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PLASMACYTOID DENDRITIC CELL-MEDIATED HUMORAL AUTOIMMUNITY

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PLASMACYTOID DENDRITIC CELL-MEDIATED HUMORAL AUTOIMMUNITY

A

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DEDICATION

To my grandmother, Elba Martinez Maldonado, who taught me to never give up.

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PLASMACYTOID DENDRITIC CELL-MEDIATED HUMORAL AUTOIMMUNITY

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Humoral autoimmunity is characterized by the breakdown of B cell immune tolerance to self-antigens and consequent production of pathogenic autoantibodies. Plasmacytoid dendritic cells (pDCs), a potent type I interferon (IFN-I) producer, have been linked to the pathogenesis of systemic lupus erythematosus (SLE), a prototypic systemic humoral autoimmune disease. However, the cellular events that stimulate the development of humoral autoimmunity as a result of pDC activation have not been characterized. Moreover, the B cell subset(s) responsible for the generation of autoantibodies remains to be clearly identified.

The immunization of DNA-containing amyloids into non-autoimmune mice triggers the activation of pDCs and induction of lupus-like disease, characterized by the production of autoantibodies. Using this lupus model that is dependent on pDC activation and IFN-I production, we delineated the B cell responses elicited during the break of tolerance and characterized the key cellular players that may influence those responses. We found that, when IgM autoantibodies were induced, germinal centers were inhibited whereas immature B cells were activated and expanded. Such interesting observation suggested that humoral autoimmunity may arise from B cells outside germinal centers. While pDCs were involved in the overt activation of immature B cells, type II interferon (IFN-II) promoted their expansion. In addition, both IFN-I and IFN-II were required for isotype-class switch of autoantibodies thereby the generation of pathogenic subtypes. We further determined that IFN-II was produced by natural killer (NK) cells, which contributed to the development of humoral autoimmunity. In contrast, NKT cells suppressed the autoimmune B cell response. Last, we demonstrated that

serum amyloid P-component, a humoral factor that binds to amyloids, prevented the activation of pDCs and IFN-I production thus may exert a protective role against humoral autoimmunity.

Our results established a functional link between IFN-I and IFN-II, where IFN-I from pDCs and IFN-II from NK cells are essential in stimulating multiple types of adaptive immune cells to coordinate the differentiation and expansion of self-reactive B cells. Selective targeting of the key cellular and molecular players may lead to innovative therapies for SLE and other autoimmune diseases.

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ABBREVIATIONS

25-OHC	7α, 25-hydroxycholesterol
ABC	Age-Associated B cell
AICD	Activation-Induced Cell Death
ANA	Anti-Nuclear Antibodies
AP-HSA	Amyloid Precursor-derived from Human Serum Albumin
Aβ	Amyloid Beta
BAFF	B Cell Activating Factor
BCL6	B cell lymphoma 6 protein
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
BST2	Bone marrow stromal cell antigen-2
CSR	Class-Switch Recombination
DC	Dendritic Cell
dsRNA	Double-stranded Ribonucleic acid
EBI-2	Epstein-Barr Virus Induced Molecule 2
EGCG	Epigallocatechin Gallate
EndoU	Endonuclease, PolyU-Specific
Eomes	Eomesdermin

GC	Germinal Center
GCSAM	Germinal Center Associated, Signaling and Motility Gene
HSA	Human Serum Albumin
IC	Immune Complex
ICOS	Inducible T Cell Co-Stimulator
IFN	Interferon
IFNAR	Type I Interferon Receptor
IFN-I	Type I Interferon
IFN-II	Type II Interferon
IKK	IκB Kinase
IRAK	IL-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
ISGF	Interferon-Stimulated Gene Factor
ISRE	Interferon-Stimulated Response Elements
Jak	Janus Family Kinase
LPS	Lipopolysaccharides
Mda-5	Melanoma Differentiation-Associated Gene 5
MHC	Major Histocompatibility Complex

Myd88	Myeloid Differentiation Primary Response Gene 88
MZ	Marginal Zone
NALP3	NOD-Like Receptor Family, Pyrin Domain Containing 3
NK	Natural Killer
NOD	Nucleotide-Binding Oligomerization
PAMP	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PC	Plasma Cell
PD-1	Programmed Cell Death 1
pDC	Plasmacytoid Dendritic Cell
PI3K	Phosphoinositide 3-kinase
PNA	Peanut Agglutinin
PRR	Pattern-Recognition Receptor
Rgs	Regulator of G-protein Signaling
RIG	Retinoic Acid Inducible Gene
SAP	Serum Amyloid-P Component
SHM	Somatic Hypermutation
Siglec-H	Sialic acid-binding immunoglobulin-type lectin-H
Slc15a4	Peptide/Histidine Transporter Solute Carrier Family 15, Member 4

SLE	Systemic Lupus Erythematosus
ssRNA	Single-stranded Ribonucleic acid
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
Tfh	T Follicular Helper
TLR	Toll-Like Receptor
TRAF	Tumor Necrosis Factor Receptor-Associated Factor
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
Tyk	Tyrosine Kinase 2

CHAPTER 1. GENERAL BACKGROUND

1.1 Humoral Autoimmunity

While protecting the host against pathogens, the immune system must also avoid a response against host cells (self). Autoimmune diseases develop when the immune system triggers an immune response against self-components, which is also referred as break of tolerance. B cells and T cells can become self-reactive and have organ-specific or systemic effects. For example, type 1 diabetes is considered an organ-specific autoimmune disease, whereas systemic lupus erythematosus (SLE) is considered a systemic disease.

The escape of self-reactive B cells from negative selection, a process that, in theory, should prevent their development, may trigger the development of antibodies that react to self-antigens. These antibodies may form immune complexes (ICs) and bind tissue self-antigens, which may lead to organ damage. This antibody-mediated autoimmune response is called humoral autoimmunity.

The innate and adaptive immune system must maintain a fine balance to react against foreign pathogens while preventing the recognition of self-proteins. A dysregulation in any arm of the immune system may trigger a cascade of events leading to the development of autoimmunity. The early events promoting an autoimmune reaction remain to be known [1]. This dissertation will focus on the identification of novel mechanisms involved during the development of humoral autoimmunity.

1.2 Systemic Lupus Erythematosus

SLE is the prototype of humoral autoimmunity characterized by the production of autoantibodies reactive to nuclear cell components. These antibodies can form ICs, composed by autoantibodies and DNA or RNA, that deposit in different tissues and trigger inflammation. SLE pathogenesis may target multiple organs including the kidney, heart, joints, skin, lungs, blood, vessels, liver and nervous system. The course of the disease is unpredictable, with

periods of more active disease, called flares, followed by remissions. Due to the complex manifestation of the disease, it can be difficult to diagnose. However, two typical organ manifestations together with the appearance of anti-nuclear antibodies (ANA) are sufficient for an SLE diagnosis [2, 3].

The etiopathogenesis of SLE is not completely understood, but is considered a combination of genetic, hormonal and environmental factors that result in the break of immune tolerance. The genetic contribution on SLE is evidenced by the finding that siblings from SLE patients are more likely to develop SLE compared with individuals without affected siblings [4]. Furthermore, single nucleotide polymorphisms in genes coding for major histocompatibility complex (MHC)-class II, Fcγ-receptors and complement factors have been associated to increased susceptibility to SLE [5]. The role of sex hormones has also been linked to SLE due to the striking incidence of SLE among women; around 90% of SLE patients are women of child-bearing age [6]. In addition, environmental factors such as increased exposure to sunlight and smoking can induce flares in SLE patients.

The appearance of autoantibodies can start years before the onset of clinical symptoms [2]. This phenomenon could facilitate the earlier diagnosis of the disease. However, the cellular mechanisms occurring at the initiation phase of the disease are not well understood. The innate immune cells, plasmacytoid dendritic cells (pDCs) are thought to play an important role at this early stage, due to their ability to produce type I interferons (IFN-I) [7, 8].

1.3 Plasmacytoid dendritic cells

Dendritic cells (DCs) are innate immune cells able to present antigens to T cells. In mice, these cells express the CD11c marker and can be subdivided in several subsets, including the pDCs. Mouse pDCs express Siglec-H and BST-2, and have the ability to produce large amounts of IFN-I upon stimulation. These rare innate immune cells preferentially express the toll-like

receptors TLR7 and TLR9 on endosomal membranes, which are activated by nucleic acids with particulate-like properties or as part of a protein complex through receptor-mediated endocytosis [9]. Signaling through these TLRs triggers the recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule. MyD88 pathway, which is used by most TLRs, except TLR3, activates a multi-protein signal complex, containing IL-1 receptor-associated kinase- (IRAK) 1 and 4, which further associates with tumor necrosis factor receptor-associated factor (TRAF)-6 and TRAF-3 and IκB kinase (IKK) -α [10]. This complex leads to the phosphorylation of interferon regulatory factor (IRF) -3, IRF7 and IRF5, which translocate to the nucleus and facilitate IFN-I gene transcription [11, 12].

The ability of pDCs to produce IFN-I enables them to modulate T cell and B cell responses, which are important during viral infection and autoimmunity (Figure 1.1) [13]. Also, pDCs can modulate immune cells independently of IFN-I. Depending on the stimuli, pDCs can also produce pro- and anti-inflammatory cytokines and chemokines, such as IL-6, IL-12, CXCL18, CXCL10, CCL3, CCL4, BAFF and APRIL, which modulate T cell, NK cell and B cell responses [13-15]. Also, they express MHC class II and co-stimulatory molecules, which make them capable of priming T cells [16, 17].

1.4 Interferons

The IFNs are a large family of cytokines that comprise three distinct classes: IFN-I, IFN-II and type III IFNs. IFN-I consist of different IFN-α subtypes, IFN-β and other novel gene products (IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ). Type II IFN (IFN-II) is composed by IFN-γ, and type III IFN consists of IFN-λ. All three classes of IFNs have the ability to interfere with viral replication [18]. However, IFN-I and IFN-II have numerous additional functions that influence innate and adaptive immune responses, making them relevant in cancer and autoimmune diseases.

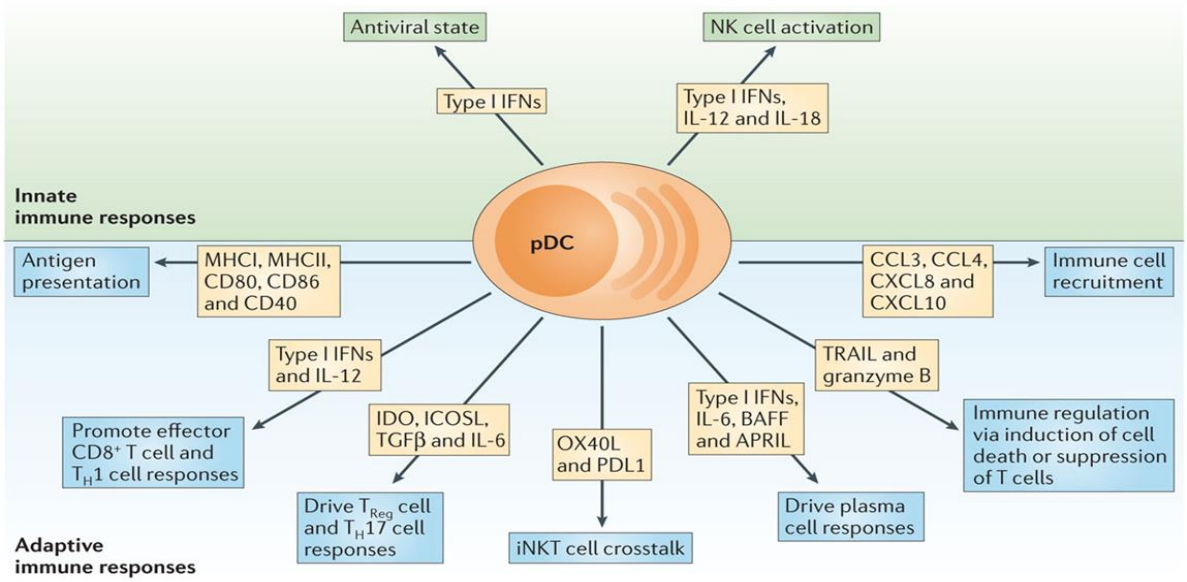

Figure 1.1. Plasmacytoid dendritic cell functions. pDCs are important drivers of both innate and adaptive immune responses. Their ability to rapidly produce type I IFNs and different cytokines enables pDCs to present antigens to T cells, promote T cell and B cell responses and induce immune cell recruitment to infection sites or to inflamed tissues. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] (Swiecki M and Colonna M., *The multifaceted biology of plasmacytoid dendritic cells*. Nat Rev Immunol, 2015. 15(8):874-7) doi:10.1038/nri3865. Copyright Clearance Center.

1.4.1 Type I Interferons

Almost all cells can produce both IFN-α and IFN-β after stimulation of pattern recognition receptors (PRRs) by microbial products or self-nucleic acids. Among the PRRs, RIG-I, the melanoma differentiation-associated gene 5 (Mda-5) (from the RIG-I-like proteins), NOD2, TLR3, TLR4, TLR7, TLR8 and TLR9 are involved in the induction of IFN-I as well as the induction of many other inflammatory cytokines (Figure 1.2) [19, 20]. In the cytosol, RIG-I and Mda-5 recognize double-stranded RNA (dsRNA), whereas NOD2 recognizes ssRNA. In endosomal compartments TLR3, TLR7 and TLR8, and TLR9 respond to dsRNA, single-

stranded RNA (ssRNA) and unmethylated CpG DNA, respectively [21-23]. Also, TLR4, present in the cytosol, recognizes lipopolysaccharides (LPS) from bacteria [24]. Whereas dendritic cells (DCs), monocytes and macrophages express TLR4, TLR7 and TLR8, pDCs preferentially express TLR7 and TLR9. The differential distribution of TLRs in different immune cell types allows a robust response against a broad range of viruses and bacteria [12, 25].

After IFN-I release, these cytokines signal through the IFN-α/β receptor (IFNAR) (Figure 1.3) [26]. Binding of IFN-I to their receptor leads to the dimerization of the two receptor subunits, IFNAR1 and IFNAR2. This triggers the activation of the Janus family kinases 1 (JAK1) and tyrosine kinase 2 (Tyk2). These activated kinases recruit and phosphorylate the transcription factors signal transducer and activator of transcription (STAT) 1 and STAT2, which associate with IRF9 forming the IFN stimulated gene-factor 3 (ISGF3) complex. The complex translocates to the cell nucleus and binds to IFN-stimulated response elements (ISRE) and activates the transcription of hundreds of interferon-stimulated genes (ISGs) [18].

IFN-I are able to modulate innate and adaptive immune responses. For example, IFN-I regulate cell migration, NK cell cytotoxicity, antigen presentation by DCs, and enhance antibody production and survival of B cells [12]. Because of the wide range of effects on different cell types, IFN-I are agents of treatment in different diseases. Due to their anti-viral capacities, IFN-I are used as treatment for hepatitis B and C [27]. Additionally, IFN-I have been used as a treatment for melanoma, leukemia and Kaposi's sarcoma [28, 29]. However, this treatment increased the susceptibility to develop autoimmune diseases [30, 31]. Also, IFN-I has been linked to the development of autoimmune diseases, which will be a major topic of this dissertation.

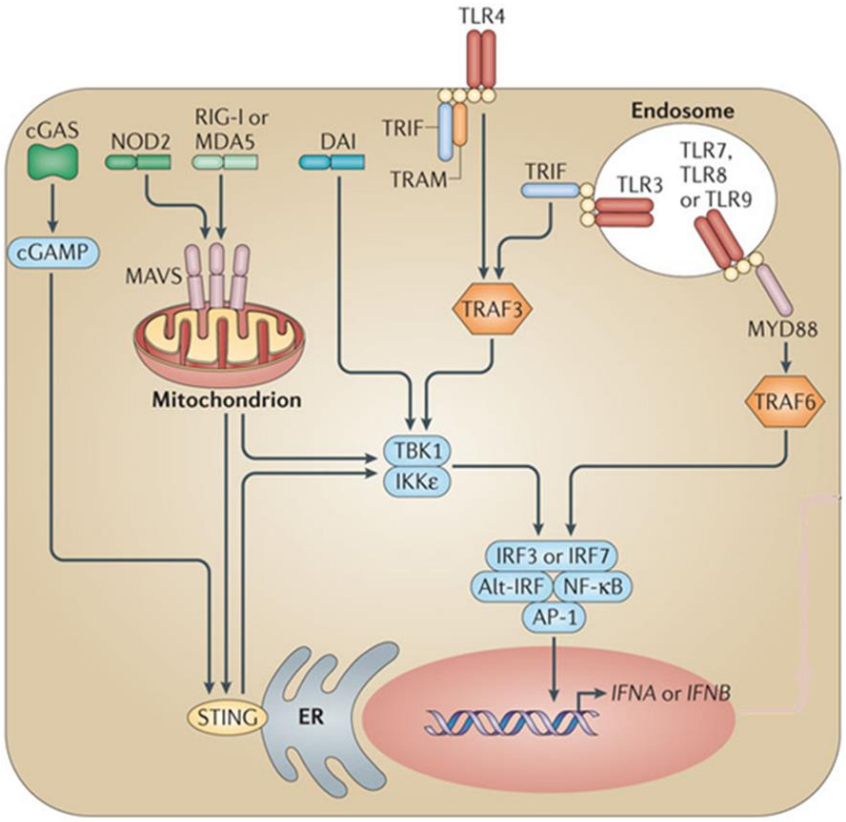

Figure 1.2. Pathways of type I IFN induction. Microbial products can be recognized by cell-surface and intracellular pattern recognition receptors, including Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I), to trigger the induction of type I interferons (IFNs) by several distinct signaling pathways. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] (McNab F, et al., *Type I interferons in infectious disease*. Nat Rev Immunol, 2015. 15(2):87-103). Copyright Clearance Center.

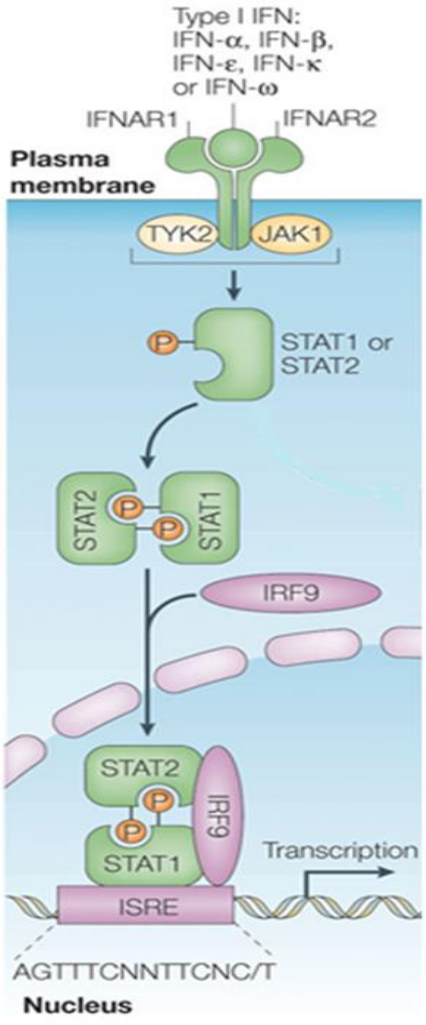

Figure 1.3. Type I IFN receptor signaling. Binding of type I IFNs to the IFNAR triggers a signaling cascade to activate the IFN-response elements which transcribe hundreds of ISGs. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] (Platanias LC., *Mechanisms of type-I- and type-II-interferon-mediated signaling*. Nat Rev Immunol, 2005. 5(5):375-86). Copyright Clearance Center.

1.4.2 Type I Interferon in systemic lupus erythematosus

In SLE and other autoimmune diseases, the activation of the IFN-I system is observed during disease. PBMCs from SLE patients show a notable upregulation of interferon-stimulated

genes (ISGs; referred as IFN-signature) suggesting the stimulation of cells by IFN-I [32]. Furthermore, a proportion of SLE patients have elevation of IFN-α in the serum and existence of IFN-α-producing cells in affected tissues [33-35]. The increased levels of IFN-α in the serum and type I IFN signature are associated with a more severe disease [36]. Additionally, IFN-I present in SLE sera can induce the *in vitro* differentiation of monocytes to dendritic cells, which can promote T cell proliferation [37]. Another mechanism by which IFN-I may be involved in SLE development is through the induction of BAFF by myeloid cells, which can further enhance the survival of B cells [38, 39]. Altogether, IFN-I may play a pathogenic role in SLE patients.

Studies in both humans and mice provide evidence that IFN-I contribute to the development of SLE. As mentioned, case reports revealed that some patients under IFN-I treatment for malignancies or chronic viral infections exhibit features of SLE including the development of anti-nuclear antibodies [27-30]. In addition, exogenous IFN-α exacerbates disease on autoimmune-prone mice, whereas IFNAR deficiency protects them from developing autoantibodies. Most of these autoimmune-prone mice do not show an IFN signature [38, 40, 41].

It has been proposed that the activation of pDCs is through nucleic acid-containing ICs. [7, 8]. These ICs can be internalized via receptor-mediated phagocytosis and activate TLR9 in pDCs to produce IFN-I. However, this event first requires the presence of autoantibodies. The role of pDCs as initiators of autoimmunity has just been recently described. Our lab first determined that pDC depletion prevented autoimmune development in an inducible lupus model [42]. Subsequently, several groups have reported that depletion of pDCs or dysfunction of pDCs prohibited autoantibody generation and lupus pathogenesis in lupus-prone mice [43]. However, the cellular events occurring after pDC activation that contribute to the development of autoantibodies are not well understood. **This dissertation aims to study the mechanism by which pDC activation stimulates humoral autoimmunity.**

1.5 B cells

1.5.1 B cell development

After birth, B cells develop in the bone marrow from hematopoietic precursor cells and pass through a rearrangement process of the immunoglobulin gene segments until a functional BCR is formed. The pairing of a rearranged heavy chain, consisting of V_H, D_H and J_H gene segments, with a rearranged light chain, consisting of V_L and J_L gene segments, will generate a complete BCR. This process is defined by 3 developmental stages (Figure 1.4) [44]. First, pro-B cells rearrange the D and J segments of the heavy chain, followed by a second rearrangement joining a V region with the DJ segment. This can form a functional μ-heavy chain that allows the entry to the second stage, known as pre-B-cell. At this stage, B cells form the pre-B cell receptor. The rearrangement of the light chain and its expression on the cell surface allows the formation of the IgM molecule and these cells enter the third stage, and are identified as immature B cells. Immature B cells leave the bone marrow and migrate to the spleen where they finalize their development and can differentiate into mature B cell subsets [45].

Immature B cells, also termed transitional B cells, home to the follicle of the spleen. There are three main transitional subsets, T1, T2 and T3, which are classified based on the expression of CD23 and IgM [46]. Although not completely clear, T1 (IgM^{hi} $CD23^{low}$) B cells are thought to give rise to follicular B cells, T2 (IgM^{high} $CD23^{+}$) B cells most likely give rise to marginal zone (MZ) B cells, whereas T3 B cells ($IgM^{-}CD23^{-}$) cells are thought to be anergic [47]

Different signals modulate the development and maturation of B cells in the bone marrow and in peripheral lymphoid organs. Signaling through the BCR by antigen modulates B cell development. Additionally, the presence of CXCL12 and IL-7 are required for B cell development in the bone marrow. In the periphery, BAFF provides survival signals to immature B cells through its binding to the BAFF receptor [48].

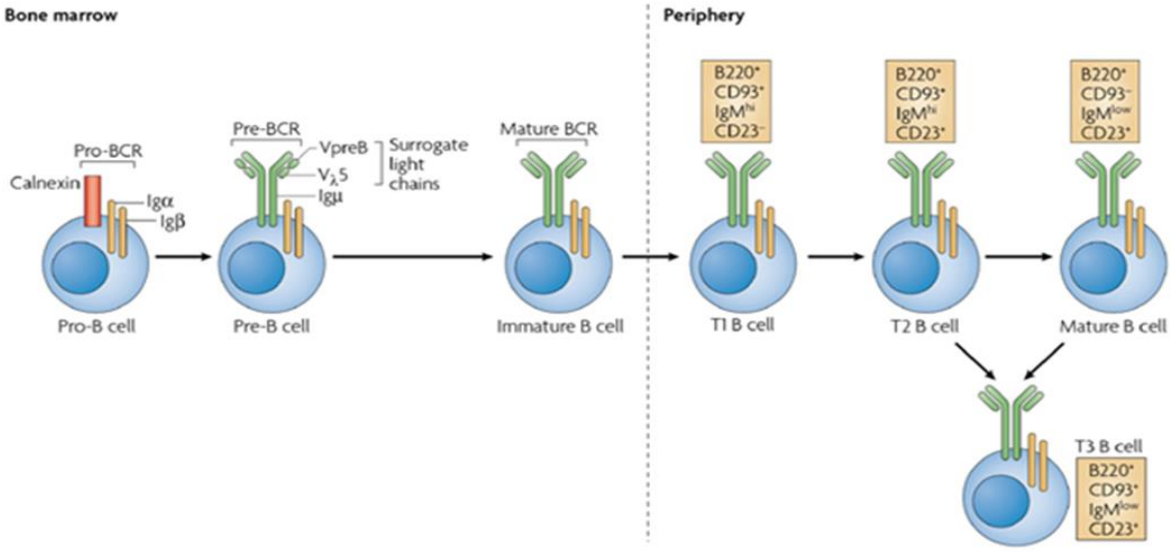

Figure 1.4. B cell development. In the bone marrow, B cells undergo a maturation process consisting of the pro-B cell, pre-B cell and immature B cell stage. After a mature BCR receptor is rearranged, immature B cells go to the periphery and continue their development. In the periphery, immature B cells develop from T1 B cell to T2 B cell, at this stage T3 B cells (anergic B cell population) may develop or they continue their maturation into mature B cells. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] Cambier JC, et al., B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol*, 2007. 7(8):874-7. Copyright Clearance Center.

The mature B cell compartment is mostly composed by follicular B cells, but MZ B cells are also present in the spleen. Follicular B cells migrate through the blood and lymphoid organs until activation. This B cell population needs antigen-mediated BCR stimulation and T cell co-stimulation for their differentiation into plasma cells. In contrast, MZ B cells can differentiate into antibody-producing following exposure to TLR ligands. Due to their ability to quickly produce antibodies, MZ B cells are considered innate-like cells, therefore, they play a major role in T cell-independent responses [49].

1.5.2 Antibody responses

Antibody responses can be divided according to the location where the B cells develop into antibody secreting cells. In most instances, T-independent responses develop at extrafollicular niches, although the presence of extrafollicular T cells may also help in the activation of B cells at this location. The movement of B cells into extrafollicular areas drives the generation of plasmablasts, which produce low affinity antibodies against invading pathogens [50]. In contrast, T-dependent responses mostly develop on germinal centers (GCs). The establishment of GCs generates long-lived plasma cells and high affinity antibodies [51, 52].

1.5.3 Germinal Center

The GC allows the formation of highly diverse and specific antibodies (Figure 1.5) [53]. After antigen exposure, activated follicular B cells migrate to the center of the B cell follicle and proliferate within the follicular DC network [54]. Along with follicular DCs, T follicular helper cells (Tfh) assist in the formation of the GC response by providing survival signals to GC B cells (expressing Fas and GL-7). After 8-10 days, the GC polarizes with proliferating B cells (centroblasts) on the T cell side and with the resting B cells (centrocytes) on the other. Centroblasts are highly proliferative and undergo somatic hypermutation (SHM), which is the basis for affinity maturation of antibodies. SHM induces high rates of mutation in the immunoglobulin variable region genes of the B cells, which produces high affinity antibodies. After the SHM process is completed, B cells undergo selection. B cells with high affinity BCR survive, while those with weak or moderate affinity either die immediately or undergo SHM again. Then, B cells may switch their Ig class expression to other classes to acquire a distinct effector function; this process is known as class-switch recombination (CSR). Lastly, the B cells that survive these processes either become memory B cells or long-lived plasma cells [55].

During this process different checkpoints occur to ensure antigen specificity of the humoral response, which will be discussed in section 1.5.6.

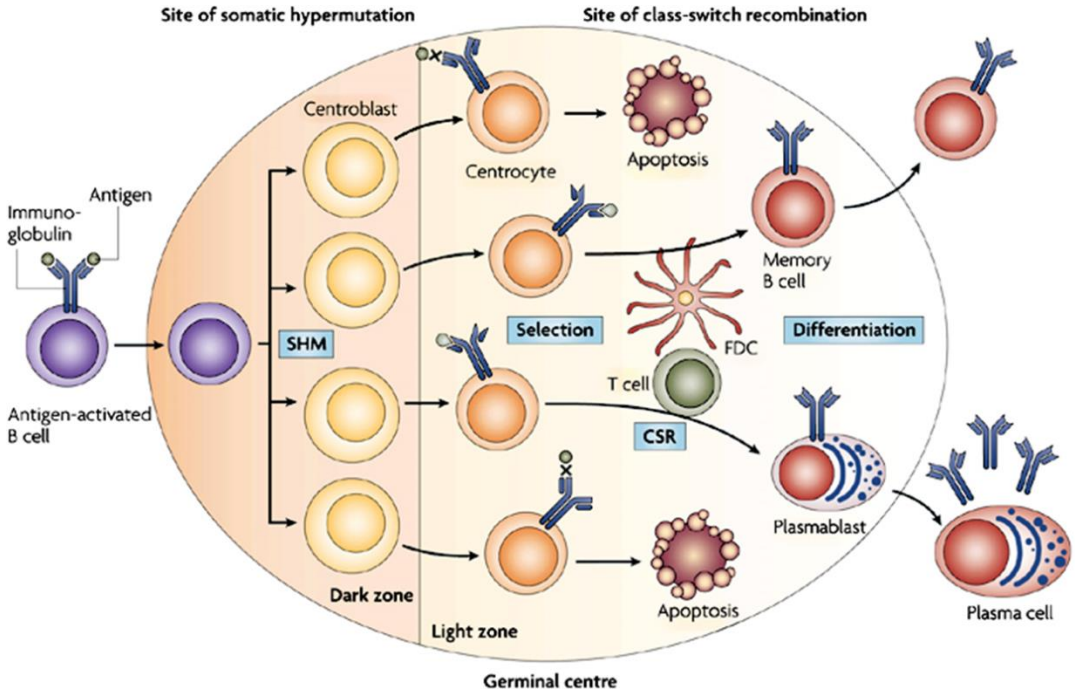

Figure 1.5. Germinal center reaction. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion and somatic hypermutation (SHM) in the dark zone of the germinal center. Centroblasts then differentiate into centrocytes and move to the light zone, where T follicular helper cells and follicular dendritic cells (FDCs) select B cells with a B cell receptor highly specific to the immunizing antigen. Centrocytes that are not specific to the antigen undergo apoptosis and are removed. Then, a subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] Klein U and Dalla-Favera R., *Germinal centres: role in B-cell physiology and malignancy*. Nat Rev Immunol, 2008. 8(1):874-7. doi:10.1038/nri2217. Copyright Clearance Center.

A specialized T cell subset modulates GC development and function. It has been well characterized that Tfh cells, which express ICOS, CXCR5 and PD-1, are required for the development of GC B cells. The help provided by Tfh cells is essential for the survival and proliferation of GC B cells. This T cell subset regulates GC size, restrict low-affinity B cell entry

into the GC while supporting high-affinity B cell selection [56, 57]. Therefore, the presence and functionality of Tfh cells is important for antibody responses.

The major transcriptional regulator of the germinal center is B cell Lymphoma 6 (Bcl6). Both GC B cells and Tfh cells express Bcl6. The disruption of the *Bcl6* gene blocks the development of GC B cells and Tfh cells, making it a master regulator of antibody affinity maturation in GCs [53]. Due to the repressive function of Bcl6, its expression modulates a broad array of genes to prevent premature activation and differentiation of GC B cells [58]. Also, Bcl6 can suppress genes that are required to drive the differentiation of alternative T helper cell lineages [59].

1.5.4 Extrafollicular Responses

Circulating B cells pass through the extrafollicular bridging channels when migrating into the follicle. Some B cell populations are positioned in this area, including transitional B cells and MZ B cells. Although not as well defined as GC structures, extrafollicular regions contain DCs and T cells, providing a microenvironment necessary for B cell interaction and activation. After B cell activation, extrafollicular B cells usually form short-lived plasma cells, called plasmablasts, mostly after T-independent responses. However after immunization with T-dependent antigens, extrafollicular responses have also been observed. A specialized $CD4^+$ T cell subset, with similar characteristics to Tfh cells, helps in the formation of extrafollicular responses. These extrafollicular T helper cells (Tefh) require ICOS and Bcl6 for their development and express CD40L and IL-21 to induce B cell maturation [50, 60].

1.5.5 B cell migration

Chemokine receptors facilitate the migration of B cells inside secondary lymphoid organs. Among these receptors, CXCR5 and CCR7 are upregulated on cells destined to go to the GC. Their respective ligands are CXCL13, which is produced by follicular DCs, and CCL19/CCL21,

produced by T cells. Recently identified, Epstein-Barr virus induced molecule 2 (EBI-2) signaling has the ability to guide naïve and activated B cells throughout the follicle. For B cells to localize in extrafollicular foci, EBI-2 is retained, CXCR5 is downregulated and CXCR4 is increased. A tight regulation of these chemokine receptors and their ligands ensures a homeostatic antibody response [61, 62]. However, little is known about the initial signals that induce cells to move towards one region or the other. Whether IFN-I directly or indirectly affects the migration of B cells into the GC and/or extrafollicular areas during autoimmunity need to be further investigated.

1.5.6 B cell tolerance

B cell tolerance is essential for maintaining unresponsiveness to self-antigens. Central and peripheral mechanisms exist to prevent the generation of self-reactive B cells and the development of humoral autoimmunity. In the bone marrow, immature self-reactive B cells can become anergic, deleted, or the specificity of their receptor can be edited (referred as receptor editing). Ligand specificity and BCR signal strength play key roles in determining the fate of the B cell during the selection process [63].

However, 50-80% of immature B cells that emerge from the bone marrow are autoreactive; therefore, peripheral tolerance checkpoints must exist to prevent further maturation of self-reactive B cells [64]. This phenomenon is noticed after analyzing immature B cells in healthy individuals, where most of them are polyreactive and capable of binding self-antigen [64]. However, only a small percentage of immature B cells survive and enter the pool of mature naïve B cells.

Indeed, in peripheral lymphoid organs, autoreactive B cells are deleted at the transitional stage and in the germinal center. The first checkpoint occurs between the immature B cell from

the bone marrow and the transitional T1 B cell from the spleen. The second is between the T1 and T2/T3 stage and the third is between T2/T3 B cells and mature B cell subsets [65].

During these checkpoints positive and negative selection processes take place, where ligand specificity, BCR signal strength and the cellular microenvironment ultimately determine the fate of the B cell. It has been proposed that soluble antigens with low valency provide a positive signal to the B cell, whereas polyvalent self-antigens cause deletion of the B cell [66]. Additionally, the presence of BAFF is thought to provide a positive signal for further B cell differentiation at the transitional stage [67]. The mechanism that modulate the positive and negative selection processes to prevent the selection of autoreactive B cells is still not very well understood.

Not clear is also the process of B cell selection inside GCs. However, two models have been proposed. In both models, follicular DCs present antigens to B cells. B cells that have affinity against self or show low affinity against foreign antigen undergo apoptosis due to lack of T cell help or inadequate BCR signaling from follicular DCs. In the first model, the different strengths of BCR signaling against foreign antigens determine survival and affinity maturation, i.e., high affinity BCRs acquire proper T cell help and further survive, divide and differentiate. In the second model, sufficient BCR signaling is required but competition for T cell help limit selection. A GC B cell that presents the highest number of antigen-MHC complexes compared with neighboring cells will be selected by antigen-specific T cells [54]. The exact mechanism by which B cell tolerance is broken inside GCs during autoimmunity it remains ambiguous.

1.5.7 B cell dysregulations in SLE

Patients with SLE show dramatic changes in their B cell subsets [68]. The most pronounced alterations are increased frequencies of transitional, memory and plasmablasts [65, 69, 70]. Among the naïve B cell population in SLE patients, 25-50% expresses self-reactive

BCR, whereas healthy individuals have from 5-20% self-reactive naïve B cells [71]. Moreover, a subpopulation of memory B cells show signs of SHM that correlates with disease activity, suggesting a GC origin [72].

In murine lupus models, dysregulated B cell subsets have been detected. One of the most affected B cell subsets are GCs. Many autoimmune-prone mice spontaneously develop GCs, and their presence correlates with the appearance of autoantibodies [73]. In addition, other B cell subsets have been found expanded *in vivo*. For example, MZ B cells are expanded in NZB/W F1 and B6.Sle1.Sle2.Sle3 mice and they generate anti-dsDNA IgM antibodies [74, 75]. Recently, transitional B cells have been shown to be expanded in TLR7-transgenic mice and the presence of anti-RNA antibodies was thought to come from this population; however the presence of this B cell subset was independent of IFNAR signaling [76]. The effect of pDC activation on the development of self-reactive B cells needs to be studied in a mouse model that exhibit a type I IFN signature.

1.6 Helper cells in humoral autoimmunity

Different immune cells can help and shape B cell responses. T cells have long been known to provide critical help to B cells in response to protein antigens. Because most B cell responses require T cell help, peripheral B cell tolerance largely depends on T cells. The specialized T helper subset, Tfh, provides positive signals to B cells inside the GC. These Tfh cells not only need to enter GCs, but also must be competent to provide appropriate co-stimulation to B cells. Overexpression of molecules expressed by Tfh cells, such as CD40L, ICOS and IL-21, induces break of B cell tolerance and autoimmunity, whereas blockade of these molecules prevents autoantibody development in autoimmune-prone mice [57, 77].

Glycolipid antigens can elicit B cell responses through the activation of invariant NKT cells. These NKT cells express an invariant T cell receptor that is Vα14-Jα18 in mice and Vα24-

Jα18 in humans. Unlike conventional T cells that recognize antigens by MHC class II, NKT cells recognize antigens presented by CD1d [78]. A specialized subset of NKT cells is also present in the GC, with similar phenotypic characteristics to Tfh cells. The activation of NKT cells with glycolipid antigens induces a rapid formation of GCs, but it can also promote the formation of extrafollicular plasmablasts [79, 80]. Despite the stimulatory role of NKT cells in normal antibody responses, NKT cells seem to have a suppressive function during autoimmunity. This is supported by several findings showing that NKT cell deficiency exacerbated autoantibody generation [81, 82].

Growing evidence has shown that non-classical helper cells can interact with B cells and provide the necessary signals for B cell activation and differentiation. Among these, neutrophils, macrophages and mast cells have been shown to directly or indirectly induce B cell activation [83]. Moreover, NK cells have been shown to induce B cell activation *in vitro* [84]. It seems that different types of antigens activate a different set of helper cells to induce the necessary antibody response. Whether different types of helper cells are induced during systemic autoimmunity needs to be further studied. Due to the complexity and multicellular players involved during autoimmunity, this is a likely scenario.

1.7 Mouse lupus models

As pointed before, different lupus mouse models share certain similarities with human SLE (Table 1.1), such as the development of autoantibodies against nuclear components, which may lead to immune complex deposition in the kidneys and proteinuria [85, 86]. However, there are some disadvantages while using these mouse models. Autoimmune-prone mice, such as NZB/W F1 and MRL-Fas^{lpr} strains which develop spontaneous lupus disease, harbor mutations in different genes that predispose them to autoimmunity. Due to effects on different immune cell types, the contribution by a specific gene to disease pathogenesis is difficult to dissect. In

contrast to human SLE, these mice lack the IFN-I signature. Although BXSB mice show a weak type I IFN signature, the development of lupus is only observed in male mice [87]. This is in contrast to human SLE, where female preponderance exists.

Animal model	IFN signature	Source of IFN	Clinical features
NZB/W, NZM2410	Not detected	N/A	ANA, severe kidney disease, splenomegaly
MRL-Faslpr	Absent	N/A	ANA, severe kidney disease, splenomegaly
B6-*Fas*^{lpr}	Absent	N/A	mild ANA, splenomegaly
BXSB male	In blood and kidney	Not known in blood, pDCs in kidney	ANA, splenomegaly, severe kidney disease,

Table 1.1. Spontaneous lupus mouse models.

Some inducible lupus models have been developed to study autoimmunity in non-autoimmune mice. Injection of pristane, a hydrocarbon oil, in C57BL/6 or Balb/c mice causes the development of autoantibodies and IC-mediated glomerulonephritis [88]. Even though these mice exhibit a type I IFN signature, IFN-α is produced by inflammatory monocytes instead of pDCs [89].

1.7.1 Amyloid-induced lupus model

Our laboratory has developed a novel inducible lupus model that is better suited for studying pDC-mediated humoral autoimmunity. Nucleic acid-containing amyloids can activate pDCs through TLR9 activation, leading to IFN-I production. More importantly, the immunization of non-autoimmune mice with DNA-containing amyloids triggers a lupus-like disease. These mice develop autoantibodies against several anti-nuclear antigens, such as DNA, histone and

Sm/RNP. Additionally, the immunized mice exhibited IC deposition in the kidneys, which correlated with proteinuria. A type I IFN signature was present in these mice, and depletion of pDCs prevented the development of autoantibodies [42]. Moreover, deficiency of *Ifnar* or impairment of pDCs' capacity to produce IFN-I prevented the development of anti-nuclear antibodies (Figure 1.6) [90]. Therefore, this model allows experimental delineation of pDC-mediated induction of humoral autoimmunity.

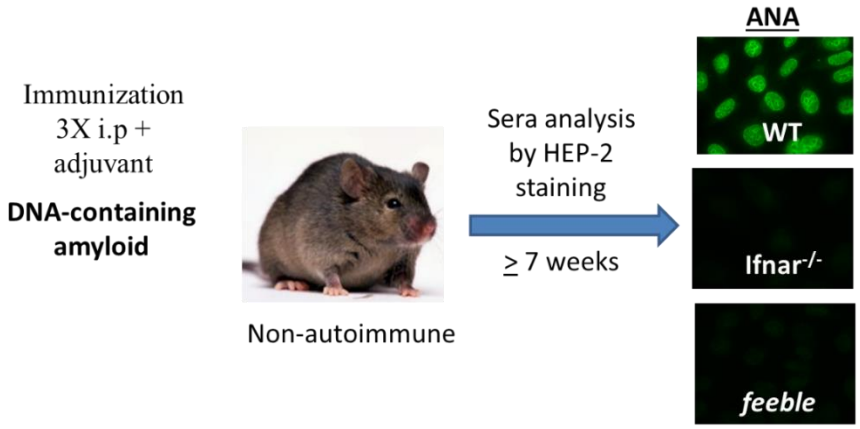

Figure 1.6. Amyloid-induced lupus model. Immunization with DNA-containing amyloids into non-autoimmune mice triggers the development of anti-nuclear antibodies (ANA). Deficiency in Ifnar1 (*Ifnar*$^{-/-}$) or mutations in TLR signaling from pDCs (*feeble*), which impairs their cytokine production, prevents the development of ANA.

Interestingly, the functional amyloid *curli*, produced during biofilm formation, can complex with DNA and activate DCs to produce IFN-I. When injected *in vivo*, curli-DNA or bacteria-producing *curli* amyloid induced lupus-like disease [91]. Since DNA-containing amyloids can certainly elicit an autoimmune reaction, it is relevant to study the immunological events occurring after amyloid stimulation *in vivo*. The amyloid-induced lupus model may help elucidate these events.

1.8 Organization of the dissertation

1.8.1 Rationale of this dissertation

Type I IFNs and pDCs have been directly linked to SLE pathogenesis; however the cellular cascade that stimulates the development of humoral autoimmunity as a result of pDC activation has not been described. Moreover, the origin of self-reactive B cells is unknown: it is not clear whether the GC, extrafollicular responses or both are necessary for the development of autoreactive B cells. Although different lupus mouse models are available, most of them lack a type I IFN signature, which is predominantly present in SLE patients [32]. In addition, spontaneous lupus mice contain polygenic mutations, which make it difficult to dissect the contribution by individual genes. Fortunately, we have developed an inducible lupus mouse model, where the immunization of non-autoimmune mice with DNA-containing amyloids induces a break of tolerance, characterized by the presence of autoantibodies and IC deposition in the kidney. These mice show a type I IFN signature and require pDCs for autoantibody development [42]. We believe that this mouse model better mimics SLE pathogenesis, thus will allow us to analyze the B cell responses and identify cellular players downstream of pDC activation, which leads to the establishment of autoimmunity. Additionally, we will determine whether humoral factors known to interact with amyloids, can affect the ability of amyloid to activate pDCs.

1.8.2 Aims

We hypothesize that pDCs stimulate the development of humoral autoimmunity by promoting a cascade of cellular events, which involve the development of self-reactive B cells that can arise from the germinal center and/or extrafollicular locations by activating immune helper cells. Separately, the presence of pentraxins may influence this process by affecting amyloid-mediated pDC activation.

We developed the following AIMS:

AIM I: Determine the role played by germinal center response in pDC-mediated humoral autoimmunity.

AIM II: Determine whether extrafollicular B cells are involved in pDC-mediated humoral autoimmunity.

AIM III: Identify immune helper cells that participate in pDC-mediated humoral autoimmunity.

AIM IV: Determine whether serum amyloid P-component (SAP) modulates amyloid-mediated pDC activation *in vitro*.

In Chapters II, III and IV, we will use the amyloid-induced lupus model, to study how pDC mediates humoral autoimmunity. On Chapter II and III, B cell responses will be analyzed. We will mostly focus on the B cell responses occurring during break of tolerance, defined as the first appearance of IgM autoantibodies. Specifically, in Chapter II we will analyze the presence of GC B cells and possible factors influencing their development during pDC-mediated humoral autoimmunity. In Chapter III, we will analyze different B cell subsets outside GCs that are activated in this autoimmune model. To complete the *in vivo* study, in Chapter IV, we will identify immune helper cells and the potential mechanisms used by these cells to influence the development of autoantibodies. The results of these 3 chapters will identify the key B cell subset that produce autoantibodies initially and the cellular players that modulate the generation of self-reactive B cells in a pDC-mediated autoimmune model.

Additionally, in Chapter V, we will study the ability of humoral factors present in serum, which are known to interact with amyloid, such as SAP, to modulate pDC activation *in vitro*. This dissertation will uncover novel mechanisms that promote and modulate humoral autoimmunity.

CHAPTER 2.

GERMINAL CENTERS ARE INHIBITED DURING PLASMACYTOID DENDRITIC CELL-MEDIATED AUTOIMMUNITY

2.1 Rationale

Humoral autoimmunity surges from the development of autoantibodies whose origin remains unknown. It has been suggested that autoantibodies arise primarily from GCs. This hypothesis is supported by several findings. First, peripheral blood from SLE patients contains an expanded B cell subset with GC characteristics, including enhanced SHM [70, 92]. In accordance, autoantibodies and plasma cells from SLE patients display extensive SHM [93]. Second, numerous mouse strains that develop SLE-like disease exhibit spontaneous GCs in the spleen. The generation of these GCs correlates with the onset of autoantibody production [94].

The role of GCs in SLE is further supported by the dysregulated phenotype of Tfh cells in patients with SLE and lupus mouse models. In some SLE patients, circulating T cells with characteristics of Tfh are expanded and correlated with autoantibody titers and severity of end-organ involvement [71]. Additionally, polymorphisms of IL-21, a major Tfh cytokine that induces GC development, and IL-21R are associated with SLE [95]. In mice, an expanded Tfh population, which produces high levels of IL-21, has been found in the *sanroque* mice, which harbored a mutation that regulates ICOS expression on T cells, and developed spontaneous GCs and aggressive lupus-like disease [96]. A haploinsufficiency of *Bcl6*, which reduced GC B cells and Tfh cells, also reduced lupus pathogenesis in *sanroque* mice, further supporting the role of GCs in SLE development [73]. Additionally, excessive production of IL-21 has been observed in MRL-Fas^{lpr} and BXSB/*Yaa* mice and their crossing with *Il21r*$^{-/-}$ prevented the development of autoantibodies [97]. However, it is unclear whether GC response is similarly affected in pDC-mediated humoral autoimmunity, as the spontaneous lupus mouse models do not exhibit a type I IFN signature.

This major caveat is overcome in the amyloid-induced lupus model. DNA-containing amyloids can activate pDCs to produce IFN-I. Interestingly, after *in vivo* immunization, DNA-

containing amyloids can induce lupus-like disease in non-autoimmune mice, characterized by the production of self-reactive antibodies and IC deposition in the kidneys. The depletion of pDCs abolished the lupus-like symptoms. Due to the presence of human serum albumin (HSA) in the amyloid backbone, we can simultaneously analyze and contrast the immunogen-reactive and the self-reactive antibody responses. By using this inducible lupus mouse model, **we intend to determine whether pDC-IFN-I axis elicits a GC reaction to promote the development of self-reactive B cells.**

2.2 Differential humoral responses to immunogen vs self-antigen

To study the events occurring during break of tolerance, we analyzed the antibody responses in non-autoimmune mice at 1-3 weeks after injection of HSA-DNA (further referred as control) or DNA-containing amyloid (further referred as amyloid) mixed with complete Freund's adjuvant (CFA) [42]. During the first two weeks after immunization, antigen-specific antibody levels were low in both control- and amyloid-immunized mice. However, at 3 weeks of immunization striking differences were observed between the two groups of immunized mice: the levels of IgG antibodies reactive to the immunogen (anti-HSA) dramatically increased in mice receiving control, whereas mice receiving amyloid did not show such increase (Figure 2.1A). In contrast, the levels of histone-specific IgM antibodies were elevated after immunization with amyloid, but not with control (Figure 2.1B). Anti-histone IgG antibodies were undetectable at this time point (data not shown). Consistent with this result, the *in vitro* culture of isolated B cells from amyloid-immunized mice produced lower levels of anti-HSA IgG and higher levels of anti-histone IgM compared to B cells from control-immunized mice after R848 stimulation, a TLR7 agonist (Figure 2.1C, 2.1D). Altogether, these data showed that pDC activation induces a break of tolerance at 3 weeks of immunization, characterized by the generation of self-reactive antibodies. Intriguingly, amyloid inhibits the immunogen-specific antibody response.

To confirm our results, we generated hybridoma clones from B cells isolated from control- and amyloid- immunized mice. The same amounts of B cells ($2x10^6$ cells) were fused to myeloma cells. As expected, higher percentage of clones from mice receiving control produced anti-HSA antibodies (Figure 2.2A), whereas higher percentage of B cells from mice receiving amyloid produced anti-histone antibodies (Figure 2.2B). Interestingly, some clones were reactive to 2 or more additional antigens. For example, some clones produced antibodies that reacted to histone and also to HSA and Sm/RNP. After comparing the polyreactivity between B cell clones from control or amyloid, we found that most of the histone-specific antibodies from amyloid immunized mice also reacted to the immunogen and/or to other self-antigen (Figure 2.2C); this is similar to the observed property of B cells from SLE patients [98]. Altogether, these data indicated that during break of tolerance, which occurred at 3 weeks in our model, self-reactive B cells are selected to produce primarily polyreactive autoantibodies, whereas immunogen-specific B cells are inhibited.

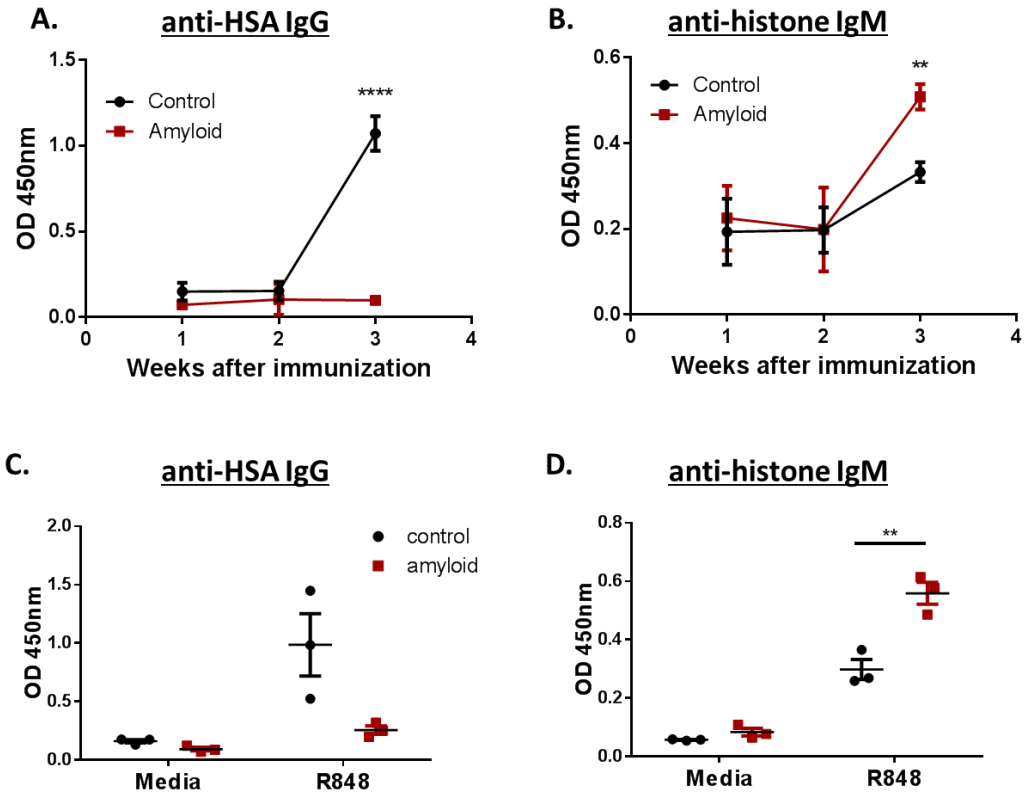

Figure 2.1. Immunogen-specific antibodies are decreased while self-reactive antibodies are increased during break of tolerance.
(A) Sera from control or amyloid immunized C57BL/6 mice was collected at different time points after immunization and anti-HSA IgG antibodies were detected by ELISA (n = 3).
B) Levels of anti-histone IgM antibodies were detected in the sera by ELISA (n = 3 – 7)
(C) After 3 weeks of immunization, enriched B cells were cultured for 7 days in media or R848 and anti-HSA IgG antibodies were detected in the supernatant. Two additional experiments were performed.
(D) Levels of anti-histone IgM antibodies were detected in the supernatant from the samples as in (C).
(A, B and D) Data represented as means $\pm$ SEM. *P* values: $^{**}p<0.005$, and $^{***}p<0.0005$. Student's *t*-test was performed to detect statistical difference between groups.

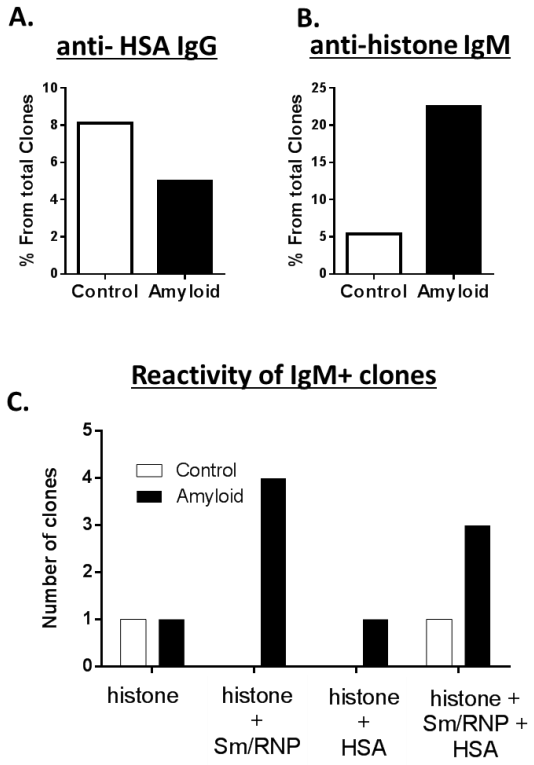

Figure 2.2. Break of tolerance induces polyreactive B cell clones.
(A) Hybridomas were prepared from sorted total CD19+B220+ cells and the levels of anti-HSA IgG were detected in the supernatant by ELISA. Graphs show % of positive clones among all clones tested. (control – n = 37 clones; amyloid – n = 40 clones)
(B) Anti-histone IgM antibodies were detected by ELISA from the samples as (A).
(C) Reactivity to HSA, histone and Sm/RNP were tested in anti-histone IgM positive clones (control – n = 2; amyloid – n = 9).
(A-C) Data from one experiment. Collaborator contributions: Long Vien from Hybridoma Core Facility at MD Anderson Cancer Center performed the fusion of sorted B cells into myeloma cells.

2.3 Germinal centers reactive to immunogen are diminished

GCs are a unique microenvironment where antigen-specific B cells are selected and develop into antibody-secreting cells. Due to the differential antibody responses induced by control- vs. amyloid-immunization, we assumed that the development of GCs may also be affected. To clearly identify GC B cells, we utilized the *Bcl6*^{yfp} reporter mice. In these mice, the yellow fluorescent protein (YFP) gene was inserted in-frame right after the initiation codon of the

Bcl6 gene, allowing the identification of *Bcl6*+ cells, mostly GC B cells and Tfh cells, by the YFP signal [99]. *Bcl6*$^{yfp/+}$ mice develop comparable GC B cells and Tfh cell numbers as C57BL/6 mice, whereas, homozygous *Bcl6*$^{yfp/yfp}$ mice contain lower number of GC-derived cells [99]. Therefore, we immunized the *Bcl6*$^{yfp/+}$ mice with control or amyloid and quantified CD19^{+}GL7^{+}Fas^{+}*Bcl6*$^{+}$ GC B cells at different time points by flow cytometry. During the first 2 weeks, GC B cell numbers were highly increased in mice that received control but not those that received amyloid (Figure 2.3A). Surprisingly, at week 3, amyloid induced a reduction of GC B cells whereas control immunization maintained high GC B cell numbers (Figure 2.3B). To confirm these results, we performed immunofluorescence staining on frozen spleens from mice 3 weeks after immunization. By staining with peanut agglutinin (PNA) and IgD, we could observe that amyloid immunization restricted the development of GCs, as the spleen from amyloid-immunized mice formed not only less number of GCs but also smaller GCs (Figure 2.3C). This occurred at the same time when immunogen-specific antibodies were decreased (Figure 2.3A), suggesting that those antibodies came from GC B cells.

To confirm this finding, we biotinylated HSA and identified B cells that were able to bind to this immunogen by flow cytometry. In fact, a high percentage of GC B cells from control-immunized mice recognized HSA, whereas GC B cells from amyloid-immunized mice barely showed positivity towards HSA (Figure 2.4A). Non-GC B cells displayed marginal reactivity towards the immunogen (Figure 2.4B). Moreover, depletion of GL7^{+}Fas^{+} GC B cells resulted in the loss of hybridoma clones that produce IgG reactive to HSA (data not shown). Altogether, we conclude that amyloid inhibits GC-derived immunogen antibodies.

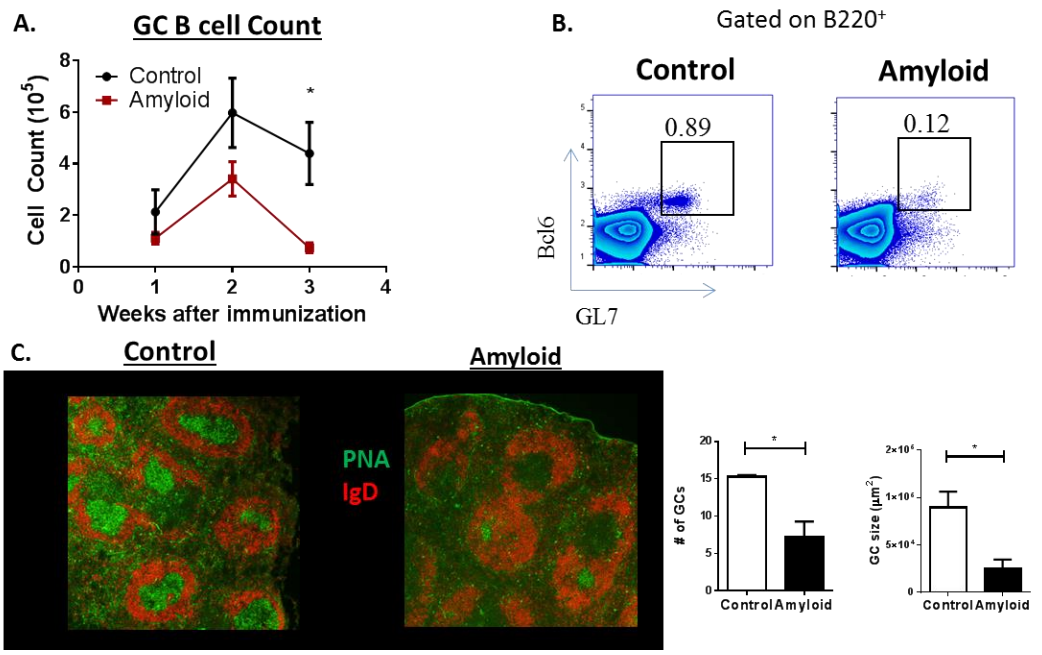

Figure 2.3. Amyloid immunization diminishes GC B cells.
(A) Spleens from Bcl6$^{yfp/+}$ mice immunized with control or amyloid were analyzed by flow cytometry to quantify GC B cells at different time points.
(B) Flow cytometry plot depicting GC B cells after 3 weeks of immunization.
(C) Frozen spleen sections from C57BL/6 mice immunized with control or amyloid after 3 weeks of immunization were stained with PNA and IgD to quantify GC numbers and GC size. Magnification x10 using Leica SP8 confocal microscope and individual images were taken to the whole spleen section to count GCs. Average GC areas were determined by using ImageJ software.
(A and C) Data represented as means ± SEM (n = 3). *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups.

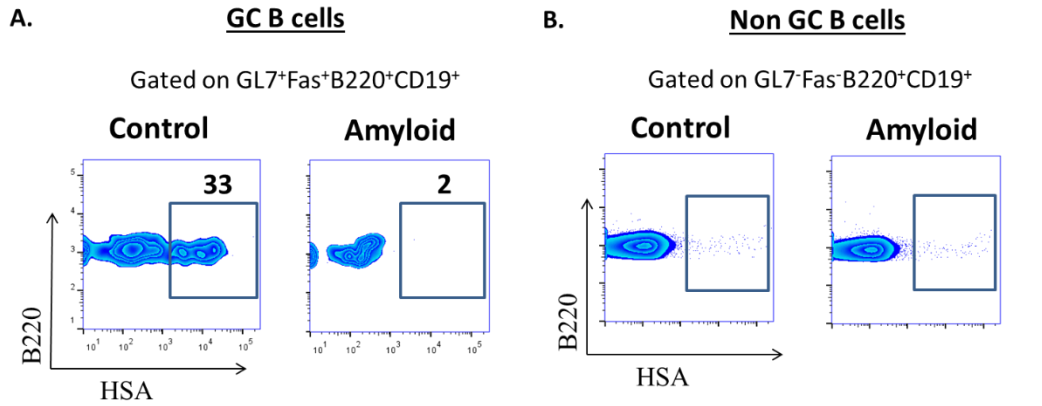

Figure 2.4. GC B cells are specific to the immunogen.
(A) Biotinylated HSA was used to identify immunogen-specific GC B cells.
(B) HSA-specific B cells, excluding GC B cells, were detected as in (A).
(A-B) Similar results obtained in 4 independent experiments, 3 replicates per experiment.

2.4 Activation-induced cell death may promote germinal center inhibition

The reduction of GC B cells by amyloid was intriguing to us; therefore we wanted to determine the mechanism responsible for this phenomenon. To determine whether proliferation of GC B cells was affected, we analyzed the expression of Ki67, a cell cycle-associated protein. Quantification by flow cytometry revealed that amyloid immunization induced lower percentage of $Ki67^{+}$ GC B cells compared to control-immunization (Figure 2.5A). This result was consistent with histological analysis, showing lower $Ki67^{+}$ cells inside GCs in amyloid-immunized spleen (Figure 2.5B). These data suggested that a decrease in B cell proliferation may explain the reduction of GC B cells during break of tolerance.

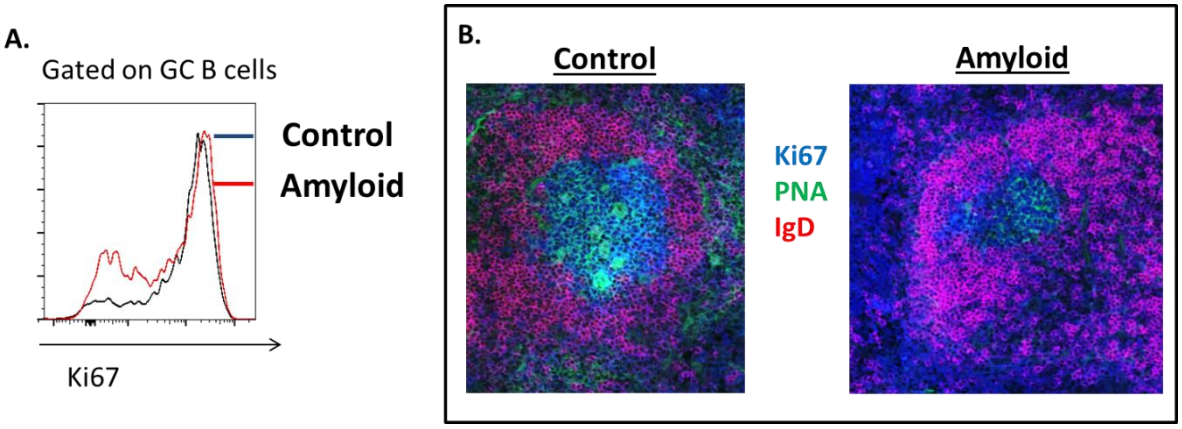

Figure 2.5. GC B cells show impaired proliferation.
(A) Ki67 expression was analyzed on GC B cells by flow cytometry after 2 weeks of immunization. Similar results were obtained from 2 additional experiments, 3 replicates each.
(B) Spleens were frozen to detect GCs and Ki67 expression by immunofluorescence. Data is representative of one experiment (n = 3).

Activation-induced cell death (AICD) is an important negative selection mechanism to prevent self-reactivity. In T cell-dependent responses, AICD is initiated in B cells after stimulation of the Fas receptor by Fas ligand on T cells [100, 101]. To determine whether GC B cells undergo AICD during break of tolerance, we analyzed the expression of Fas and activation markers on GC B cells by flow cytometry and RT-PCR. Interestingly, GC B cells from amyloid-immunized mice showed enhanced surface (Figure 2.6A) and mRNA expression (Figure 2.6B) of Fas compared to control-immunized mice. Additionally, the surface expression of CD80 was increased on GC B cells after amyloid immunization, which correlated with increased mRNA expression (Figure 2.6C and 2.6D).

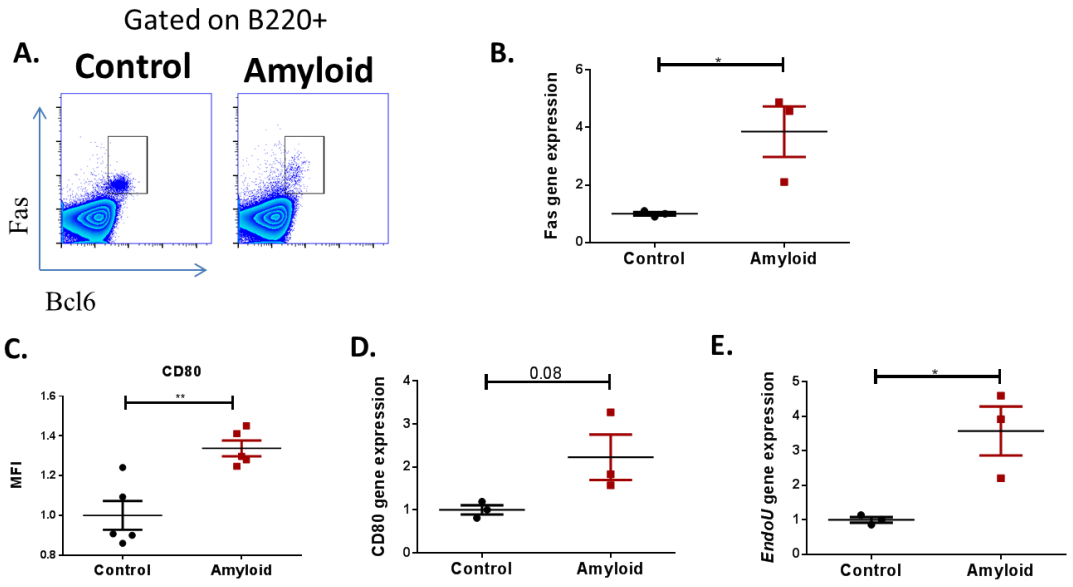

Figure 2.6. Amyloid induces markers of AICD on GC B cells.
(A) The surface expression of Fas was analyzed on GC B cells by flow cytometry after 3 weeks of immunization. Similar results were obtained in 3 additional experiments, 3 replicates per experiment.
(B) The mRNA expression of Fas was quantified by RT-PCR on cell sorted GC B cells after 2 weeks of immunization. Data represents 2 pooled experiments.
(C) The surface expression of CD80 was analyzed on GC B cells by flow cytometry after 3 weeks of immunization.
(D) The mRNA expression of CD80 was quantified on GC B cells as in (B).
(E) EndoU mRNA expression was quantified as in (B and D).
(B, C, D and E) Data represented as means ± SEM. *P* values: **p*<0.05, ***p*<0.005. Student's *t*-test was performed to detect statistical difference between groups.

Recently, endonuclease PolyU-Specific (EndoU), a novel RNA-binding protein, has been identified as an important positive regulator of AICD on B cells. Therefore, we wanted to determine whether EndoU is expressed by GC B cells. Interestingly, GC B cells from amyloid-immunized mice upregulated *EndoU*, implying that the reduction of GC B cells relates to AICD (Figure 2.6E).

2.5 pDC-IFN-I pathway is required for autoantibody development but dispensable for GC inhibition

The peptide/histidine transporter solute carrier family 15, member 4 (Slc15a4) is crucial for cytokine production triggered by TLR7, TLR9 and NOD1 [102, 103]. Mice carrying a mutation on Slc15a4 gene, named "*feeble*", showed defects in pDCs' ability to produce cytokines, including IFN-I [104]. To determine whether pDC activation and IFN-I signaling influence the GC reaction during break of tolerance, we immunized *feeble* and *Ifnar1*$^{-/-}$ mice and quantified GCs, as previously described. As noted in Figure 2.7A, amyloid-immunized C57BL/6 mice showed decreased numbers of GCs whereas *feeble* mice, but not *Ifnar1*$^{-/-}$ mice, showed a partial rescue of GC B cells. This suggests a putative role of pDC activation on GC regulation, independent of IFN-I. It could be noted that both *feeble* and *Ifnar1*$^{-/-}$ mice developed less GCs with control-immunization compared to C57BL/6 mice, suggesting a requirement of IFN-I in the full development of GCs in immunogen responses. After analyzing antibody production, no difference was detected in anti-HSA IgG antibodies from *feeble* and *Ifnar1*$^{-/-}$ mice receiving amyloid-immunization (Figure 2.7B). Altogether, these data suggested that pDC activation may not be responsible for the GC inhibition triggered by amyloid.

In a previous study, we have demonstrated that *feeble* and *Ifnar1*$^{-/-}$ mice did not develop class-switched autoantibodies [90] (Figure 1.6). To determine whether pDC activation is required to induce break of tolerance, we analyzed autoantibody development in *feeble* and

Ifnar1$^{-/-}$ mice after 3 weeks of immunization with amyloid. Interestingly, we observed that anti-histone IgM was induced in C57BL/6 mice but not in *feeble* or *Ifnar1*$^{-/-}$ mice (Figure 2.7C). Our data showed that pDCs are required for the development of self-reactive clones and their further isotype class-switching.

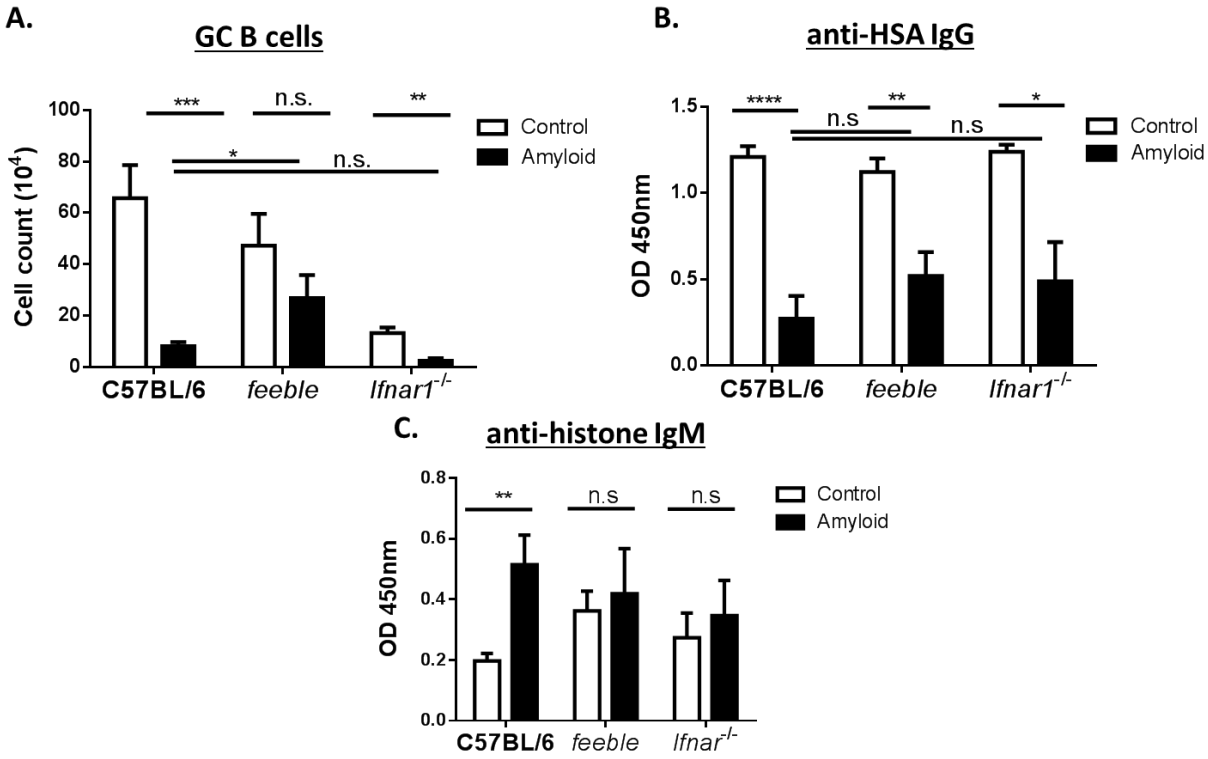

Figure 2.7. pDC-IFN are required for the initial autoantibody development but may not be involved in GC B cell inhibition.
(A) Numbers of GC B cells on spleens after 3 weeks of immunization in the corresponding mouse strains.
(B) Levels of anti-HSA IgG antibodies in the serum after 3 weeks of immunization.
(C) Levels of anti-histone IgM was quantified as in (B).
(A-C) Data represented as means $\pm$ SEM (n = 5 – 8). *P* values: *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005, n.s. is > 0.05. Student's *t*-test was performed to detect statistical difference between groups.

2.6 Defective GC development does not affect IgM autoantibody response but hinders isotype-class switching of autoantibodies

The dramatic reduction of GCs during break of tolerance suggested that GCs may be dispensable for autoantibody development. To assess the role of GCs on the development of humoral autoimmunity, we analyzed mice with impaired *Bcl6* function, a major transcriptional factor involved in the development of GCs. *Bcl6*$^{yfp/yfp}$ mice have reduced numbers of GC B cells and Tfh cells [99]. To analyze long-term autoantibody development, mice received an injection of control or amyloid with CFA, followed by 2 booster immunizations in incomplete Freund's adjuvant (IFA) 2 weeks apart. After amyloid immunization, GC B cells were almost undetectable in *Bcl6*$^{yfp/yfp}$ mice (data not shown). Interestingly, these mice developed similar anti-histone IgM antibodies compared to WT mice after 7 weeks of amyloid immunization (Figure 2.8A). However, impairment of *Bcl6* significantly reduced the production of anti-nuclear IgG antibodies (Figure 2.8B). Moreover, *Bcl6*$^{yfp/yfp}$ mice showed a significant impairment on the development of histone-specific pathogenic IgG isotypes, such as IgG2b and IgG2c (Same as IgG2a in Balb/c mice), suggesting a defect in isotype class-switching (Figure 2.8C). These data demonstrated that, while *Bcl6* is dispensable for autoreactive IgM production, it is alternatively involved in subsequent IgG class-switching.

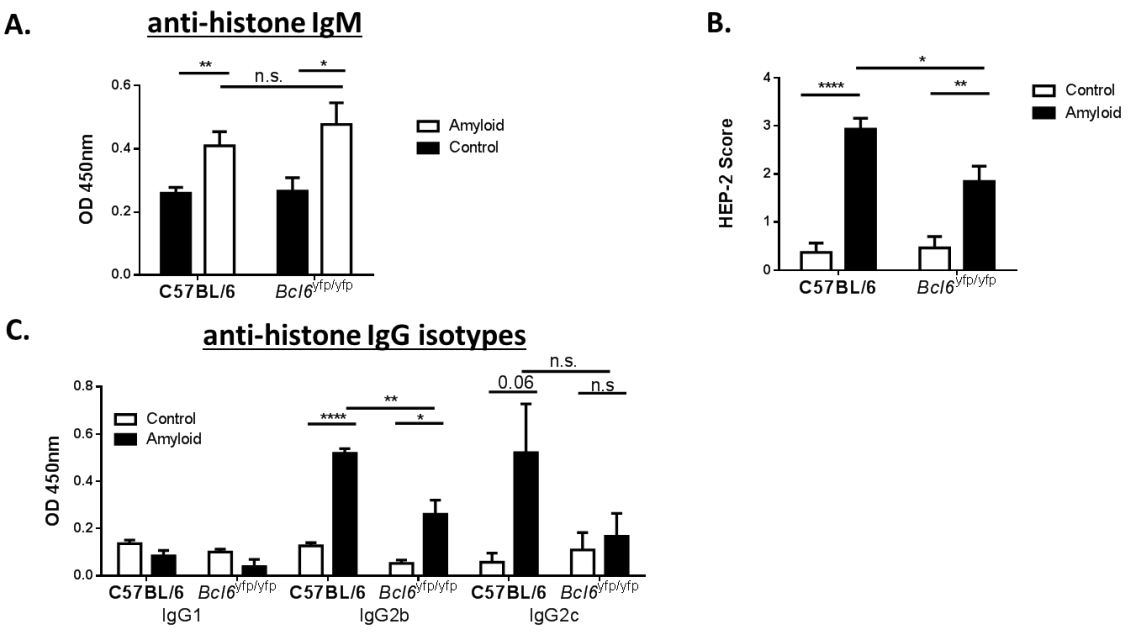

Figure 2.8. Bcl6 may be required for isotype class switching to pathogenic autoantibodies
(A) Levels of anti-histone IgM were detected in sera from C57BL/6 or *Bcl6*$^{yfp/yfp}$ mice after 7 weeks of immunization (n = 11 – 16).
(B) Sera from (A) were tested for anti-nuclear reactivity on HEP-2 cells.
(C) Different anti-histone IgG subtypes in the sera were analyzed by ELISA (n = 4 – 5). Data from one experiment.
(A-C) Data represented as means ± SEM. *P* values: **p*<0.05, ***p*<0.005, ****p*<0.0005, *****p*<0.00005, n.s. is > 0.05. Student's *t*-test was performed to detect statistical difference between groups.

2.7 Discussion

It is still unknown how the pDC-IFN axis promotes the positive selection and expansion of self-reactive B cells and what the cellular origin of autoantibodies is. We have determined that, in the amyloid-induced autoimmune model, pDCs and IFN-I play distinct roles in immunogen and self-reactive humoral responses. While pDCs do not seem to affect immunogen-specific antibodies, they appear to promote the production of autoantibodies at the initiation phase of the disease, and also the class-switching of autoantibodies. Interestingly, defective GCs did not

affect the IgM autoantibody response; however, they were likely required for the class-switching of autoantibodies. Our data suggests that the break of tolerance may occur initially outside GCs.

Several studies in mouse and human have suggested that autoantibodies can arise outside GCs. In MRL-Fas^{lpr} mice, rheumatoid factor and anti-dsDNA antibodies are produced in extrafollicular sites [105, 106]. In BXSB/Yaa mice, both follicular and extrafollicular responses drive the generation of autoantibodies [97]. More importantly, not all self-reactive plasma cells from SLE patients contain SHM mutations, suggesting that some have differentiated outside GCs [98]. By analyzing the amyloid-induced lupus model, we have determined that self-reactive antibodies can arise in the absence of GCs, suggesting an extrafollicular origin. This is supported by several findings. First, GCs are reduced after amyloid immunization and *Bcl6* impairment failed to affect IgM autoantibody development. This result needs to be further confirmed by analyzing Bcl6-deficient mice. Second, upon fusion of splenic B cells from amyloid-immunized mice with myeloma cells, the generation of self-reactive B hybridoma clones was unaffected by the depletion of GC B cells (data not shown). **We would speculate that GCs are not the initial source of self-reactive B cells, but rather a second location to enhance autoantibody production and/or promote isotype class-switching to more pathogenic isotypes.** In support of this claim, at 7 weeks of immunization, GC B cells are recovered in amyloid immunized mice (data not shown), suggesting that at later stages they are a possible source of autoantibodies. In the following chapter, non-GC B cells will be analyzed to identify a potential self-reactive B cell subset responsible for the initial break of tolerance in this model.

The mechanism by which GCs are reduced is still unclear, but antigen availability, dysregulation of Tfh cells or apoptosis of B cells may all affect GC response. Lower antigen availability can prevent GC development [107]. In our model, the sequestration of the immunogen HSA in the amyloid structure might prevent their availability to induce GCs. This possibility needs to be further study. The role of Tfh cells will be examined in Chapter 4.

Programmed cell death might be triggered and result in GC reduction [108]. One type of programmed cell death occurs after engagement of TLR7 and BCRs, which triggers activation of B cells and necroptosis. However, this mechanism is not likely to happen in our model, since TLR7 deficiency did not rescue GCs from amyloid-induced inhibition (data not shown). Moreover, the upregulation of *Fas* and *EndoU* implies that AICD, an important negative selection mechanism, occurs in GC B cells. Further confirmation of GC B cell death, through analysis of caspase activity and detection of dying GC B cells, is needed to substantiate this conclusion. We will speculate that the inhibition of GC development might relate to an enhanced negative selection process to censor the generation of self-reactive B cells.

Our study and recent investigations support the concept that polyreactive antibodies can be generated during autoimmunity. The idea that immune responses generated against foreign antigens may give rise to cross-reactive antibodies that bind both self-antigen and foreign antigen is called “molecular mimicry” [109]. These polyreactive antibodies have been found not only in autoimmune diseases following infections, but also in SLE patients undergoing flares [98]. The reason why an antibody can react to multiple unrelated antigens is still a matter of speculation. It is thought that low affinity antibodies have a more flexible antigen-binding pocket that can accommodate different antigens [110]. Therefore, it is likely that the initial autoantibodies produced during break of tolerance are of low affinity.

Our data supports the hypothesis that IFN-I and Bcl6 play a pathogenic role in the development of autoantibodies. Therefore, **therapeutic strategies aiming to block IFN-I or BCL6 may help control disease severity and progression in SLE patients.**

CHAPTER 3.

ACTIVATION OF NON-GERMINAL CENTER B CELLS IN PLASMACYTOID DENDRITIC CELL-MEDIATED HUMORAL AUTOIMMUNITY

3.1 Rationale

Many tolerance checkpoints exist during B cell development and maturation to prevent the generation of self-reactive clones. At these checkpoints, the BCR is tested for self-reactivity and may undergo negative selection if its affinity against self-antigen is above threshold. Defects at any of these checkpoints may release self-reactive B cells even before they enter the GC.

Different B cell subsets outside GCs have been found to be dysregulated in humoral autoimmunity. For example, MZ B cells are known to produce anti-DNA antibodies in several lupus mouse models, most likely in a T-cell independent manner [74, 75]. In TLR7-transgenic mice, which mostly develop anti-RNA antibodies, T1 B cells were found to be expanded *in vivo* and produced high levels of autoantibodies *in vitro* [76]. However, the dysregulation of T1 B cells was independent of IFN-I in this mouse model [76]. In SLE patients, transitional B cells are hyper-responsive to IgM crosslinking and IFN-α; the latter stimulation decreases apoptosis and increases proliferation of the B cells [111]. Additionally, age-associated B cells (ABCs), originally found in aging mice, are expanded in MRL-Fas^{lpr}, NZB/W F1 and BXSB mice [104, 112]. This B cell population proliferates robustly in response to TLR9 or TLR7 agonists and can quickly produce IgG class-switched autoantibodies. A corresponding ABC subset has been found in patients with scleroderma and rheumatoid arthritis, but not on SLE patients [112]. At this time, whether pDC-IFN axis induces the activation of non-GC B cell subsets to establish autoimmunity is not clear. **In this chapter, we will characterize different B cell subsets in the amyloid-induced lupus model and identify potential non-GC B cells that are activated by pDCs.**

Chemotaxis of B cells through different areas of lymphoid organs is essential for proper B cell responses. The migration of B cells to outer follicular areas is partially regulated by the EBI-2 signaling pathway. The ligand for EBI-2 is the oxysterol 7α,25-hydroxycholesterol (25-OHC)

[61]. The expression of EBI-2 and the two enzymes required for synthesis of 25-OHC, cholesterol 25-hydroxylase (Ch25h) and oxysterol 7α-hydroxylase (Cyp7b1), are upregulated under inflammatory conditions. Additionally, Ch25h is induced by IFN-I [62]. Mice deficient in *Ch25h* or *Cyp7b1*, which have impaired production of 25-OHC, develop defective T cell-dependent plasma cell responses [113, 114]. We hypothesize that in the amyloid-induced lupus model, Ch25h is upregulated therefore promoting B cell localization into extrafollicular areas. By analyzing autoantibody development in *Ch25h*-deficient mice, **we will determine whether the blockade of extrafollicular responses would affect the generation of self-reactive antibodies.**

3.2 Immature B cells are expanded during break of tolerance

To identify the potential B cell subset that is activated during break of tolerance, we immunized mice with control or amyloid, and quantified the numbers of different B cell subsets. Interestingly, after 3 weeks of amyloid immunization, spleens were bigger (Figure 3.1A) and showed an increased number of B cells (Figure 3.1B) compare to control-immunized mice, suggesting an overt B cell activation. To differentiate between different stages of B cell development and B cell subsets, we utilized the markers CD93, IgM, CD21 and CD23 for their identification by flow cytometry. Notably, immature B cells ($B220^{+}CD93^{+}CD138^{-}$) were increased after amyloid immunization, whereas mature B cells ($B220^{+}CD93^{-}CD138^{-}$) did not differ between the two immunized groups (Figure 3.2A). Among immature B cell subsets, all of the transitional B cells were increased after amyloid immunization; however, T1 B cells ($IgM^{+}CD23^{-}$) were expanded most significantly (Figure 3.2B). The numbers of mature B cell subsets, follicular B cells ($CD23^{+}CD21^{int}$), marginal zone B cells ($CD23^{-}CD21^{+}$) and extrafollicular B cells ($CD23^{-}CD21^{-}$) did not dramatically change after amyloid immunization (Figure 3.2C). ABCs, which have been found expanded in some autoimmune-prone mice, were not altered in our model

(Figure 3.2D). Our data suggested that, during break of tolerance, immature B cells, preferentially T1 B cells, are expanded.

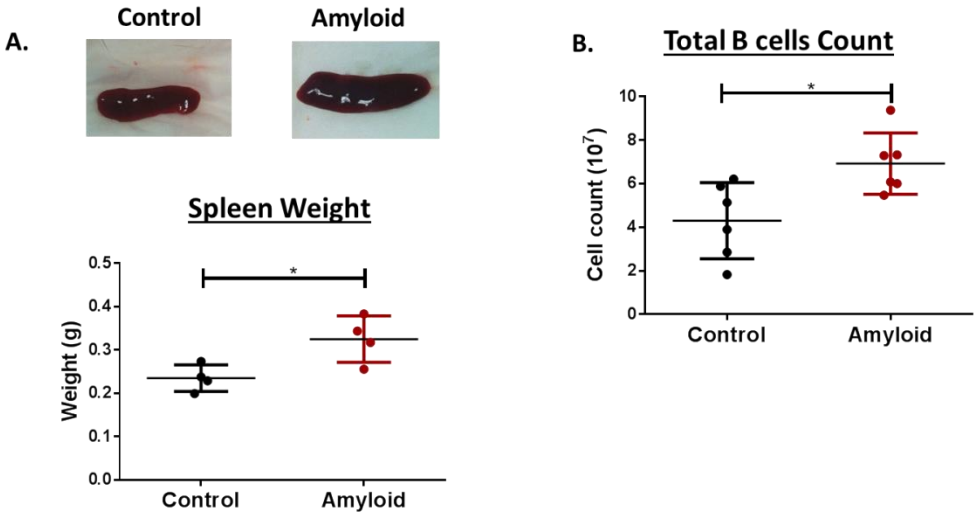

Figure 3.1. B cells are expanded after amyloid immunization.
(A) Balb/c mice were immunized with control or amyloid, and after 3 weeks of immunization spleens were weighted.
(B) $B220^+$ B cells from the spleen were quantified after 3 weeks of immunization.
(A and B) Data represented as means ± SEM. *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups. Similar results were obtained from three additional experiments.

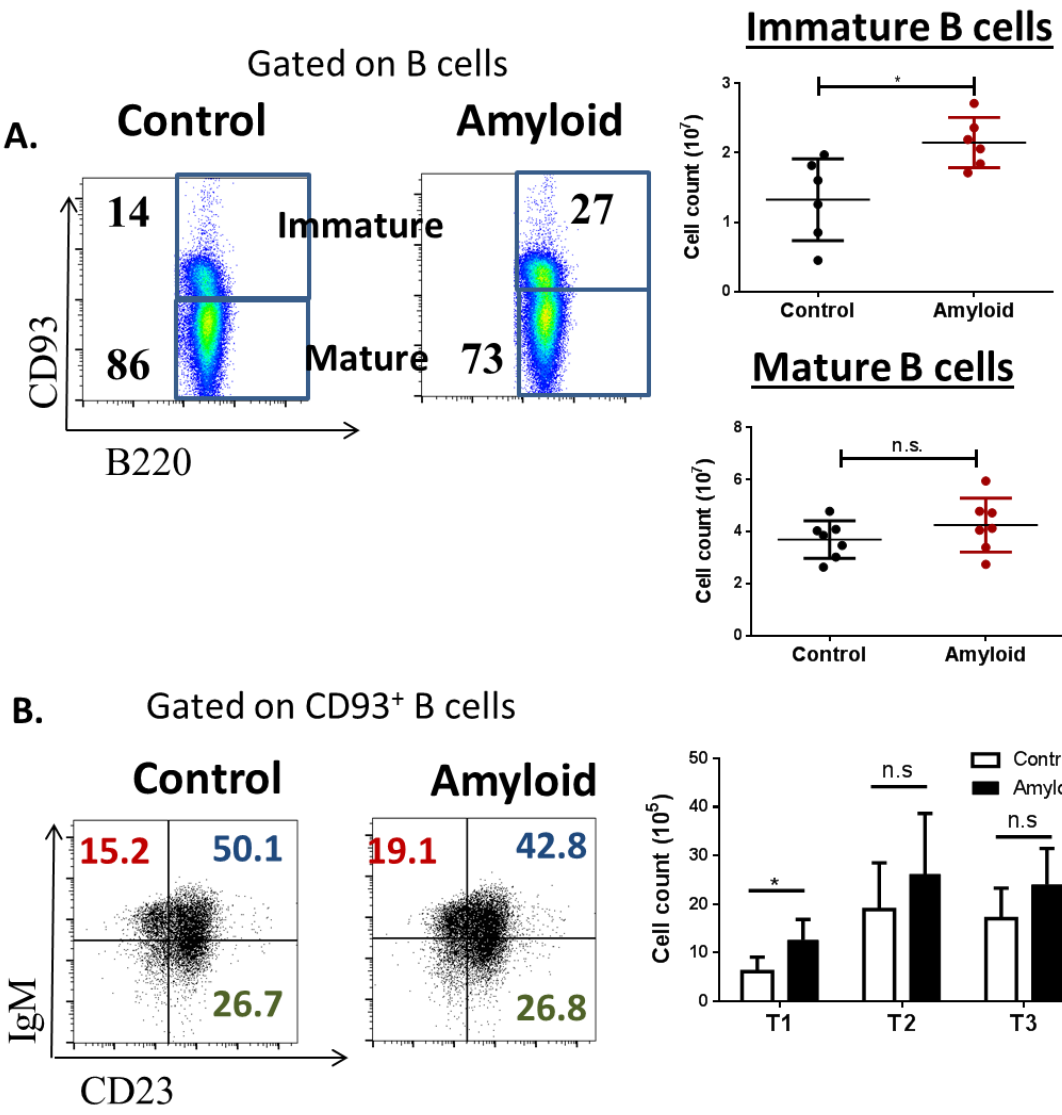
A.
Gated on B cells
Control
Amyloid
14
Immature
27
86
Mature
73
CD93
B220
Immature B cells
Cell count (10^7)
Control
Amyloid
Mature B cells
n.s.
B.
Gated on CD93+ B cells
Control
Amyloid
15.2
50.1
19.1
42.8
26.7
26.8
IgM
CD23
Cell count (10^5)
n.s
T1
T2
T3
C.
Mature B subsets
Cell count (10^6)
FO
MZ
EF
D.
ABCs
% from B220+

Figure 3.2. Transitional B cells are expanded during break of tolerance.
(A) After 3 weeks of immunization, immature ($B220^+CD138^-CD93^+$) and mature ($B220^+CD138^-CD93^-$) B cells were quantified in Balb/c mice. Data from 2 experiments (n = 6).
(B) Transitional B cells were quantified, after gating $B220^+CD138^-CD93^+$, according to their expression of CD23 and IgM (T1 = $CD23^-IgM^+$, T2 = $CD23^+IgM^+$, T3 = $CD23^-IgM^-$)
(C) Mature B cell subsets were quantified in Balb/c mice, after gating $B220^+CD138^-CD93^+$, according to their expression of CD23 and CD21 (FO = $CD23^+CD21^{int}$, MZ = $CD23^-CD21^+$, EF = $CD23^-CD21^-$).
(D) Age-associated B cells were identified as $CD19^+B220^+CD11b^{mid}CD11c^{mid}$ in Balb/c mice from the same samples as in (A).
(B-D) Data from 1 experiment (n = 3). Similar results were obtained from three additional experiments.
(A-D) Data represented as means ± SEM. *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups.
Abbreviations: FO (follicular), MZ (marginal zone), EF (extrafollicular).

3.3 Immature B cells are functionally activated during break of tolerance

To directly determine whether immature B cells are activated during break of tolerance, we sorted different B cell subsets and analyzed mRNA expression of different markers related to IFN signaling, activation and somatic hypermutation (Figure 3.3A). As expected, amyloid stimulated the expression of the ISG *Mx1* in immature B cells (Figure 3.3B). Additionally, immature B cells showed enhanced expression of *Cd80* and *Cd86,* demonstrating activation induced by amyloid (Figure 3.3B). Intriguingly, these cells showed upregulation of *Rag1* and *Rag2*, important genes involved in receptor editing in peripheral lymphoid organs; a process that occurs in self-reactive B cells (Figure 3.3C) [115]. Also, the upregulation of *Aicda* suggests that SHM and class-switching is occurring in these cells (Figure 3.3C). These data suggested that immature B cells responded to IFN, were highly activated and may be undergoing receptor editing, SHM and class-switching during break of tolerance.

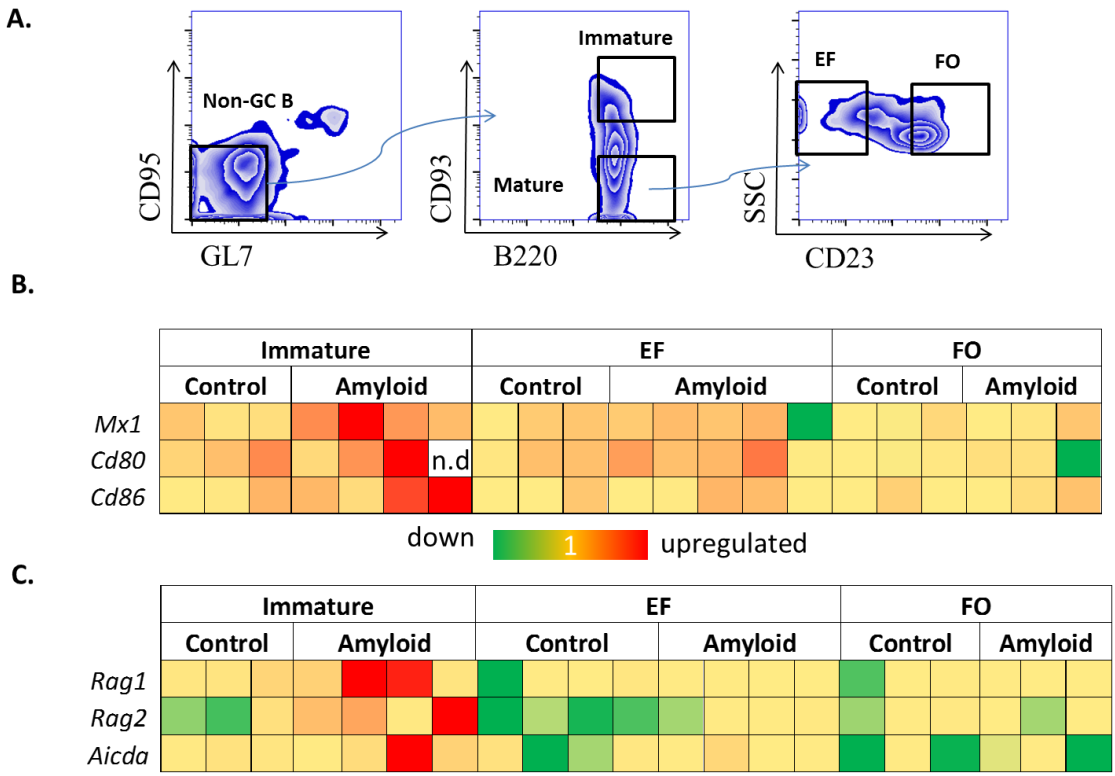

Figure 3.3. Immature B cells show an activated gene profile.
(A) Sorting strategy to isolate different B cell subsets after 2 weeks of immunization. Cells were first gated as $B220^{+}CD138^{-}$.
(B-C) Immature ($CD93^{+}$), extrafollicular ($CD93^{-}CD23^{-}$) and follicular ($CD93^{-}CD23^{+}$) B cells were sorted from WT mice and the mRNA expression of different genes was analyzed by RT-PCR. Data from 2 experiments.
Abbreviations: EF (extrafollicular), FO (follicular).

3.4 Transitional B cells show a type I IFN signature and upregulate genes related to B cell migration

To comprehensively assess the signaling pathways being activated on immature B cells, we sorted T1, T2, and follicular B cells, from control- and amyloid-immunized mice and performed gene expression profiling analysis by microarray (Figure 3.4A). The pathway analysis showed that T1 B cells have enhanced expression of genes related to chemotaxis, cell viability,

proliferation and activation. Not surprisingly, several ISGs were upregulated in immature B cells after amyloid immunization, such as *Iigp1*, *Isg20*, *Ifi44*, *Xaf1*, *Ifitm2*, and *Oasl2* (Figure 3.4B). Additionally, genes involved in B cell trafficking, such as *S1pr2*, *S1pr3*, *Ch25h* and C*yp7b1,* were upregulated after amyloid immunization (Figure 3.4C). Therefore, immature B cells, mostly transitional B cells, have an IFN signature and show changes in their migratory gene profile during the break of tolerance induced by amyloid.

Unexpectedly, several germinal center-specific genes were upregulated on immature B cells after amyloid immunization. The germinal center associated, signaling and motility (Gcsam) gene and the regulator of G-protein signaling (Rgs) -13 were upregulated specifically on T1 and T2 B cells in amyloid-immunized mice (Figure 3.4D). Both *Gcsam*, also known as HGAL, and *Rgs13* are highly expressed on GC B cells [116, 117]. Thus, during break of tolerance, a GC-like program may be induced in transitional B cells.

The pathway analysis also showed that amyloid induces several signaling pathways that are activated by IFN-α and/or IFN-γ (data not shown). It is now clear that many ISGs are induced by both IFN-α and IFN-γ; therefore this was not completely surprising. However, some of the ISGs induced are specifically upregulated by IFN-γ. For example, *iigp1* was highly upregulated on T1 and T2 B cells compared to other B cell subsets after amyloid immunization (Figure 3.4B). These data suggested that both IFN-α and IFN-γ may be produced during break of tolerance and they may impact B cell responses, a topic that will be discussed in Chapter 4.

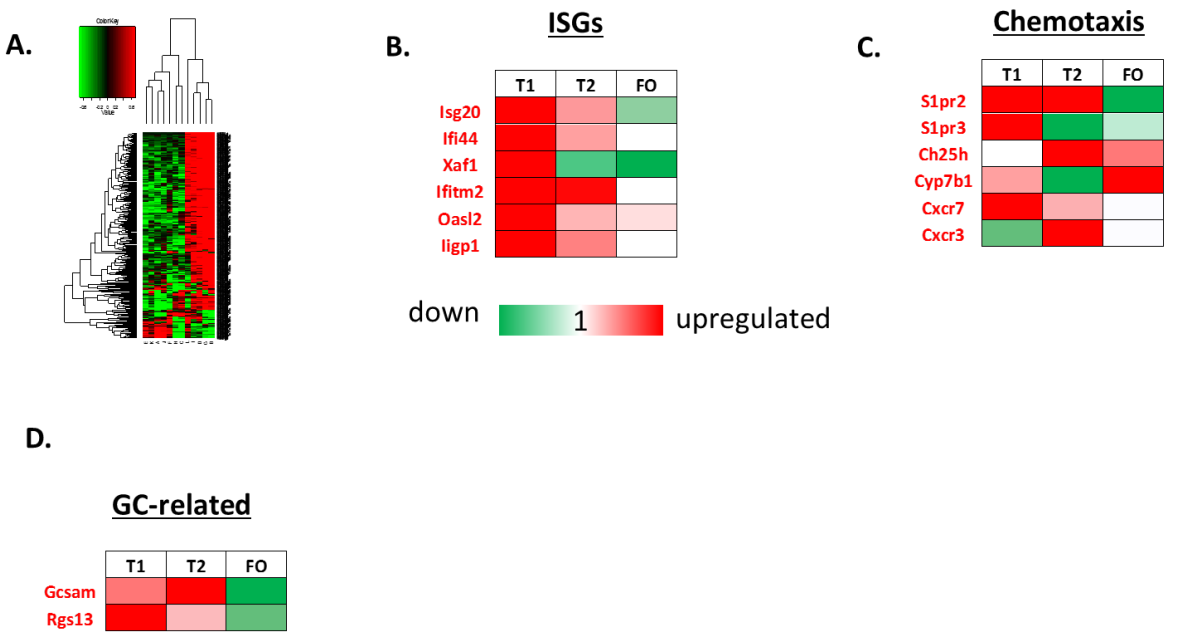

Figure 3.4. Transcriptome of immature B cells.
(A) A microarray analysis was performed on sorted T1, T2 and follicular (FO) B cells after 2 weeks of immunization. The color map of the microarray data is depicted.
(B) The expression of ISGs in the three B cell populations is depicted. Gene expression is compared to each B cell subset from control immunized mice.
(C) The expression of chemotaxis-related genes was determine as in (B).
(D) GC-related genes were analyzed as in (B and C).
(A-D) Collaborators: Philip Brohawn and Christopher A. Morehouse from MedImmune LLC performed the microarray analysis.

3.5 Plasmacytoid dendritic cells are required for the activation of immature B cells

To determine whether pDCs are involved in the activation profile of immature B cell subsets, we sorted T1 and T2 B cells from WT or *feeble* mice, which have defective TLR signaling on pDCs, and analyzed the expression of relevant genes in B cells by amyloid (Figure 3.3 and 3.4). We could observe that *Mx1*, *Rag1*, *Rag2* and Aicda were upregulated on both transitional B cell subsets from C57BL/6 mice but not in *feeble* mice (Figure 3.5A and B). In addition, upregulation of the GC-related genes *Rgs13* and *Gcsam* in T1 B cells required pDC function (Figure 3.5). These data demonstrates that pDCs are required for the activation of immature B cells.

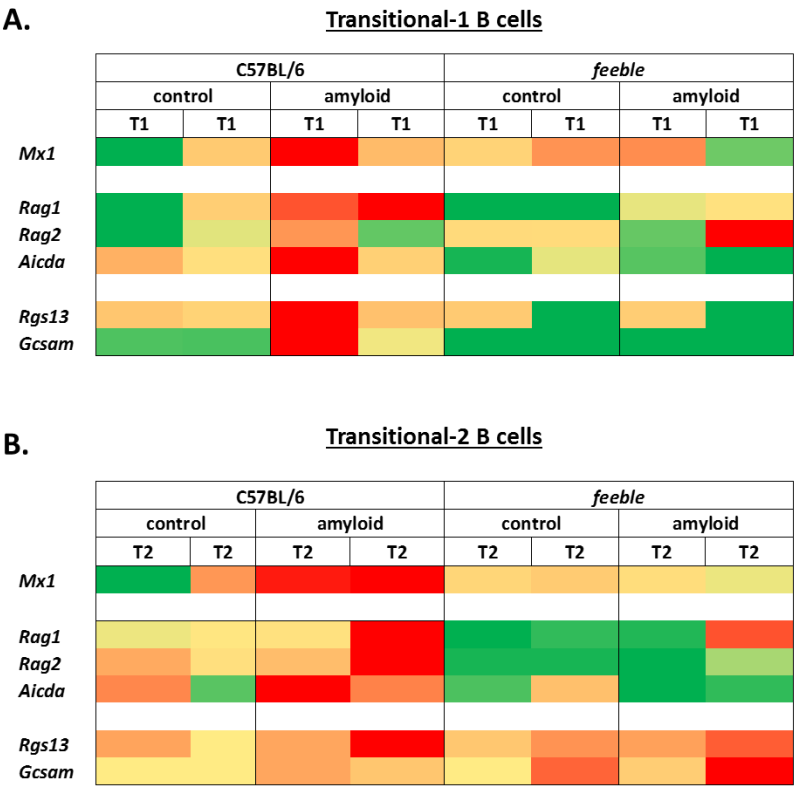

Figure 3.5. pDCs promote the activation of transitional B cells.
(A) T1 ($CD93^+CD23^-IgM^+$) B cells were sorted after 2 weeks of immunization from C57BL/6 or *feeble* mice and the mRNA expression of different genes was analyzed by RT-PCR.
(B) Gene expression was analyzed in T2 B cells ($CD93^+CD23^+IgM^+$) as in (A). Data from 1 experiment.

3.6 The oxysterol pathway and Rgs13 are redundant for autoantibody development.

The microarray data showed that immature B cells upregulated genes related to migration during break of tolerance. Therefore, we wanted to determine the role of novel chemotaxis pathways in humoral autoimmunity. Interestingly, transitional B cells expressed higher levels of *Ch25h* and *Cyp7b1* after amyloid immunization (Figure 3.4C). We further confirmed this observation by RT-PCR and showed that immature B cells significantly upregulated *Ch25h* and *Cyp7b1* after amyloid immunization (data not shown). The expression of these enzymes, which

are known to stimulate EBI-2 signaling, may instruct a preferential positioning of immature B cells to extrafollicular areas.

Deficiency in Ch25h prevents the transient movement of B cells outside the follicles at early stage of B cell response [113]. To determine whether the oxysterol pathway is involved in the initiation of autoimmunity, we immunized *Ch25h*$^{-/-}$ mice with control or amyloid and analyzed GC development and generation of autoantibodies in the serum. Although we observed an increase in GC B cell numbers after control immunization in Ch25h-deficient mice, amyloid-mediated GC inhibition was not affected in these mice (Figure 3.6A). In addition, the levels of anti-histone IgM antibodies were similar between WT and *Ch25h*$^{-/-}$ mice at 3 weeks of immunization (Figure 3.6B). We also observed that Ch25h deficiency did not affect the development of class-switched autoantibodies (Figure 3.6C). These data suggested that, even though *Ch25h* is upregulated on B cells during break of tolerance, EBI-2-mediated chemotaxis pathway may be redundant for autoantibody generation and other mechanisms may be involved in B cell migration.

Rgs13 belongs to the RGS family, which modulates the activity of the heterotrimeric G proteins, including all chemokine receptors [118]. We confirmed by RT-PCR that Rgs13 was upregulated on immature B cells after amyloid immunization (Figure 3.5). To determine whether Rgs13 modulates B cell responses, we induced autoimmunity in Rgs13-deficient mice, which were previously generated by Hwang IY, 2013 [117]. However, GC B cells were similar between WT and knockout mice after control or amyloid immunization (Figure 3.6D). Furthermore, *Rgs13* deficiency did not affect the early nor late autoantibody production (Figure 3.6E, 3.6F). These data suggested that Rgs13 signaling may be redundant for B cell responses in pDC-mediated autoimmunity.

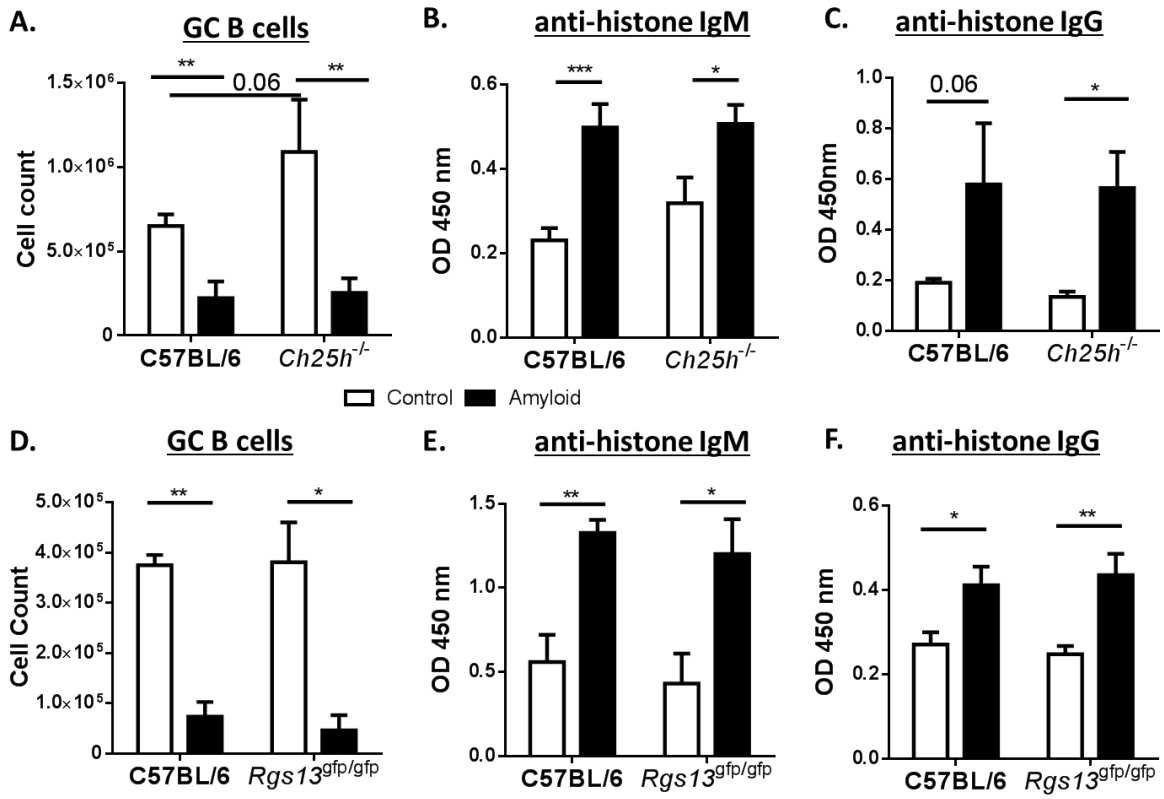

Figure 3.6. The oxysterol pathway and Rgs13 may be redundant on controlling GC migration and autoantibody generation.
(A and D) After 3 weeks of immunization, GC B cells were quantified on spleens by flow cytometry on the corresponding mouse strains.
(B and E) The levels of anti-histone IgM antibodies were detected on the sera by ELISA on the corresponding mouse strains after 3 weeks of immunization.
(C and F) The levels of anti-histone IgG antibodies were detected on the sera by ELISA on the corresponding mouse strains after 7 weeks of immunization.
(A – F) Data represented as means ± SEM (n = 3 – 6). *P* values: **p*<0.05, ***p*<0.005 and ****p*<0.0005. Student's *t*-test was performed to detect statistical difference between groups.

3.7 Discussion

The origin of B cells responsible for the break of tolerance and humoral autoimmunity in response to type I interferon stimulation has not been identified. We have determined that, in the amyloid-induced lupus model, the expansion of immature B cells correlated with the development of self-reactive antibodies. This B cell subset was activated and displayed a transcriptional profile of dysregulated lymphocyte trafficking and SHM. pDCs seemed to promote overt activation of these immature B cells.

Interestingly, not only IFN-I but also IFN-II have been linked to SLE development. Recently, a study using modular repertoire analysis revealed that SLE patients not only contain a type-I IFN signature but also involves IFN-γ [119]. This was not completely surprising due to the largely overlapping inducible gene signature between type-I and type-II IFNs [120]. In fact, both IFN-α and IFN-γ have been found elevated in SLE serum [121]. Also, like IFN-α, IFN-γ treatment occasionally triggers lupus-like disease. Interestingly, IFN-γ is required for the development of autoimmunity in MRL-Fas^{lpr}, NZB/W F1 mice, and in pristane-induced and chemically-induced lupus models [122-124]. In the amyloid-induced model, we have determined that dual type I and type II IFN signatures exist in immature B cells during break of tolerance. However, the exact mechanism by which both IFN-I and IFN-II trigger self-reactivity is still unclear. In the following chapter, the role of IFN-II in the development of autoantibodies will be studied.

Immature B cells have been identified as a potential self-reactive population. In peripheral blood of SLE patients, increased frequencies of pre-immune B cells, including transitional B cells, were detected previously [69, 70]. These transitional B cells are hyper-responsive to IgM crosslinking and IFN-α, which induces enhanced survival and proliferation *in vitro* [111]. Interestingly, immature B cells were found expanded and activated during break of tolerance

after amyloid immunization. This is similar to the expansion of T1 B cells observed in TLR7-transgenic mice [76]. Thus, we propose that immature B cells are the self-reactive B cell subset after IFN-I and IFN-II production during break of tolerance. Due to the low frequency of self-reactive B cells and mostly low affinity BCRs towards the self-antigen, the direct identification of autoimmune B cells has been challenging. Recently, tetramers from linear autoepitopes have been developed to identify La and small nuclear ribonucleoprotein reactive B cells in mice [125]. The utilization of similar tools can help us identify self-reactive B cells in our lupus model.

The mechanism by which IFN-I promotes the activation of immature B cells is not well understood. However, IFN-I upregulates BAFF expression by myeloid cells [126]. Moreover, BAFF signaling in immature B cells promotes their survival and proliferation [67]. Therefore, it is likely that in our system IFN-I promotes the proliferation of immature B cells through the induction of BAFF. The direct role of BAFF in the amyloid-induced lupus model needs to be further investigated to confirm our hypothesis.

Ch25h seemed redundant for GC development and autoantibody production in the amyloid-induced autoimmune model. Ch25h catalyzes the production of 25-OHC, the most potent ligand for EBI-2, which is an important pathway for B cell migration. In EBI2-deficient mice, GC size was not affected; however, plasmablast-derived antibodies were reduced only transiently after antigen immunization [61]. Therefore, it has been suggested that EBI-2 signaling mostly affect the early migratory events occurring after B cell activation and that additional chemokine receptors, such as CXCR4, CXCR5 and CCR7 participate after GCs are formed. The possible redundancy of chemokine and oxysterol pathways complicates the previous study and our current analysis alike. The examination of mice deficient in two or more components of B cell trafficking would help elucidate the importance of B cell positioning in autoantibody production during break of tolerance.

Similarly, Rgs13 is redundant in modulating B cell responses during autoimmunity. Rgs13 is highly expressed by GC B cells and lymphomas; however, there are conflicting reports regarding the role of Rgs13 in GC development and autoimmunity. One study indicates that Rgs13 limits the GC response, whereas another publication suggests that Rgs13 prolongs the GC program and exacerbate autoimmunity [117, 127]. In our study, we did not observe major differences on GC size or autoantibody production in Rgs13-deficient mice after amyloid immunization. These results could be due to the redundant functions of the 20 different RGS; thus their overall physiological roles remain to be dissected [128].

Surprisingly, another GC-specific gene was found upregulated on immature B cells, during break of tolerance. GCSAM, also known as HGAL, is specifically expressed in GC B cells and GC-derived B cell lymphomas. It has been demonstrated that GCSAM enhances Syk activation after BCR signaling, which increases B cell proliferation [129]. It is interesting to note that Syk hyperactivation has been previously observed in B cells from SLE patients [130]. Mice transgenic for human *GCSAM* developed polyclonal B cell lymphoproliferation and amyloidosis, suggesting a defect in B cell selection [116]. We speculate that the upregulation of *Gcsam* on T1 B cells after amyloid immunization relates to an enhanced B cell activation and reduced negative selection during break of tolerance. Further studies need to be performed to determine the role of Gcsam during autoimmunity.

Our data suggest that pDC activation may be involved in the overt activation of immature B cells and further development of self-reactive B cell clones.

CHAPTER 4.

IMMUNE HELPER CELLS INVOLVED IN PLASMACYTOID DENDRITIC CELL-MEDIATED HUMORAL AUTOIMMUNITY

Portions of this chapter are based on Huang X., Li J., Dorta-Estremera S., Di Domizio J., Anthony SM., Watowich SS., Popkin D., Liu Z., Brohawn P., Yao Y., Schluns KS, Lanier LL., Cao W., "Neutrophils regulate humoral autoimmunity by restricting interferon-γ production via the generation of reactive oxygen species". Cell Reports (2015) 12(7): 1120-32. doi:10.1016/j.celrep.2015.07.021 [90]. This article is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND). Collaborator efforts are outlined in the Figure legends whenever necessary.

4.1 Rationale

Different immune helper cells are known to promote B cell responses. Among these cells, T cells provide a second signal for efficient B cell activation. While inside GCs, Tfh cells can provide co-stimulation to GC B cells; in extrafollicular areas, an extrafollicular T helper (Tefh) cell population is present. Whether Tefh cells are functionally relevant in autoimmunity is still a matter of speculation [131, 132]. Due to the presence of additional immune populations in extrafollicular areas, it is possible that non-classical helper cells may modulate extrafollicular responses.

Both IFN-α and IFN-γ are known to be involved in mouse and human SLE. Moreover, deficiency of IFN-I or IFN-II signaling prevents the development of autoimmune phenotype in mice [38, 133]. In the amyloid-induced lupus model, we have identified both IFNs as major inducers of B cell activation. Therefore, it is relevant to determine the mechanisms how IFN-I and IFN-II promote humoral autoimmunity.

T cells, NK cells and NKT cells are the major producers of IFN-γ; however, **the source of IFN-γ that triggers autoimmunity is still unclear.** As for T cells, a larger number of the IFN-γ-producing T_{H1} cells have been found in SLE patients and autoimmune-prone mice [134-136]. Also, an increase proportion of $CD56^{bright}$ NK cells, which have lower cytotoxicity and increased IFN-γ-secreting capacity, is observed on the periphery of patients with active SLE [137, 138]. NKT cells have also been found to produce both IL-4 and IFN-γ in type 1 diabetes and experimental allergic encephalomyelitis mouse models [139]. Whether these immune cell subsets are activated downstream of pDC-IFN-I axis and modulate humoral autoimmunity is not clear.

In this Chapter, we will identify the key cellular players that modulate B cell responses in pDC-mediated humoral autoimmunity. Since IFN-γ is induced in our model, we will study the

contribution of this cytokine and the function of IFN-γ-producing cells in autoimmune development in the amyloid-induced lupus model.

4.2 T cells are required for humoral immune responses to immunogen and self-antigens

T cells are required for the development of GC-derived antibody responses. To determine the role of T cells in humoral responses in our inducible lupus model, we immunized TCRβ/δ-deficient mice with control or amyloid. As shown in Figure 4.1A, these mice did not develop anti-nuclear antibodies. Not surprisingly, they did not develop anti-HSA antibodies (Figure 4.1B). Thus, our data suggested that the development of IgG-class switched autoantibodies and immunogen-specific antibodies are dependent on T cells.

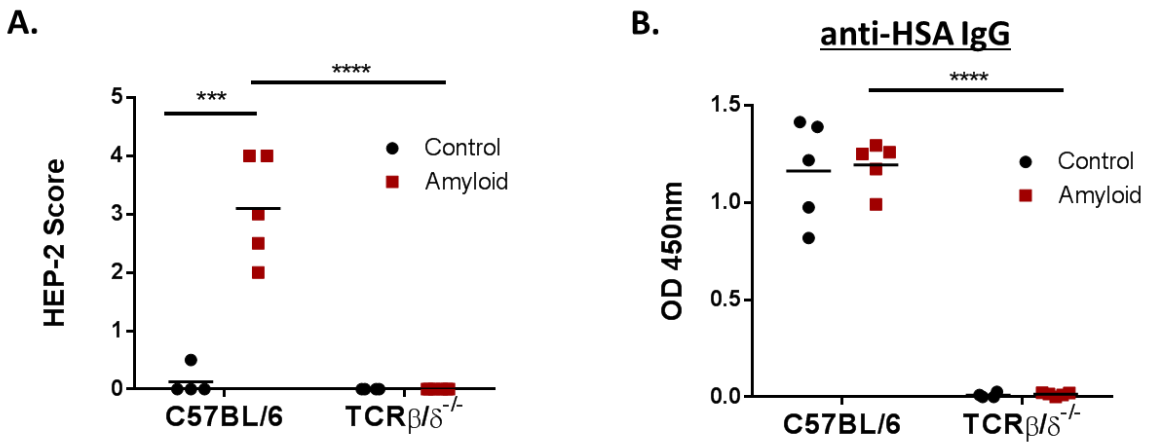

Figure 4.1. T cells are required for the development of self- and immunogen-specific antibodies.
(A) Sera from C57BL/6 or TCRβ/δ-/- mice were collected after 7 weeks of immunization and tested for anti-nuclear reactivity on HEP-2 cells. Data from 2 experiments.
(B) Levels of anti-HSA IgG antibodies were detected by ELISA after 7 weeks of immunization.
(A and B) Data represents one experiment. Similar results in 2 additional experiments; 4 replicates per experiments. Data represented as means. *P* values: ****p*<0.0005, *****p*<0.00005. Student's *t*-test was performed to detect statistical difference between groups.

4.3 Amyloid immunization induces Tfh cell development

Tfh cells are an essential helper population involved in GC development. Since amyloid can activate pDCs to produce IFN-I, we wanted to determine whether amyloid-induced pDC activation induces the development of Tfh cells *in vitro*. We set up a co-culture system, where CD11c+ cells, which included both cDCs and pDCs, were mixed with CD4+ T cells from $Bcl6^{yfp/+}$ mice. After 3 days of stimulation, we analyzed surface markers by flow cytometry to quantify the Tfh cells. Interestingly, amyloid, but not control, effectively induced the generation of $CD4^+CD44^+CXCR5^+PD1^+$ Tfh cells (Figure 4.2A). To confirm this finding *in vivo*, we examined the number of Tfh cells in spleen after amyloid immunization. Strikingly, Tfh cells, identified as $CD4^+CD44^+ICOS^+CXCR5^+PD1^+$*Bcl6*$^+$, were significantly increased 1 week after immunization (Figure 4.2B). However, the numbers of Tfh cells dramatically reduced after 2 weeks and became comparable with the mice receiving control-immunization (Figure 4.2C), which follows normal GC kinetics [99]. At 3 weeks, there were no significant differences between Tfh cells from control- or amyloid-immunized mice, although the number of GC B cells was reduced in mice immunized with amyloid (Figure 2.3).

Also, Tfh cell population after 1 week of immunization showed a normal transcriptional program: when compared with naïve T cells, Tfh cells upregulated *Bcl6*, *Icos*, *Cxcr5* and *Il21*. Although a slight reduction on *Bcl6* expression is observed in Tfh cells from amyloid-immunized mice, these cells showed increased expression of *Icos*, *Cxcr5* and *Il21* compared to control immunized mice, indicating a strong functional activation (Figure 4.2D) [140]. Altogether, these data suggest that the inhibition of GCs, triggered after pDC-mediated break of tolerance, is independent of Tfh cells. Additionally, the number of $CXCR5^-$ T cells, which contain Tefh cells, dramatically reduced after 3 weeks of amyloid immunization (Figure 4.2E), implying that Tefh cells were not likely induced by amyloid. This is consistent with our preliminary data showing that T cell-deficiency did not affect IgM autoantibody production at early stages of autoimmunity

(data not shown). Thus, another helper cell type may modulate the extrafollicular responses generated at the initiation of autoimmunity.

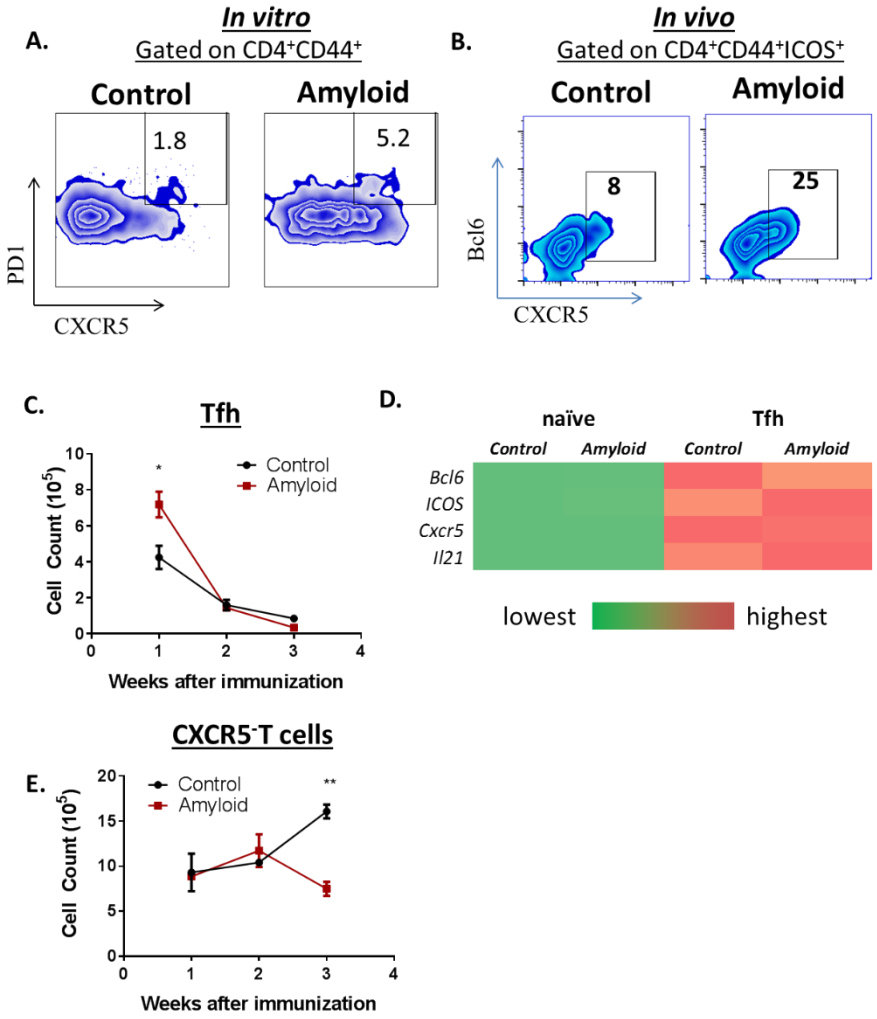

Figure 4.2. Amyloid induces Tfh cell expansion.
(A) $CD4^+CD44^+PD1^+CXCR5^+$ T cells were detected by flow cytometry after 3 days of co-culture of $CD11c^+$ cells and $CD4^+$ T cells with control or amyloid. Similar results were obtained in 2 additional experiments; 2 replicates per experiment.
(B) Spleens from $Bcl6^{yfp/+}$ mice immunized with control or amyloid were analyzed by flow cytometry to detect Tfh cells after 1 week of immunization.
(C) Tfh cells were quantified as in (B) at different time points. Data representative of one experiment (n = 3). Similar results were obtained in 2 additional experiments; 3 replicates per experiment.
(D) $CD4^+CD44^-ICOS^-$ (naïve) or $CD4^+ICOS^+CD44^+CXCR5^+PD1^+$ (Tfh) cells were sorted after 1 week of immunization and the expression of several genes was determined by RT-PCR. Data from one experiment.
(E) Cell numbers of $CD4^+ICOS^+CD44^+CXCR5^-$ T cells were quantified at different time points from the samples in (C).
(C and E) Data represented as means ± SEM. *P* values: **p*<0.05, ***p*<0.005. Student's *t*-test was performed to detect statistical difference between groups.

4.4 Interferon-γ is produced by NK cells during break of tolerance

We previously demonstrated that B cells show a type II interferon signature after amyloid immunization (Figure 3.4). To determine whether IFN-γ is actually produced after amyloid inoculation, we quantified its levels in the peritoneal cavity of mice receiving control or amyloid. After 24 hrs, IFN-γ was detected in mice injected with amyloid (Figure 4.3A). To determine whether IFN-γ is produced in the spleen, we examined IFN-γ production by different immune cell types by flow cytometry. Interestingly, NK cells, identified as $CD3^{-}NK1.1^{+}NKp46^{+}$, but not $CD4^{+}$ or $CD8^{+}$ T cells, produced elevated IFN-γ protein after amyloid immunization (Figure 4.3B). NK cells not only produced cytokines, but were also expanded in the spleen (Figure 4.3C). Interestingly, these cells upregulated the NK cell receptor, NKp46, after amyloid immunization (Figure 4.3D). NK cells are known to be cytotoxic; however, this expanded NK cell population expressed low levels of CD107a, a marker for degranulation, and TRAIL, an effector molecule mediating cytotoxicity (Figure 4.3E and 4.3F). Thus, NK cells, rather than T_{H1} cells, are the predominant IFN-γ-producing cell in our model.

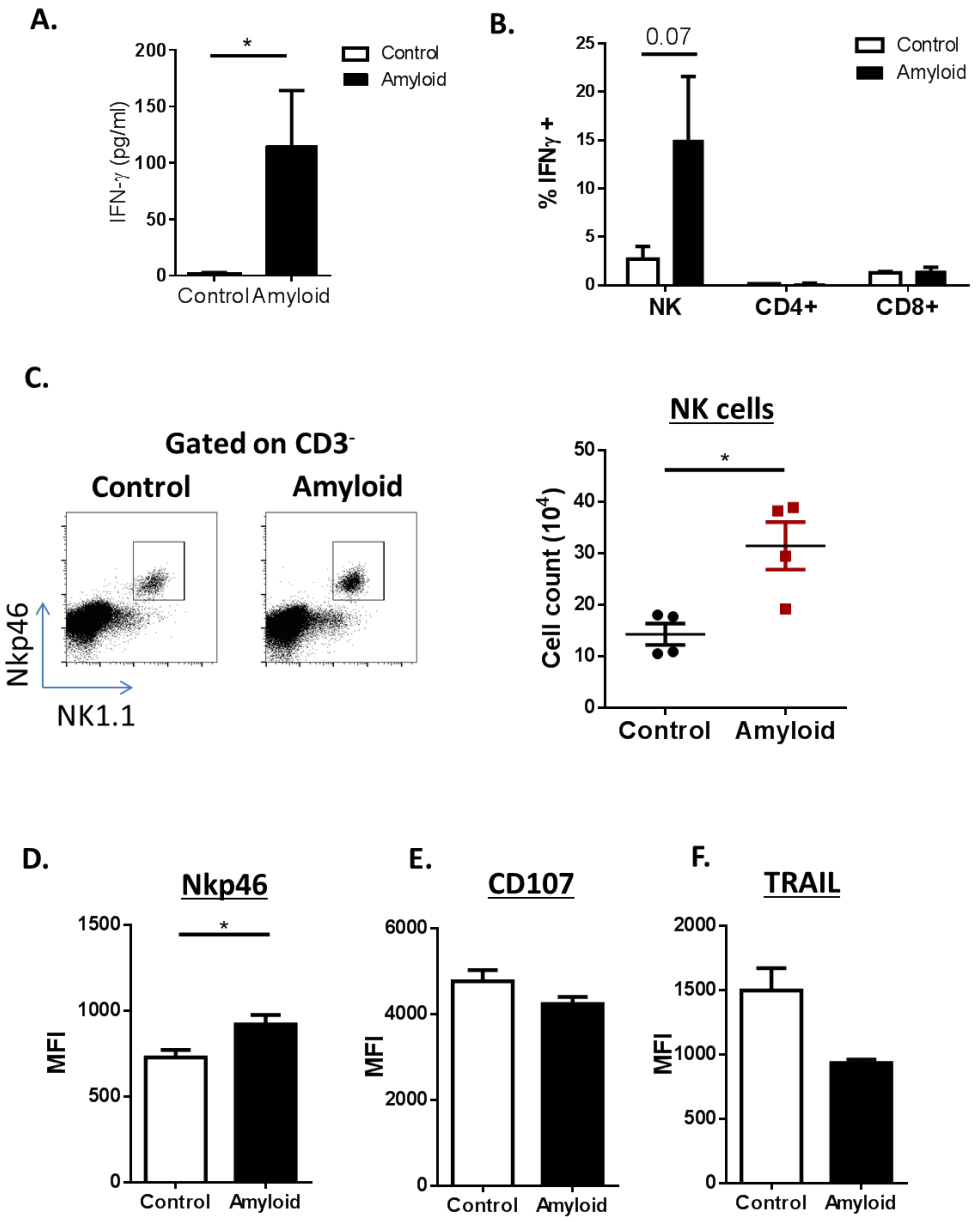

Figure 4.3. IFN-γ is produced by NK cells during break of tolerance.
(A) The levels of IFN-γ were quantified in the peritoneal fluid after 24 hrs of control or amyloid inoculation in C57BL/6 mice. (n = 6). Collaborators: Dr. Jingjing Li performed the analysis on peritoneal fluids
(B) The percentage of IFN-γ^+ cells was quantified in the spleen after 1 week of control or amyloid injection. Data represents one experiment; means ± SEM (n = 2-3).
(C) NK cells were quantified after 2 weeks of immunization. Data represents 2 pooled experiments.
(D-F) The expression levels of D) Nkp46, E) CD107 and F) TRAIL on NK cells was determined by flow cytometry (n = 4).
(A-F) Data represented as means ± SEM. *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups.

4.5. Type I and type II IFNs enhance T cell and B cell activation *in vitro*

Since IFN-γ is produced in our *in vivo* model, we wanted to determine whether IFN-γ affects the differentiation of IFN-α-induced Tfh cells. To induce IFN-α, we stimulated $CD11c^+$ cells with the TLR-9 agonist, CpG A. By using a co-culture system similar to the one described in Figure 4.2A, we determined that CpG A-stimulated $CD11c^+$ cells induced the development of Tfh cells. Although IFN-γ alone did not induce *Bcl6*$^+$ Tfh cells, IFN-γ enhanced the development of Tfh cells after CpG A stimulation (Figure 4.4). This result demonstrates a cooperative effect between IFN-α and IFN-γ in promoting Tfh cell differentiation.

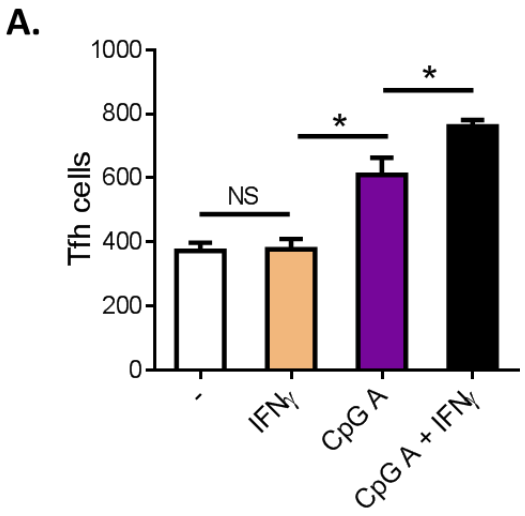

Figure 4.4. Type I and type II interferon cooperate to generate Tfh cells *in vitro*.
(A) Numbers of Tfh cells in the mixed culture of *Bcl6*$^{yfp/+}$ $CD4^+$ T cells and $CD11c^+$ DCs were quantified after 3 days of culture with the corresponding stimuli. Live cells were gated on $CD4^+CD44^+ICOS^+CXCR5^+$*Bcl6*$^+$ population. Data represents 2 pooled experiments as means ± SEM (n=4). *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups.

To determine whether IFN-I and IFN-II directly affect autoantibody production by B cells, we cultured enriched B220^{+} cells from the autoimmune-prone strain NZB/W F1 in the presence or absence of IFN-α and/or IFN-γ. IFN-γ alone, but not IFN-α, enhanced autoantibody production (Figure 4.5). Interestingly, the combination of IFN-α and IFN-γ significantly boosted the secretion of anti-histone IgM and anti-ssDNA IgM. B cells from WT mice did not produce autoantibodies even in the presence of two IFNs *in vitro* (data not shown), suggesting that additional mechanisms must exist to break B cell tolerance. Altogether, our data shows that IFN-α and IFN-γ cooperate to enhance autoantibody production *in vitro*.

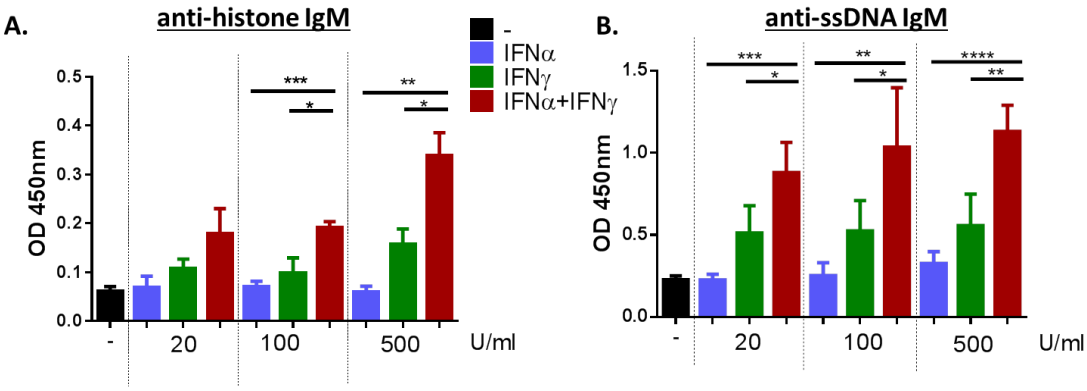

Figure 4.5. IFN-I enhances IFN-II induction of autoantibodies *in vitro*.
(A) Enriched B220+ cells from NZB/W F1 mice were cultured with recombinant IFN-α and/or IFN-γ at different doses. The supernatant was collected after 7 days of culture and levels of anti-histone IgM antibodies were detected by ELISA.
(B) Levels of anti-ssDNA IgM were detected from the samples in (A).
(A and B) Data represented as means ± SEM (n = 4). *P* values: *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005. Student's *t*-test was performed to detect statistical difference between groups.

4.6 Interferon-γ is required for the development of humoral autoimmunity

To determine the role of IFN-γ in humoral autoimmunity, we analyzed the ability of *Ifng*$^{-/-}$ mice to develop humoral autoimmunity induced by amyloid. In contrast to WT mice, *Ifng*-

deficient mice did not develop anti-nuclear antibodies, as analyzed by HEP-2 staining (Figure 4.6A). Similarly, we have observed that the anti-self antibodies induced in our model are IgG2a/c and IgG2b subclasses (Figure 2.8C), which are known to be the most pathogenic isotypes in lupus [141]. Interestingly, *Ifng*$^{-/-}$ mice failed to develop self-reactive antibodies of the IgG2a and IgG2b subclasses after amyloid immunization (Figure 4.6B). These observations are consistent with the prevailing role of IFN-γ in driving pathogenic antibody isotypes [142].

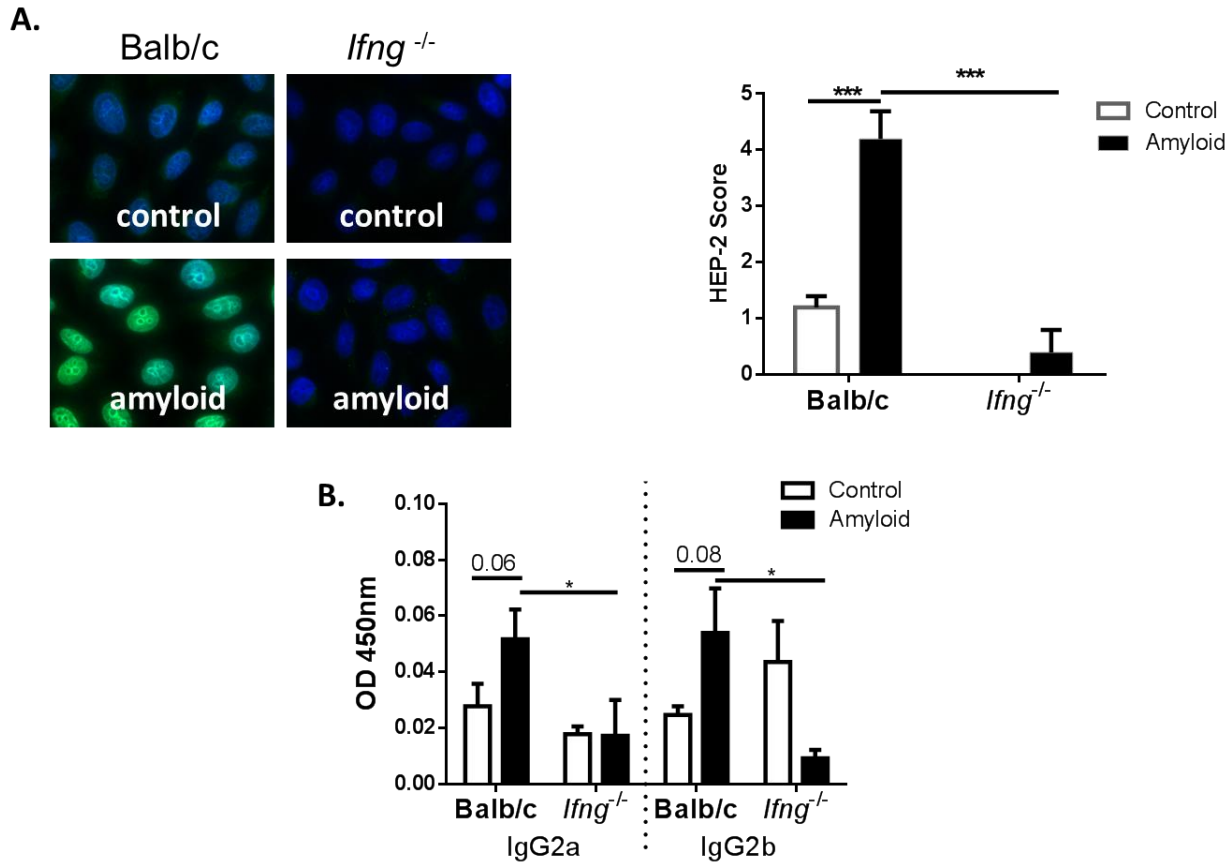

Figure 4.6. IFN-γ is required during humoral autoimmunity.
(A) Anti-nuclear IgG antibodies were detected in sera from Balb/c or *Ifng*$^{-/-}$ immunized with control or amyloid (n=5).
(B) The levels of anti-histone IgG subclasses were detected in the sera after 7 weeks of immunization. Data represents one experiment (n=4).
(A and B) Data represented as means ± SEM. *P* values: *p<0.05, ***p<0.0005. Student's *t*-test was performed to detect statistical difference between groups.

To determine the role of IFN-γ during break of tolerance, we immunized WT or *Ifng*$^{-/-}$ mice with control or amyloid after 3 weeks of immunization. Interestingly, *Ifng*$^{-/-}$ mice showed a decrease in anti-histone IgM antibodies after amyloid immunization compared to WT mice (Figure 4.7A). Since we have observed that T1 B cell expansion correlates with anti-histone IgM antibody levels, we wanted to determine whether this population is affected in mice deficient on IFN-γ. Intriguingly, T1 B cells from *Ifng*$^{-/-}$ mice failed to expand to the same extent as T1 B cells from WT mice (Figure 4.7B). Thus, our data revealed that IFN-γ play an essential role in the break of tolerance by inducing the expansion of T1 B cells and enabling pathogenic class switch of self-reactive antibodies.

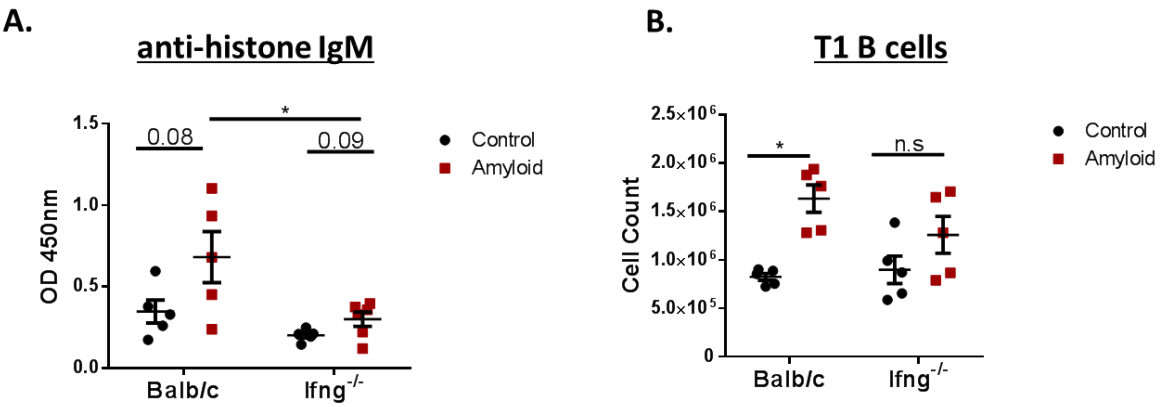

Figure 4.7. IFN-γ is required for the break of tolerance and generation of T1 B cells.
(A) After 3 weeks of immunization, levels of anti-histone IgM antibodies were detected from the sera of the corresponding mouse strains.
(B) Numbers of T1 B cells ($B220^+CD93^+CD23^-IgM^+$) were quantified in the spleen of the respective mouse strains.
(A and B) Data represents 2 pooled experiments. Data represented as means ± SEM. *P* values: **p*<0.05. Student's *t*-test was performed to detect statistical difference between groups.

4.7 NKT cells suppress the development of humoral autoimmunity

Remarkably, NK cells, instead of Th1 cells, were the producers of IFN-γ in our inducible model (Figure 4.3B). To determine whether NK cells are involved in the development of autoimmunity, we injected mice with anti-NK1.1 antibody PK136 prior to control- or amyloid-immunization. Surprisingly, this treatment increased the development of anti-nuclear antibodies (Figure 4.8A). This result may be due to the depletion of not only NK cells but also NKT cells that express NK1.1. Since NKT cells have been found to be suppressive during humoral autoimmunity [81, 82], it is important to dissect the role of NKT cells in our model.

To determine the role of NKT cells in humoral autoimmunity, we immunized CD1d-deficient mice that lack NKT cells, with control or amyloid [143]. Interestingly, *Cd1d*$^{-/-}$ mice developed higher anti-histone IgG subtypes, specifically IgG2a and IgG2b (Figure 4.8B). This is similar to previous reports showing that CD1d-deficiency worsened autoantibody production [81, 82]. Moreover, Vα14-Jα18-transgenic mice, which have an expanded population of NKT cells, showed a reduction in anti-nuclear autoantibodies after amyloid immunization (Figure 4.8C) [144]. A similar reduction was also observed when comparing anti-histone IgG (Figure 4.8D). Therefore, the enhanced autoantibody levels observed after PK136 treatment is likely due to the depletion of NKT cells. Altogether, we determined that NKT cells inhibit isotype-class switching of autoantibodies.

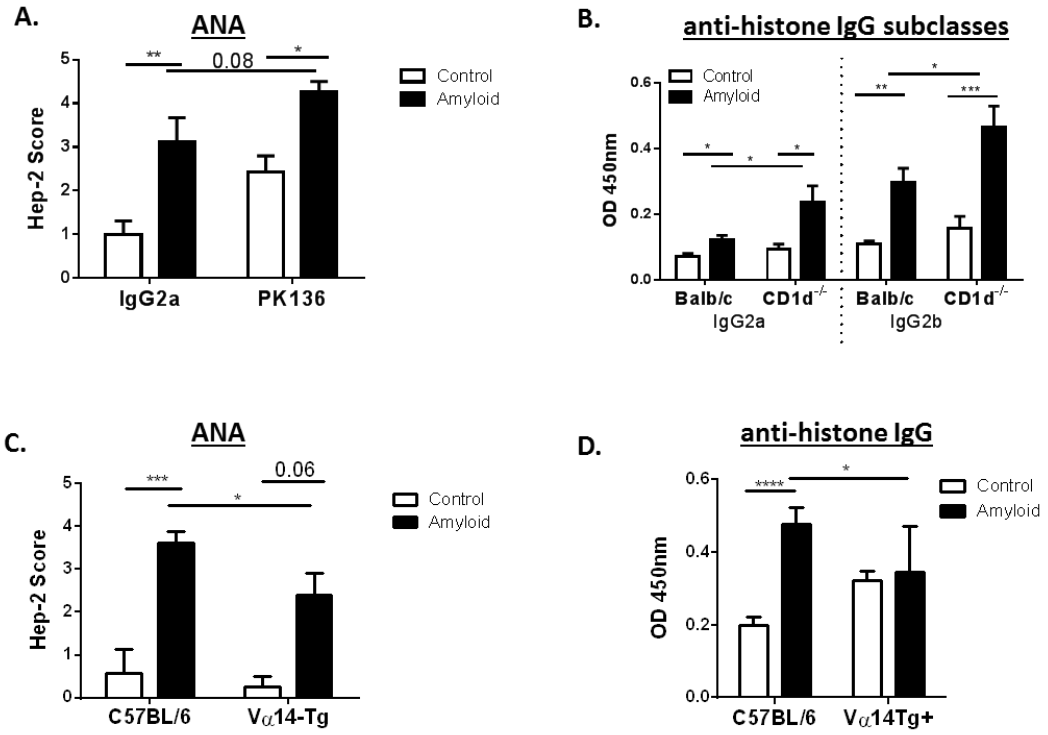

Figure 4.8. NKT cells suppress autoantibody development.
(A) After 7 weeks of immunization, anti-nuclear antibodies were detected in the sera of mice pre-treated with PK136 mAB or IgG2a mAb 24 hrs prior to immunization (n = 7 – 8). Collaborator: Dr. Jingjing Li performed this experiment.
(B) The levels of anti-histone IgG subclasses were detected in the sera of Balb/c or *CD1d*$^{-/-}$ after 7 weeks of immunization (n = 8 – 17).
(C) ANA were detected in the sera after 7 weeks of immunization on C57BL/6 or Vα14-Tg mice (n = 2 – 10).
(D) Levels of anti-histone IgG antibodies from were detected in the sera from (C) by ELISA .
(A-D) Data represented as means ± SEM. *P* values: **p*<0.05, **p<0.005, ***p<0.0005, ****p<0.00005. Student's *t*-test was performed to detect statistical difference between groups.

4.8 NK cells promote humoral autoimmunity partly through NKp46

NK cells express several lineage-specific receptors, which are responsible for NK cell activation. Mouse NK cells only express *Ncr1* (known as NKp46). Interestingly, we have observed an upregulation of NKp46 on NK cells after amyloid immunization (Figure 4.3D), suggesting a role of this receptor in NK cell function. To determine the function of this activating

receptor during humoral autoimmunity, we analyzed mice deficient of NKp46 (referred as *Nkp46*$^{gfp/gfp}$). The insertion of a green fluorescent protein (GFP) cassette into the *Ncr1* locus rendered a non-functional *Ncr1* [145]. *Nkp46*$^{gfp/gfp}$ mice are susceptible to tumor spread and influenza infection but resistant to develop type 1 diabetes [145-147]. To determine the role of this receptor in our inducible lupus model, we immunized C57BL/6 mice and *Nkp46*$^{gfp/gfp}$ mice side by side and detected the self-reactive antibodies in the sera. Interestingly, WT mice developed anti-histone specific antibodies, whereas deficiency in Nkp46 reduced their development (Figure 4.9A). Moreover, *Nkp46*-deficiency specifically diminished the production of pathogenic isotypes, such as IgG2b and IgG2c (Figure 4.9B-C). These data suggest that NK cells may promote the development of autoantibodies partly through NKp46.

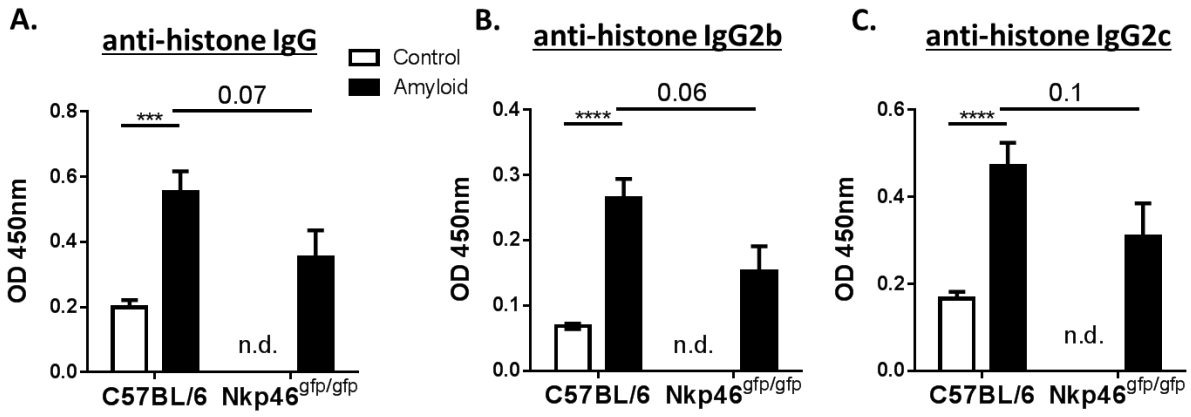

Figure 4.9. Nkp46-deficiency inhibits isotype class-switching.
(A) Levels of anti-histone IgG antibodies were detected in the sera by ELISA from the corresponding mouse strains after 7 weeks of immunization.
(B-C) Levels of the anti-histone IgG subclasses, (B) IgG2b and (C) IgG2c were detected in the sera from the same samples as in (A).
(A-C) Data represented as means ± SEM (n = 4 – 6). *P* values: ***p<0.0005, ****p<0.00005. Student's *t*-test was performed to detect statistical difference between groups.

4.9 Discussion

Loss of B cell tolerance occurs early in SLE, noticed by the presence of autoantibodies years before the onset of SLE [2]. However, the initial events triggering the break of tolerance are not well understood. This study revealed an important role of IFN-γ in initiating autoimmunity, probably through the induction of break of tolerance in T1 B cells. It also proposes that NK cells provide critical help in the establishment of humoral autoimmunity.

It has been widely accepted that IFN-γ is required for autoantibody development, as shown in our study and others [133]. The pathogenicity of IFN-γ is likely due to its ability to promote IgG class switching to more pathogenic antibodies [148]. However, the mechanism by which IFN-γ triggers break of tolerance remains unclear. We have determined that IFN-γ was required for the development of T1 B cells and autoantibody production during the early phase of lupus disease. Similarly, TLR7-transgenic mice show expansion of T1 B cells, which correlated with autoantibody development [76].

Tfh cells, and not T_{H1}, were developed after amyloid immunization, probably through the interplay between IFN-I and IFN-II. Recently published data showed that addition of IFN-I in cell culture induced a Tfh-like cell through STAT1 activation [149]. Also, during chronic viral infection, Tfh differentiation was dependent on IFN-I signaling [150]. IFN-γ has also been found to induce Tfh differentiation. Excessive IFN-γR signaling in *sanroque* mice was sufficient to drive Tfh development [151]. Moreover, IFN-γ induced the upregulation of Bcl6 in *in vitro* activated T cells through STAT1 [152]. Therefore, IFN-I and IFN-II may synergize to enhance Tfh development through STAT1 activation.

The mechanism by which IFN-I and IFN-II cooperate to enhance antibody production by B cells is not well understood. However, both cytokines activate STAT1, which is known to be required for plasma cell differentiation [153]. Moreover, the activation of PI3K by both cytokines

is important for B cell proliferation. We would speculate that STAT1 signaling and PI3K activation may be involved in the enhanced activation of B cells.

We have identified NK cells as the source of IFN-γ during pDC-mediated autoimmunity. Our laboratory has recently determined a mechanism by which IFN-I induce NK cell activation. We proposed that IFN-I activates cDCs to produce IL-15, which further triggers the secretion of IFN-γ by NK cells [90]. IFN-γ secretion by NK cells can induce isotype class-switching of B cells *in vitro* through IFN-γ-dependent and –independent mechanisms [84]. Moreover, we determined that IFN-γ enhanced Tfh development and autoantibody production *in vitro*. Thus, IFN-γ from NK cells likely provides critical help to B cells directly and/or through the activation of T cells.

The role of NK cells in SLE pathogenesis remains unclear. Several groups have shown that NK cell numbers from blood are significantly lower compared with healthy individuals, and this correlated with elevated serum levels of IFN-α [154-156]. Importantly, genotype combinations of killer cell immunoglobulin-like receptors and their HLA class I ligands that favor NK cell activation predispose individuals to certain autoimmune disorders [157]. Furthermore, genetic polymorphisms in the activating NK cell receptor NKp30 that results in reduced gene transcription conveys protection from primary Sjogren's syndrome, whereas NKp30-dependent IFN-γ secretion by NK cells is significantly elevated in Sjogren's patients [158].

We have determined that *Nkp46*-deficiency diminishes the development of autoantibodies, suggesting a pathogenic role for NK cells during autoimmunity. Interestingly, NK cells from *Nkp46*$^{gfp/gfp}$ mice have been shown to produce less IFN-γ in a model of delayed-type hypersensitivity. These mice showed reduce levels of antigen-specific IgG2a antibodies which correlated with reduced allergic responses [159]. Whether *Nkp46*-deficiency diminished IFN-γ production in NK cells in our autoimmune model needs to be further investigated. Moreover, the

analysis of NK cell-deficient mice, which are not currently available, would definitively determine whether NK cells can stimulate humoral autoimmunity.

The depletion of NK1.1+ cells revealed the prominent role of NKT cells during autoimmunity. NKT cells are believed to play a regulatory role in lupus pathogenesis [160]. In lupus mouse models and in human SLE, NKT cells are reduced. As for MRL/lpr mice, a reduction of NKT cells started to be observed right before autoantibody development and this trend continued through the progression of the disease until complete disappearance of these cells [161]. Adoptive transfer of NKT cells delayed onset of disease [161]. Similar to our results, *CD1d*-deficiency enhanced autoantibody development in NZB/W F1 mice and pristane-induced lupus model [81, 82].

The mechanism by which NKT cells suppress humoral autoimmunity is not well understood. It has been suggested that the presentation of self-reactive peptides by CD1d-positive B cells may trigger a suppressive signal to NKT cells [162]. Interestingly, autoreactive B cells have been found to upregulate CD1d expression, suggesting an additional tolerance mechanism where NKT cells may play a significant role. In type 1 diabetes and experimental allergic encephalomyelitis (EAE), NKT cells produced both IL-4 and IFN-γ, and inhibited autoimmune development [163]. Also, NKT cells can limit the inhibitory cytokine IL-10 from autoreactive B cells [164]. Whether IL-4 and/or IL-10 play a significant role in NKT-mediated inhibition of humoral autoimmunity needs to be further investigated.

Altogether, this study revealed a novel immunological cascade occurring downstream of pDC-IFN-I axis during humoral autoimmunity. Early events are comprised by the production of IFN-I by pDCs, IFN-γ production by NK cells and the activation of T1 B cells, which triggers the production of self-reactive antibodies that further undergo Ig isotype class-switching. Moreover, NKT cells can inhibit this autoimmune reaction.

Many therapies have been developed to block IFN-I in SLE; however, our results suggest that IFN-II might be a better target. Although, there are concerns regarding the safety of IFN-γ, given its importance during infection, a humanized monoclonal antibody against IFN-γ was well tolerated and showed some efficacy in patients with Crohn's disease [165]. Also, another anti-IFN-γ antibody is being evaluated for safety in patients with SLE [133]. However, these therapies may trigger various side effects; thus, conditional targeting of immune cell subsets to decrease their cytokine production may be a better approach.

CHAPTER 5.

HUMAN SERUM AMYLOID P-COMPONENT INTERACT WITH MISFOLDED PROTEINS AND INHIBIT THE PRODUCTION OF TYPE I INTERFERON BY NUCLEIC-ACID CONTAINING AMYLOIDS

This chapter is based on Dorta-Estremera SM., Cao W., “Human pentraxins bind to misfolded proteins and inhibit production of type I interferon induced by nucleic acid-containing amyloid”. J Clin Cell Immunol (2015) 6:332 doi: 10.4172/2155-9899.1000332 [166].

5.1 Rationale

The misfolding of monomeric polypeptides that assemble into insoluble amyloid fibrils is linked to protein misfolding diseases [167]. A number of proteins have been identified that exhibit amyloidogeneic potential; among the most studied are amyloid-beta (Aβ) in Alzheimer's disease (AD) and islet amyloid polypeptide in type 2 diabetes [168-170]. Recent data suggest that the self-assembly of amyloidogenic proteins occurs through formation of misfolded intermediates that have an oligomeric structure [171, 172]. These aggregate species, also known as amyloid precursors (APs), are soluble and display inherent cytotoxicity towards live cells, presumably cause neuronal damage in neurodegenerative diseases [173, 174].

Furthermore, it is now evident that misfolded proteins have aberrant innate immune stimulatory capability. For example, amyloid and possibly AP can activate NALP3 inflammasome and induce IL-1β secretion [175, 176]. In addition, our laboratory has demonstrated that amyloid fibrils containing DNA or RNA are potent to activate pDCs to produce IFN-I. This activity can be pathogenic as it induces the breakdown of humoral immune tolerance *in vivo* [42]. Similarly, a complex between amyloid protein curli and bacterial DNA, present in biofilms, induced IFN-I from dendritic cells and triggered autoimmunity in non-autoimmune mice [91]. To date, the mechanism by which the host minimizes the harmful effects of misfolded proteins is not clear. Therefore, it is important to elucidate the fundamental mechanism that is protective against the pathogenicity triggered by the various forms of misfolded proteins.

Pentraxins represent an important element of the humoral innate immune system. They are characterized by a common structural organization in five or ten identical subunits arranged with pentameric radial symmetry [177, 178]. These pattern-recognition molecules include the short pentraxins serum amyloid-P component (SAP) and C-reactive protein, and the long pentraxin 3. All pentraxins are able to interact with components of the complement pathway

[177, 179]. In addition, short pentraxins can bind to membrane phospholipids and nuclear components [180]. Their binding to different ligands is critically promoted by calcium ions, which triggers changes in the conformation of SAP [177]. The aggregated form of SAP in Ca^{2+}-containing solutions interacts with Fcγ receptor, complement, microbes and cell debris [177, 179, 181, 182]. The binding of pentraxins with diverse ligands is important for host defense and removal of damaged cells and nuclear components [179].

It is well known that SAP can bind to amyloid fibrils *in vitro* and *in vivo*, which renders it a universal constituent of amyloid deposits [183, 184]. It has been shown that SAP binding stabilizes the amyloid fibrils, whereas antibodies against SAP can facilitate the phagocytosis and clearance of amyloids [185, 186]. However, it is not known whether SAP modulates pDC activation triggered by amyloids. Also, whether SAP interacts with amyloid intermediates has not been investigated. Therefore, by studying a stabilized model of AP, we intend to determine whether SAP is capable of interacting with the precursor form of amyloid and identify the biological impact of SAP through its interaction with DNA-containing amyloids [187, 188].

5.2 Serum amyloid P-component recognizes co-factor-containing amyloids

SAP can bind to different types of ligands in a calcium-dependent manner [177]. To verify that the commercially obtained SAP was functionally active, ELISA was performed to confirm that SAP bound to Aβ (1-42), an interaction that was enhanced by the presence of calcium (Figure 5.1A). Given that amyloid fibrils may contain various cofactors such as nucleic acids and glycosaminoglycans [189, 190], we first determined whether SAP binds to *in vitro* generated cofactor-containing amyloids. To do that, we prepared heparin-containing amyloid, DNA-containing amyloid and protein-only amyloid as demonstrated previously [188]. The resulting insoluble fibrils did not coat ELISA plates evenly (data not shown), therefore a dot blot analysis was performed. Briefly, SAP or BSA, an irrelevant protein used as a control, was incubated with the amyloid-containing blots in the presence or absence of Ca^{2+}. Our results showed that SAP, but not BSA, readily bound to all types of amyloid complexes examined (Figure 5.1B). This binding predominantly occurred in the presence of Ca^{2+}. These data demonstrated that, in addition to protein-only amyloid, SAP readily binds to hybrid amyloid fibrils containing different cofactors.

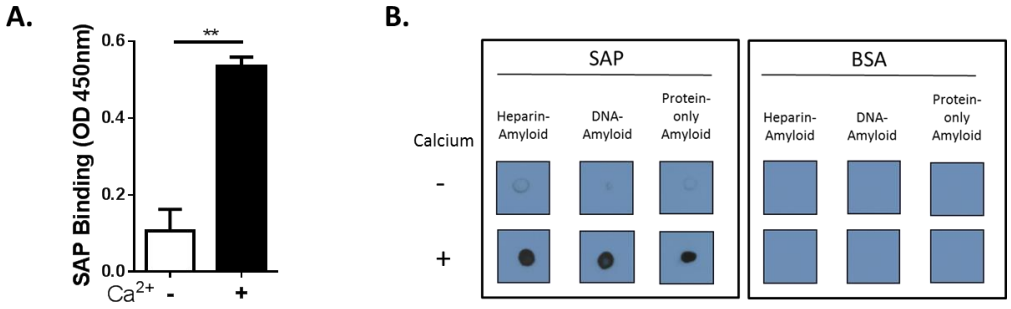

Figure 5.1. SAP binding to amyloid fibrils-containing cofactors. (A) Binding of SAP to Aβ (1-42) (10 μg/ml) in the absence or presence of 2mM Ca^{2+} was assessed by ELISA. Error bars are means ± SEM of duplicate wells (**p<0.005 compared with no Ca^{2+}). Similar results were obtained from five independent experiments. (B) Heparin-containing amyloid, DNA-containing amyloid, and protein-only amyloid were mixed with biotinylated SAP or biotinylated BSA in the absence or presence of 2mM Ca^{2+}. After several washes, the precipitates were dotted on a membrane, and binding was detected by chemiluminescence. Similar results were obtained from three independent experiments. Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amyloid*. J Clin Cell Immunol 6:332.

5.3 SAP binds to amyloid precursor protein in the absence of divalent cations

The AP of amyloidogenic proteins is only formed transiently in solution [191], which made it challenging to study their biochemical properties. However, we have recently generated a form of stabilized AP derived from HSA, referred to as AP-HSA, which displays partially misfolded structure and is capable of converting to amyloid [187]. To determine whether AP-HSA is recognized by SAP, we performed an ELISA-based assay. In contrast to what was observed regarding SAP interaction with amyloid, SAP failed to bind to native HSA or to AP-HSA in the presence of Ca^{2+} (Figure 5.2A). Instead, SAP bound considerably to AP-HSA, but not native HSA, in the absence of Ca^{2+} (Figure 5.2B). These data suggested that SAP can recognize amyloid precursor proteins under a condition distinct from its interaction with amyloid.

Given that SAP bound to AP-HSA in the absence of Ca^{2+}, we tested whether Ca^{2+} would influence the interaction between SAP and AP-HSA. As shown in Figure 2C, Ca^{2+} blocked the binding of SAP to AP-HSA in a dose-dependent manner. EDTA is a chelating agent that can sequester metal ions such as Ca^{2+}. After the incubation of SAP with different concentrations of EDTA in the presence of Ca^{2+}, we showed that EDTA neutralized the inhibitory effect of Ca^{2+} on the binding of SAP to AP-HSA (Figure 5.2D). These results demonstrated that Ca^{2+} prohibited the binding of SAP to AP.

It is established that the binding of SAP to its ligands can be affected by different divalent cations. Similar to Ca^{2+}, Cu^{2+}, but not Mg^{2+}, can promote SAP binding to its ligands through the induction of conformational changes in SAP structure [192]. Therefore, we determined whether these divalent cations affected SAP-AP interaction. Expectedly, Cu^{2+} blocked SAP interaction with AP-HSA (Figure 5.2E). In contrast, Mg^{2+} did not affect the binding of SAP to AP-HSA (Figure 5.2F). These findings suggested that cations that enable conformational changes in SAP effectively inhibit its interaction with AP.

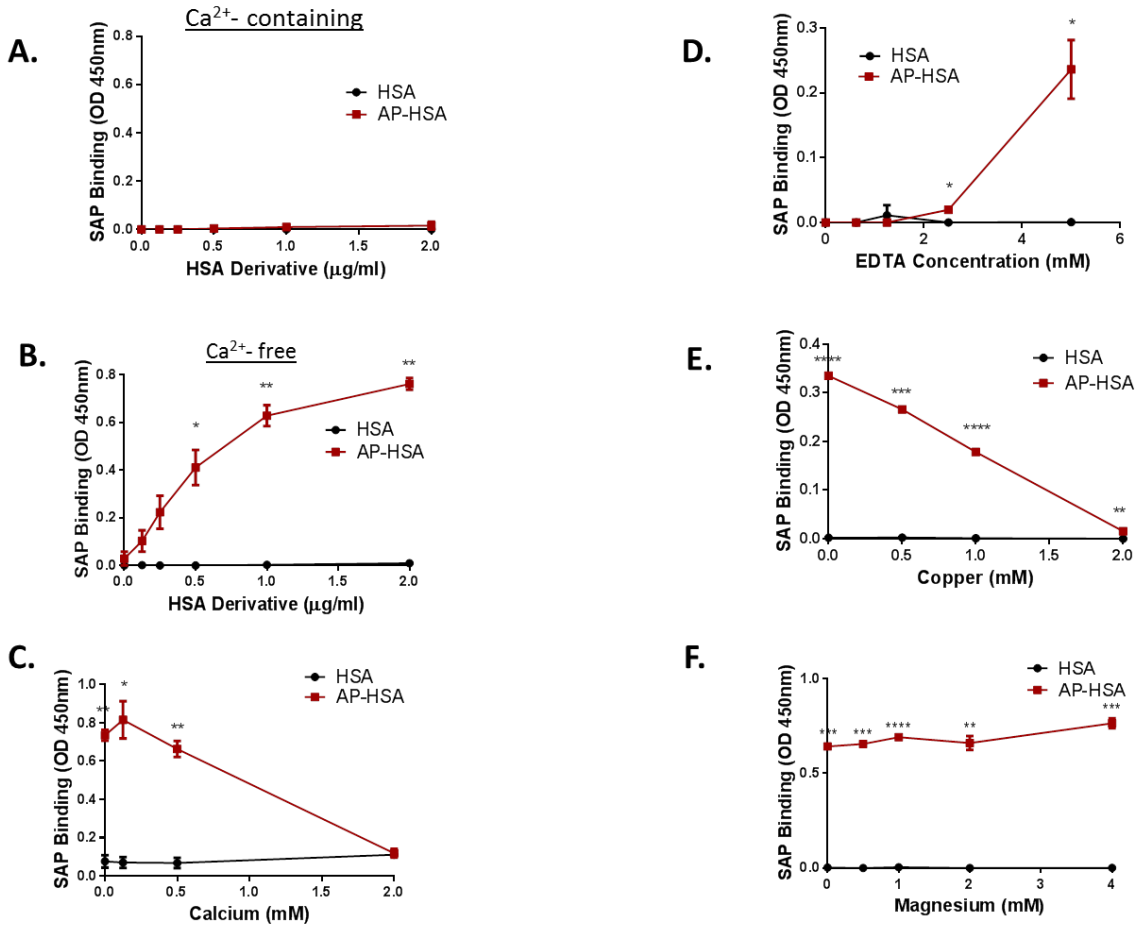

Figure 5.2. SAP binding to amyloid precursor protein. (A-F) Binding of SAP to HSA and AP-HSA in the presence of 2mM Ca^{2+} (A), in PBS (B) in PBS with different concentrations of Ca^{2+} (C), in 2mM Ca^{2+} plus different concentrations of EDTA (D), or with different concentrations of Cu^{2+} (E) or Mg^{2+} (F) was assessed by ELISA. Error bars are means ± SEM of duplicate wells. Similar results were obtained from at least 2 independent experiments (*p<0.05, **p<0.005, ***p<0.0005 and ****p<0.00005 compared with HSA). Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amyloid*. J Clin Cell Immunol 6:332.

5.4 SAP decamer preferentially binds to amyloid precursor protein

Human SAP can undergo conformational changes according to the presence of calcium, pH, ligand availability and albumin concentration [193-195]. In the absence of Ca^{2+}, SAP forms a decamer composed of two cyclic pentamers whereas in the presence of calcium, SAP aggregates into high molecular weight complexes [194]. If high concentrations of proteins are present in Ca^{2+}-containing conditions, such as HSA in human serum, SAP reverses to a pentameric form [195]. To determine the conformation of SAP during its interaction with AP-HSA, we incubated SAP in the presence of Ca^{2+} and tested the effect of different concentrations of HSA (native monomeric form). As shown earlier (Figure 5.2A), SAP, in the presence of Ca^{2+} but at low levels of HSA, failed to bind AP-HSA. However, it regained the ability to interact with AP-HSA in the presence of high doses of HSA (Figure 5.3A). These data hint to us that, when interacting with AP, SAP may adopt a pentameric conformation.

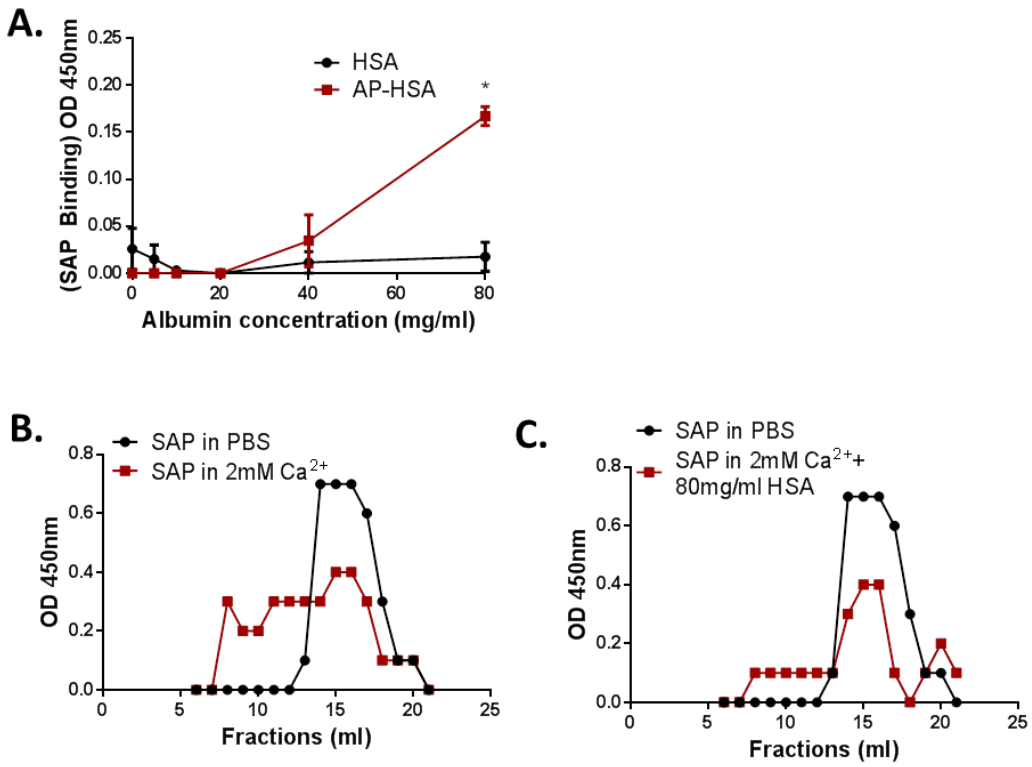

Figure 5.3. **SAP conformation during amyloid precursor protein binding.** (A) Effect of different doses of HSA, in the presence of 2mM Ca^{2+}, on SAP binding to HSA or AP-HSA was assessed by ELISA. Error bars are means ± SEM of duplicate wells (*$p<0.05$ compared with HSA). Similar results were obtained from at least two independent experiments. (B, C) Biotinylated SAP was incubated in the presence or absence of Ca^{2+} (B) or 80 mg/ml HSA plus Ca^{2+} (C), and the molecular weight of SAP was assessed by gel filtration. Fractions were collected and analyzed by ELISA. Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amyloid*. J Clin Cell Immunol 6:332.

To further identify the assembly of SAP, gel filtration of purified SAP in different buffer conditions was performed. SAP monomer has a molecular weight of 25 kD, therefore SAP pentamer has a molecular mass of 127 kD and SAP decamer is 254 kD [195]. In PBS, SAP eluted between fractions 13 and 17, within the expected range of decamers. When 2mM Ca^{2+} was added, SAP appeared earlier, in fractions 7 to 16, suggesting the formation of heterogeneous higher molecular weight aggregates (Figure 5.3B). These results are consistent with our earlier assumption that, SAP exists primarily as a decamer in buffer lacking Ca^{2+}, a

conformation enabling its binding to AP-HSA (Figure 5.2B), whereas in the presence of Ca^{2+}, SAP autoaggregates and gained capacity to interact with amyloid fibrils (Figure 5.1A and 5.1B). Given that high concentration of HSA promoted the binding of SAP to AP-HSA in Ca^{2+}-containing solution (Figure 5.3A), we further fractionated SAP under this condition. The presence of 80 mg/ml HSA in Ca^{2+}-containing PBS solution, reverted the majority of the aggregated SAP back into the fractions 13 to 16, which contain lower molecular weight species (Figure 5.3C). These data supported the notion that SAP decamer predominantly interacts with AP whereas highly aggregated SAP binds to amyloids.

5.5 Misfolded structure in amyloid precursor protein is crucial for SAP binding

EGCG is a natural compound that interferes the formation of β-sheet structure by complexing with AP [196]. To determine whether the misfolded structure of AP-HSA is critical for its interaction with short pentraxins, different amounts of EGCG were pre-incubated with HSA or AP-HSA. By dot blot analysis, we found that high doses of EGCG prohibited the binding of SAP to AP-HSA (Figure 5.4A). These data suggested that a misfolded structure of APs might be crucial for their recognition by SAP.

Acidic conditions can promote structural misfolding of proteins; therefore, acidic conditions can facilitate the formation of APs *in vitro* [197, 198]. Consistently, the generation of stabilized AP-HSA by crosslinking requires an acidic pH. Conversely, other crosslinkers, such as glutaraldehyde and DMP, that only react at basic pH, failed to produce AP [188]. Therefore, we crosslinked HSA with glutaraldehyde (referred as HSA-Glut) and DMP (referred as HSA-DMP) to determine the specific determinant enabling pentraxin binding. Even though all three crosslinked HSA products had similar HSA oligomerization (data not shown), SAP selectively recognized EDC-stabilized AP-HSA but not glutaraldehyde or DMP-crosslinked proteins (Figure

5.4B). Therefore, instead of indiscriminately interacting with any form of aggregated proteins, SAP likely recognize specific misfolded structure present within AP.

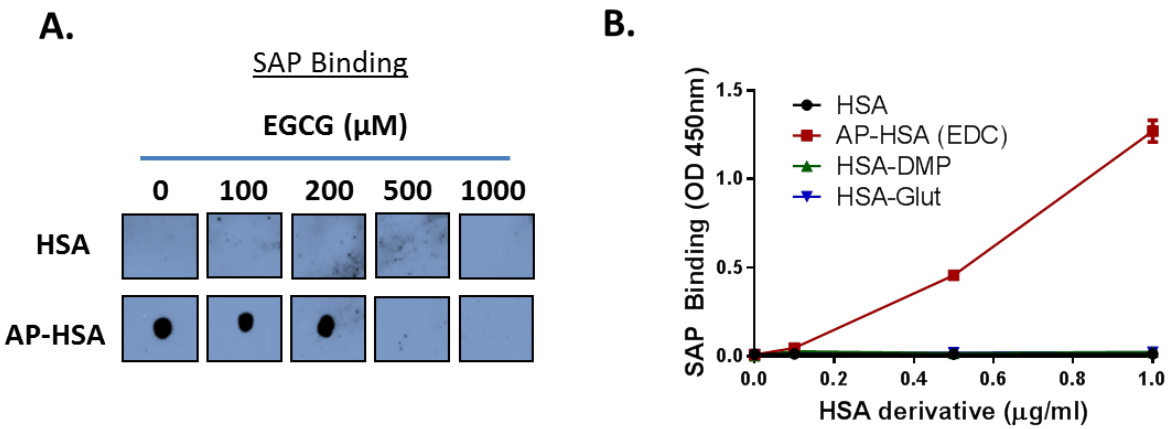

Figure 5.4. **Misfolded structure in amyloid precursor protein is crucial for SAP binding.** A) EGCG was incubated with HSA or AP-HSA for 1 hr and binding of SAP to HSA or AP-HSA was assessed by dot blot. Similar results were obtained from at least two independent experiments. B) Binding of SAP to HSA or HSA crosslinked with EDC (AP-HSA), DMP (HSA-DMP) or glutaraldehyde (HSA-Glut) was assessed by ELISA. Error bars are means ± SEM of duplicate wells. Similar results were obtained from at least two independent experiments. Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amvloid*. J Clin Cell Immunol 6:332.

5.6 SAP binding does not affect amyloid precursor-mediated cytotoxicity

In light of our findings on pentraxin binding to APs, we wanted to determine whether such interactions have functional consequence on the cytotoxicity of APs. As previously shown, AP-HSA can bind to the cell membrane and exert cytotoxic function [188]. First, to determine whether SAP interfere with the binding of AP-HSA to the cell membrane, we incubated SAP with biotinylated HSA or AP-HSA before adding it to RPMI 8226 cells, a human plasmacytoma cell line. After 1 hr on ice, the cells were washed to remove unbound HSA or AP-HSA followed by staining with Alexa Fluor 488-labeled neutravidin. The binding of HSA or AP-HSA to RPMI 8226 cells was then examined by flow cytometry. At a concentration up to 50 μg/ml, SAP had no

significant effect on the surface attachment of AP-HSA (Figure 5.5A). Second, to determine whether SAP affects cell death induced by AP, we stained RPMI 8226 with propidium iodide to quantify dead cell population by flow cytometry. SAP (Figure 5.5B) did not affect the cytotoxicity of AP-HSA at the dose tested (up to 50 μg/ml). These data suggested that, at the doses tested, SAP binding to APs does not affect AP-induced cytotoxicity.

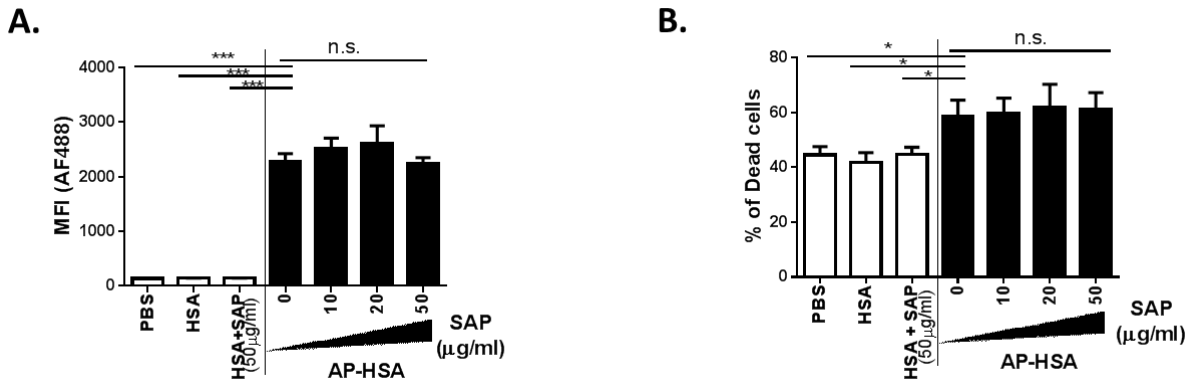

Figure 5.5. **Effect of SAP binding to amyloid precursor protein in cellular cytotoxicity.** (A-B) Biotinylated HSA or AP-HSA (1 μg/ml) preincubated with different concentrations of SAP was added to RPMI 8226 cells. After 1 hr on ice, streptavidin-AF488 and propidium iodide were added to analyze binding of HSA or AP-HSA (A) and cell death (B) by flow cytometry. Error bars are means ± SEM of 3 pooled experiments (*p<0.05 and ***p<0.0005). Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amyloid*. J Clin Cell Immunol 6:332.

5.7 Binding of SAP to amyloids prevents type I interferon production by plasmacytoid dendritic cells

Earlier observation that SAP binds to diverse types of amyloids (Figure 1B) prompted us to investigate whether the association of SAP might affect the innate immune property of amyloid fibrils. Given that DNA-containing amyloid can potently activate pDCs [42], we investigated whether SAP binding could impact the ability of pDCs to produce IFN-α triggered by DNA-containing amyloid. First, we pre-incubated SAP with comparable amounts of DNA-containing amyloid or native HSA plus DNA (referred to as control) then added it to the culture

of PBMC. DNA-containing amyloid induced significant amounts of secreted IFN-α; in contrast, SAP at 50 μg/ml decreased the levels of IFN-α considerably (Figure 5.6A). No IFN-α was detected in cultures incubated with control. SAP had no effect on the production of IL-6 and TNFα, two proinflammatory cytokines, in PBMC culture (Figure 5.6B and 5.6C). To further confirm the inhibitory effect of SAP, we isolated primary human pDCs from peripheral blood and cultured with DNA-containing amyloid together with SAP. Consistently, pDCs produced less amounts of IFN-α when exposed to SAP-amyloid complexes (Figure 5.6D). These data revealed that SAP binding can limit the activation of pDCs and inhibit the production of type I interferon stimulated by nucleic acid-containing amyloids.

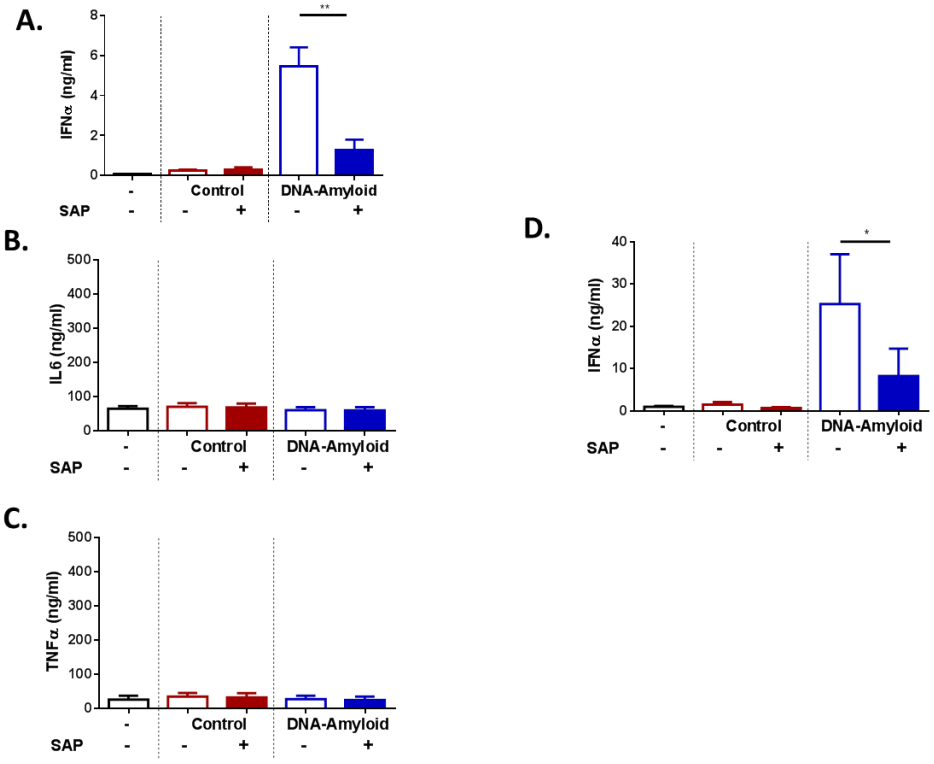

Figure 5.6. **SAP inhibits IFN-α production triggered by DNA-containing amyloids.** (A-C) PBMC were incubated with control (HSA+DNA) or DNA-amyloid with or without SAP (50 μg/ml) for 24 hrs. Supernatants were analyzed by ELISA for IFN-α (A), IL-6 (B) and TNFα (C) secretion. Error bars are means ± SEM of six donors. (D) Purified pDCs were incubated with control or DNA-amyloid, and IFN-α was quantified by ELISA after 24 hrs of culture. Error bars are means ± SEM of six donors (*$p<0.05$ and **$p<0.005$ compared with DNA-amyloid without SAP). Dorta-Estremera SM and Cao W., *Human pentraxins bind to misfolded proteins and inhibit production of interferon induced by nucleic acid-containing amyloid*. J Clin Cell Immunol 6:332.

5.8 Discussion

In this study, we discovered a previously unrecognized interaction between SAP and amyloid precursor protein. Our investigation further reveals a molecular mechanism by which SAP differentially recognizes amyloid fibrils and AP by adopting different conformations. Additionally, we demonstrated that SAP binding can inhibit the innate immune function of amyloid, particularly IFN-α production triggered by nucleic acid-containing amyloid.

Proteins lose structural integrity due to genetic mutations or when subjected to assorted stress, which may lead to cellular damage and eventually diseases if not controlled [167]. Therefore, mechanisms must exist to prevent the accumulation of extracellular misfolded proteins. Here, we report that SAP can interact with terminal amyloid fibrils and/or their soluble precursor species, suggesting a fundamental role of this pentraxin in protein homeostasis. Interestingly, we observed that the interaction between SAP and amyloid required Ca^{2+}, whereas SAP binding to AP was inhibited by divalent cations. It is known that inflammation may increase the extracellular levels of calcium ion in the tissue [199]. Therefore, an inflammatory condition may favor Ca^{2+}-mediated SAP aggregation and complex formation between SAP and amyloid. Since neuroinflammation is exceedingly associated with AD [200], deposition of SAP with amyloid is therefore promoted in the brain of AD patients. On the contrary, human peripheral blood contains high levels of HSA and Ca^{2+}, in which SAP predominantly exist as pentamers [201].

Suggested by our study, AP may be recognized by pentameric SAP; thus it is likely that SAP complexes with AP primarily in the blood. Given that pentraxins readily activate complement to facilitate cargo clearance, it is reasonable to hypothesize that circulating pentraxins mediate the removal of misfolded proteins. Indeed, we have observed that AP-HSA was rapidly cleared from circulation after intravenous injection in mice (data not shown).

Therefore, the interaction between pentraxins and misfolded proteins may serve as an important protective mechanism to eliminate pathogenic misfolded proteins.

Although it was unexpected initially to observe that SAP bound to AP, several published reports have suggested a unique affinity between these short pentraxins and proteins with altered structures. For example, SAP binds to denatured lactate dehydrogenase independently of calcium ion [202]. We present experimental evidence to suggest that SAP specifically recognize the misfolded structure within AP, a novel finding worthy of further investigation. It is likely that the exposed surface of SAP pentamers contains binding sites for the misfolded structures on APs.

We have shown that SAP binding significantly inhibited the magnitude of IFN-α production induced by DNA-containing amyloid, despite the fact that, at the same dose, SAP had no effect on the cytotoxicity of AP. DNA has been detected within the amyloid plaques in AD brain; however, its biological relevance is vague and only under speculation [189]. Several recent studies have established a remarkable link between IFN-α in the brain and the pathogenesis of cognitive decline and AD [203-205]. However, the molecular entity that triggers IFN-α production in CNS has not been identified. We would speculate that DNA-containing amyloid fibrils likely serve as a self-derived ligand to induce IFN-α in AD brain, a process that can be modulated by SAP.

SAP has been implicated in the process of autoimmune pathogenesis, playing largely regulatory roles. In systemic autoimmune conditions such as systemic lupus erythematosus (SLE), dead cell material and nuclear antigens may accumulate and stimulate autoimmune reactions [206]. Shown in studies where SAP was transferred or over-expressed in autoimmune prone mice, this pentraxin potently ameliorate the disease progression presumably by promoting debris clearance [207-210]. However, the relevance of these results in human

disease has been difficult due to differences between mouse and human pentraxins [177]. For example, SAP is an acute phase protein in mouse; whereas in humans, SAP is constitutively expressed [211].

Here we provide another potential mechanism by which SAP dampen the innate immune activation pathway critical for autoimmune response. We have shown that amyloid fibrils containing DNA induced the development of anti-nuclear antibody and a lupus-like syndrome, mimicking SLE, after inoculated into non-autoimmune mice [42]. Interestingly, not only SAP inhibits IFN-α production by pDCs as we have shown here, but also human CRP limits the pDCs' interferon response to autoimmune complexes [212]. Hence, by regulating type I interferon response and debris removal, short pentraxins may play an important role in guarding against the development of autoimmunity at multiple stages.

The novel interaction and biological effect we have observed suggest that pentraxins may function as key players in controlling the pathogenesis of protein misfolding diseases as well as interferon-mediated autoimmune manifestation.

CHAPTER 6.

SUMMARY, SIGNIFICANCE AND FUTURE DIRECTIONS

6.1 Summary

The pDC-IFN-I axis has been linked to the pathogenesis of SLE; however, the cellular events occurred during the break of tolerance and the mechanisms modulating pDC-mediated autoimmunity remain to be fully delineated. In this dissertation, we have identified different cellular players and cytokines involved in the development of humoral autoimmunity. Among these, the roles played by pDCs, NK cells, NKT cells, IFN-I and IFN-II were investigated. Also, we were able to identify a differential regulation of GC B cells versus immature B cells during break of tolerance. We have had several important observations:

1) pDC activation and IFN-I are required for the initial generation of IgM autoantibodies and isotype-class switched autoantibodies.
2) GC B cells are inhibited whereas immature B cells are expanded during break of tolerance.
3) pDCs promote the activation of immature B cells.
4) IFN-I from pDCs induces Tfh cell development and IFN-II enhances IFN-I mediated Tfh development.
5) The kinetics of NK cells and immature B cells correlated with the onset of IgM autoantibodies (Figure 6.1A).
6) IFN-γ is produced by NK cells.
7) IFN-II is required for T1 B cell expansion, IgM autoantibodies and isotype-class switching.
8) NK cells promote autoantibody development partly through NKp46.
9) NKT cells suppress isotype-class switched autoantibodies.
10) SAP binds to amyloid and inhibits IFN-I production by pDCs.

We propose a cascade of events that occur downstream of pDC activation, where IFN-I promotes IFN-γ production by NK cells, which further triggers the activation and expansion of immature B cells that produce the initial self-reactive antibodies (Figure 6.2). In contrast to the pathogenic role of pDCs and NK cells, NKT cells have a suppressive effect in this process. In addition, the presence of SAP may prevent the activation of pDCs by DNA-containing amyloids (Figure 6.2). In this complex process, it is likely that dysregulation of any of the key steps may result in the break of B cell tolerance.

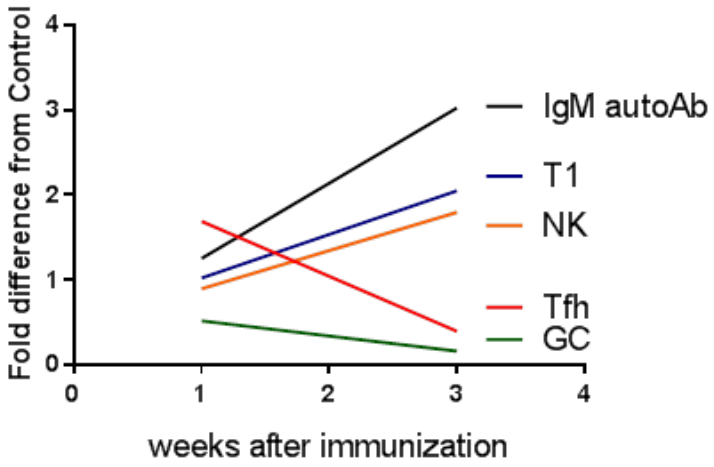

Figure 6.1. **Kinetics of cellular players involved in pDC-mediated break of tolerance.** Representation of the numerical changes occurring at different time points after amyloid immunization.

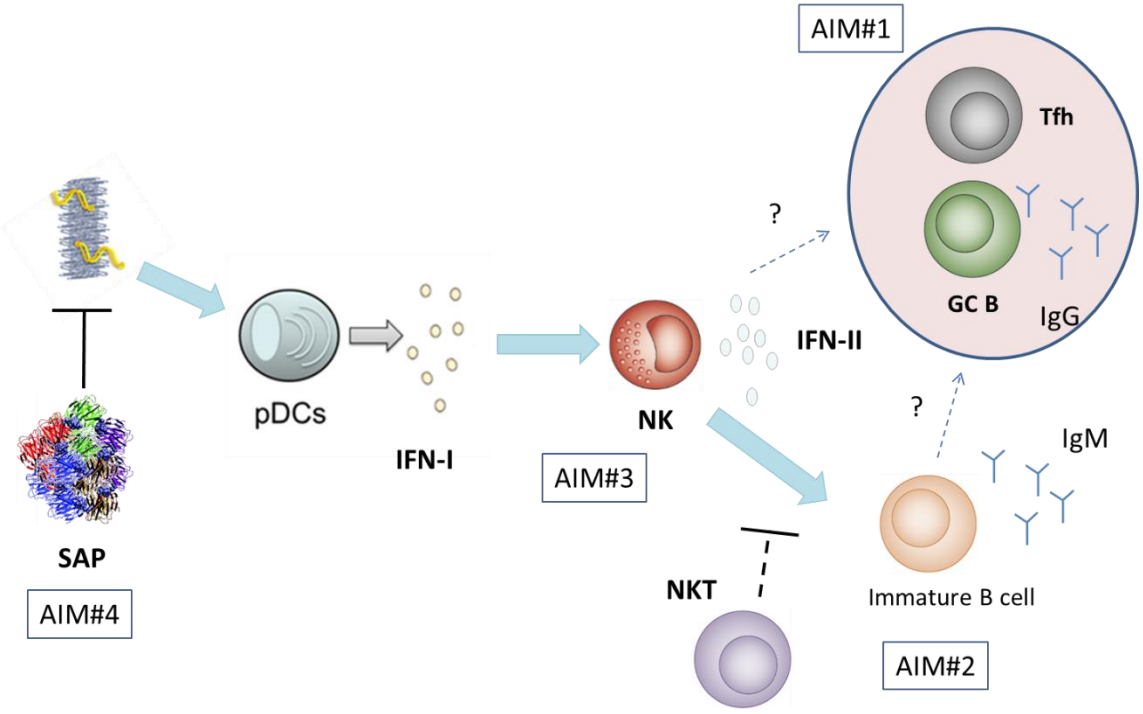

Figure 6.2. **Model for pDC-mediated humoral autoimmunity.** DNA-containing amyloid activates pDCs to produce IFN-I, which triggers the activation of NK cells to produce IFN-II. This further promotes the activation of immature B cells to produce IgM autoantibodies. IFN-I, IFN-II and NK cells are required for the development of class-switched autoantibodies, whether these antibodies arise directly from GCs or immature B cells needs to be further investigated. Also, we have determined that NKT cells suppress the development of autoantibodies. The exact mechanism by which this occurs needs to be further studied. Additionally, SAP can prevent the activation of pDCs by DNA-containing amyloids.

6.2 Significance

SLE causes a heavy physical, emotional and economic burden for affected patients and their families. SLE is characterized by prolonged morbidity and significant mortality [213]. The treatment of SLE patients has been limited by the use of immunosuppressant drugs to control the severe manifestations, which in many cases show partial or no response. Also, these drugs lead to generalized immunosuppression and increased their predisposition to infection, malignancy and infertility [214]. In view of this poor benefit-risk profile, new drugs need to be

developed to target specific immune cells or cytokines that are thought to be central to the disease pathogenesis. To develop better diagnosis and treatment, we need a deeper understanding of the complex mechanisms involved during disease progression. This dissertation revealed the roles played by diverse immune cell types and cytokines, which may serve as targets for treating SLE.

SLE is a highly heterogeneous disease, where different autoantibodies can be present and different organs can be affected. Therefore, it is likely that these differences in lupus patients are due to different genetic and extrinsic mechanisms. Our model may mimic a specific set of patients, where both IFN-I and IFN-II are highly induced. The presence of these two cytokines need to be consider at the time of treatment, mostly now that both IFN-I and IFN-II are being tested as therapies for SLE. A targeted treatment to patients, depending on the presence of type I IFN or type II IFN signatures may provide a better response.

According to our data, targeting the specific immune cell subsets that produce IFN-I and IFN-II may prevent disease pathogenesis or decrease disease burden in SLE patients. Human pDCs express several surface receptors, such as BDCA2 and ILT7, as well as intracellular signaling adaptors, such as IRF7 and PACSIN-1 that are essential for IFN-I induction [12]. Targeting any of these molecules on pDCs may serve useful to control pDC-mediated autoimmune pathogenesis. Being the main producer of IFN-γ, NK cells through specific depletion may also provide benefits in the treatment of SLE patients.

The positive correlation between serum titer, disease activity and deposition of immune complexes in end-organ pathology implies a pathogenic role for certain autoantibodies. As the primary autoantibody-producers, therapies to deplete B cells have been developed; however they failed to show effectiveness in the treatment of SLE patients [215]. A more targeted approach may be needed to accomplish a positive response. Importantly, this dissertation

identified immature B cells as the possible initial source of autoantibodies during humoral autoimmunity. Also, GC B cells may play a significant role in the development of pathogenic autoantibody isotypes. These B cell subsets may be good therapeutic targets in humoral autoimmunity.

6.3 Future Directions

While our study revealed the involvement of different cellular players in the development of autoimmunity in a lupus mouse model, it also raised many new questions that may be answered in the short term by using our inducible lupus model and other murine autoimmune models.

We determined that the expansion and activation of immature B cells correlated with the appearance of autoantibodies; however direct evidence is required to determine whether this B cell subset is in fact the autoantibody-producing cell. The analysis of self-reactive B cells has been hindered by the lack of reliable tools for identification. It is possible that by using phenotypic and microarray analysis, together with the detection of self-reactive clones, we could increase our understanding of the development of autoreactive B cells in our lupus model. Also, recently generated, self-reactive tetramers may be useful to identify the self-reactive B cell subset in our model [125].

Another important question to answer is whether immature B cells are the ones that undergo class-switching and produce IgG autoantibodies. There are two possibilities - immature B cells move to GCs and go through isotype-class switching, or a newly developed autoreactive B cell in GCs undergo class-switching and start producing autoantibodies at later stages after pDC activation. By transferring labeled immature B cells, we could identify their phenotypic changes after amyloid immunization. Also, by localizing these cells in the spleen, we could determine whether they go to GCs or stay outside GCs.

IFN-α and IFN-γ were shown to be required for immature B cell activation and expansion in our inducible lupus model; however the molecular mechanism is not known. IFN-α induces BAFF expression, and BAFF is known to be involved in the proliferation of immature B cells [67, 126]. Therefore, we propose to identify the presence of BAFF in our model and the *in vivo* role of BAFF in the development of autoantibodies at early and late stages of autoimmunity. Since NK cells are the IFN-γ-producing cell in our model, we propose that these cells may move from extrafollicular regions, where they are usually localized, to the marginal zone or GCs to influence the differentiation of follicular B cells. The transfer of labeled NK cells into immunized mice may help elucidate the mechanism by which NK cells modulate B cell responses *in vivo*.

The formation of immune foci are known to induce immune cell activation, thus the identification of these immune centers can reveal the cellular players involved at different times of disease. Thus, the location of B cells and immune helper cells within the spleen during break of tolerance is currently under investigation. Additionally, the study of complex *in vitro* cultures containing different immune cell types and cytokines may expand the understanding of the cross-talk between cells and the key signals triggering overt B cell activation. Lastly, conditional ablation of cytokines in specific immune cell subsets can confirm the pathogenic or protective role of pDCs, NK cells and NKT cells *in vivo*.

In the long term, the major impact of this dissertation will be its translation to benefit SLE patients. It is essential to determine the role played by the cytokines and immune cells characterized in this dissertation during SLE development. Dysregulation of IFN-I, IFN-II, pDCs, NK cells, NKT cells and B cells have been found on peripheral blood of SLE patients [157, 216, 217]. However, these individual observations do not reflect the complexity of the disease. I propose that a personalized approach must be used to classify patients according to their immunological profile and disease pathogenesis. The analysis of the peripheral blood of SLE patients, although not completely representative of the systemic disease, can provide relevant

information regarding the disease pathogenesis. Thus, it is important to analyze type I IFN signature, type II IFN signature and numerical and molecular changes in immune cell types from SLE patients and correlate these parameters with disease prognosis, organ involvement, and autoantibody specificity. A comprehensive analysis of this kind may help identify cellular and molecular targets that can be used as diagnostic markers and/or as treatment.

6.4 Conclusions

A very fine regulation between the two arms of the immune system, *i.e.* innate and adaptive, is needed to prevent autoimmunity. We now know that dysregulation of not one rather many different immune cell types, likely stimulates a break of tolerance and development of SLE. As mentioned in this dissertation, a shift towards the production of IFN-I and IFN-II can promote autoimmunity, whereas a shift towards the activation of NKT cells may prevent it. We foresee that a more personalized approach for disease identification will open new opportunities to treat patients according to their immune profile. Therefore, a deeper understanding of the events triggering autoimmunity, as we have aimed in this dissertation, will accelerate the development of more targeted therapies.

CHAPTER 7.

MATERIALS AND METHODS

Portions of this chapter are based on Huang X., Li J., Dorta-Estremera S., Di Domizio J., Anthony SM., Watowich SS., Popkin D., Liu Z., Brohawn P., Yao Y., Schluns KS, Lanier LL., Cao W., “Neutrophils regulate humoral autoimmunity by restricting interferon-γ production via the generation of reactive oxygen species”. Cell Reports (2015) 12(7): 1120-32. doi:10.1016/j.celrep.2015.07.021 [90]. This article is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND). Collaborator efforts are outlined in the Figure legends whenever necessary.

Portions of this chapter are also based on Dorta-Estremera SM., Cao W., “Human pentraxins bind to misfolded proteins and inhibit production of type I interferon induced by nucleic acid-containing amyloid”. J Clin Cell Immunol (2015) 6:332 doi: 10.4172/2155-9899.1000332 [166]. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Reagents. Materials and their suppliers were as follows: purified human serum albumin (HSA), Sigma-Aldrich; bacterial DNA (endotoxin-free, *Ecoli* K-12), Invivogen; Aβ(1-42) peptide, EMD Biosciences; Smith antigen/ribonucleoprotein complex (Sm/RNP), Meridian Life Sciences; epigallocatechin gallate (EGCG), Sigma-Aldrich; human SAP, Calbiochem. ANA antigen substrate slides were purchased from MBL International. Oligonucleotide CpG2006 (TCGTCGTTTTGTCGTTTTGTCGTT) was synthesized by Sigma-Genosys. HSA and SAP were biotinylated by using EZ-link Sulfo-NHS-LC-Biotin (Invitrogen) according to the manufacturer's instructions. Mouse IFN-γ, human interferon α (IFN-α), human interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were detected by using R&D ELISA kits. RPMI 8226 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood provided by the Gulf Coast Blood Center. pDCs were isolated by BDCA-4 positive selection (Miltenyi Biotech) from buffy coats provided by the Gulf Coast Blood Center.

Preparations of AP and amyloid. HSA-derived AP (AP-HSA) was prepared as described before [187]. Briefly, HSA was crosslinked with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) in MES buffer at pH 4.7. To produce DNA- and heparin-containing amyloid, AP-HSA was mixed at a 1:3 ratio with DNA (Sigma) or heparin (Lovenox, Sanofi-Aventis) and after 1 hr, precipitates were centrifuged to remove soluble components. To prepare protein-only amyloid, 10 mg/ml HSA was reconstituted in MES buffer and incubated for 4 hrs at 65°C. To crosslink HSA with dimethyl pimelimidate (DMP), further referred as HSA-DMP, a 10 fold molar excess of DMP (Thermo Scientific) was added to 5 mg/ml of HSA in 0.2M triethanolamine pH 8. After 1 hr, the reaction was stopped with glacial acetic acid. To prepare glutaraldehyde-crosslinked HSA (HSA-Glut), 0.05% glutaraldehyde (Sigma Aldrich) was added to 1 mg/ml HSA for 10 min. Tris-HCl was added to terminate the reaction.

Antibodies. The following antibodies were used: anti-CD45R/B220 (clone: RA3-6B2) (BD Biosciences), anti-CD11c (clone: HL3) (BD Biosciences), anti-CD11b (clone: M1/70) (BD Biosciences), anti-CD3 (clone: 145-2C11) (BD Biosciences), anti- NK1.1 (clone: PK136) (BD Biosciences), anti-CD49b (clone: DX5) (BD Biosciences), anti-CD335 (NKp46) (clone: 29A1.4) (eBioscience), anti-CD19 (clone: 1D3) (BD Biosciences), anti-CD4 (clone: RM4-5) (BD Biosciences), anti-CD8 (clone: 53-6.7) (BD Biosciences), anti-CD44 (clone: IM7) (BD Biosciences), anti-CXCR5 (clone: 2G8) (BD Biosciences), anti-CD80 (clone: 16-10A1) (Tonbo Biosciences), anti-CD278/ICOS (clone: 7E.17G9) (BD Biosciences), anti-IFN-γ (clone: XMG1.2) (BD Biosciences), anti-GL7 (clone: GL-7) (ebioscience), anti-Fas (clone: 15A7) (ebioscience), anti-CD93 (clone: AA4.1) (ebioscience), anti-IgM (clone: II141) (ebioscience), anti-CD138 (clone: 281-2) (BD Biosciences), anti-CD23 (clone: Cy34.1) (BD Biosciences), anti-CD21 (clone: 7G6) (BD Biosciences), anti-CD107 (clone: 1D4B) (Biolegend), anti-TRAIL (clone: N2B2) (Biolegend), anti-Ki67 (clone: SolA15) (ebiosciences) and anti-IgD (clone: 11-26c2a) (Biolegend).

Mice. All experiments were conducted with sex- and age-matched mice. Animal studies were approved by the **Institutional Animal Care and Use Committees** of University of Texas MD Anderson. C57BL/6, BALB/cByJ, *Ifng*$^{-/-}$ (B6.129S-*Ifng*^{tm1Ts}), *Ch25h*$^{-/-}$ (B6.129S6-Ch25h^{tm1RUS}/J), Vα14-Tg (C57BL/6-Tg (CD4-TcraDN32D3), *Nkp46*$^{gfp/gfp}$ (NCR1 KI/J) and *Cd1d*$^{-/-}$ (C.129S2-Cd1^{tm16ru}/J) mice were purchased from The Jackson Laboratory. Dr. W. Overwijk (University of Texas M.D. Anderson Cancer Center, Houston, TX) generously provided *Ifnar1*$^{-/-}$ C57BL/6 mice, Dr. SC. Sun (University of Texas M.D. Anderson Cancer Center, Houston TX) generously provided *TCRβ/δ*$^{-/-}$ C57BL/6 mice and Dr. T Okada (RIKEN, Research Center for Allergy and Immunology, Yokohama, Japan) generously provided *Bcl6*^{yfp} C57BL/6 mice. *Rgs13*$^{KI/KI}$ were generously provided by Dr. J Kehrl (NIH/NIAID). All animal experiments were conducted on 8-12 weeks old mice.

Mice injections. The amyloid-induced autoimmune mouse model was performed as previously described [42]. Briefly, mice were injected i.p. with control (HSA+DNA) or amyloid (HSA-amyloid+DNA) in PBS mixed 1:1 in CFA. Serum and spleen cells were analyzed after 7-21 days to analyze events occurring during break of tolerance. For long term autoimmune development, booster injections with IFA were performed 2 and 4 wks after. For depletion of NK cells, 250μg of anti-NK1.1 antibody or control IgG2a were i.p. injected 24hrs before the initial amyloid immunization and before every booster immunization.

Analysis of humoral autoimmune responses. Blood samples were collected bi-weekly from immunized mice. For anti-nuclear antibodies, sera were analyzed on ANA antigen substrate slides containing fixed Hep-2 cells (MBL International). The dilution of sera was 1:100 for C57BL/6J or 1:200 for BALB/cByJ mice unless specified. To measure antigen-specific autoantibodies, sera (diluted at 1:1000) were first incubated on 96-well polystyrene assay plates (Greiner Bio-one) coated with histone type II-A or HSA (all from Sigma-Aldrich), and then detected with either horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) or biotin-conjugated anti-mouse IgM (Southern Biotech). For isotype analysis, sera were incubated (diluted at 1:250) on the antigen-coated ELISA plate then detected with an ELISA kit (Bethyl Laboratories).

Flow cytometry. Single-cell suspensions from spleenocytes were obtained and stained with fluorophore-conjugated antibodies. For staining of intracellular IFN-γ, cells were cultured with Golgi Plug (3 mM) for 4 hrs, to enable the accumulation of intracellular proteins. The cells were then harvested and were fixed for 30 min at 4°C with BD Cytofix/Cytoperm kit (BD Biosciences) and subsequently were permeabilized 30 min at 4°C in Perm buffer (BD Biosciences). LSR II or FACSFortessa (Becton-Dickinson) was used for flow cytometry analysis and FACSAria™ for cell

sorting (The Flow Cytometry Core at MD Anderson Cancer Center assisted during cell sorting). Data were analyzed with FlowJo software (Treestar). GC B cells were gated as $B220^+CD19^+GL7^+Fas^+$ or $B220^+CD19^+GL7^+Fas^+Bcl6^+$, Tfh cells as $CD4^+CD44^+ICOS^+PD\text{-}1^+CXCR5^+$, NK cells as $CD3^-NK1.1^+NKp46^+$ or $CD3^-DX5^+NKp46^+$ and immature B cells as $CD19^+CD138^-CD93^+$ unless specified.

Analysis of Peritoneal Fluid. Peritoneal exudate cells were harvested after injection of control or amyloid. Cells were spined down and the supernatant was used to analyze the presence of cytokines in the peritoneal fluid.

Hybridoma development. $CD19^+B220^+$ B cells were sorted from control or amyloid immunized mice. 2 million B cells were mixed with myeloma cells at 1 : 0.8 ratio and fused with polyethylene glycol and plated into 96 well plates. (Collaborator: Long Vien from Hybridoma Core Facility). After 10 days, cells were expanded into 24 well plates. After 7-15 days, the supernatant was collected and tested by ELISA for reactivity against HSA, histone and Sm/RNP.

Immunofluorescence Staining. Spleens were snap-frozen in methylbutane and sections were cut at 6µm. Slides were fixed in acetone, blocked with 1% BSA in PBS and then antibodies against PNA, IgD and Ki67 (clone: 16A8) (Biolegend) were used to detect GCs. After several washes in PBS, a secondary antibody against rat IgG (Life Technologies) was added. Finally, Prolong Gold Antifade Mountant (Thermo Scientific) was applied for further analysis in a Leica TCS SP8 confocal microscope.

Quantitative RT-PCR. Total RNA was isolated from lysed cells by using a PureLink™ RNA Mini kit (Life Technologies). RNA was reverse-transcribed into cDNA with iScript reverse transcription supermix (Bio-Rad). Real-time quantitative PCR was performed by using iCycler Sequence Detection System (Bio-Rad) and iQTM SYBR®Green Supermix (Bio-Rad). The expression of individual genes was calculated by normalizing with the levels of S18 and displayed as relative expression to that of bone marrow of naive C57BL/6 mice. A list of the primers used is listed in Table 1.2.

Gene	Forward	Reverse
fas	aaaccagacttctactgcgattct	gggttccatgttcacacga
cd80	tacctgctttgcttccggg	tccaaccaagagaagcgagg
EndoU	cgtcaacgagaagctgttctccaag	ccacatgttcttcaaatcgtccac
isg15	acggtcttacccttccagtc	ccccttcgttcctcaccag
mx1	ttcaaggatcactcatacttcagc	gggaggtgagctcctcagt
cd86	caagcttatttcaatgggactgc	agcctttgtaaatgggcacg
rag1	ggctagggtcagcagcaagga	cacgggatcagccagaatgtgttc
rag2	cagaacttcaggatgggctgtcttt	tttgagtgaggattgcactggagac
aicd	cgtggtgaagaggagagatagtg	cagtctgagatgtagcgtaggaa
ch25h	ctgcctgctgctcttcgaca	ccgacagccagatgttaatca
cyp27a	ccctttggaagcgatacctg	gtcagtgtgttggatgtcgtgt
cyp27b	tcaggaaaggcaagatctgctga	cctgttgactgcaggaaactgtca
bcl6	ttccgctacaagggcaac	cagcgatagggtttctcacc
icos	cggcagtcaacacaaacaa	tcaggggaactagtccatgc
cxcr5	gaatgacgacagaggttcctg	gcccaggttggcttcttat
il21	ggagtgaccccgtcatctt	aggagcagcagcatgtgag

Table1.2. **List of primers.**

In vitro **induction of T_{FH} cells.** DCs were isolated from the spleen of C57BL/6 mice using FITC-labeled anti-mouse CD11c followed by anti-FITC microbeads. T cells were isolated from the spleen of $Bcl6^{yfp/+}$ mice using anti-mouse CD4 microbeads. T cells and DCs were co-cultured at the ratio of 10:1 in the presence of 0.5 ug/ml anti-CD3 (BD Biosciences). The cells

were cultured in media, with different combinations of CpG A 2216 (0.1 μM), recombinant IFN-α (R&D Systems) or recombinant mouse IFN-γ (R&D Systems). After 3 days, cells were harvested and analyzed by flow cytometry to evaluate T_{FH} development.

***In vitro* B cell cultures.** B220+cells were isolated from the spleen of C57BL/6 mice using FITC-labeled anti-mouse B220 followed by anti-FITC microbeads. The cells ($1X10^6$/ml) were cultured in media or, with different concentrations of recombinant IFN-α and/or recombinant IFN-γ. In some experiments R848 (1μg/ml) was added.

Microarray. T1, T2 and Follicular B cells were sorted after 2 weeks of immunization with control or amyloid. RNA was isolated from the cells using the RNeasy Mini Kit on-colum Dnase digestion (Qiagen) and in collaboration with MedImmune Inc, the Affymetrix Mouse 430.2.0 array was used to detect the expression of genes in these B cell subsets.

SAP ELISA. To detect the binding of SAP to different ligands, ELISA was performed. Different concentrations of Aβ (1-42), Aβ (42-1), Sm/RNP, HSA, AP-HSA, HSA-DMP or HSA-Glut in carbonate buffer (45.3mM $NaHCO_3$, 18.2mM Na_2CO_3 pH 9.6) were coated on ELISA plates overnight at 4°C. The plates were blocked with blocking buffer [150mM NaCl, 25mM Tris (TBS), 1% bovine serum albumin (BSA)] for 1 hr at room temperature. Biotinylated human SAP or CRP (4 μg/ml) was added in TBS + 0.01% Tween 20 at different conditions, such as Ca^{2+}, EDTA, copper (Cu^{2+}) or magnesium (Mg^{2+}), for 2 hrs. Then, streptavidin-horseradish peroxidase (streptavidin-HRP) was added and the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was used to detect color development with maximum absorbance of 450 nm.

Dot blot analysis. To identify the binding of pentraxins to cofactor-containing amyloids, 30 µg of protein-only amyloid, DNA-containing amyloid or heparin-containing amyloid was made as described above and incubated with biotinylated SAP or biotinylated BSA (5 µg/ml) in PBS or 2mM Ca^{2+} in PBS overnight. After several washes, the precipitates were resuspended in 100 µl of PBS and then 4 µl of this solution was spotted onto activated Immobilon-P membrane (Millipore). The blot was then blocked in 1% BSA in TBS and streptavidin-HRP was added. After several washes, the blot was developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotech). To determine the effect of EGCG on pentraxin binding to AP-HSA, EGCG was incubated with 1 µg of HSA or AP-HSA for 1 hr and then 4 µl of the mixture was spotted onto an activated membrane as described above. After blocking with 1% BSA in TBS for 1 hr, biotinylated SAP or biotinylated CRP was incubated in blocking buffer overnight. After several washes, streptavidin-HRP was added and the blot was developed as described above.

Gel filtration. To identify the conformation of SAP at different buffer conditions, a 24 ml Superose 6 10/300 GL column was run on an AKTA (Amersham Bioscience) liquid chromatography system. After equilibration with at least 4 column volumes of buffer, 3 µg of biotinylated SAP in PBS, 2mM Ca^{2+} or 2mM Ca^{2+} plus 80 mg/ml HSA was loaded into the column. One ml fractions were collected and used to coat ELISA plates. Streptavidin-HRP was used to detect SAP in each fraction.

Binding and cytotoxicity *in vitro* assays. To determine the effect of SAP binding on the cytotoxicity of AP-HSA, RPMI 8226 cells were plated at 0.5 X 10^6/ml in PBS and biotinylated HSA or AP-HSA preincubated with SAP or CRP was added. One hour later, cells were washed

and then FITC-conjugated streptavidin and propidium iodide were added to detect binding of HSA or AP-HSA and cell death, respectively.

Human *in vitro* cell culture. Human PBMC (10 X 10^6/ml) or isolated pDCs was cultured overnight in complete medium with 1 μg/ml HSA or AP-HSA mixed with 1 μg/ml *E. coli* DNA with or without SAP. Supernatants were then analyzed by ELISA for cytokine production.

Statistical analysis. Two-tailed, unpaired or paired Student's *t*-tests were used for statistical comparison between two groups, with the assumption of equal sample variance, by using GraphPad Prism software. When more than two groups of samples were analyzed, one-way ANOVA test was performed. Differences with a *P* value of <0.05 were considered statistically significant.

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