis

Since results from the *in vitro* experiment were rather inconclusive, we were interested to see whether stimulation of RF+ B cells with the PL2-3 hybridoma *in vivo* and for a longer period would replicate the results from the *in vitro* experiments or provide more distinct trends. AM14 WT mice were subjected to four different conditions. As a positive control, mice were injected with the PL2-3 hybridoma (n=3). As an experimental condition, mice were injected with PL2-3 and the TLR7 inhibitor (IRS661) (n=2). The TLR7 inhibitor was used to suppress signaling through TLR7 thus, mimicking AM14 TLR7$^{-/-}$ mice. And as negative controls, one mouse each was injected with IRS661 only and PBS only. Mice were injected on alternate days with PL2-3 and IRS661 starting with the inhibitor for a total of three days per reagent. The mice were sacrificed on the seventh day. The PBS only mouse was injected on each day.

As differences in expression of most cells surface markers between AM14 WT and AM14 TLR7$^{-/-}$ mice were not observed in the *in vitro* experiments, only the expression of CD22 and CD138 were assessed in 4-44+ B cells from the four conditions. While there were no differences in CD138 expression (data not shown), an interesting observation for CD22 expression was made. On the basis of levels of CD22 expression, B cells were divided into low and high populations (Figure 13). The proportion of low and high CD22 expressing 4-44+ B cells was the lowest for the mouse injected with PBS only (Figure 13B) with a slight

increase for the mouse injected with IRS661 only (Figure 13A). For mice injected with PL2-3 and PL2-3 and IRS661, the average proportion of cells expressing high and low levels of CD22 was similar (Figure14). While there was little variability in the expression of CD22 in the mice injected with PL2-3, the mice injected with PL2-3 and IRS661 differed considerably (data not shown). One mouse injected with PL2-3 and IRS661 supported the results from *in vitro* experiments. When comparing this one mouse from the PL2-3 and IRS661 injected group (Figure 13D) with one from PL2-3 injected group (Figure 13C) we see that while the proportion of 4-44+ B cells expressing low levels of CD22 was similar in the two conditions, a larger proportion of mice injected with PL2-3 and the inhibitor expressed high levels of CD22 (Figure 14). The implications of this will be examined in the discussion.

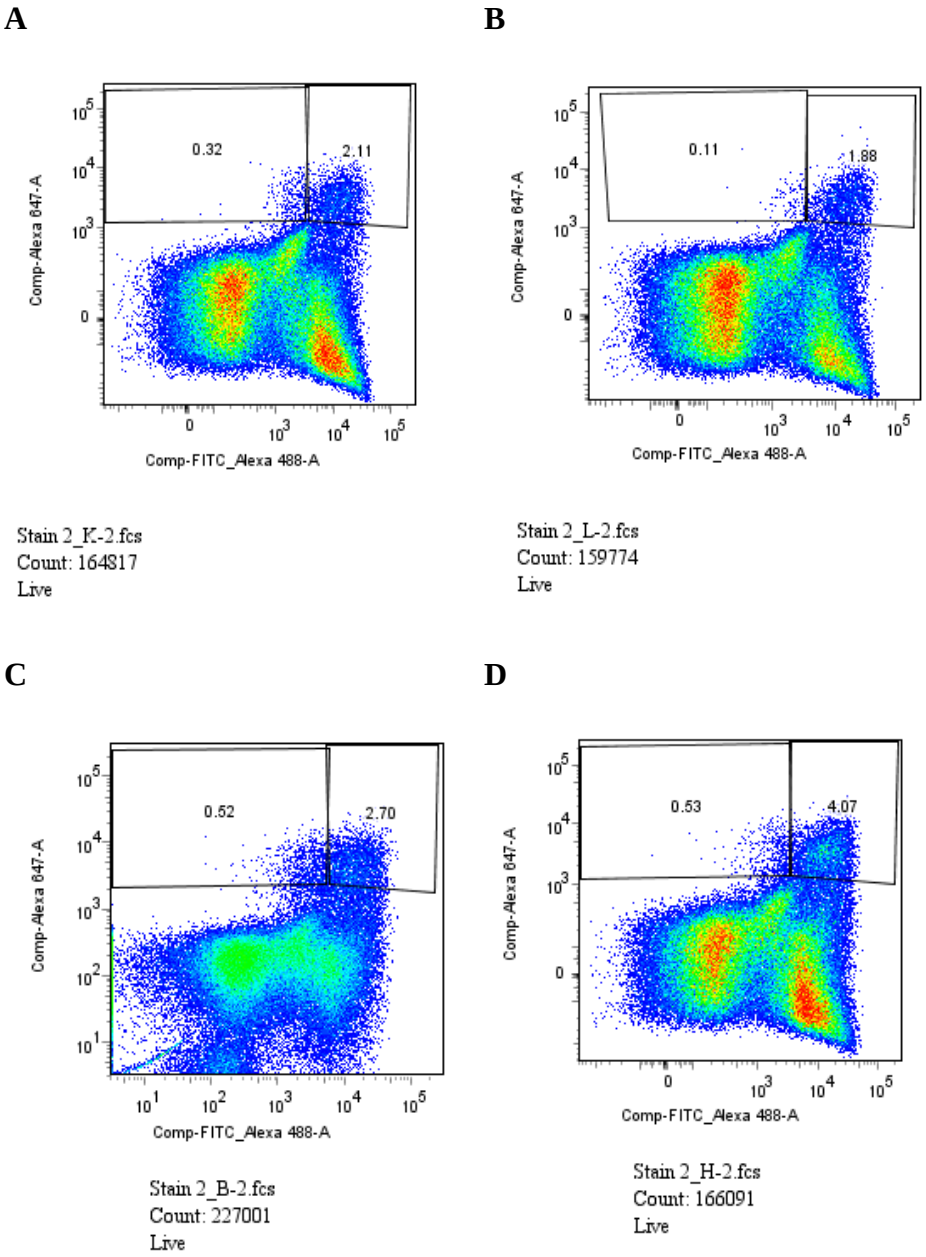

Figure 13. TLR7 deficiency increases the population of 4-44+ B cells expressing high levels of CD22. FACS plots of 4-44+ B cells expressing CD22 in mice injected with A) the TLR7 inhibitor (IRS661) only, B) PBS only, C) PL2-3 hybridoma only and D) PL2-3 and the TLR7 inhibitor. Each plot is representative of one mouse.

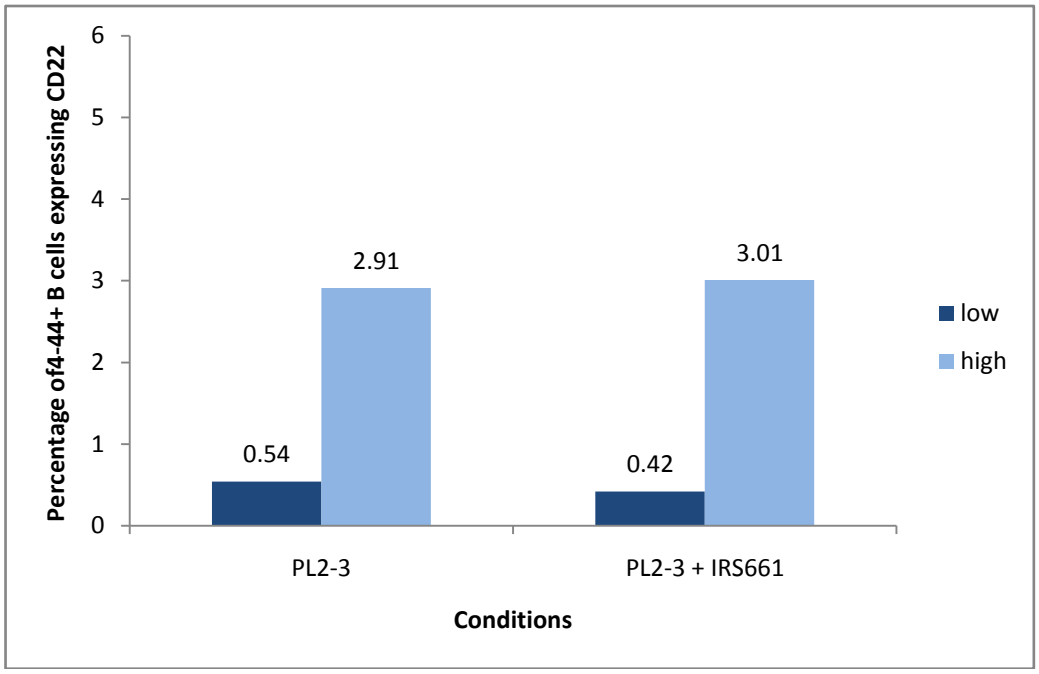

Figure 14. 4-44+ B cells expressing high and low levels of CD22. Bar graph of the average percentage of 4-44+ B cells expressing high and low levels of CD22 from mice injected with PL2-3 hybridoma only and PL2-3 and the TLR7 inhibitor.

ELISPOT Assay

An ELISPOT assay was conducted to detect the number of 4-44+ antibody producing B cells or activated RF+ B cells. ELISPOT (Enzyme-linked Immunosorbent Spot) is a technique similar to ELISA (Janeway, 2005). In an ELISPOT assay, antibodies specific for the antigen (including cytokines and antibody) of interest is attached to a surface, cells which may be secreting the antigen are added and plates are incubated for hours in a CO_2 incubator. Secreted antigens or antibodies become bound to the plate at the precise site of where a

secreting cell was located. The plates are then washed and an enzyme linked antibody is then used to bind the secreted antigen of interest (called a capture antibody). The enzyme is activated by the addition of a substrate and the antigen/antibody complex is developed to present colored spots on the plate where antigen- or antibody-secreting cells were present. Therefore, each spot represents one cell that secreted the captured antigen of interest (Janeway, 2005).

As expected, the PBS injected mouse did not produce any 4-44+ antibody secreting B cells but, the IRS661 injected mouse produced a low number of 4-44+ antibody secreting B cells. Compared to mice injected with PBS and IRS661 only, mice injected with the PL2-3 hybridoma and PL2-3 and IRS661 produced more 4-44+ antibody secreting B cells (Table 3). However, it was interesting to find that the proportion of 4-44+ antibody secreting B cells in mice injected with the PL2-3 hybridoma and PL2-3 and IRS661 was similar (Figure 15).

Table 3. Numbers of 4-44+ antibody secreting B cells per million cells in mice injected with PL2-3 hybridoma only, PL2-3 and IRS661, IRS661 only and PBS only.

PL2-3	PL2-3 + IRS661	IRS661	PBS
318	1134	34	0
828	1134	-	-
1458	-	-	-

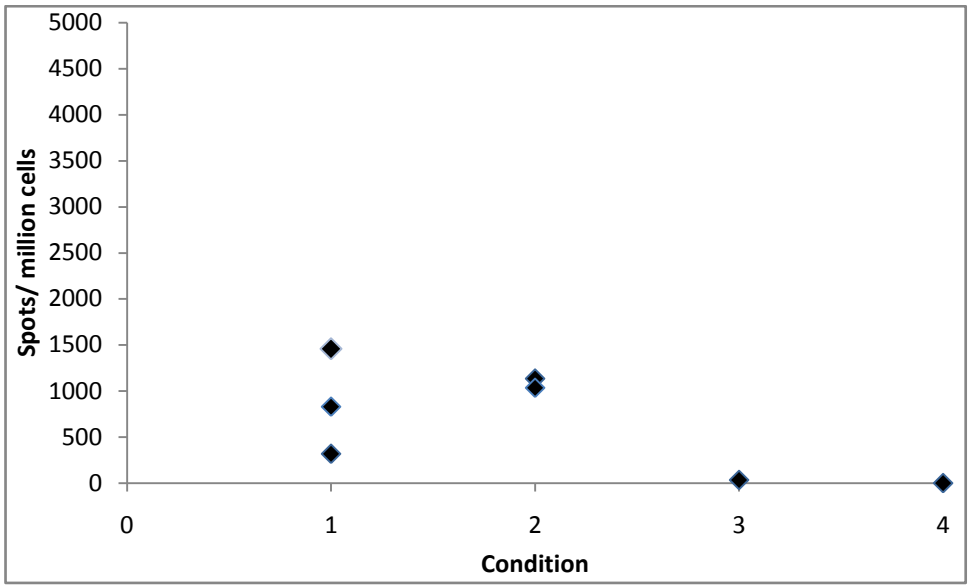

Figure 15. AM14 WT mice injected with PL2-3 hybridoma and PL2-3 and IRS661 induced the same number of 4-44+ antibody secreting B cells. Numbers of 4-44+ antibdoy secreting B cells per million cells, in mice injected with 1) PL2-3 hybridoma (n=3), 2) PL2-3 hybridoma and the TLR7 inhibitor, IRS661 (n=2), 3) IRS661 only (n=1) and 4) PBS only (n=1).

DISCUSSION

Importance of TLR7 in Activating Auto-reactive B cells

In SLE, T-independent activation of auto-reactive B cells through TLR7 and TLR9 signaling has been established (Herlands et al., manuscript submitted for publication). Furthermore, the inflammatory role of TLR7 has also been demonstrated by the fact that the absence of this innate immune receptor results in less severe disease of common target organs in autoimmune prone mice (Christensen et al., 2006). However, the specific pathway by which TLR7 influences the disease progression in SLE has yet to be explored, particularly at early time stages. In one previous study, plasmablasts from AM14 TLR7 deficient mice had an increased expression of CD45 (Herlands et al., 2008, unpublished data). CD45 is a B cell marker down-regulated on differentiated plasma cells and low on extrafollicular plasmablasts (Ardvin et al., 1999; Shapiro-Shelef et al., 2003; William et al., 2005b). Since extrafollicular plasmablasts are central to an autoimmune response during SLE in mice, this finding suggests that TLR7 plays a role in the initial activation events of auto-reactive B cells.

In vitro Experiments Confirm Importance and Enhance Understanding of the Role of TLR7

The well characterized AM14 heavy chain system was used to explore expression of differentiation markers by activated RF+ B cells in an attempt to elucidate TLR7 activation pathway. While trends in the expression of CD22, CD44, CD38 and CD86 could not be reproduced in the *in vitro* experiments an interesting observation was made in regards to the expression of CD138. CD138 is a marker for plasma cells, in particular the antibody secreting ability of this cell type (Sanderson et al., 1989; Lalor et al., 1992). CD138 expression was shown to be up-regulated on extrafollicular 4-44^{high} CD22^{low} plasmablasts in spontaneous SLE on AM14 Tg MRL/lpr mice (Williams et al., 2005). Elevated CD138 expression is also seen in AM14 Tg MRL/lpr mice treated with the PL2-3 hybridoma, but this was only examined after 7 days of treatment (Herlands et al., 2007).

The *in vitro* results show that RF+ B cells express low, medium and high levels of CD138. In AM14 WT mice, a larger proportion of RF+ B cells were CD138^{high} when stimulated by CpG and anti-IgM F(ab')$_2$ or the PL2-3 hybridoma. In TLR7 deficient mice however, the proportion of CD138^{high} cells from cultures treated with CpG and anti-IgM F(ab')$_2$ and the PL2-3 hybridoma were similar to that from cultures treated with the PL2-8 hybridoma. This important result suggests that TLR7 signaling induces activated B cells to differentiate into CD138

expressing auto-antibody secreting plasmablasts, something previously unknown and with important implications.

The fact that TLR7 plays a role in the expression of CD138 fits the previously reported finding that TLR7 deficiency results in a reduced form of disease of common target organs (Christensen et al., 2006). From our examination of CD138 expression, we conclude that absence of TLR7 restricts activated B cells from differentiating into auto-antibody producing plasmablasts as early as 48 hours post activation. We hypothesize that this hold in differentiation, over time, would lead to a reduction in the production of auto-antibodies and would decrease their accumulation in and damage of organ tissues. This hypothesis is supported by results from a one week long study, where it was shown that while AM14 TLR7 knockout mice treated with the PL2-3 hybridoma had similar levels of 4-44+ $CD22^{low}$ cells as AM14 WT mice, the number of auto-antibody secreting B cells was reduced in comparison to WT mice (Herlands et al., manuscript submitted for publication). Unfortunately, CD138 specifically was not examined in these experiments, though we hypothesize that there would be a lack of $4\text{-}44^{high}$ $CD138^{high}$ cells in AM14 TLR7-/- mice treated with the PL2-3 hybridoma. From our data in combination with these results, we may further infer that TLR7 is involved in generating functional auto-antibody secreting plasmablasts by inducing the up-regulation of CD138.

Problems with the *In Vitro* System

Despite our ability to successfully interpret some data regarding CD138 and the role of TLR7 in plasmablast differentiation, the *in vitro* system was far from perfect and much of our data was difficult or impossible to interpret. The major problems with the *in vitro* method were the short time-frame in which activation was investigated, the very small population of cells of interest in these cultures, and suboptimal reagents.

Since we were interested in exploring the role of TLR7 in the initial activation events of auto-reactive B cells, we cultured the cells for 48 hours only. Thus far, *in vivo* studies with the AM14 system have not been carried out this early possibly because B cells require a longer period of exposure to the activators: CpG and anti-IgM $F(ab')_2$ or the PL2-3 hybridoma. This short time-frame post-treatment could have been insufficient in activating RF+ B cells as reflected in the large population of $CD138^{low}$ cells in cultures treated with CpG and anti-IgM $F(ab')_2$ or the PL2-3 hybridoma .

The small size of the RF+ B cell population could have contributed to the difficulty in determining differential expression and replicating results of surface markers. $CD22^{high}$ cells are only known to make up 1-3% of the splenic live cell population (Williams et al., 2005). That means that there were a lot of non-relevant B cells in our cultures, and that cell surface marker analysis of these cells meant collecting a large number of events during FACS analysis. Even with these

efforts, sometimes the number of 4-44^{high} cells we had to analyze was very small and this could contribute to the variability we saw in different experimental trials and conditions. Switching to "heavy-light" mice where all the B cells are auto-reactive (all would be 4-44+) since they only express BCRs comprising the AM14 heavy chain Tg and the Vκ8 light chain Tg, may allow us to see a more demonstrated effect of the different culture conditions as there would be many more cells for analysis. This adaptation to the procedure may improve our ability to provide insight to initial activation events by increasing the RF+ B cell pool.

Some of the reagents were not ideal or did not work ideally in our experiments. In the case of the positive control, the reagent was not correct for investigating the role of TLR7 in activating auto-reactive B cells. We activated RF+ B cells by the combined binding of anti-IgM F(ab')$_2$ to the 4-44+ BCR and CpG to TLR9, creating a dual BCR-TLR activation signal. Since we induced activation through TLR9 rather than TLR7, the results for surface marker expression in the CpG and anti-IgM F(ab')$_2$ culture was not determined by TLR7. Using a TLR7 agonist, imiquimod for example, rather than CpG would provide a positive control specific for the role of TLR7 in the expression of the surface markers of interest.

An additional reagent problem was with our negative control, PL2-8. The PL2-8 hybridoma is a type of IgG2b, which does not bind the AM14 BCR thus, cannot activate RF+ B cells. However, in most instances, results from cultures of

AM14 WT mice treated with the PL2-3 hybridoma and PL2-8 hybridoma looked very similar which suggested that the stimulatory effect of PL2-3 hybridoma was not as strong as it should have been or that the PL2-8 hybridoma were stimulatory when they should not have been, perhaps due to some kind of contamination. An example of this from our experiments is in the case of CD86. PL2-3 and PL2-8 cultures from AM14 WT mice were found to be $CD86^{high}$ (Figure 10), which is unusual since CD86 is a co-stimulatory molecule up-regulated on activated B cells.

The PL2-3 and PL2-8 hybridomas could have been contaminated as they were obtained from ascites, fluid of the peritoneal cavity. B cells producing PL2-3 and PL2-8 antibody were originally captured from the spleen of an MRL/lpr mouse (Losman et al., 1993). These B cells were fused with myelomas or cancer cells thus producing hybrid cells also known as hybridomas (Losman et al., 1993). These hybridomas were intraperitoneally injected in RAG-/- mice for amplification of the antibodies and the ascites was used to extract the antibodies which are also referred to as hybridomas (Losman et al., 1993). In our experiments, filtered PL2-3 and PL2-8 hybridomas from ascites were used but these may still be contaminated. Hybridomas rather than purified proteins are a quicker and cost-effective way of producing antibodies in large quantities thus, hybridomas were used our experiments. However, it may be worth purifying antibodies from this ascites fluid to eliminate the possibility of contamination in the future.

Lastly, we had some difficulties successfully examining all the surface markers we wanted to due to problems with the LSRII machine we used for FACS analysis at University of Massachusetts at Amherst. Due to the configuration of the LSRII filters, some of the antibodies for the surface markers of interest were not effective. During one experiment, detection of anti-CD22 antibody coincided with that of the 4-44 anti-idiotype antibody, a phenomenon called "spectral overlap" because the detectors of the LSRII could not distinguish signals that were supposed to be coming from two very different fluorophores. This overlap prevented the analysis of a very important marker and rendered the experiment uninterpretable. For further experiments, a more thorough inspection of the LRSII capabilities should be done and trial runs with various antibody combinations should be attempted prior to analyzing important experimental trials. Antibodies with fluorophores that do not restrict the detection of other useful markers should be used in determining whether TLR7 plays a role in the expression of those markers.

Suggestions for Future *In Vitro* Studies

A number of changes to the *in vitro* studies could improve the chance of obtaining reliable results to elucidate the role of TLR7 in the initial activation events of RF+ B cells. The culture conditions could be optimized by using the "heavy-light" strain, which would increase the RF+ B cell pool and examining

cells after a longer time-frame period post-treatment, which would activate a larger number of auto-reactive B cells thus including more cells of interest in our analysis. The reagents should also be optimized, such as using imiquimod as the positive control for stimulating TLR7 signaling and increasing the dosage of the PL2-3 and PL2-8 hybridomas as they may be contaminated or ineffective in the currently used concentrations. Since i*n vivo* studies have shown that TLR7 plays a role in the secretion of the cytokines IL-6 and IL-12, it would be interesting to see whether this result could be replicated *in vitro*.

In vivo Pilot Experiment using TLR7 Inhibitor IRS661 was Inconclusive

The *in vivo* study was an attempt to replicate observations from the *in vitro* studies. Since 48 hours did not appear sufficient to present any discernable differences, we predicted that a week long exposure would provide sufficient time to stimulate RF+ B cells. As we received a lot of variability in surface marker expression in the *in vitro* studies we only assessed the expression of two surface markers, CD22 and CD138 in the *in vivo* study. Mice were injected with PL2-3 hybridoma only, PL2-3 and the TLR7 inhibitor (IRS661), IRS661 only or PBS only. The purpose of IRS661 was to see whether this inhibitor would restrict signaling through TLR7, thereby resulting in less RF+ B cell activation and antibody secretion.

We ran into technical difficulties in determining CD138 expression due to the incompatibility of the anti-CD138 antibody with the LSRII. The other marker of activation, CD22, yielded complex results with data from only one mouse injected with PL2-3 hybridoma and one injected with PL2-3 and IRS661 fitting results from *in vitro* experiments. We expected the population of $CD22^{high}$ and $CD22^{low}$ cells from mice injected with PL2-3 and IRS661 to resemble that from the mouse injected with PBS. Instead, the proportion of $CD22^{low}$ cells in the mouse injected with PL2-3 and IRS661 was higher than that for the mouse injected with PBS but similar to the mouse injected with PL2-3 hybridoma only. However, the proportion of $CD22^{high}$ cells were higher in the mouse injected with PL2-3 and IRS661 than in that injected with PL2-3 hybridoma only. Since $CD22^{high}$ cells are activated RF+ B cells that differentiate into antibody secreting $CD22^{low}$ plasmablasts we may infer that TLR7 deficiency prevents activated RF+ B cells from fully differentiating into antibody secreting $CD22^{low}$ plasmablasts. However, this experiment needs to be repeated for consistency in the results.

The ELISpot assay was conducted to determine the quantity of 4-44+ antibody secreting B cells in mice subjected to the four conditions. As expected the mouse injected with IRS661 only showed negligible 4-44+ antibody secreting B cells and that injected with PBS only did not show any presence of auto-reactive B cells. Mice injected with the PL2-3 hybridoma and those injected with PL2-3 and IRS661 showed similar counts of 4-44+ AFCs (antibody forming cells) and these were dramatically higher than those of the control mouse injected with

IRS661 only (Figure 7). This result suggests that the PL2-3 hybridoma activated RF+ B cells however, IRS661 was not successful at inhibiting signaling through TLR7. A much less likely conclusion would be that TLR7 inhibition does not affect 4-44+ AFC formation, but this is contrary to previously studied reports in TLR7-/- mice.

Future Directions for *In Vivo* Studies

One major concern in the *in vivo* pilot was whether or not the IRS 661 actually inhibited TLR7, since this had not been tested *in vivo* in the manner in which we used IRS661. The concentration of the IRS661 inhibitor may not have been sufficient to prevent signaling through TLR7, or may not have been enough to reach the AM14 B cells in the spleen (dendritic cells and macrophages could take up a large portion of what is injected). A study to determine the most effective dose of IRS661 would be helpful and required for future studies with this reagent.

A major improvement would be to extend the study. In one previous study, mice injected with the inhibitor and then stimulated with a TLR7 agonist, imiquimod, for twelve weeks reduced the release of cytokines such as IL-6, IL-12 and TNF-α by spleen monocytes as well as the level of autoantibodies: anti-dsDNA, IgG2a, IgG2b, and anti-Smith IgG (Pawar et al., 2007). While the concentration of the inhibitor was increased in our experiment (50ug compared to

40ug), the delivery method was different (subcutaneous versus intra peritoneal) and the exposure period at one week may not have been sufficient to activate RF+ B cells at the concentration we used (Pawar et al., 2007). It could take much longer for the TLR7 inhibitor to take effect. As brought up earlier, it is also possible that at one week, the inhibitor may have been taken up by splenic monocytes before it reached RF+ B cells. A longer period of exposure may insure inhibition of TLR7 on RF+ B cells.

Conclusions

The *in vitro* studies presented in this paper suggest that TLR7 signaling induces activated B cells to differentiate into CD138 expressing auto-antibody secreting plasmablasts. This result fits the previously reported finding that TLR7 deficiency results in a reduced form of SLE (Christensen et al., 2006). However, the study could be improved by increasing the RF+ B cell pool, the population of activated auto-reactive B cells, a TLR7 specific positive control, and optimal forms of the experimental condition (the PL2-3 hybridoma) and negative control (the PL2-8 hybridoma).

The *in vivo* pilot study, though largely inconclusive, could support the role of TLR7 in inducing activated B cells to functional antibody producing plasmablasts. However, the study needs to be improved for optimal dosage of the TLR7 inhibitor and the use of the TLR7 agonist, imiquimod, to fully elucidate the

role of TLR7 in the initial activation events of auto-reactive B cell activation in systemic lupus erythematosus.

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